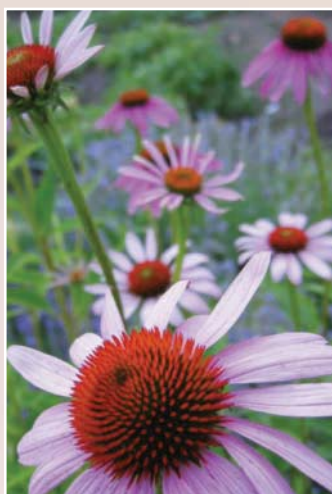


Encyclopedia of Dietary Supplements

Second Edition



Edited by
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Marc R. Blackman
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Encyclopedia of Dietary Supplements

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Preface

Welcome to the second edition of *Encyclopedia of Dietary Supplements*, reflecting the combined efforts of more than 100 authors from 13 countries on 97 topics. Response to the first edition, published in 2005 and then supplemented by a series of online chapters, prompted us to revise and expand the Encyclopedia. There has been considerable expansion in research on many dietary supplements and their ingredients. We expect that this Encyclopedia will continue to be a valuable reference for students and researchers in physiology and chemistry, for health care providers, and for consumers who are interested in understanding the kind of science that is—or is not—behind the claims that are made for dietary supplements that are sold throughout the world, where standards of government regulation differ from country to country. In the United States, sales of products in the dietary supplement market approached \$25 billion in 2009. Their form and their labeling are regulated by the Food and Drug Administration (FDA) as a result of legislation passed in 1994 called the Dietary Supplement Health and Education Act (DSHEA). The dietary supplement category in the United States includes vitamins, minerals, and other ingredients that are found in foods, as well as ingredients not ordinarily found in foods—such as extracts of herbs and other natural products—that are used by consumers for their potential health-promoting, disease-preventing, or performance-enhancing properties. Many of these are represented in the chapters of this book.

The Encyclopedia is not just for consumers in the U.S. market, although we acknowledge that the term “dietary supplements” is an American expression. We are not aware of any other single term that describes all of the substances that we wish to include in this Encyclopedia, although terms such as food supplements, nutritional supplements, or natural health products have been applied as well. Sometimes the claims for benefit of specific products are borne out by well-documented scientific studies. In other cases, they are not, or the science to support their use is still at an early stage. Enthusiasm for their use may be based on popular legend or on longstanding patterns of use in traditional healing systems. In this book, we hope that readers will be able to examine the types of evidence that have been used to support claims of benefit and safety.

The goal of this book is to provide readers with comprehensive, yet accessible, information on the current state of science for individual supplement ingredients or extracts. To this end, each entry reviews basic information available about the ingredient including, where applicable, its chemistry and functions, before detailing the preclinical and clinical literature. Articles conclude with references to the relevant literature.

Given the large number of dietary supplement products in commerce, this book covers only a small fraction of them, with selection based primarily on the frequency of their use and the availability of a sufficient science base to discuss their efficacy and safety. It is clear that the level of scientific information available differs markedly among the various entries. For many ingredients, the chemistry and physiology, preclinical and clinical information, and mechanism of action are well known. For others, by contrast, some or many pieces of these data are missing. The preparation of some commercial products is of high quality and follows good agricultural, laboratory, and manufacturing practices. Again, by contrast, the preparations for others have not been reliable, making them subject to high variability in content and possible contamination. As dietary supplement use becomes more widespread, there are growing concerns about safety of some ingredients, including possible harmful interactions between supplements and prescribed drugs. When known, this information is included in the chapters of this book. These issues should form the basis for future research.

The field of dietary supplements is a rich one, and the science related to this large class of ingredients is expanding all the time. All the chapters that appeared in the first edition have been revised and updated for this edition. In addition to providing these updated chapters, we have included 12 additional chapters on topics not previously covered, reflecting the emergence of dietary supplements in the marketplace, as well as the science behind them. There is also a new chapter on the challenges of dietary supplement research. Additional changes involve gathering several related chapters under “umbrella” topics: *Carotenoids* and *Polyphenols*. Two of the chapters in this edition of the Encyclopedia, on *Ephedra* and *Androstenedione*, were commissioned before their status as dietary supplements in the U.S. market was changed. In 2004, the FDA banned ephedra-containing products from the dietary supplement market in the United States. Also in 2004, the FDA issued warning letters to companies then marketing products containing androstenedione; the regulatory status of these products as dietary supplements has therefore changed. Nevertheless, until recently, both ephedra and androstenedione were widely consumed in the United States. We felt, therefore, that discussion of the science of these ingredients was important. The chapters have been updated to reflect the new regulatory status of these ingredients.

Where possible and applicable, chapter names for botanical ingredients have been adapted to conform to the standardized common names in the American Herbal

Products Association's *Herbs of Commerce*, Second Edition (2000). The accepted scientific names (with authority) and additional synonyms may be found in the individual chapters.

We express our thanks to the authors of the individual chapters. This is a challenging and somewhat controversial field, but we believe that our authors have provided a balanced and current view of the literature. We also acknowledge with gratitude the hard work and guidance of Informa Healthcare's editorial staff, particularly the project editor, Timothy DeWerff.

Finally, we wish to emphasize that the inclusion of chapters on particular dietary supplements in this Encyclopedia does not imply that we endorse them.

Paul M. Coates
Joseph M. Betz
Marc R. Blackman
Gordon M. Cragg
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The Challenges of Dietary Supplement Research and Considerations for Future Studies

D. Craig Hopp and Catherine M. Meyers

INTRODUCTION

The American public and the popular press have considerable interest in the use of dietary supplements (1,2). In view of observed widespread use, there is a need for further information regarding dietary supplement products and their potential clinical applications. This report presents recently compiled data on dietary supplement use in the United States and discusses primary considerations for further research in this area. These considerations focus largely on the need for a standard approach to product characterization and the need to develop an appropriate knowledge base for individual products, prior to embarking on large multicenter trials assessing product efficacy.

BACKGROUND ON DIETARY SUPPLEMENT USE IN THE UNITED STATES

Findings from the 2007 National Health Interview Survey (NHIS), conducted by the Centers for Disease Control and Prevention's National Center for Health Statistics, have provided extensive information on dietary supplement use by the American public (1). The NHIS is an annual in-person survey of Americans regarding their health- and illness-related experiences. The 2007 NHIS included a complementary and alternative medicine (CAM) section and collected information from nearly 24,000 adults, as well as nearly 9500 children under the age of 18 years.

The 2007 NHIS data (Table 1) reveal that approximately 38% of adults, nearly 39 million Americans, use some form of CAM therapy and further that nearly 18% of adults use at least one nonvitamin, nonmineral dietary supplement (1). Similarly, approximately 12% of children less than 18 years of age use some form of CAM therapy, with nearly 4% using at least one dietary supplement (1). The most common reason provided for dietary supplement use is for enhancing wellness (40%). Another 35% of respondents indicate that dietary supplements are used for both wellness and for treatment of a specific condition, whereas only 20% relate that dietary supplements are used to treat a specific condition. The most common health conditions related to CAM product use are those associated with chronic pain, largely of musculoskeletal origin (1).

The most commonly used dietary supplements reported in the 2007 NHIS are listed in Table 2 (1). The 10

Table 1 The 10 Most Common CAM Therapies Used in U.S. Adults—2007^a

Therapy	Prevalence (%)
Dietary supplements	17.7
Deep breathing	12.7
Meditation	9.4
Chiropractic and osteopathic	8.6
Massage	8.3
Yoga	6.1
Diet-based therapies	3.6
Progressive relaxation	2.9
Guided imagery	2.2
Homeopathic treatment	1.8

^aSource: Adapted from Ref. 1.

Table 2 The 10 Most Common Natural Products Used in the United States—2007^a

	Prevalence (%)
Adults	
Fish oil/ ω -3	37.4
Glucosamine	19.9
Echinacea	19.8
Flaxseed oil/pills	15.9
Ginseng	14.1
Combination herb pills	13.0
Ginkgo biloba	11.3
Chondroitin	11.2
Garlic supplements	11.0
Coenzyme Q10	8.7
Children	
Echinacea	37.2
Fish oil/ ω -3	30.5
Combination herb pills	17.9
Flaxseed oil/pills	16.7

^aSource: Adapted from Ref. 1.

most commonly used products in adult respondents are fish oil or ω -3 fatty acids, including docosahexaenoic acid, glucosamine, echinacea, flaxseed oil or pills, ginseng combination herb pills, ginkgo biloba, chondroitin, garlic supplements, and coenzyme Q10. Most dietary supplement use reported for children in the United States is focused on four products: echinacea (37.2%), fish oil or ω -3 fatty acids (30.5%), combination herb pills (17.9%), and flaxseed oil or pills (16.7%) (1).

Use of CAM therapies, including dietary supplements, is widespread across all demographic groups of the U.S. population (1,2) and is more prevalent in women

than in men, with regional variability, in that the use is more prevalent in the West than in the Midwest, Northeast, or Southern regions of the United States. Greater use of CAM therapies is observed between the ages of 30 and 69 years and is also associated with higher levels of education, former smokers, and reported regular levels of physical activity. CAM therapy use is also higher in respondents who report more health conditions or doctor visits, although 20% of CAM users did not report underlying health conditions (1,2).

In view of this extensive use, there is a need for further study of dietary supplements. Rigorous testing of individual dietary supplements, however, is frequently limited because of lack of critical information on several product attributes. In particular, lack of information on product characterization, purity, active ingredients, pharmacokinetics, potential mechanisms of action, or biomarkers for activity limits early phase testing of products. Lack of dosing information and definition of appropriate clinical outcome measures also limit planning of clinical trials. A more standardized approach to product characterization and development of a richer knowledge base on individual supplements will be essential to advancing investigative efforts in this field.

PRODUCT INTEGRITY ISSUES FOR DIETARY SUPPLEMENT RESEARCH

One of the unique challenges inherent to dietary supplement research is that the product complexity is highly variable. This issue poses a serious challenge to establishing a "standard" list of quality control procedures for these products. Although single-component supplements such as resveratrol or melatonin can be accurately characterized and exactly reproduced, plant extracts are much more complex. Furthermore, as has been widely documented, there can be considerable inconsistency in batch-to-batch, bottle-to-bottle, and brand-to-brand content of "off-the-shelf" dietary supplements (3). For botanical products, there is a high level of complexity and natural variability, which prevent investigators from entirely characterizing or exactly reproducing a particular extract. It is estimated that individual plant species are capable of producing thousands of metabolites at varying concentrations. Additional variables for these products include the observation that the same species grown in different places, or even different years in the same place, will not generate the same metabolic profile. It is therefore apparent that a certain amount of product variability, for some supplements, is to be expected. Despite these obstacles, researchers must still strive to conduct a thorough analysis of products used for research purposes. Extensive characterization of research materials is a necessary initial step so that subsequent study results can be appropriately interpreted and reliably reproduced.

It is also apparent that comprehensive characterization, especially for botanical products, can require an enormous amount of effort and expense. Products typically pass through several hands from the grower to the processor and the distributor, prior to arrival at the vendor, and possibly others before reaching consumers. It can be very difficult and sometimes impossible to trace a

given product back to its origins. Furthermore, the identity of every minor component in an extract is almost never known. However, with some important exceptions, this degree of detail in product characterization is neither necessary nor practical. A pragmatic approach is to establish quality control methods that are appropriate for the complexity of the product, the proposed research plan, and product's intended use.

A clinical trial testing a herbal extract will require a substantial dossier of information to document safety, stability, and reproducibility of that product. This dossier will include detailed knowledge about every step in the chain of custody of that material from the time it was grown to the time it was administered to patients. The U.S. Food and Drug Administration (FDA) released a guidance document for botanical drugs in 2004, which is an appropriate resource on quality control procedures to follow for randomized controlled trials (RCTs) of herbal products (4). Investigators intending to conduct clinical studies are strongly encouraged to contact FDA and determine whether an IND (investigational new drug) application is needed for the study of a product in the United States. If an IND is needed for a given study, FDA will provide specific guidance regarding type of information required and level of detail for product characterization.

The characterization requirements for complex products used for *in vitro* studies are perhaps less clear. Similarly, the requirements for early-stage clinical studies on refined products that are botanically derived, but far less complex than the parent extract from which they originated, are less well defined. In these examples, it might be argued that the focus should be more on accurate product characterization. High-pressure liquid chromatography is the analytical technique most commonly employed for generating a product "fingerprint," but there are other methods that could be appropriate depending on the sample. This fingerprint, regardless of the method used to generate it, establishes the identity of a given product without having to know the identity of every product component. Furthermore, it sets a reference point that can be used to document batch-to-batch reproducibility and assess product stability over time. Whichever analytical technique is chosen, the fingerprint must be unique enough to distinguish it from related products and sensitive enough to detect significant changes over time. As the particular dietary supplement research progresses into animals and ultimately humans, progressively more information will be needed regarding product origin and its manufacturing process. A realistic balance should be sought between the need for further studies of dietary supplements and the need for extensive product characterization prior to beginning research studies. Such a balance will ensure the feasibility of future research efforts on these products.

Investigators need to be cognizant of the need for product characterization even in early stages of dietary supplement research. Part of this effort involves independent product analysis, either by the investigator or third party laboratory, to confirm specifications provided by the supplier. This early-stage testing must be conducted regardless of product complexity. Even "pure" compounds from widely known manufacturers have been noted to be mislabeled, in that the content analysis demonstrated that the marketed product was not consistent

with label specifications. For products that have not been extensively studied, it may not be feasible to have an independent analysis performed as validated methods may not be available. Moreover, developing or implementing new methods for such products can represent an appropriate independent research endeavor. In such cases, the information provided requires close scrutiny to determine whether additional product concerns remain.

Finally, another important product consideration for investigating dietary supplements focuses on familiarity with the product supplier. Whenever possible, investigators should begin cultivating relationships with the product supplier at early stages of their research and start acquiring information that will be required for future studies. It is important to determine early in the course of investigations whether the supplier has stringent quality control procedures in place and whether they will provide the requisite product documentation. This is especially true if the ultimate goal is to develop a knowledge base necessary for performing clinical studies. It is therefore prudent to select suppliers or vendors that have provided products for other research studies and have a track record of supplying test materials with the requisite documentation.

CONSIDERATIONS FOR CLINICAL STUDIES OF DIETARY SUPPLEMENTS

Clinical studies are an essential tool for assessing safety and efficacy of therapeutic interventions, whether they are conventional drugs, medical devices, or dietary supplements (5). Similar to standards for assessing efficacy of pharmaceuticals, RCTs play a major role in determining whether a compound or product is safe and effective for a specific indication (5). Prior to initiating (phase III) RCTs, however, there is substantial information that should be collected on a given product. In the pharmaceutical industry, extensive preliminary preclinical and clinical studies (i.e., pharmacokinetics, dosing strategies) are typically undertaken prior to performing large multicenter trials, due to regulatory requirements enforced by FDA. There is a similar need for extensive preliminary studies for dietary supplement investigations, particularly when the research question for the study includes treatment of a disease or condition.

It is important to develop a knowledge base for individual dietary supplements, which will provide direction for further clinical investigations. The optimum knowledge base for a product includes information on mechanism(s) of action, clinical chemistry, biomarkers for in vivo effect, appropriate clinical outcome measures, and the target patient population for the product. For many dietary supplements, information is lacking on many aspects of this knowledge base, which has hampered progress in conducting definitive clinical studies.

As discussed in the previous section, it is essential to have standardized data collection on product characterization, as well as pharmacokinetics, prior to embarking on clinical trials of dietary supplements. In addition, collecting adequate data regarding dosing, potential toxicity, and development of an appropriate placebo for a given product are also requisite early tasks prior to designing

clinical trials. For some dietary supplements, product taste or odor may significantly limit the ability to generate an acceptable placebo for clinical testing.

Understanding the putative mechanism of action of a given product is also an important aspect of the knowledge base, as it strengthens the plausibility of the intervention, and, most importantly facilitates identification of biomarkers to document in vivo effect of the dietary supplement. Availability of a biomarker that can be used to document activity of the agents is of great value. A biomarker facilitates a rational approach to dosing, makes it possible to determine which patients are responding to the intervention, and can assist in identification of outcome measures that are maximally sensitive. The absence of this information can limit expansion of clinical studies beyond early phase testing, particularly for products such as dietary supplements that are generally anticipated to have mild to modest clinical effects.

In planning informative large RCTs, it is essential to have standardized outcome measures that are maximally sensitive and can reliably be implemented in the context of a clinical trial (5). It is also important to have adequate preliminary data on the target patient population before embarking on a large clinical trial (5). Although the primary standard for establishing safety and efficacy remains the RCT, early-phase investigations can exploit other design strategies. For example, adaptive trial designs or n-of-1 designs could be used for expanding the knowledge base on individual products prior to planning subsequent larger studies.

Recent trends in clinical trial design have attempted to facilitate methods for improving trial strategies for medical product development. In clinical studies of new potential therapies, investigators and regulatory agencies have considered adaptations in early-phase trials before planning a large-scale confirmatory phase III RCT (6). To facilitate optimizing final trial design, adaptations in interventional studies may include changes in sample size, enrollment criteria (target subject population), product dose, study end points, and statistical methods for analysis of clinical outcome data (6). As previously discussed, the knowledge base for many products is lacking in several critical aspects, including target subject population, dose, and appropriate end points. Although adaptive design methods provide a mechanism for informed changes to study design after study initiation, appropriate analytic methods must be implemented in the planning of studies such that the scientific validity and integrity of the study are maintained (6).

As dietary supplements are frequently used for chronic conditions, individualized medication effectiveness tests (n-of-1 trials) have been considered a potential strategy for specific products (7,8). Unlike the RCT design, n-of-1 trials are individualized within-patient, are randomized and placebo-controlled, and include multiple crossover comparisons of product versus placebo, or versus another active treatment (7,8). Also unlike the RCT, the n-of-1 trial provides a mechanism for assessing intervention effects in individual patients who might not otherwise be included in the targeted RCT subject population (7,8). The use of such less commonly employed designs can provide a means for adequate data collection, markedly enhancing a product's knowledge base, such

that more definitive clinical trials can be optimally designed and implemented.

CONCLUSION

In developing productive research programs for dietary supplements, it is important to build a hierarchy of evidence for individual supplements, including understanding essentials of individual product characterization, basic product clinical chemistry, and subsequent rigorous testing in the setting of clinical studies. Multiple lines of investigation can then be coordinated for enhancing the knowledge base on a product, with the goal of informing practitioners and the public on safety and efficacy of dietary supplement use.

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S-Adenosylmethionine

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ABBREVIATIONS

CSF, cerebrospinal fluid; GNMT, glycine *N*-methyltransferase; GSH, glutathione; HCC, hepatocellular carcinoma; Hcy, homocysteine; MAT, methionine adenosyltransferase; MTA, 5'-deoxy-5'-methylthioadenosine; MTHFR, 5,10-methylenetetrahydrofolate reductase; NASH, nonalcoholic steatohepatitis; SAH, (S)-adenosylhomocysteine; SAME, (S)-adenosylmethionine.

INTRODUCTION

Common and Scientific Name

S-Adenosyl-L-methionine, also known as 5'-[(3-Amino-3-carboxypropyl) methylsulfonio]-5'-deoxyadenosine; (S)-(5'-desoxyadenosin-5-yl) methionine; $[C_{15}H_{23}N_6O_5S]^+$, is abbreviated in the scientific literature as AdoMet, SAM, or SAME. In the early literature, before the identification of its structure, SAME was known as "active methionine."

General Description

SAME was discovered in 1953 and since then has been shown to regulate key cellular functions such as differentiation, growth, and apoptosis. Abnormal SAME content has been linked to the development of experimental and human liver disease, and this led to the examination of the effect of SAME supplementation in various animal models of liver disease and in patients with liver disease. Both serum and cerebrospinal fluid (CSF) levels of SAME have been reported to be low in depressed patients, which has led to the examination of the effect of SAME treatment in this condition. The effect of SAME in the treatment of other diseases, such as osteoarthritis, has also been investigated. This chapter reviews (i) the biochemistry and functions of SAME; (ii) altered SAME metabolism in liver disease; (iii) SAME deficiency in depression; and (iv) the effect of SAME treatment in liver disease, depression, and osteoarthritis.

BIOCHEMISTRY AND FUNCTIONS

SAME Discovery

Although SAME was discovered by Giulio Cantoni in 1953, the story of this molecule begins in 1890 with Wilhelm His when he fed pyridine to dogs and isolated *N*-methylpyridine from the urine and emphasized the need to demonstrate both the origin of the methyl group as well as the mechanism for its addition to the pyridine (1). Both questions were addressed by Vincent du Vigneaud who, during the late 1930s, demonstrated that the sulfur atom of methionine was

converted to cysteine through the "transsulfuration" pathway and discovered the "transmethylation" pathway, that is, the exchange of methyl groups between methionine, choline, betaine, and creatine. In 1951, Cantoni demonstrated that a liver homogenate supplemented with ATP and methionine converted nicotinamide to *N*-methylnicotinamide. Two years later, he established that methionine and ATP reacted to form a product, that he originally called "Active Methionine," capable of transferring its methyl group to nicotinamide, or guanidoacetic acid, to form *N*-methylmethionine, or creatine in the absence of ATP, which, after determination of its structure, he called "AdoMet" (Fig. 1). Subsequently, Cantoni and his colleagues discovered the enzyme that synthesizes SAME, methionine adenosyltransferase (MAT); (S)-adenosylhomocysteine (SAH), the product of transmethylation reactions; and SAH hydrolase, the enzyme that converts SAH into adenosine and homocysteine (Hcy). At about the same time, Bennett discovered that folate and vitamin B₁₂ could replace choline as a source of methyl groups in rats maintained on diets containing Hcy in place of methionine, a finding that led to the discovery of methionine synthase (MS). In 1961, Tabor demonstrated that the propylamino moiety of SAME is converted via a series of enzymatic steps to spermidine and spermine. In the biosynthesis of polyamines, 5'-deoxy-5'-methylthioadenosine (MTA) was identified as an end product. Thus, by the beginning of the 1960s, Laster's group could finally provide an integrated view, similar to that depicted in Figure 2, combining the transmethylation and transsulfuration pathways with polyamine synthesis.

Since then, SAME has been shown to donate (i) its methyl group to a large variety of acceptor molecules including DNA, RNA, phospholipids, and proteins; (ii) its sulfur atom, via a series of reactions, to cysteine and glutathione (GSH), a major cellular antioxidant; (iii) its propylamino group to polyamines, which are required for cell growth; and (iv) its MTA moiety, via a complex set of enzymatic reactions known as the "methionine salvage pathway," to the resynthesis of this amino acid. All these reactions can affect a wide spectrum of biological processes ranging from metal detoxication and catecholamine metabolism to membrane fluidity, gene expression, cell growth, differentiation, and apoptosis (2), to establish what Cantoni called the "AdoMet Empire."

SAME Synthesis and Metabolism

MAT is an enzyme extremely well conserved through evolution with 59% sequence homology between the human and *Escherichia coli* isoenzymes. In mammals, there are

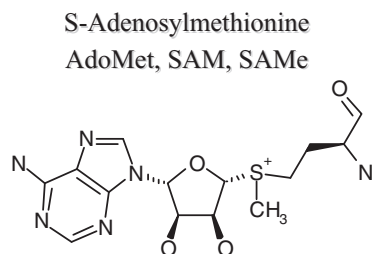


Figure 1 Structure of SAm. (S)-adenosylmethionine (SAm) has been shown to donate: (i) its methyl group to a large variety of acceptor molecules including DNA, RNA, phospholipids, and proteins; (ii) its sulfur atom, via a series of reactions, to cysteine and glutathione, a major cellular antioxidant; (iii) its propylamino group to polyamines, which are required for cell growth; and (iv) its MTA moiety, via a complex set of enzymatic reactions known as the “methionine salvation pathway,” to the resynthesis of this amino acid.

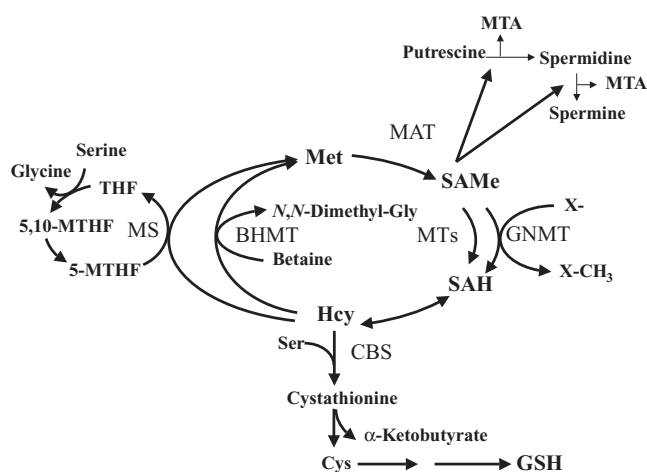


Figure 2 Hepatic metabolism of SAm. Methionine (Met) is converted into homocysteine (Hcy) via (S)-adenosylmethionine (SAm) and (S)-adenosylhomocysteine (SAH). The conversion of Met into SAm is catalyzed by methionine adenosyltransferase (MAT). After decarboxylation, SAm can donate the remaining propylamino moiety attached to its sulfonium ion to putrescine to form spermidine and methylthioadenosine (MTA) and to spermidine to form spermine and a second molecule of MTA. SAm donates its methyl group in a large variety of reactions catalyzed by dozens of methyltransferases (MTs), the most abundant in the liver being glycine-*N*-methyltransferase (GNMT). The SAH thus generated is hydrolyzed to form Hcy and adenosine through a reversible reaction catalyzed by SAH hydrolase. Hcy can be remethylated to form methionine by two enzymes: methionine synthase (MS) and betaine homocysteine methyltransferase (BHMT). In the liver, Hcy can also undergo the transsulfuration pathway to form cysteine via a two-step enzymatic process. In the presence of serine, Hcy is converted to cystathionine in a reaction catalyzed by cystathionine β -synthase (CBS). Cystathionine is then hydrolyzed by cystathionase to form cysteine, a precursor of the synthesis of glutathione (GSH). In tissues other than the liver, kidney, and pancreas, cystathionine is not significantly converted to GSH due to the lack of expression of one or more enzymes of the transsulfuration pathway. The expression of BHMT is also limited to the liver. All mammalian tissues convert Met into Hcy, via SAm and SAH, and remethylate Hcy into Met via the MS pathway. *Abbreviations:* THF, tetrahydrofolate; 5,10-MTHF, methylenetetrahydrofolate; 5-MTHF, methyltetrahydrofolate; Ser, serine; Gly, glycine; X, methyl acceptor molecule; X-CH₃, methylated molecule.

three isoforms of MAT (MATI, MATII, and MATIII) that are encoded by two genes (*MAT1A* and *MAT2A*). MATI and MATIII are tetrameric and dimeric forms, respectively, of the same subunit (α_1) encoded by *MAT1A*, whereas the MATII isoform is a tetramer of a different subunit (α_2) encoded by *MAT2A*. A third gene, *MAT2 β* encodes for a β subunit that regulates the activity of MATII (lowering the K_m and K_i for methionine and SAm, respectively) but not of MATI or MATIII (2). Adult differentiated liver expresses *MAT1A*, whereas extrahepatic tissues and fetal liver express *MAT2A*. *MAT1A* expression is silenced in HCC. It is an intriguing question why there are three different MAT isoforms in the liver. The predominant liver form, MATIII, has lower affinity for its substrates, a hysteric response to methionine (a hysteric behavior, defined as a slow response to changes in substrate binding, has been described for many important enzymes in metabolic regulation), and higher V_{max} , contrasting with the other two enzymes. On the basis of the differential properties of hepatic MAT isoforms, it has been postulated that MATIII is the truly liver-specific isoform. Under normal conditions, MATI would, as MATII outside the liver, synthesize most of the SAm required by the hepatic cells. However, after an increase in methionine concentration, that is, after a protein-rich meal, conversion to the high-activity MATIII would occur and methionine excess will be eliminated (Fig. 2). This will lead to accumulation of SAm and activation of glycine *N*-methyltransferase (GNMT), the main enzyme involved in hepatic SAm catabolism. Consequently, the excess of SAm will be eliminated and converted to homocysteine via SAH. Once formed, the excess of homocysteine will be used for the synthesis of cysteine and α -ketobutyrate as a result of its transsulfuration. This pathway involves two enzymes: cystathionine β -synthase (CBS), that is activated by SAm, and cystathionase. Cysteine is then utilized for the synthesis of GSH as well as other sulfur-containing molecules such as taurine, while α -ketobutyrate penetrates the mitochondria where it is decarboxylated to carbon dioxide and propionyl CoA. Because SAm is an inhibitor of 5,10-methylene-tetrahydrofolate-reductase (MTHFR), this will prevent the regeneration of methionine after a load of this amino acid. At the mRNA level, SAm maintains *MAT1A* and *GNMT* expression while inhibiting *MAT2A* expression. This modulation by SAm of both the flux of methionine into the transsulfuration pathway and the regeneration of methionine maximizes the production of cysteine and α -ketobutyrate, and consequently of ATP, after a methionine load minimizing the regeneration of this amino acid (oxidative methionine metabolism).

ALTERED SAm METABOLISM AND DISEASE

Altered SAm Metabolism in Liver Disease

Accumulating evidence supports the importance of maintaining normal SAm level in mammalian liver, as both chronic deficiency and excess lead to liver injury, steatosis, and development of hepatocellular carcinoma (HCC) (2,3). Majority of the patients with cirrhosis have impaired SAm biosynthesis because of lower *MAT1A* mRNA levels and inactivation of MATI/III (4,5). However, patients with *GNMT* mutations have been identified and they also

have evidence of liver injury (6). In mice, loss of GNMT results in supraphysiological levels of hepatic SAmE and aberrant methylation (7). The molecular mechanisms responsible for injury and HCC formation are different in *MAT1A* and *GNMT* knockout mice but these findings illustrate the importance of keeping SAmE level within a certain range within the cell.

In contrast to normal nonproliferating (differentiated) hepatocytes, which rely primarily on MATI/III to generate SAmE and maintain methionine homeostasis, embryonic and proliferating adult hepatocytes as well as liver cancer cells instead rely on MATII to synthesize SAmE (2). Liver cancer cells often have very low levels of *GNMT* and *CBS* expression and increased expression of *MAT2 β* , which, as mentioned earlier, lowers the K_m for methionine and the K_i for SAmE of MATII. Consequently, proliferating hepatocytes and hepatoma cells tend to utilize methionine into protein synthesis regardless of whether methionine is present in high or low amounts and to divert most homocysteine away from the transsulfuration pathway by regenerating methionine and tetrahydrofolate (THF) (aerobic methionine metabolism). *MAT2A/MAT2 β* -expressing hepatoma cells have lower SAmE levels than cells expressing *MAT1A*, which also favors the regeneration of methionine and THF. From these results, it becomes evident that proliferating hepatocytes and hepatoma cells do not tolerate well high SAmE levels for converting methionine via the transsulfuration pathway to cysteine and α -ketobutyrate.

The finding that *MAT1A*, *GNMT*, *MTHFR*, and *CBS* knockout mice spontaneously develop fatty liver (steatosis) and, in the case of *MAT1A*- and *GNMT*-deficient animals, HCC also (3) demonstrates the synchronization of methionine metabolism with lipid metabolism and hepatocyte growth.

The medical implications of these observations are obvious, since the majority of cirrhotic patients, independent of the etiology of their disease, have impaired metabolism of methionine and reduced hepatic SAmE synthesis and are predisposed to develop HCC (4,5); and individuals with *GNMT* mutations that lead to abnormal SAmE catabolism develop liver injury (6). Moreover, the observation that genetic polymorphisms that associate with reduced *MTHFR* activity and increased thymidylate synthase activity, both of which are essential in minimizing uracyl misincorporation into DNA, may protect against the development of HCC in humans (8) further supports that this synchronization may be an adaptive mechanism that is programmed to fit the specific needs of hepatocytes, and that alterations in the appropriate balance between methionine metabolism and proliferation may be at the origin of the association of cancer with fatty liver disease.

An explanation for these observations connecting methionine metabolism with the development of fatty liver and HCC has remained elusive because the association of SAmE with lipid metabolism and hepatocyte proliferation is, at first glance, not intuitive. During the past years, a signaling pathway that senses cellular SAmE content and that involves AMP-activated protein kinase (AMPK) has been identified to operate in hepatocytes (9,10). AMPK is a serine/threonine protein kinase that plays a crucial role in the regulation of energy home-

ostasis and cell proliferation. AMPK is activated by stress conditions leading to an increase in the AMP/ATP ratio, such as during liver regeneration. Once activated, AMPK shuts down anabolic pathways that mediate the synthesis of proteins, fatty acids, lipids, cholesterol, and glycogen and stimulates catabolic pathways such as lipid oxidation and glucose uptake restoring ATP levels and keeping the cellular energy balance. The finding that in the liver AMPK activity is tightly regulated by SAmE (9,10) has provided a first link between methionine metabolism, lipid metabolism, and cell proliferation. Moreover, excess SAmE can induce aberrant methylation of DNA and histones, resulting in epigenetic modulation of critical carcinogenic pathways (7). Finally, there is evidence indicating that SAmE regulates proteolysis, widening its spectrum of action. In hepatocytes, the protein levels of prohibitin 1 (PHB1) (11), the apurinic/apyrimidinic endonuclease (APEX1) (12), and the dual specificity MAPK phosphatase (DUSP1) (13) are stabilized by SAmE through a process that may involve proteasome inactivation. PHB1 is a chaperone-like protein involved in mitochondrial function, APEX1 is a key protein involved in DNA repair and genome stability, and DUSP1 is a member of a family of mitogen-activated protein kinases (MAPKs) phosphatases, which simultaneously dephosphorylates both serine/threonine and tyrosine residues.

SAmE Deficiency in Depression

Major depression has been associated with a deficiency in methyl groups (folate, vitamin B₁₂, and SAmE) (14,15). Thus, depressed patients often have low plasma folate and vitamin B₁₂ and reduced SAmE content in the CSF. Moreover, patients with low plasma folate appear to respond less well to antidepressants. The mechanism by which low SAmE concentrations may contribute to the appearance and evolution of depression is, however, not well known. SAmE-dependent methylation reactions are involved in the synthesis and inactivation of neurotransmitters, such as noradrenaline, adrenaline, dopamine, serotonin, and histamine; and the administration of drugs that stimulate dopamine synthesis, such as L-dihydroxyphenylalanine, cause a marked decrease in SAmE concentration in rat brain and in plasma and CSF in humans. Moreover, various drugs that interfere with monoaminergic neurotransmission, such as imipramine and desipramine, reduce brain SAmE content in mice (14,15). As in the liver, abnormal SAmE levels may contribute to depression through perturbation of multiple metabolic pathways in the brain. Interestingly, alterations in methionine metabolism that lead to a decrease in the brain SAmE/SAH ratio associate with reduced leucine carboxyl methyltransferase-1 (LCMT-1) and phosphoprotein phosphatase 2A_B (PP2A_B) subunit expression, and accumulation of unmethylated PP2A (16). PP2A enzymes exist as heterotrimeric complexes consisting of catalytic (PP2A_C), structural (PP2A_A), and regulatory (PP2A_B) subunits (17). Different PP2A_B subunits have been described that determine the substrate specificity of the enzyme. PP2A_C subunit is methylated by SAmE-dependent LCMT-1 and demethylated by a specific phosphoprotein phosphatase methylesterase (PME1). PP2A_C methylation has no effect on PP2A activity but has a crucial role in the recruitment of specific PP2A_B subunits

to the PP2A_{A,B} complex and therefore PP2A substrate specificity. Downregulation of LCMT-1 and PP2A_B and accumulation of unmethylated PP2A are associated with enhanced Tau phosphorylation and neuronal cell death (16).

INDICATIONS AND USAGE

SAMe Treatment in Animal Models of Liver Disease

The importance of the metabolism of methyl groups in general, and SAMe in particular, to normal hepatic physiology, coupled with the convincing body of evidence linking abnormal SAMe content with the developmental of experimental and human liver disease, led to the examination of the effect of SAMe supplementation in various animal models of liver disease. SAMe administration to alcohol-fed rats and baboons reduced GSH depletion and liver damage (2,18). SAMe improved survival in animal models of galactosamine-, acetaminophen- and thioacetamide-induced hepatotoxicity, and in ischemia-reperfusion-induced liver injury (18). SAMe treatment also diminished liver fibrosis in rats treated with carbon tetrachloride (18) and reduced neoplastic hepatic nodules in animal models of HCC (19,20). Similar to the liver, SAMe can block mitogen-induced growth and induce apoptosis in human colon cancer cells (21,22).

SAMe Treatment in Human Diseases

SAMe has been used in humans for the past 20 years for the treatment of osteoarthritis, depression, and liver disease. In 2002, the Agency for Healthcare Research and Quality (AHRQ) reviewed 102 individual clinical trials of SAMe (23). Of these 102 studies, 47 focused on depression, 14 focused on osteoarthritis, and 41 focused on liver disease. Of the 41 studies in liver disease, 9 were for cholestasis of pregnancy, 12 were for other causes of cholestasis, 7 were for cirrhosis, 8 were for chronic hepatitis, and 4 were for various other chronic liver diseases.

Pharmacokinetics of SAMe

Orally administered SAMe has low bioavailability, presumably because of a significant first-pass effect (degradation in the gastrointestinal tract) and rapid hepatic metabolism. Peak plasma concentrations obtained with an enteric-coated tablet formulation are dose related, with peak plasma concentrations of 0.5 to 1 mg/L achieved three to five hours after single doses ranging from 400 to 1000 mg (23). Peak levels decline to baseline within 24 hours. One study showed a significant gender difference in bioavailability, with women showing three- to sixfold greater peak plasma values than men (23). Plasma-protein binding of SAMe is no more than 5%. SAMe crosses the blood-brain barrier, with slow accumulation in the CSF. Unmetabolized SAMe is excreted in urine and feces.

Parenterally administered SAMe has much higher bioavailability. However, this form is currently not approved for use in the United States.

SAMe Treatment in Liver Diseases

Out of the 41 studies in liver disease analyzed by AHRQ, 8 studies were included in a meta-analysis of the efficacy of SAMe to relieve pruritus and decrease elevated

serum bilirubin levels associated with cholestasis of pregnancy (23). Compared with placebo, treatment with SAMe was associated with a significant decrease in pruritus and serum bilirubin levels. Similar results were obtained when six studies were included in a meta-analysis of the efficacy of SAMe to relieve pruritus and decrease bilirubin levels associated with cholestasis caused by various liver diseases other than pregnancy.

In 2001, the Cochrane Hepato-Biliary Group analyzed eight clinical trials of SAMe treatment of alcoholic liver disease including 330 patients (24). This meta-analysis found SAMe decreased total mortality [odds ratio (OR) 0.53, 95% confidence interval (CI): 0.22 to 1.29] and liver-related mortality (OR 0.63, 95% CI: 0.25 to 1.58). However, because many of the studies were small and the quality of the studies varied greatly, the Cochrane Group concluded, "SAMe should not be used for alcoholic liver disease outside randomized clinical trials" (24). The AHRQ reached a similar conclusion, "For liver conditions other than cholestasis additional smaller trials should be conducted to ascertain which patient populations would benefit more from SAMe, and what interventions (dose and route of administration) are most effective" (23). The Cochrane Hepato-Biliary Group also concluded that only one trial including 123 patients with alcoholic cirrhosis used adequate methodology and reported clearly on mortality and liver transplantation. In this study (25), mortality decreased from 30% in the placebo group to 16% in the SAMe group ($P = 0.077$). When patients with more advanced cirrhosis (Child score C) were excluded from the analysis (eight patients), the mortality was significantly less in the SAMe group (12%) as compared with the placebo group (25%, $P = 0.025$). In this study, 1200 mg/day was administered orally. Unfortunately, new controlled prospective double-blind multicenter studies on the benefits of SAMe for liver diseases are lacking.

SAMe Treatment in Depression

Out of the 39 studies in depression analyzed by the AHRQ, 28 studies were included in a meta-analysis of the efficacy of SAMe to decrease symptoms of depression (23). Compared with placebo, treatment with SAMe was associated with an improvement of approximately six points in the score of the Hamilton Rating Scale for Depression measured at three weeks (95% CI: 2.2 to 9.0). This degree of improvement was statistically as well as clinically significant. However, compared with the treatment with conventional antidepressant pharmacology, treatment with SAMe was not associated with a statistically significant difference in outcomes. With respect to depression, the AHRQ report concluded, "Good dose-escalation studies have not been performed using the oral formulation of SAMe for depression" (23). The AHRQ report also concluded, that "Additional smaller clinical trials of an exploratory nature should be conducted to investigate uses of SAMe to decrease the latency of effectiveness of conventional antidepressants and to treat of postpartum depression" (23). Unfortunately, these clinical studies are still lacking.

SAMe Treatment in Osteoarthritis

Out of the 13 studies in osteoarthritis analyzed by the AHRQ, 10 studies were included in a meta-analysis of

the efficacy of SAME to decrease pain of osteoarthritis (23). Compared with placebo, one large randomized clinical trial showed a decrease in the pain of osteoarthritis with SAME treatment. Compared with the treatment with nonsteroidal anti-inflammatory medications, treatment with oral SAME was associated with fewer adverse effects while comparable in reducing pain and improving functional limitation. In 2009, the Cochrane Osteoarthritis Group analyzed 4 clinical trials including 656 patients, all comparing SAME with placebo (26). The Cochrane Group concluded, "The effects of SAME on both pain and function may be potentially clinically relevant and, although effects are expected to be small, deserve further clinical evaluation in adequately sized randomized, parallel-group trials in patients with knee or hip osteoarthritis. Meanwhile, routine use of SAME should not be advised" (26).

Adverse Effects

The risks of SAME are minimal. SAME has been used in Europe for more than 20 years and is available under prescription in Italy, Germany, United Kingdom, and Canada, and over the counter as a dietary supplement in the United States, China, Russia, and India. The most common side effects of SAME are nausea and gastrointestinal disturbance, which occurs in less than 15% of treated subjects. Recently, SAME administration to mice treated with cisplatin has been found to increase renal dysfunction (27). Whether SAME increases cisplatin renal toxicity in humans is not known.

Interactions with Herbs, Supplements, and Drugs

Theoretically, SAME might increase the effects and adverse effects of products that increase serotonin levels, which include herbs and supplements such as Hawaiian Baby Woodrose, St. John's wort, and L-tryptophan, as well as drugs that have serotonergic effects. These drugs include tramadol (Ultram), pentazocine (Talwin), clomipramine (Anafranil), fluoxetine (Prozac), paroxetine (Paxil), sertraline (Zoloft), amitriptyline (Elavil), and many others. It is also recommended that SAME should be avoided in patients taking monoamine oxidase inhibitors or within two weeks of discontinuing such a medication.

CONCLUSIONS

Although evidence linking abnormal SAME content with the development of experimental and human liver disease is very convincing, the results of clinical trials of SAME treatment of liver disease are not conclusive. Consequently, SAME should not be used outside clinical trials for the treatment of liver conditions other than cholestasis. A new clinical study enrolling a larger number of patients should be carried out to confirm that SAME decreases mortality in alcoholic liver cirrhosis. This is important because if SAME improves survival, SAME will become the only available treatment for patients with alcoholic liver cirrhosis.

Although depression has been associated with a deficiency in SAME, it is not yet clear whether this is a consequence or the cause of depression. To clarify this point, more basic research and the development of new exper-

imental models are needed. Clinical trials indicate that SAME treatment is associated with an improvement of depression. Dose studies using oral SAME should be performed to determine the best dose to be used in depression. New studies should also be carried out where the efficacy of SAME is compared with that of conventional antidepressants.

With respect to osteoarthritis, at present there is no evidence associating a deficiency in SAME with the appearance of the disease. Moreover, the efficacy of SAME in the treatment of osteoarthritis is also not convincing at present.

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Aloe Vera

Santiago Rodriguez, Steven Dentali, and Devon Powell

INTRODUCTION

Aloe vera is one of the oldest known medicinal herbs with a history of use that spans thousands of years. Today, aloe vera is cultivated and used in a large variety of commercial preparations. It is an economic driver in the food, dietary supplement, and personal care industries worldwide. The two main commercial materials derived from aloe vera are aloe vera juice and aloe latex. Aloe vera juice is used for various dietary, cosmetic, and medical purposes such as burn treatment, wound healing, and skincare. It is available in several forms including liquid juice, juice powder, and concentrates. Aloe latex was formerly recognized as an over-the-counter (OTC) laxative drug in the United States. It has seen limited use in dietary supplements as a laxative and in the personal care industry as a skin lightener.

Confusion among consumers, researchers, and regulatory bodies has arisen from the fact that products from aloe latex are often referred to as simply “aloe” or “aloe juice” (including in pharmacopoeias and other official documents around the world), which is physically, chemically, and biologically distinct from products made from the charcoal filtered whole leaf or inner leaf aloe vera juice. These latex-free juice products represent the vast majority of aloe products on the market. Regardless, the prominence of, interest in, and use of aloe vera products for centuries attests to the plant’s myriad value and benefits.

BACKGROUND

Aloe vera (L.) Burm. f. is one of more than 400 known *Aloe* species in the Asphodelaceae family, though it is sometimes classified in Aloaceae. Because most aloe species are indigenous to Africa, it is most likely that aloe vera also originated from that continent. However, because of its now worldwide cultivation, its origin is difficult to establish. Linnaeus classified aloe vera as the “true aloe” hence the name “vera,” meaning true in Latin. Although it has also been known as *Aloe barbadensis*, *Aloe chinensis*, *Aloe indica*, *Aloe vulgaris*, and others, *A. vera* (L.) Burm. f. has precedence (1). Its standardized common name is “aloe vera” though it has also been called Barbados aloe, Curaçao aloe, true aloe, West Indian aloe, Ghrita kumari, or simply aloe (2).

The plant is cactus-like in appearance with succulent leaves that grow in a spiral form from a basal rosette (Fig. 1). An inflorescence is produced annually (typically December through March) with yellow flowers in a trident configuration from a single central stalk with many

flowers in each of the three branches. Aloe vera does not normally reproduce from seeds but from offshoots often called “pups” that grow out from the mother plant. When the green outer rind of the leaves is cut or damaged, a bitter yellow exudate from pericyclic tubules located between the outer rind and the inner leaf is released. This sap is commonly referred to as “aloe latex” (3) and contains several anthraquinone glycosides that have powerful stimulant laxative properties.

When the rind is completely removed, a semitransparent, semicrystalline gel-like layer composed of large thin-walled parenchyma cells is revealed. This inner leaf material is often called “aloe gel,” or “inner leaf fillet,” because of its similarity in shape to a fish fillet. When crushed, it produces a very viscous fluid usually containing approximately 98.5% water. The solids are composed mainly of polysaccharides and other carbohydrates, pectin, and organic acids.

As mentioned earlier, aloe latex-derived products are used as a laxative agent and the processed leaf or inner leaf is often employed topically for the treatment of burns and injury. More recent applications range from skin-moisturizing agents to the management of cancers in animals to impregnation in articles of clothing and mattresses for its softening and moisturizing properties. Aloe vera juice is also orally ingested to manage digestive ailments and for its immune-modulating activities and is sold worldwide in beverage form as a food-based drink product available in various flavors. Aloe vera is also used widely in Ayurvedic medicine (4).

CULTIVATION

Aloe vera is cultivated in subtropical regions around the globe for commercial use and is widely grown by indoor and outdoor plant enthusiasts as an ornamental plant because of its hardiness and beauty. The species is resistant to most insect pests and needs very little maintenance or care to flourish, given appropriate temperature conditions (5).

Because of its very low inner leaf solid content of 0.5% to 1.5%, aloe vera plants are highly susceptible to freezing, which causes extensive damage, even killing them when the temperature falls below 32°F. For this reason, commercial cultivations are typically carried out in warm weather areas (USDA zones 8–11). *Aloe vera* is the most cultivated species of the various *Aloe* species because it produces the largest, thickest leaves and therefore yields the greatest amount of juice. It is cultivated extensively in



Figure 1 Aloe vera flowering. Source: Courtesy of Santiago Rodríguez, Houston, Texas.

Mexico, the Dominican Republic and other Caribbean Islands, Central America, Venezuela, the southern border areas of Texas, New Mexico, Arizona, and California, as well as Tanzania, Uganda, South Africa, Australia, Southern China, Thailand, and India. Some small acreage is also present in the Canary Islands as well as Southern Spain and Southern Italy and recently new commercial operations have been proposed in areas such as Greece, Iran, Pakistan, and other countries in the Middle East.

Aloe vera prefers very well-drained soils, such as sandy loam, but can grow in almost any type of soil. Although aloe vera is naturally adapted to survive in very dry climates, water must be supplied to the plant year-round in order to keep the leaves succulent enough for a good commercial juice yield. Aloe vera is typically planted at a density of 10,000 plants per acre yielding approximately 40 metric tons of leaves per year. Commercial growers should not make the mistake of planting these large plants too densely. The suggested row spacing is a standard, 42-inch-wide row with approximately 60 cm of spacing between plants.

Harvesting usually begins two years after planting. The plants are harvested year-round by carefully remov-

ing most of the outer, lower, older leaves. Typically one to four leaves are removed at a time per plant per harvest. This way it is possible to obtain three to six harvests in a year depending on how many leaves are collected from each plant at harvest.

Cultivation practices for the industrial production of aloe extracts made from the yellow latex sap are radically different from those used to grow aloe vera for juice. In the case of sap production, plants are not irrigated and are grown in arid regions. The leaves turn brown and thin under these conditions but when cut produce the maximum amount of an anthraquinone glycoside-rich latex, the principal constituents of which are the compounds known as "aloins A and B." The sap exudates are collected and further processed to produce two main products, aloe latex concentrate also known as "aloin paste" and a product commercially known as "aloin spray dried."

DESCRIPTION

The leaf of aloe vera is normally described as consisting of three major parts that are used in commercial products: the outer mesophyll (rind or cuticle), the interior parenchyma (inner leaf, gel or gel fillet, inner gel, inner leaf gel fillet), and the aloe latex (sap, bitter element, yellow sap, yellow latex). Researchers, raw material manufacturers, and finished goods manufacturers have utilized all three plant parts separately or in combination for aloe vera research and in the formulation of consumer products.

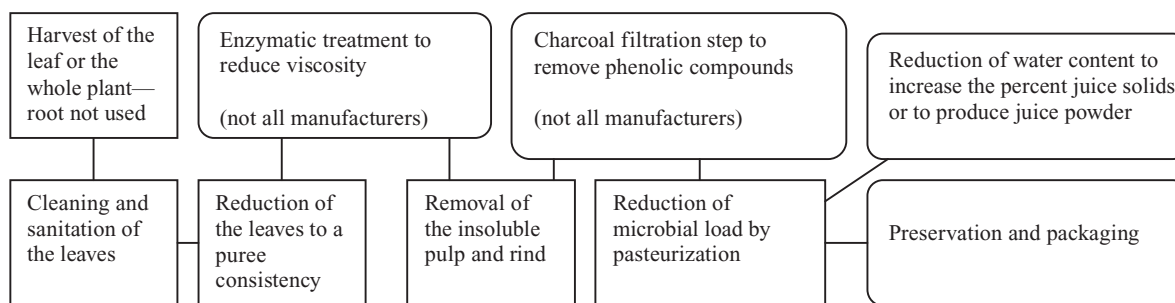
Outer Mesophyll (Rind)

Aloe vera rind or cuticle is the site of photosynthesis and primarily consists of cellulose, monosaccharides, water soluble and insoluble carbohydrates, chlorophyll, amino acids, proteins, and lipids.

Interior Parenchyma (Inner Leaf)

Aloe vera inner leaf is the colorless, mucilaginous parenchyma of the aloe vera plant leaf consisting of water, monosaccharides, water-soluble carbohydrates, water-soluble polysaccharides, and water-insoluble fibrous pulp. The compound β -(1-4)-acetylated mannan, a polysaccharide also known as "acemannan" or "acetylated polymannose," is widely considered to be the biologically most important component of the inner leaf. After removal of fibrous pulp from the inner leaf, the resulting juice contains about 0.5% to 1.5% solids.

Histological examination of aloe vera inner leaf pulp has shown it to be composed of large cells made up of 16% cell walls, about 1% microparticles, and 83% of a viscous gel on a dried weight basis. The carbohydrate portion of each of these components was distinct, with the cell walls composed of 34% galacturonic acid (an unusually high level), the microparticles composed of galactose-rich polysaccharides, and the liquid gel contained mannan (6). These findings showed that different pulp structures are associated with different polysaccharides and may therefore confer different biological activities.



Flowchart 1 Aloe vera whole leaf processing.

Aloe Latex (Aloe Sap, Aloe Bitters)

Aloe latex is a yellow-green bitter exudate that contains the anthraquinone glycosides aloins A and B, formerly known as “barbaloin” and “isobarbaloin,” respectively. The aloin content of aloe latex changes with the season and the age of the leaf but usually makes up 10% to 25% of the dried latex by weight. Products made from aloe latex have been used historically as a laxative. The source plant is most commonly *Aloe ferox* from Africa or Argentina.

COMMERCIAL RAW MATERIAL PROCESSING

Aloe Vera Juice

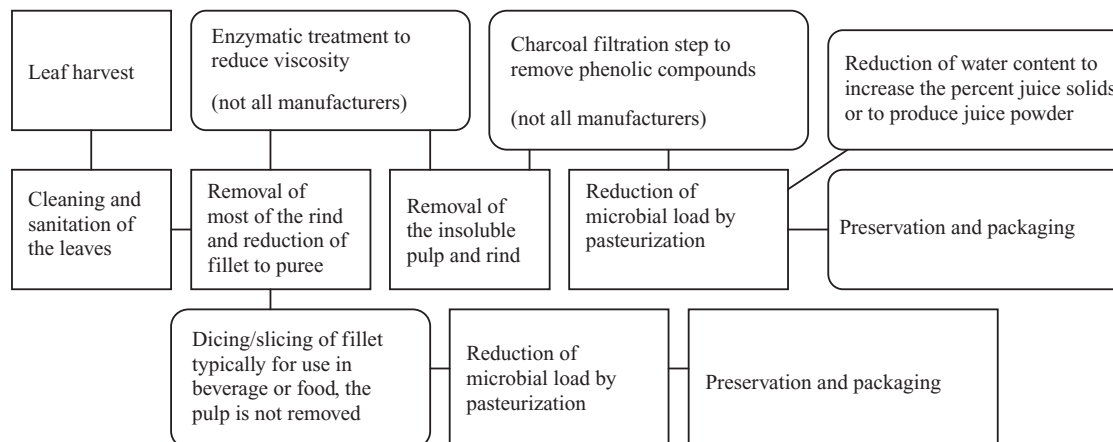
Aloe vera juice can be manufactured from raw leaves in two ways—from the entire leaf or from only the inner leaf material. In both cases, the leaves are first processed to remove the side thorns and tips. For aloe vera juice made from the entire leaf, the leaves are macerated in a grinder into what is commonly called “guacamole” and then further processed by enzymatic treatment (usually with cellulase) to break up cell walls and then charcoal filtered to remove anthraquinones and other phenolic constituents. The resulting aloe vera juice is commonly referred to as “filtered aloe vera juice” or “purified whole leaf aloe vera juice.” See flowchart 1 for more detail.

When creating juice from only the inner leaf material, the inner leaf is separated from the outer rind either manually with a knife or by machine and then washed to rinse away any aloe latex present. The remaining material is crushed and further processed to produce the aloe vera juice. See flowchart 2 for more detail.

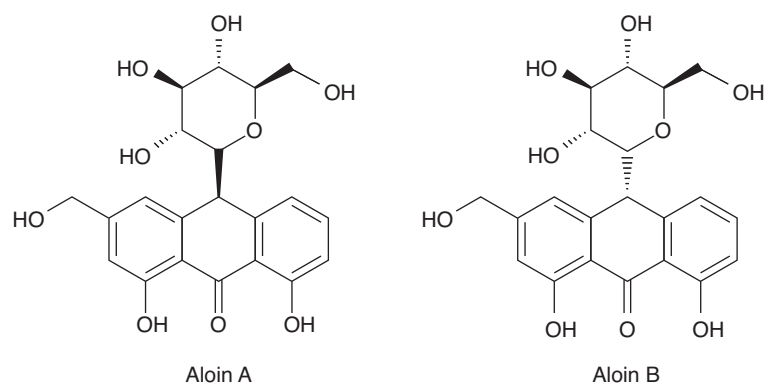
At this stage, regardless of starting material, the now-processed aloe vera juice is typically called “single-strength.” The juice from the leaf or inner leaf can also be further processed to produce concentrates and powders and are often spray or freeze dried. Some heat is usually applied in the industrial production of aloe vera juice to deactivate enzymes that would break down the mannans into oligosaccharides and simple sugars. Heating also serves to control the normal microbial load present on the plant. Enzymatic treatment can be used to further break down cell walls, with filtration removing any remaining insoluble fiber. The resulting filtered juice contains all the major groups of components from the original aloe vera inner leaf.

Aloin-Rich Materials

The commercial production of aloin-rich materials starts with the specialized cultivation practices mentioned earlier. In contrast to aloe juice production, the leaves are



Flowchart 2 Aloe vera inner leaf processing.

**Figure 2** Structures of Aloin A & B.

gathered and the whole plant is cut at the base, producing a transverse cut on all the leaves of the plant at the same time. These leaves are placed in a V-shaped collection device and the aloin-rich sap is allowed to drain. The resulting yellow sap is then concentrated by applying heat until it becomes viscous and forms a solid upon cooling. This aloin paste product contains approximately 25% of the anthraquinone glycosides aloins A and B (Fig. 2). A more sophisticated production method takes the yellow sap, precipitates the resins by adding acid, and concentrates the soluble fraction under vacuum at reduced temperature. The concentrate is then spray dried to produce "spray-dried aloin," which typically contains about 50% aloins. Aloin-rich aloe extracts have been used worldwide mostly in laxative preparations though it has other uses including as a bitter flavoring agent, especially in the wine industry.

Acemannan

Mannan is a generic name for polysaccharides that are polymers of the sugar mannose. In aloe vera juice, the mannose moieties are connected by β -(1-4) linkages, which are partially substituted with acetate units and with galactose-rich side chains on the mannose backbone. This β -(1,4)-acetylated-polymannose material is also known by other names such as "aloverose" and "acemannan"; the latter is also a name given to a proprietary substance covered by many patents (7) and has been assigned as a generic name by the United States Adopted Names Council (8). It is based on the chemical name as it refers to the acetylated *mannan* found in all aloe vera inner leaf fillets (Fig. 3).

Acemannan is not sold as a pure material; however, many commercial products contain varying amounts of it depending on the processing of the aloe vera leaf as men-

tioned earlier. The therapeutic properties of aloe vera juice have been largely attributed to its polysaccharide component and acemannan in particular. This high-molecular-weight material is perhaps the most studied component of the aloe vera plant aside from the anthraquinone glycosides. Many industrial methods have been developed to stabilize the aloe vera juice and prevent polysaccharide degradation. Drying the juice at temperatures over 60°C has been shown to cause deleterious changes in acemannan and also pectin from the cell walls (9).

ANALYSIS OF COMMERCIAL PRODUCTS

Analysis of 32 commercial products showed wide variations in polysaccharide content when compared by molecular weight (10), which was attributed to different manufacturing procedures. A second study of nine commercial powders used a method that hydrolyzed the mannan into mannose as a rapid way to measure the total polysaccharide content in the powder. One sample was found to have an abnormally high concentration of free glucose, four showed signs of spoilage, and all but three were found to have low levels of polymannose present (11). Both studies found all samples to have a significantly lower amount of aloins than the raw unwashed inner leaf fillet, with a high of 16 ppm of aloin A found in one sample in one of the studies. Because of the widespread use of aloe vera juice in personal care products, a voluntary industry limit of 50 ppm aloin content for use in cosmetics as a topical agent has been established (12).

PRECLINICAL STUDIES

General

Many of the biological properties of aloe vera have been attributed to acemannan. This compound has been studied

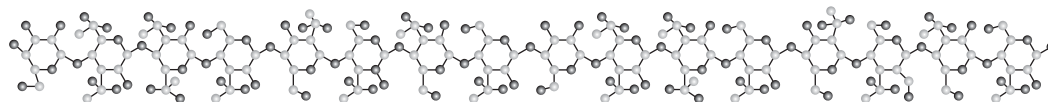
**Figure 3** Partial molecular model of acemannan [β -(1,4)-acetylated-polymannose]. Source: Courtesy of Santiago Rodriguez, Houston, Texas.



Figure 4 Aloe vera plant. Source: Courtesy of Devon Powell, Columbia, Maryland.

for its ability to effect changes in a mouse macrophage cell line and was found to stimulate cytokine production, nitric oxide release, surface molecule expression, and cause changes to cell morphology. This, coupled with the finding that the effect on cytokine production was dose dependent (13), suggests that acemannan may function in part through macrophage activation.

The dermatological activities of aloe vera were investigated in a systematic review that extracted data from 40 studies in a predetermined standardized manner (14). Orally administered to mice, it was found to be effective in promoting wound healing and reducing the incidence of tumors and leishmania parasites by greater than 90% in selected tissues. Its antiviral and antimicrobial properties as well as positive effects on inflammation, frostbite, and burns were reported. Topical application was found not to protect against sunburn in this review. Clinical effectiveness for the use of aloe vera for dermatological conditions was not thought to be sufficiently explored.

Another review examined purported biological properties of aloe vera leaf juice, namely promotion of wound healing, and antifungal, antidiabetic, anti-inflammatory, anticancer, immunomodulatory, and gastroprotective properties and focused on more recently discovered effects and applications such as the ability of aloe vera juice to increase the bioavailability of coadministered compounds and to enhance skin permeability (15).

Wound Healing

The wound healing activity of topical and oral aloe vera was studied in rodent models of anti-inflammatory effects and wound healing (16). Both supernatant and precipitate fractions of a 50% ethanol extract of aloe vera decreased inflammation but only the high-molecular-weight precipitate

fraction demonstrated wound healing activity (17), suggesting that more than one aloe compound could be useful in treating both inflammation and wounds.

Skin Moisturizing

Aloe vera applied topically has a moisturizing effect on skin (18) and has been used for this purpose and as a conditioning agent.

Antidiabetic Activity

Rodent studies have shown blood glucose regulating activity of an aloe vera alcoholic extract (19) and processed aloe vera inner leaf juice (20), suggesting its utility in treating non-insulin-dependent diabetes.

Antitumor Activity

Acemannan has shown significant antitumor activity via immune system activation. In a mouse model, IP injection of acemannan at the time of implantation of sarcoma cells resulted in a 40% survival rate in the treated animals versus 0% of the controls, most likely because of the production of monokines from macrophage peritoneal stimulation. The data suggested that this acemannan-stimulated synthesis "resulted in the initiation of immune attack, necrosis, and regression of implanted sarcomas in mice" (21). A study involving acemannan treatment as an adjunct to surgery and radiation in confirmed fibrosarcoma in dogs and cats showed tumor shrinkage in one-third of the animals after four to seven weeks of treatment administered by intraperitoneal and intralesional injections (22). An earlier study by the same group showed similar results (23).

Studies on Aloin-Rich Materials

Aloin-rich extracts derived from aloe latex belong to the stimulant laxatives drug class. Aloins are inactive until deglycosylated by intestinal flora to form aloe-emodin, the putative active compound (24). Their mechanism of action is believed to involve increasing peristalsis and water accumulation in the colon (25). Aloe latex has been subjected to a human clinical trial as a laxative in combination with other ingredients (26).

The potential toxicities of aloin and its metabolites are not well established though studies have shown it does not promote colon cancer in a mouse model (27) and induces cell changes that could be a sign of anticancer activity (28). Selective activity against certain cancers has also been demonstrated by aloe-emodin, a metabolite of aloins A and B (29,30).

CLINICAL STUDIES

Wound Healing

Although one study showed a delay in healing wound complications after cesarean delivery or gynecological surgery following treatment with "aloe vera dermal wound gel" (31), another recorded a 6-day statistically significant reduction (from 18 to 12 days) in the healing time of partial thickness burns. A systematic review of the literature for the use of topical aloe vera in treating burn wounds included four controlled clinical trials involving 371 patients. A meta-analysis based on the time for healing showed almost nine fewer days required for

the aloe vera–treated group over the controls (32). No specific conclusions could be drawn because of the difference in preparations and outcome measures. Further studies with well-characterized materials were called for by the authors as cumulative evidence tended to support the use of aloe vera for the treatment of first- and second-degree burns. A bioadhesive patch of an aloe vera preparation was evaluated in an open uncontrolled trial for the management of mouth ulcers in children with apparent good results (33).

Ulcerative Colitis and Irritable Bowel Syndrome (IBS)

Ulcerative colitis is caused by a dysfunction of the immune system (34). A 2004 clinical trial involving 44 patients with mild or moderately active ulcerative colitis compared 100 mL twice-daily oral aloe vera juice treatment with placebo for four weeks. The aloe vera–consuming patients showed positive clinical responses more often than placebo. Clinical remission was seen in 30% of the active group, clinical improvement in 37%, and a clinical response in 47% of patients compared with 7%, 7%, and 14% in the placebo group, respectively. Histological scores and the Simple Clinical Colitis Activity Index did not change in the placebo group but decreased significantly for those who consumed aloe vera. No significant differences were seen between the two groups with regard to laboratory values or sigmoidoscopic scores (35).

A human clinical study using aloe vera for treatment of irritable bowel in refractory secondary care patients failed to show a benefit, though the authors could not rule out that diarrhea-predominant patients were helped (36).

Antidiabetic Activity

Aloe dried sap has demonstrated hypoglycemic activity in a study involving five patients with non-insulin-dependent diabetes (37).

Antitumor Activity

A preliminary clinical trial on the use of orally administered “aloe vera tincture” for untreatable metastatic solid tumor patients with and without melatonin treatment showed a significantly higher percentage of nonprogressing patients in the group that received the aloe treatment (50% vs. 27%, $P < 0.05$) (38). Another human trial on 240 patients treated with *Aloe aborescens* (used because of purported immunostimulating activity from this plant owing to its acemannan component) suggested that oral aloe therapy may be a successful adjunct to chemotherapy in patients with metastatic solid tumors. Tumor regression rate and survival time were improved in this study (39). No conclusions can be drawn from this study because details on characterization of the test material were not provided.

SAFETY

A four-week subacute oral toxicity study in mice administered a freeze-dried aloe juice product reported no remarkable subacute toxic effects but did note a decrease in male kidney weights. The report also provided a review of several adverse reaction case studies associated

with aloe vera (40). They ranged from skin irritation from topical use to one report of acute hepatitis in a 73-year-old female taking oral aloe vera capsules for constipation. A second case of acute hepatitis involving a 26-year-old man who had been drinking “aloe vera tea” has also been reported (41).

The National Toxicology Program of the U.S. government nominated “aloe vera gel” for study in 1998 (42). No long-term carcinogenicity studies of aloe vera gel in animals were identified at that time. NTP subsequently chose to conduct a two-year carcinogenicity study on mice and rats with a “whole leaf extract” (43) that includes a considerable amount of latex aloins. The majority of aloe vera juice products intended for long-term internal use are either charcoal filtered whole leaf preparation or are made from washed inner leaf juice with aloin concentrations typically under 10 parts per million. The NTP report was still in progress at the time of publication of this chapter.

REGULATORY STATUS

United States

Aloe and aloin are present in the first approved food additives list published by the U.S. Food and Drug Administration in 1959. Although initially approved in the United States in 1975 as an OTC drug to treat chronic constipation, aloe latex is no longer approved for such use in the United States as of May 9, 2002 (44). Standard quality tests for aloe latex have been described in detail in many official pharmacopeias including the *United States Pharmacopeia*, *Japanese Pharmacopoeia*, and the *European Pharmacopoeia*, though, as mentioned in the introduction, these texts typically define aloe latex as simply “aloe” or incorrectly as “aloe juice.”

Aloe vera juice products can be labeled and marketed as dietary supplements. Aloe latex may also be used in dietary supplements in the United States with laxative or constipation claims as long as such claims are not for the treatment of chronic constipation.

Australia

Aloe vera inner leaf (called “aloe barbadensis”) is eligible for use as an active or excipient ingredient in Australia in “Listed” medicines in the Australian Register of Therapeutic Goods. Acemannan is approved as a component. Components are not approved as substances for use in their own right and can only be used in conjunction with an approved source.

Some aloe vera juice and juice concentrate beverages are viewed as “nontraditional foods” and not as “novel foods” and there are some listed medicines described as “aloe vera drinking gel” or as “aloe vera juice.”

Canada

Aloe vera inner leaf, when included as a Natural Health Product (NHP) active ingredient, requires premarket authorization and a product license number for OTC human use. Such products must comply with the minimum specifications outlined in the current NHPD Compendium of Monographs (45).

European Community

Aloe vera inner leaf was listed as “currently not on the priority list” in the inventory of herbal substances for assessment by the European Medicines Agency as of March 2009. There is an EU regulatory limit established for aloin content of 0.1 ppm in orally ingested products based on a flavoring regulation in which the aloin is defined as an added ingredient as opposed to naturally occurring. The International Aloe Science Council (IASC) (a trade association) has taken a position that these regulations are not applicable to aloe vera juice products.

Japan

Aloe vera juice is regulated as a food beverage product in Japan and is not to contain more than 0.60 mg/kg of benzoic acid. Various forms of aloe vera and extracts thereof are used as components of functional food products or in Foods for Specified Health Use such as in fortified waters and fermented yogurt drinks.

South Korea

Aloe products, known as “edible aloe concentrate” and “edible aloe gel,” are regulated as food products by the Korean Food and Drug Administration. Juice or concentrate from the inner leaf or dried and powdered inner leaf material containing not-less-than 30 mg/g of total aloe polysaccharides is able to carry the health claim of “smoothing the evacuation” on the basis of 20 to 30 mg delivered as aloe polysaccharides. Processed aloe vera leaf or concentrates thereof, after removal of the inedible parts, and containing 2.0 to 50 mg/g of anthraquinones (as anhydrous barbaloin), is permitted to make the same health claim at the specified daily intake. Aloe vera is also one of the four botanical ingredients allowed to make immune system enhancement claims in South Korea.

CONCLUSION

Of the 400 known species of aloe, *Aloe vera* is the most commonly used in commerce and is cultivated in many different areas of the world. The plant yields two raw materials for use in various consumer products including foods, dietary supplements, cosmetics, and drugs, namely aloe vera juice and aloe latex. Aloe vera juice can be made from processing either the entire leaf or only the inner leaf material. Aloe vera juice is often further processed into a powder or concentrate.

Preliminary scientific evidence suggests that aloe vera has therapeutic benefits; however, more studies need to be conducted to definitively demonstrate efficacy. Consumers should be aware and informed when buying aloe vera products; although there are many quality products on the market, there are also many products that may bring little or no benefit to the user. The IASC maintains a certification program using validated analytical methods to determine and ensure products displaying the IASC program seal contain aloe vera of a particular quality. It is recommended that consumers verify that products displaying the IASC seal are current participants in the IASC certification program.

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Androstenedione

Benjamin Z. Leder

INTRODUCTION


Androstenedione (chemical name: 4-androstene-3,17-dione) is a steroid hormone produced primarily in the reproductive system and adrenal glands in men and women. It circulates in the bloodstream and is the immediate precursor to the potent anabolic/androgenic hormone testosterone in the steroid synthesis pathway. Despite this well-known physiological classification, as well as a growing body of evidence demonstrating that orally administered androstenedione is converted to more potent steroid hormones, the United States Food and Drug Administration originally classified the hormone as a “dietary supplement.” As such, it was available to the general public without a prescription and for nearly a decade could be easily purchased in health clubs, nutrition stores, and over the Internet. This over-the-counter availability of androstenedione came to an end when Food and Drug Administration banned its sale in early 2004. The ban was then codified with the passing of the 2004 Anabolic Steroid Control Act. This law reclassified androstenedione as an anabolic steroid and hence a controlled substance.

GENERAL DESCRIPTION

The original and seemingly contradictory classification of androstenedione as a dietary supplement was based on the definition set forth in the 1994 Dietary Supplement Health and Education Act (DSHEA). According to the DSHEA, a substance was defined as a dietary supplement if it was a “product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, mineral, amino acid, herb or other botanical. . . or a concentrate, metabolite, constituent, extract, or combination of any ingredient described above.” Hence, because androstenedione could be synthesized from plant products, it fell under that umbrella. Furthermore, the DSHEA specified that the Department of Justice could not bring action to remove a product unless it was proven to pose “a significant or unreasonable risk of illness or injury” when used as directed. Not surprisingly, after the passing of the DSHEA, the use of dietary supplements increased dramatically. In fact, by 1999, the dietary supplement industry in the United States was generating annual sales of \$12 billion (1).

Initially, androstenedione use was primarily confined to athletes in strength and endurance-related sports, an interest that seems to have sprung from reports of

its use in the official East German Olympic athlete doping program. The event that most dramatically sparked widespread curiosity in androstenedione, however, was the media report that the St. Louis Cardinals baseball player Mark McGwire had used androstenedione in the 1999 season (during which he broke the record for most home runs in a season). The publicity that surrounded this supplement also prompted an increased interest in related “prohormones,” such as norandrostenedione and androstenediol. This then led to a proliferation of claims concerning the potential benefits of androstenedione use. Manufacturers credited these products not only with promoting muscle growth and improving athletic performance but also with increasing energy, libido, sexual performance, and general quality of life. Additionally, androstenedione was often packaged in combination with other substances as part of an intensive nutritional approach to performance enhancement. An example of such a combination is shown in Figure 1. Clearly, the use of androstenedione and related compounds during that time went well beyond the accumulation of data that could provide a rational basis for their use.



PROHORMONE FACTORS
4-Androstenedione: 100 mg
19-Nor-5-Androstenedione: 50 mg
5-Androstenediol: 50 mg
DHEA: 50 mg

GH/IGF FACTORS
L-Arginine Pyroglutamate: 2500 mg
L-Ornithine Alpha-Ketoglutarate: 1250 mg
Taurine: 750 mg
Colostrum: 250 mg

LH BOOSTER
Tribulus: 250 mg
Acetyl-L-Carnitine: 250 mg
L-Carnitine: 100 mg

DHT BLOCKERS
Saw Palmetto: 200 mg
Beta Sitosterol: 200 mg
Pygeum Africanum: 50 mg

ESTROGEN BLOCKERS
Kudzu: 100 mg
Chrysin: 250 mg

Figure 1 A typical combination dietary supplement product.

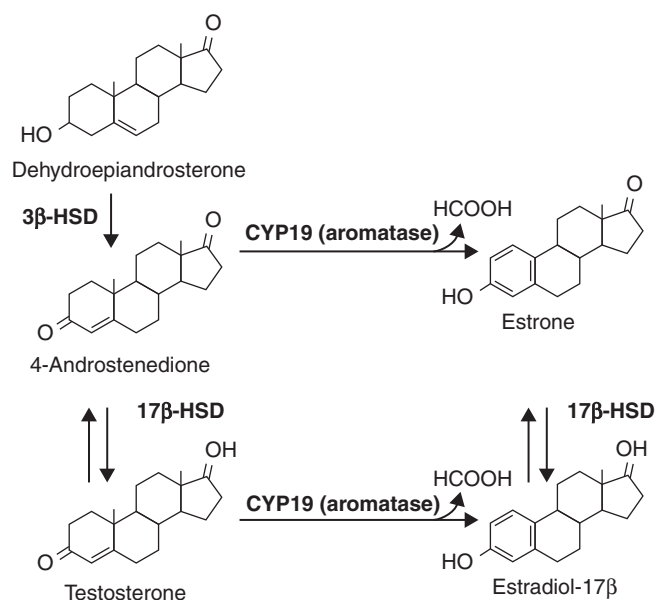


Figure 2 Androstenedione's relationship to other steroid hormones. Enzyme abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.

BIOCHEMISTRY AND PHYSIOLOGY

Androstenedione is a steroid hormone that is produced primarily in the adrenals, testes, and ovaries. It is classified as a "weak androgen" because it binds to the body's receptor for androgen hormones in a much less potent fashion than classic anabolic/androgenic steroids such as testosterone (2). It is synthesized from the precursor hormone dehydroepiandrosterone (itself a dietary supplement) and is the direct precursor to testosterone. In normal physiological circumstances, androstenedione can also be converted to potent feminizing hormones such as estrone and estradiol (both members of the "estrogen" class of hormones). The relationship between androstenedione, other steroid hormones, and the enzymes involved in the conversion of androstenedione to testosterone and estrogens is shown in Figure 2.

Importantly, the enzymes that convert androstenedione to potent hormones such as testosterone and estradiol are active not only in endocrine glands but also in many peripheral body tissues such as muscle, bone, liver, and brain (3). Thus, if orally administered androstenedione has biological activity, it may act either directly or by conversion to these more potent agents.

ANDROSTENEDIONE USE

There were no precise data concerning the prevalence of androstenedione use in the general population during the time that it was widely available. Our best estimates were based on industry sales figures and extrapolations from data on classic anabolic/androgenic steroid use in specific populations. For example, in 1997, it was estimated that

4.9% of male and 2.4% of female adolescents in the United States had used illegal anabolic steroids (4). Because these substances were so readily available, there was concern that androstenedione use in this particularly susceptible population may have greatly exceeded these numbers. In fact, in a survey administered in five health clubs in Boston, Massachusetts, in 2001, 18% of men and 3% of women respondents admitted to using androstenedione or other adrenal hormone dietary supplements at least once. These percentages suggested that as many as 1.5 million U.S. health club members alone may have used these substances (5).

PHARMACOKINETICS AND HORMONAL EFFECTS OF ANDROSTENEDIONE IN MEN

Because so many of the claims that surrounded androstenedione were based on the premise that oral administration increases serum testosterone levels, it may be surprising to some that prior to 1999, there was only a single published study investigating the ability of orally administered androstenedione to be converted to more potent steroid hormones (6). In this study, two women were given a single dose of androstenedione, and the levels were subsequently measured over the next several hours. Since 1999, however, numerous small studies (mostly in men) have investigated the effects of the supplement (6–16). In general, these studies report that serum androstenedione levels increase dramatically after oral administration and thus confirm that a significant portion of the supplement is absorbed through the gastrointestinal tract after ingestion. However, the answer to the more important question, namely, whether it is then converted to more potent steroid hormones such as testosterone and estradiol, appears to be complex. In general, these studies suggest that the ability of oral androstenedione to increase estrogen and testosterone levels in men is dose dependent and is possibly related to the age of the study population as well. Specifically, the bulk of the research indicates that when androstenedione is administered to men in individual doses between 50 and 200 mg, serum estrogen levels increase dramatically. However, larger individual doses (e.g., 300 mg) are required to increase serum testosterone levels.

For example, King and colleagues studied the effects of a single 100-mg oral dose of androstenedione in 10 men between the ages of 19 and 29 and reported that although serum androstenedione and estradiol levels increased significantly, testosterone levels did not change (13). These investigators then specifically measured the portion of circulating testosterone that is not bound to protein and considered the "bioactive" portion (called "free testosterone") and similarly saw no effect of the supplement. In a separate study, Leder and colleagues gave 0, 100, or 300 mg of androstenedione to normal healthy men between the ages of 20 and 40 for seven days and took frequent blood samples on days 1 and 7 (14). As in the study by King, they also found that men receiving both the 100- and 300-mg doses of androstenedione experienced dramatic increases in serum estradiol that were often well above the normal male range. Another similarity was that 100-mg dose

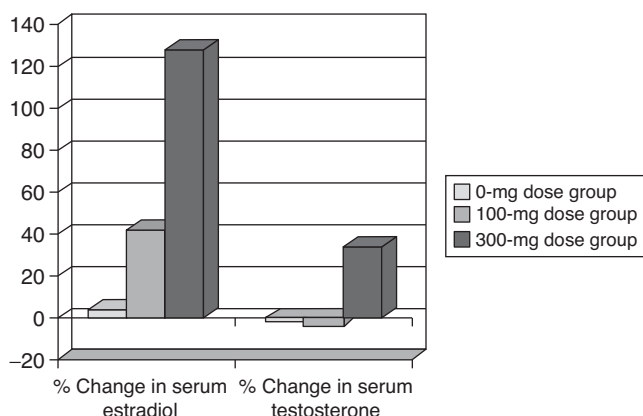


Figure 3 Percentage change in serum testosterone and estradiol in healthy men after a single androstenedione dose (as measured by eight hours of frequent blood sampling). Source: Adapted from Ref. 14.

did not affect serum testosterone levels. As shown in Figure 3, however, the novel finding of this study was that 300 mg of androstenedione increased serum testosterone levels significantly, even though by only a modest amount (34%).

Leder and colleagues further observed that there was a significant degree of variability among men with regard to their serum testosterone response after androstenedione ingestion. As shown in Figure 4, some subjects, even in the 300-mg dose group, experienced relatively little change in testosterone levels, whereas serum testosterone levels doubled in other men. This finding suggests that there may be individual differences in the way androstenedione is metabolized that could impact any one person's physiological response to taking the supplement.

Brown and colleagues investigated the hormonal response in a group of men between the ages of 30 and

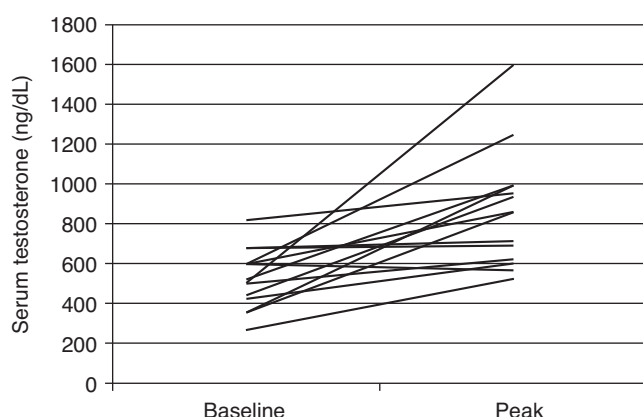


Figure 4 Individual variability in the peak serum testosterone level achieved after a single 300-mg dose of androstenedione in men. Each line represents one study subject. Source: Adapted from Ref. 14.

56 (10). In this study, subjects consuming 100 mg of androstenedione three times daily experienced increases in serum estrogens but not in serum testosterone. However, unlike in the study by King and colleagues discussed in the previous text, free testosterone did increase significantly (even though again by only a small amount).

Finally, several studies have compared the hormonal effects of androstenedione with those of other "prohormone" dietary supplements. Broeder and colleagues studied the results of a 100-mg twice-daily dose of oral androstenedione, androstenediol (a closely related steroid hormone), or placebo in men between the ages of 35 and 65 (7). They found that both compounds increased estrogen levels but neither affected total serum testosterone levels. Similarly, Wallace and colleagues studied the effects of 50-mg twice-daily doses of androstenedione and DHEA in normal men and reported no increases in serum testosterone levels with either (16).

EFFECTS ON MUSCLE SIZE AND STRENGTH IN MEN

The results of the studies discussed earlier suggest that androstenedione use in men would be less likely to promote the muscle building and performance-enhancing effects associated with testosterone use and more likely to induce the undesirable feminizing effects associated with estrogens. Several studies have assessed the ability of androstenedione (with or without exercise) to increase muscle size and strength and have been uniformly disappointing (7,9,13,15,16). For example, Broeder and colleagues, in the study described earlier, also measured changes in body composition and strength in subjects taking 100 mg androstenedione twice daily in combination with a 12-week intensive weight-training program (7). Despite using sensitive methods that can detect small changes in body composition, they found no differences in muscle mass, fat mass, or strength in the subjects receiving androstenedione compared with those receiving a placebo tablet. Importantly, however, in this study as well as all of these studies referenced earlier, the supplement was given in doses that were not sufficient to increase testosterone levels. It thus remains unknown whether doses of androstenedione sufficient to increase testosterone levels enhance muscle mass or athletic performance.

METABOLISM OF ANDROSTENEDIONE IN MEN

One of the consistent findings of the various androstenedione studies in men is the inefficiency of conversion of the supplements to testosterone. Leder and colleagues explored this issue further by investigating the pattern of androstenedione metabolism in healthy men (17). Specifically, they measured the concentration of inactive testosterone metabolites (also called "conjugates") in the urine of subjects ingesting androstenedione and found an increase of over 10-fold compared with their baseline levels. This finding was in direct contrast to the much more modest changes in serum testosterone they had observed. It suggests that although much of the androstenedione

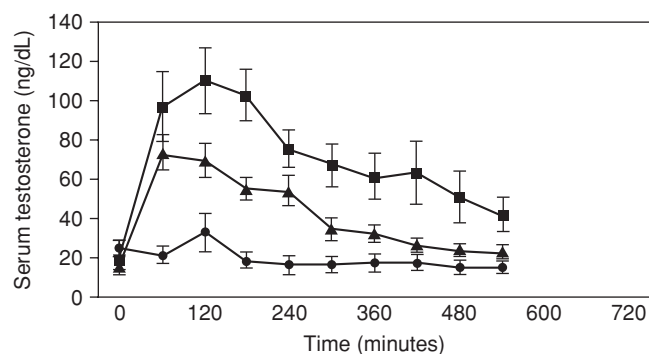


Figure 5 Serum testosterone levels during 12 hours of frequent blood sampling in postmenopausal women. Circles represent control subjects receiving no supplement, triangles those receiving 50 mg of androstenedione, and squares those receiving 100 mg. Source: Adapted from Ref. 18.

that is absorbed after oral administration is converted to testosterone, it is then immediately further metabolized to inactive compounds in the liver. The investigators confirmed this hypothesis by directly measuring the concentration of one of these inactive metabolites (testosterone glucuronide) in the serum of these subjects. As expected, they found that testosterone glucuronide levels increased by 500% to 1000% (as opposed to the 34% increase in biologically active serum testosterone after a single 300-mg dose of oral androstenedione). Together, these findings demonstrate the effectiveness of the liver in inactivating steroid molecules when taken orally.

PHARMACOKINETICS AND HORMONAL EFFECTS OF ANDROSTENEDIONE IN WOMEN

Since the initial report of androstenedione administration in two women in 1962 (6), research into the effects of the supplement has focused largely on the hormonal response to oral administration in young men. Between 2002 and 2003, however, two studies on women were published. The first of these studies examined the effects of a single dose of 0, 50, or 100 mg of androstenedione in postmenopausal women (18). The findings of this study were surprising. In contrast to the effects observed in men, even these low doses increased testosterone levels significantly in women (Fig. 5).

Also, unlike the results seen in men, estradiol levels were unaffected by androstenedione administration. In the other study, 100 mg of androstenedione was administered to young, premenopausal, healthy women. Similar to postmenopausal women, these subjects experienced significant increases in serum testosterone levels after androstenedione administration (estradiol was not measured) (19). Importantly, in both of these studies, the peak testosterone levels achieved by the older and younger women taking androstenedione were often significantly above the normal range. Together, these results predict that the physiological effects of the supplement may be different in men and women, as might their potential toxicities. To date, however, there have been no

published reports investigating the long-term physiological effects in women.

ADVERSE EFFECTS AND TOXICITY

Ever since the publicity surrounding androstenedione exploded in 1999, many reports in the lay press have focused on the potential dangerous side effects. Nonetheless, with the exception of a single case description of a man who developed two episodes of priapism in the setting of androstenedione ingestion (20), there have been no published reports of androstenedione-associated serious adverse events. This fact should be only partially reassuring, however, because androstenedione's prior classification as a dietary supplement (as opposed to a drug) allowed manufacturers to avoid responsibility for rigorously monitoring any potential toxicity of their product.

It is well known that oral administration of certain testosterone derivatives can cause severe liver diseases, and anabolic steroid use in general is associated with anecdotal reports of myocardial infarction, sudden cardiac death, and psychiatric disturbances ("roid rage"). Nonetheless, despite androstenedione's close chemical similarity to these substances, it is important to note that it is not a potent anabolic steroid nor does it have a chemical structure similar to those specific compounds that cause liver problems. Thus, the potential of androstenedione to cause these particular serious side effects appears to be limited. Of more pressing concern to clinicians are the possible long-term effects in specific populations. In clinical trials, the supplement was generally well tolerated, though several studies did report that it reduces high-density lipoprotein (or "good cholesterol") levels in men. Importantly, however, even the longest of these studies lasted only several months. It thus remains quite possible that androstenedione use, especially at high doses, could cause subtle physiological changes over prolonged periods that could directly lead to adverse health consequences. In men, for example, the dramatic increase in estradiol levels observed with androstenedione administration could, over time, lead to gynecomastia (male breast enlargement), infertility, and other signs of feminization. In women, because the supplement increases testosterone levels above the normal range, it could cause hirsutism (excess body hair growth), menstrual irregularities, or male-like changes in the external genitalia. In children, increases in both testosterone and estrogen levels could cause precocious puberty or premature closure of growth plates in bone, thereby compromising final adult height.

PURITY OF COMMERCIALLY AVAILABLE ANDROSTENEDIONE

During its period of over-the-counter availability, androstenedione was available from multiple manufacturers and could be purchased as a tablet, capsule, sublingual tablet, or even a nasal spray. Often, it was combined with other products that claimed to limit its potential side effects (such as chrysin, for example, which is purported to decrease androstenedione's conversion to estrogens). Because the manufacture of dietary supplements was not

Table 1 Analysis of Nine Common Brands of Androstenedione Supplements

Amount of androstenedione listed (mg)	Amount of androstenedione found (mg)
100	93
100	83
100	103
100	90
100	88
100	85
50	35
50	0 (no steroid compounds identified)
250	168 (10 mg of testosterone was also present)

Source: From Ref. 21.

subject to the same regulations as pharmaceuticals, the purity and labeling of androstenedione-containing products were often inaccurate. Catlin and colleagues, for example, reported that urine samples from men treated with androstenedione contained 19-norandrosterone, a substance not associated with androstenedione metabolism but rather with the use of a specific banned anabolic steroid (21). Further investigation revealed that the androstenedione product used contained a tiny amount of the unlabeled steroid "19-norandrostenedione." Though the amount of 19-norandrostenedione was not physiologically significant, it was enough to cause a "positive" urine test for illegal anabolic steroid use when tested in the standard fashion. In fact, it is precisely this type of contamination that may have explained increases in positive tests for 19-norandrosterone among competitive athletes in the past decade. Additionally, it is now common for athletes who test positive for norandrosterone or other androgenic metabolites to point to dietary supplement contamination as the potential explanation.

Catlin and colleagues also analyzed nine common brands of androstenedione and showed that there was considerable variation and mislabeling among products in terms of both purity and content (Table 1).

REGULATORY STATUS AND DETECTION

Androstenedione was available over-the-counter from 1994 (when the DSHEA was passed) until it was reclassified as an anabolic steroid by the Anabolic Steroid Control Act in 2004. It is important to note that this reclassification came without any evidence that androstenedione increased muscle mass or strength, which was the previous legal definition of an anabolic steroid. Virtually all sports organizations, including the National Football League, the National Collegiate Athletic Association, and the International Olympic Committee, have banned androstenedione. Despite these prohibitions, detection of androstenedione has not been standardized. Specifically, the method used most often to detect testosterone use, measurement of the urinary testosterone-to-epitestosterone ratio, has not proven to be reliable in establishing androstenedione use (22). Further study is still needed to define novel testing procedures that are able to detect androstenedione use reliably.

CONCLUSIONS

Androstenedione is a steroid hormone, which, until 2004, was a popular over-the-counter dietary supplement. Since then, however, it has been classified as an anabolic steroid, and hence a controlled substance. It is purported to increase strength, athletic performance, libido, sexual performance, energy, and general quality of life. Studies indicate that when taken orally by men, small doses are converted to potent estrogens and larger doses to both testosterone and estrogens. Comparatively, there appears to be a much more physiologically important increase in estrogens compared with testosterone in men. In women, the effects are reversed. Studies have thus far failed to confirm any effect on muscle size or strength, though the dosing regimens were modest. Although documentation of adverse side effects among users of androstenedione is scarce, there is considerable concern over potential long-term toxicity, especially in women and adolescents.

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L-Arginine

Mauro Maccario, Guglielmo Beccuti, Valentina Gasco, Mariangela Seardo, Gianluca Aimaretti, Emanuela Arvat, Fabio Lanfranco, and Ezio Ghigo

INTRODUCTION

Arginine was first isolated in 1895 from animal horn. It is considered a nonessential amino acid under physiological conditions; however, it may be classified as semi-essential (or conditioned) in newborns, young children, or other circumstances characterized by accelerated tissue growth (e.g., infection, sepsis, trauma) when its production may be too slow and not sufficient to meet the requirements (1). Arginine is physiologically active in the L-form (L-Arg) and participates in protein synthesis in cells and tissues. It is essential for the synthesis of urea, creatine, creatinine, and pyrimidine bases. It also strongly influences hormonal release and has an important role in vasculature dynamics, participating in the synthesis of nitric oxide (NO).

BIOCHEMISTRY

Dietary arginine is particularly abundant in wheat germ and flour, buckwheat, oatmeal, dairy products (cottage cheese, ricotta cheese, nonfat dry milk, skimmed yogurt), chocolate, beef (roasts, steaks), pork, nuts (coconut, pecans, walnuts, almonds, hazel nuts, peanuts), seeds (pumpkin, sesame, sunflower), poultry (chicken, turkey), wild game (pheasant, quail), seafood (halibut, lobster, salmon, shrimp, snails, tuna), chick peas, and soybeans (2).

L-Arg, delivered via the gastrointestinal tract, is absorbed in the jejunum and ileum of the small intestine. A specific amino acid transport system facilitates this process and participates also in the transport of the other basic amino acids, L-lysine and L-histidine. About 60% of the absorbed L-Arg is metabolized by the gastrointestinal enterocytes, and only 40% remains intact reaching the systemic circulation.

An insufficient arginine intake produces symptoms of muscle weakness, similar to muscular dystrophy (3). Arginine deficiency impairs insulin secretion, glucose production, and liver lipid metabolism (4). Conditional deficiencies of arginine or ornithine are associated with the presence of excessive ammonia in the blood, excessive lysine, rapid growth, pregnancy, trauma, or protein deficiency and malnutrition. Arginine deficiency is also associated with rash, hair loss and hair breakage, poor wound healing, constipation, fatty liver, hepatic cirrhosis, and hepatic coma (4).

Depending on nutritional status and developmental stage, normal plasma arginine concentrations in humans and animals range from 95 to 250 $\mu\text{mol/L}$. Toxicity and

symptoms of high intake are rare, but symptoms of massive dosages may include thickening and coarsening of the skin, muscle weakness, diarrhea, and nausea.

The proximal renal tubule accounts for much of the endogenous production of L-Arg from L-citrulline. In the tubule, arginine reacts via the Krebs cycle with the toxic ammonia formed from nitrogen metabolism, producing the nontoxic and readily excretable urea (Fig. 1) (5). If this mechanism does not efficiently handle metabolic byproducts and if L-Arg intake is insufficient, ammonia rapidly accumulates, resulting in hyperammonemia.

L-Arg undergoes different metabolic fates. NO, L-citrulline, L-ornithine, L-proline, L-glutamate, and polyamine-like putrescine are formed from L-Arg. Moreover, the high-energy compound NO-creatinine phosphate, which is essential for sustained skeletal muscle contraction, is also formed from L-Arg (Fig. 2).

L-Arg, its precursors, and its metabolites are deeply involved in the interaction of different metabolic pathways and interorgan signaling. The amino acid influences the internal environment in different ways: disposal of protein metabolic waste; muscle metabolism; vascular regulation; immune system function; healing and repair of tissue; formation of collagen; and building of new bone and tendons.

A leading role for arginine has been shown in the endocrine system, vasculature, and immune response.

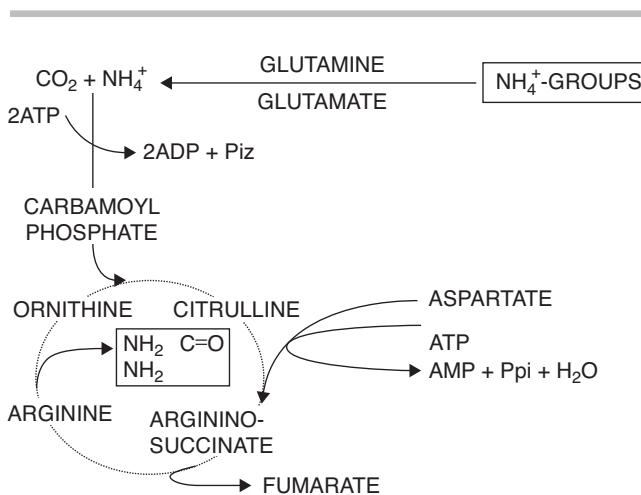


Figure 1 L-Arginine and Krebs cycle in the renal tubule.

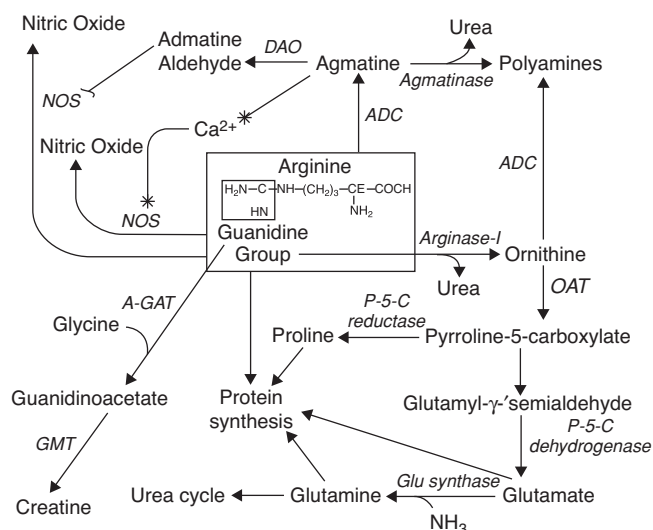


Figure 2 L-Arginine metabolites. *Abbreviations:* ADC, arginine decarboxylase; A:GAT, arginine:glycine amidinotransferase; DAO, diamine oxidase; Glu synthase, glutamine synthase; GMT, guanidinoacetate-*N*-methyltransferase; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; P-5-C dehydrogenase, pyrroline-5-carboxylate dehydrogenase; P-5-C reductase, pyrroline-5-carboxylate reductase.

PHYSIOLOGY

Endocrine Actions

L-Arg functions as a secretagogue of a number of important hormones at the pituitary, pancreas, and adrenal levels. The effects on growth hormone (GH), prolactin (PRL), adrenocorticotrophic hormone (ACTH), and insulin secretion will be discussed in detail.

GH Secretion

Among the various factors modulating somatotropin function, arginine is well known to play a primary stimulatory influence. Arginine has been shown to increase basal GH levels and to enhance the GH responsiveness to growth hormone releasing hormone (GHRH) both in animals and in humans throughout their life span (6-9); its GH-stimulating activity occurs after both IV and oral administration and is dose dependent; 0.1 and 0.5 g/kg are the minimal and the maximal IV effective doses, respectively. Moreover, a low orally administered arginine dose has been shown to be as effective as a high IV dose in enhancing the GH response to GHRH both in children and in elderly subjects (10,11).

Arginine, directly or indirectly via NO, is likely to act by inhibiting hypothalamic somatostatin (SS) release. It has been shown that arginine—but not isosorbide-dinitrate and molsidomine, two NO donors—stimulates GH secretion (12,13), suggesting that it does not exert its effects through the generation of NO. Moreover, arginine does not modify either basal or GHRH-induced GH increase from rat anterior pituitary (14). On the contrary, it potentiates the GH response to the maximal GHRH dose in humans. Arginine can elicit a response even when the response has been previously inhibited by a GHRH ad-

ministration, which induces an SS-mediated negative GH autofeedback (7,8,15). Moreover, arginine counteracts the GH-inhibiting effect of neuroactive substances that act by stimulating SS release; it does not modify the GH-releasing activity of stimuli acting via SS reduction (8). Again, in favor of an SS-mediated mechanism is also the evidence that ornithine, the active form of arginine, is unable to modify plasma GHRH levels in humans (16). Moreover, arginine fails to potentiate the increased spontaneous nocturnal GH secretion, which is assumed to reflect circadian SS hyposecretion and GHRH hypersecretion, respectively (8). Arginine does not influence the strong GH-releasing action of ghrelin, the natural ligand of GH secretagogue receptors, which is supposed to act as a functional antagonist of SS at both the pituitary and the hypothalamic levels (17,18).

The GH-releasing activity of arginine is sex dependent but not age dependent, being higher in females than in males but similar in children, young, and elderly subjects (8,19–23). Moreover, it has been clearly demonstrated that arginine totally restores the low somatotrope responsiveness to GHRH in aging, when a somatostatinergic hyperactivity is likely to occur (20–23). This evidence clearly indicates that the maximal secretory capacity of somatotrophic cells does not vary with age and that the age-related decrease in GH secretion is due to a hypothalamic impairment (20–23). This also points out the possible clinical usefulness of this substance to rejuvenate the GH/insulin-like growth factor-I (IGF-I) axis in aging. In fact, the reduced function of the GH/IGF-I axis in aging may account for the changes in body composition, structure, and function. In agreement with this assumption, it has been reported by some, but not all, authors that elderly subjects could benefit from treatment with rhGH to restore IGF-I levels within the young range (21,24). As it has been demonstrated that the GH releasable pool in the aged pituitary is basically preserved and that the age-related decline in GH secretion mostly reflects hypothalamic dysfunction (21,23), the most appropriate, that is, “physiological,” approach to restore somatotroph function in aging would be a treatment with neuroactive substances endowed with GH-releasing action. Among these GH secretagogues, arginine received considerable attention. In fact, the coadministration of arginine (even at low oral doses) with GHRH (up to 15 days) enhanced the GH responsiveness to the neurohormone in normal aged subjects (11). However, the efficacy of long-term treatment with oral arginine to restore the function of the GH/IGF-I axis in aging has never been shown in elderly subjects.

Following the evidence that GHRH combined with arginine becomes the most potent and reproducible stimulus to diagnose GH deficiency throughout the life span (25), GHRH + arginine is, at present, one of the two gold standard tests for the diagnosis of GH deficiency (25,26). In fact, the GH response to a GHRH + arginine test is approximately threefold higher than the response to classical tests and does not vary significantly with age (25,26). Because of its good tolerability and its preserved effect in aging, the GHRH + arginine test is currently considered to be the best alternative choice to the insulin-induced tolerance test (ITT) for the diagnosis of GH deficiency throughout the life span (25).

PRL Secretion

Among the endocrine actions of arginine, its PRL-releasing effect has been shown both in animals and in humans after IV but not after oral administration (10,27). The PRL response to arginine is markedly lower than the response to the classical PRL secretagogues, such as dopaminergic antagonists or thyrotropin-releasing hormone (TRH) (6) but higher than that observed after secretion of GH and other modulators of lactotrope function (17).

The mechanisms underlying the stimulatory effect of arginine on PRL secretion are largely unknown, but there is evidence that this effect is not mediated by galanin, a neuropeptide with PRL-releasing effect. In fact, galanin has been shown to potentiate PRL response to arginine, suggesting different mechanisms of action for the two substances (28).

ACTH Secretion

Although some excitatory amino acids and their agonists have been demonstrated to differently modulate corticotropin-releasing hormone and arginine vasopressin release in vitro and influence both sympathoadrenal and hypothalamo-pituitary-adrenal (HPA) responses to hypoglycemia in animals (29,30), little is known about arginine influences on HPA axis in humans. Many studies have shown that mainly food ingestion influences spontaneous and stimulated ACTH/cortisol secretion in normal subjects and that central α_1 -adrenergic-mediated mechanisms are probably involved (31). In humans free fatty acids inhibit spontaneous ACTH and cortisol secretion, but no data exist regarding the effect of each nutrient component on HPA function. Previous studies demonstrated that arginine is unable to exert an ACTH-stimulatory effect in humans via generation of NO (12) and our unpublished preliminary data failed to demonstrate a significant effect of arginine (30 g IV) on either ACTH or cortisol secretion in normal subjects.

Insulin Secretion

Arginine is one of the most effective known insulin secretagogue and it may be used with glucose potentiation to determine a patient's capacity to secrete insulin (32). Arginine acts synergistically with glucose, and to a much lesser extent with serum fatty acids, in stimulating insulin release. A synergistic effect of arginine and glucose on insulin secretion has been shown in humans (33,34), and the combined administration of these two stimuli has been studied in an attempt to test β -cell secretory capacity in diabetic patients (35).

A protein meal leads to a rapid increase in both plasma insulin and glucagon levels (36). Administration of arginine has a similar effect. An arginine transport system is present in the β -cell plasma membrane (37). When arginine enters the β cell, it causes ionic changes that depolarize the β cell and trigger Ca^{2+} uptake and exocytosis of insulin-containing granules.

Several mechanisms for arginine-induced β -cell stimulation have been proposed. These include the metabolism of L-Arg leading to the formation of ATP (38,39), the generation of NO (40,41), and the direct depolarization of the plasma membrane potential due to the accumulation of the cationic amino acid (42–44).

A sustained Ca^{2+} influx is directly related to insulin secretion following arginine uptake by β cells. The arginine-induced increase in Ca^{2+} concentration is inhibited by the activation of ATP-sensitive potassium (K-ATP) channels with diazoxide and seems dependent on the nutritional status. These observations suggest that the K-ATP channels, when fully open, act to prevent membrane depolarization caused by arginine. The presence of a nutrient, such as glucose, produces sufficient closure of K-ATP channels to allow arginine-induced membrane depolarization and activation of the voltage-activated Ca^{2+} channels (37).

Nonendocrine Actions

Cardiovascular System

Increasing interest has been recently focused on NO. This mediator, which is synthesized from L-Arg (45) by nitric oxide synthases (NOS) (46), is a potent vasodilator (47) and inhibitor of platelet adhesion and aggregation (48). Three isoforms of NOS are described: neuronal NOS (nNOS—NOS-1), inducible NOS (iNOS—NOS-2), and endothelial NOS (eNOS—NOS-3). NOS-1 and NOS-3 are expressed constitutively and they produce NO at low rates (49). NOS-3 is responsible for a consistent vasodilator tone and, although constitutive, can be regulated by endothelial shear stress (50) and substances such as acetylcholine, histamine, serotonin, thrombin, bradykinin, and catecholamines. Calcium is required for NOS-3 activation (51). NO production is mainly dependent on the availability of arginine and NOS is responsible for the biochemical conversion of L-Arg to NO and citrulline in the presence of cofactors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH_4), flavin mononucleotide, and flavin adenine nucleotide. Reduced NO production, leading to vasoconstriction and increases in adhesion molecule expression, platelet adhesion and aggregation, and smooth muscle cell proliferation has been demonstrated in atherosclerosis, diabetes mellitus, and hypertension (52–54)—conditions known to be associated with an increased mortality because of cardiovascular disease. Taken together, these observations lead to the concept that interventions designed to increase NO production by supplemental L-Arg might have a therapeutic value in the treatment and prevention of the endothelial alterations of these diseases. Besides several actions exerted mainly through NO production, arginine also has a number of NO-independent properties, such as the ability to regulate blood and cellular pH, and the effect on the depolarization of endothelial cell membranes.

The daily consumption of arginine is normally about 5 g/day. Arginine supplementation is able to increase NO production, although the K_m for L-Arg is 2.9 μmol and the intracellular concentration of arginine is 0.8 to 2.0 mmol. To explain this biochemical discrepancy, named "arginine paradox," there are theories that include low arginine levels in some diseases (e.g., hypertension, diabetes mellitus, and hypercholesterolemia), and/or the presence of enzymatic inhibitors (55), and/or the activity of the enzyme arginase (which converts arginine to ornithine and urea, leading to low levels of arginine).

Recently attention has been given to the methylated forms of L-Arg, generated by the proteolysis of

methylated proteins; they are represented by asymmetric dimethylarginine (ADMA) and two symmetric dimethylated derivatives: symmetric dimethylarginine (SDMA) and monomethylarginine (MMA) (56). Only ADMA and MMA, but not SDMA, exert inhibitory effects on NOS-3 activity (57). For this reason, ADMA is now recognized as a new emerging cardiovascular risk marker and likely as a causative factor for cardiovascular disease (58).

L-Arginine therapy in cardiovascular pathologies showed contradictory results. However, it is now clear that individual response to L-Arg may be influenced by SDMA. In fact, no effects of L-arg therapy are demonstrated in patients with low ADMA levels, whereas in patients with high ADMA level, L-Arg normalizes the L-Arg to ADMA ratio, thus normalizing the endothelial function (59).

Several studies demonstrated that L-Arg infusion in normal subjects and patients with coronary heart disease (60), hypercholesterolemia (61), and hypertension (62) is able to improve the endothelial function, but the results, although encouraging, are not conclusive because of the short-term effects of IV arginine. However, arginine does not affect endothelial function in patients with diabetes mellitus. On the other hand, oral L-Arg has a longer half-life and longer-term effects than L-Arg given intra-arterially or intravenously (63). Thus, in the setting of long-term health maintenance or symptom management, the oral route would be preferred. Studies in animals documented that oral L-Arg supplementation is able to reduce the progression of atherosclerosis, preserving endothelium function (64) and inhibiting circulating inflammatory cells (65) and platelets (66) in animals with hypercholesterolemia, and to decrease blood pressure and wall thickness in animals with experimental hypertension (67). On the other hand, studies in humans *in vivo* are not so widely positive as the animal experimental data. Actually, although the majority of the data is in normal subjects, individuals with a history of cigarette smoking and patients with hypercholesterolemia and claudication demonstrate beneficial effects of oral L-Arg administration on platelet adhesion and aggregation, monocyte adhesion, and endothelium-dependent vasodilation (68,69). Other studies do not show any benefit (70,71); therefore, no definitive conclusions can be drawn. Taken together, the studies show a major effect when L-Arg supplementation was given in subjects with hypercholesterolemia, probably because of an increase in NO production via reduction of the ADMA intracellular concentration, which is increased in the presence of LDL hypercholesterolemia.

In conclusion, despite several beneficial effects on intermediate end points, particularly in hypercholesterolemic patients, there is no evidence for a clinical benefit in the treatment or prevention of cardiovascular disease. More data, derived from large-scale prospective studies evaluating the effect of long-term treatment with L-Arg, are needed. Future perspectives of pharmacological intervention are represented by the regulation of the enzyme dimethylarginine dimethylaminohydrolase responsible for the ADMA metabolism (57), the arginase (72), and the endothelial cell L-Arg transporter (73).

Immune System

Many studies, in animals as well as in humans, have shown that arginine is involved in immune modulation. In

fact, this amino acid is a component of most proteins and the substrate for several nonprotein, nitrogen-containing compounds acting as immune modulators.

There is clear evidence that arginine participates in the cell-mediated immune responses of macrophages and T lymphocytes in humans through the production of NO by inducible nitric oxide synthase (iNOS—NOS-2), which occurs mostly in the macrophage (74,75), and through the modulation of T-lymphocyte function and proliferation (76,77). At intracellular levels, arginine is metabolized by two different enzymatic pathways: the arginase pathway, by which the guanidino nitrogen is converted into urea to produce ornithine, and the NOS pathway, which results in oxidation of the guanidino nitrogen to produce NO and other substances (78,79).

It has been shown that macrophage superoxide production, phagocytosis, protein synthesis, and tumoricidal activity are inhibited by high levels of arginine *in vitro* and that sites of inflammation with prominent macrophage infiltration, such as wounds and certain tumors, are deficient in free arginine (80). In particular, a decrease in arginine availability due to the activity of macrophage-derived arginase rather than the arginine/NO pathway may contribute to the activation of macrophages migrating at inflammatory sites (80). Arginine metabolism in the macrophages is activity dependent. At rest, macrophages exhibit minimal utilization of arginine and lower NOS-2 expression or arginase activity, whereas in activated cells, arginine is transported into the cell, and NOS-2 expression and arginase are induced by cytokines and other stimuli (81). The types of stimuli that induce NOS-2 and arginase are quite different. *In vitro* and *in vivo* studies demonstrated that NOS-2 is induced by T-helper I cytokines (IL-1, TNF, and γ -interferon) produced during activation of the cellular immune response, such as severe infections or sepsis (74,75), whereas arginases are induced by T-helper II cytokines (IL-4, IL-10, and IL-13) and other immune regulators aimed at inducing the humoral immune response (82,83). Thus, in disease processes, where inflammatory response predominates, NOS-2 expression and NO production prevail. Under biological circumstances where T-helper II cytokine expression is prevalent, arginase activity and the production of ornithine and related metabolites would predominate.

In vitro studies in animals demonstrated depressed lymphocyte proliferation in cultures containing low levels of arginine and maximal proliferation when arginine is added at physiological plasma concentration (77,84), but the molecular details have not been completely defined.

It has also been shown that supplemental arginine increased thymic weight in rodents because of increased numbers of total thymic T lymphocytes. On the other hand, in athymic mice, supplemental arginine increased the number of T cells and augmented delayed-type hypersensitivity responses, indicating that it can exert its effects on peripheral lymphocytes and not just on those within the thymus (76).

The immunostimulatory effects of arginine in animal studies have suggested that this amino acid could be an effective therapy for many pathophysiological conditions in humans, able to positively influence the immune response under some circumstances by restoring cytokine balance and reducing the incidence of infection.

In healthy humans, oral arginine supplementation shows many effects on the immune system, including increase in peripheral blood lymphocyte mitogenesis, increase in the T-helper-T-cytotoxic cell ratio and, in macrophages, activity against microorganisms and tumor cells (85). Furthermore, the delayed-type hypersensitivity response as well as the number of circulating natural killer (NK) and lymphokine-activated killer cells are increased (85–87). Therefore, it has been hypothesized that arginine could be of benefit to patients undergoing major surgery after trauma and sepsis and in cardiovascular diseases, HIV infection, and cancer (88). In fact, short-term arginine supplementation has been shown to maintain the immune function during chemotherapy; arginine supplementation (30 g/day for 3 days) reduced chemotherapy-induced suppression of NK cell activity, lymphokine-activated killer cell cytotoxicity, and lymphocyte mitogenic reactivity in patients with locally advanced breast cancer (89). It must be noted that chronic administration of arginine has also been shown to promote cancer growth by stimulating polyamine synthesis in both animal and human studies (89). On the other hand, NO has been shown to inhibit tumor growth. Thus, the real effect on cancer processes depends on the relative activities of NOS and arginase pathways that show variable expression, depending on the stage of carcinogenesis (91).

These data clearly indicate the involvement of arginine in immune responses in both animals and humans. Large clinical trials are needed to clarify the clinical application and efficacy of this amino acid in immunity and immunopathology.

SUPPLEMENTAL ARGININE

The available form of supplemental L-Arg is represented by the free base, the Cl⁻ salt (L-Arg hydrochloride—L-Arg-HCl) and the aspartate salt of the amino acid (92).

L-Arg is stable under sterilization condition and its administration is safe for mammals in an appropriate dose and chemical form (91).

Oral L-Arg (up to 9 g of Arg-HCl per day for adults) has no adverse effects on humans but higher doses can lead to gastrointestinal toxicity, theoretically increasing local production of NO and impairing intestinal absorption of other basic amino acids (91). Moreover, the local NO production may be particularly dangerous if intestinal diseases are present (92).

Oral L-Arg supplement is commonly used to increase GH release and consequentially physical performance; moreover, it has been hypothesized that L-Arg supplement could lead to improved muscular aerobic metabolism and less lactate accumulation, enhancing NO-mediated muscle perfusion.

However, in a clinical trial, arginine supplement in endurance-trained athletes did not show any difference from placebo in endurance performance (maximal oxygen consumption, time to exhaustion), endocrine (GH, glucagon, cortisol, and testosterone concentrations), and metabolic parameters (93).

In another study, the association “arginine plus exercise” produced a GH response approximately 50% lower than that observed with exercise alone, suggesting that

the acute use of oral L-Arg prior to exercise blunts the GH response to subsequent exercise (94).

No effects on NO production, lactate and ammonia metabolism, and physical performance in intermittent anaerobic exercise were shown in well-trained male athletes after short-term arginine supplementation (95). It has been hypothesized that NO production is not modified by arginine supplementation in athletes because they may have higher basal concentrations of NO than general population; in fact, basal NO production can be increased by regular exercise training, without any pharmacological intervention (95).

There are many interesting clinical perspectives on arginine supplementation therapy, especially in critical care setting (96), treatment and prevention of pressure ulcers (97), hypertension (59), and asthma and chronic obstructive pulmonary disease (98), but further studies are required to clarify which categories of patients may benefit from this treatment (99).

CONCLUSIONS

From an endocrinological point of view, the simple classification of arginine as an amino acid involved in peripheral metabolism is no longer acceptable. In fact, besides other nonendocrine actions, it has been clearly demonstrated that arginine plays a major role in the neural control of anterior pituitary function, particularly in the regulation of somatotrophin secretion. One of the most important concepts regarding arginine is the existence of an arginine pathway at the CNS level, where this amino acid represents the precursor of NO, a gaseous neurotransmitter of major importance. On the other hand, NO does not necessarily mediate all the neuroendocrine or the peripheral arginine actions.

In the past years, new discoveries have led to a rapid increase in our knowledge of the arginine/NO system, from a neuroendocrine and nonendocrine point of view. Up to now, there is no evidence for the utility of L-Arg supplement for muscle strength or exercise performance in humans. However, several other aspects still remain to be clarified; the potential clinical implications for arginine have also never been appropriately addressed and could provide unexpected results both in the endocrine and in the cardiovascular fields.

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Astragalus

Roy Upton

INTRODUCTION

Astragalus root (*Astragalus membranaceus* and *Astragalus mongholicus*) (Figs. 1 and 2; flowers are shown in Fig. 2) is one of the most important plant products used in traditional Chinese medicine (TCM) for supporting immune resistance (衛氣; wei qi) and energy production (補氣; bu qi). Astragalus is also one of the most popular ingredients in botanical dietary supplements for its putative effect of supporting healthy immune function. Despite the widespread use of this botanical among TCM practitioners and its extensive use in botanical supplements, there are few clinical trials supporting its use, though those that are available are positive. Numerous preclinical studies provide evidence for a number of pharmacological effects that are consistent with the traditional and modern use of astragalus.

BACKGROUND

Traditional and Modern Uses

In Asia, astragalus is commonly used according to both its traditional Chinese medical indications as a general tonifier and specifically for immune enhancement and for modern biomedical indications such as immune, liver, and cardiovascular support. It has been used for the prevention of the common cold and upper respiratory tract infections and is widely prescribed to children for prevention of infectious disease, though formal clinical English language studies regarding this use are lacking. In the West, astragalus is primarily used as an immune modulator. Astragalus potentiates recombinant interleukin-2 (rIL-2) and recombinant interferon-1 and -2 (rIFN-1 and -2) immunotherapy and by lowering the therapeutic thresholds, may reduce the side effects normally associated with these therapies. The data and opinion of those expert with the use of the botanical suggest that astragalus is useful as a complementary treatment during chemotherapy and radiation therapy and in immune deficiency syndromes. There is some modern evidence for its use in hepatitis and the treatment of cardiovascular disease.

In TCM and Western clinical herbal medicine, astragalus is most commonly used in combination with other botanicals and is very seldom used as a single agent. There are numerous studies of some of the classic combinations of astragalus (e.g., astragalus and *Angelica sinensis*). These have not been reviewed, but use of formulas is more consistent with the use of the astragalus than with the use of the herb alone according to traditional Chinese medical principles.

CHEMISTRY AND PREPARATION OF PRODUCTS

The primary compounds of interest in astragalus are triterpenes, polysaccharides, and flavonoids. The triterpene astragaloside IV is a relatively unique marker for astragalus species used in Chinese medicine. A variety of preparations are utilized in clinical practice and in herbal supplements. A number of preparations, including crude extracts, isolated polysaccharides, and triterpene saponins, have been subject to study and correlated with activity. Clinically, in China and among some practitioners in the United States, decoctions are frequently given. However, due to the time required for cooking and the subsequent smell and taste of Chinese herb preparations in general (though astragalus is very agreeable), many consumers and practitioners prefer crude powder or extract preparations (capsules, tablets), freeze-dried granules, or liquid extracts. Astragalus is also used as a relatively common ingredient in soups, especially during winter months.

Polysaccharides (12–36 kD) have been most often correlated with immune activity, while triterpene saponins have been predominantly associated with cardiovascular and hepatoprotective effects. Astragalus polysaccharides are generally composed of a mixture of D-glucose, D-galactose, and L-arabinose or D-glucose alone. The glucose units appear to be primarily α -(1,4)-linked with periodic α -(1,6)-linked branches (1,2). The triterpene glycosides vary by position, number, and type of sugar residues at positions 3, 6, and 25. Several of these “astragalosides” (e.g., astragaloside IV; Fig. 3) are composed of a single xylopyranosyl substituent at the 3-position, which may or may not be acetylated. Others possess either disaccharide or trisaccharide substituents (3–5). Primary flavonoids of astragalus for which activity has been reported include calycosin, formononetin (Fig. 3), and daidzein (Fig. 3) and additionally include isorhamnetin, kaempferol, and quercetin, among others (6).

PRECLINICAL STUDIES

Pharmacokinetics

Pharmacokinetic data available in English language publications on astragalus, its crude extracts, or its constituents are very limited. In the most detailed study to date, the pharmacokinetics of a decoction of astragalus, the preparation most used traditionally were investigated in four models: four complement in silico, a cacao-2 intestinal cell model, an animal, and a human volunteer ($n = 1$). Intestinal absorption was demonstrated for several flavonoids including calycosin and formononetin, along with their aglycone metabolites in all four

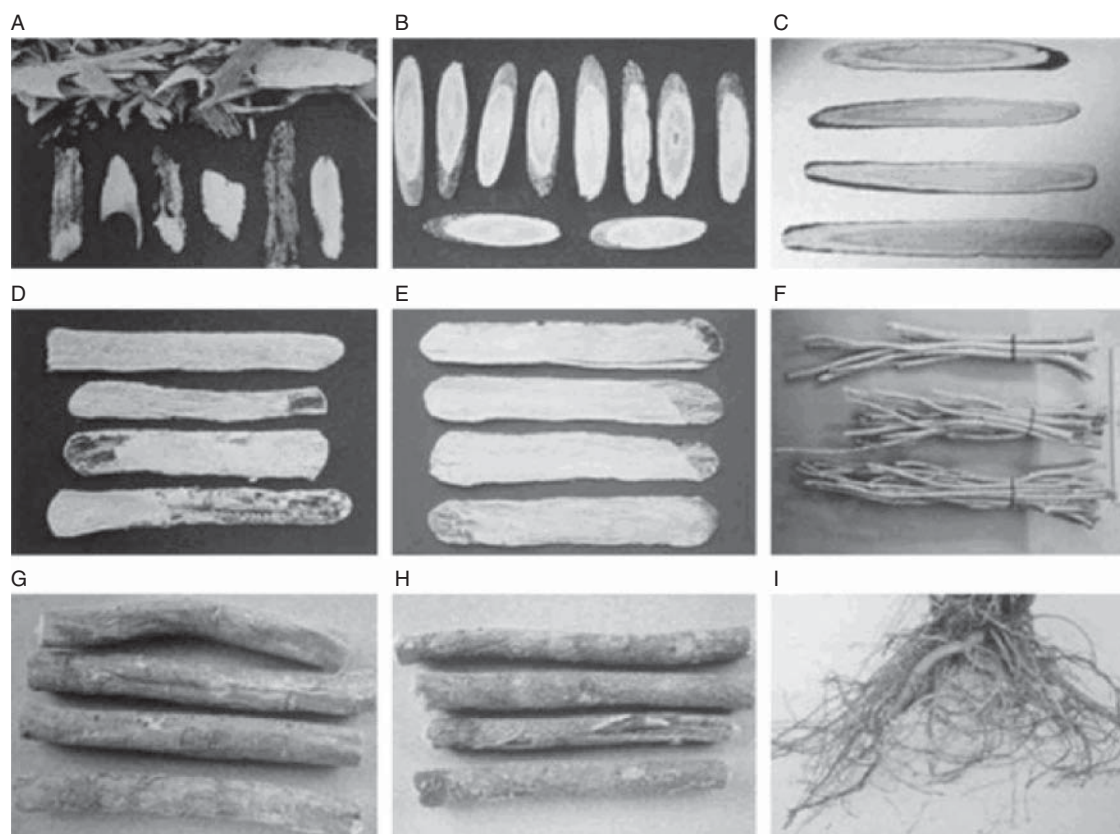


Figure 1 Different forms and quality of astragalus on the American market. Source: Photo courtesy of Roy Upton, Soquel, CA.

models. Triterpene saponins, used as chemical markers of astragalus (e.g., astragaloside I and IV) in the *Pharmacopoeia of the People's Republic of China* and the *American Herbal Pharmacopoeia*, were lacking, likely due to their low concentrations in the preparation. In the human volunteer, nine flavonoids, including calycosin, formononetin, and the isoflavone daidzein, were detected

in urine (7). In animal models (rats and dogs), astragaloside IV, which has demonstrated cardioprotective activity, showed moderate-to-fast elimination. The half-life in male rats was from 67.2 to 98.1 minutes, in female rats 34.0 to 131.6 minutes, and was linear at the intravenous doses given. The highest concentration of astragaloside IV was found in the lungs and liver. Only 50% of the compound was detected in urine and feces. Binding to plasma protein was also linear at the concentration of 250–1000 ng/mL. Slow systemic clearance of astragaloside IV occurred via the liver at approximately 0.004 L/kg/min (8).

In another pharmacokinetic study, a two-compartment, first-order pharmacokinetic model was used to describe the pharmacokinetics of intravenous-administered astragaloside IV. Systemic clearance of this triterpene was reported as moderate and distribution into peripheral tissues was limited (9).

Pharmacodynamics

A large percentage of research on astragalus has focused on its immunostimulatory activity and its purported ability to restore the activity of a suppressed immune system. More recently, interest in its potential as a cardioprotective agent has been shown. Reviews of a limited number of clinical trials and preclinical data provide some evidence for its usefulness in the prevention of the common cold and as an adjunct to cancer therapies. There is limited evidence to suggest a benefit to the cardiovascular



Figure 2 Astragalus flowers. Source: Photo courtesy of Bill Brevoort, American Herbal Pharmacopoeia.

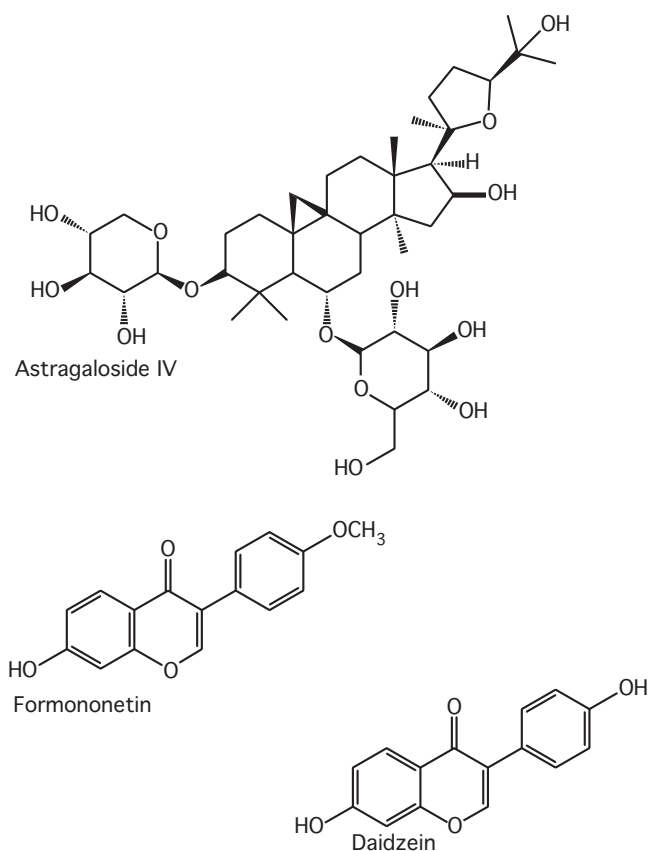


Figure 3 Some major constituents of Astragalus.

system, with improvement in clinical parameters associated with angina, congestive heart failure, and acute myocardial infarct. There is also some indication from animal studies supporting its use in the treatment of hepatitis and diabetes.

Immunomodulatory Effects

There are relatively strong preclinical data of pharmacological mechanisms that provide support for the putative immunomodulatory effects of astragalus.

Cancer

In a rat study, animals were pretreated orally for 50 days with a low or high dose of astragalus extract (3.3 or 10 g/kg/day) prior to IP injection of doxorubicin (cumulative dose of 12 mg/kg over a 2-week period). After 5 weeks of the final injection of doxorubicin, a significant inhibition of cardiac diastolic function was observed. This was accompanied by ascites, catexia, decreased heart weight, and increased mortality. Treatment with astragalus at both doses significantly attenuated the negative effects of doxorubicin on cardiac functions and ascites, while the high dose also improved survival. This protective effective was at least partially associated with the ability of astragalus to attenuate changes in cardiac SERCA2a mRNA expression (10).

A broad array of immunomodulatory effects has been demonstrated in numerous preclinical studies that

suggest a substantial value of astragalus in conjunction with conventional cancer therapies. The most relevant of these was a series of investigations conducted by researchers at the MD Anderson Cancer Center that found that astragalus extract restored to normal the immune response of patients' mononuclear cells that were grafted into rats immunocompromised by cyclophosphamide. These researchers concluded that astragalus and its polysaccharide fraction reversed the immunosuppressive effect of cyclophosphamide (11–15). In other studies, astragalus and its various fractions were shown to stimulate macrophage phagocytosis (16) and hematopoiesis (17).

One study reported on the gastroprotective effects of astragalus extract (characterization not reported) in human peritoneal mesothelial cells (HPMCs) subjected to gastric cancer cell lines. Upon incubation with cancer cell lines, apoptosis of the HPMC cells was observed. The astragalus preparation, via regulation of Bcl-2 and Bax, partially inhibited apoptosis. The authors interpreted these findings as a potential that astragalus may slow down the metastasis of the primary cancer and is therefore a potential treatment for gastric cancer (18).

The ability of an astragalus fraction to potentiate the effects of rIL-2 has been demonstrated in *in vitro* assays. Lymphokine-activated killer (LAK) cells were treated with a combination of the astragalus fraction and 100 units/mL of IL-2. The combination therapy produced the same amount of tumor-cell-killing activity as that generated by 1000 units/mL of rIL-2 on its own, thus suggesting that the astragalus fraction elicited a 10-fold potentiation of rIL-2 in this *in vitro* model (19). These findings were confirmed in a follow-up study by the MD Anderson researchers using LAK cells from cancer and AIDS patients. In this study, the cytotoxicity of a lower dose of 50 μ g/mL of rIL-2 given with the astragalus fraction was comparable to that of a higher dose of 500 μ g/mL of rIL-2 alone against the Hs294t melanoma cell line of LAK cells. With the combination, the effector-target cell ratio could be reduced to one-half to obtain a level of cytotoxicity that was equivalent to the use of rIL-2 alone. In addition, the astragalus fraction was shown to increase the responsiveness of peripheral blood lymphocytes that were not affected by rIL-2. In this study, and in another by the same researchers, it was concluded that the fraction potentiated the activity of LAK cells and allowed for the reduction in rIL-2, thus minimizing the toxicity of rIL-2 therapy (20). Other groups of researchers reported almost identical findings (a 10-fold potentiation) and concluded that astragalus is effective in potentiating IL-2-generated LAK cell cytotoxicity *in vitro* (21,22). Astragalus was also found to enhance the secretion of tumor necrosis factor (TNF) from human peripheral blood mononuclear cells (PBMCs). A polysaccharide fraction (molecular weight 20,000–25,000) increased secretion of TNF- α and TNF- β after isolation of adherent and nonadherent mononuclear cells from PBMCs (23).

Other investigations support the role of astragalus polysaccharides as immunomodulating agents. In an *in vitro* study, astragalus polysaccharides significantly induced the proliferation of BALB/c mouse splenocytes resulting in subsequent induction of interleukin 1 β and tumor necrosis factor- α and the activation of murine macrophages. The researchers concluded

that astragalus had an intermediate-to-high affinity for membrane immunoglobulin (Ig) of β lymphocytes (24).

Cardiovascular Effects

In animal studies, astragalus or its compounds were reported to elicit antioxidant (25), mild hypotensive (26), and both positive (27) (50–200 $\mu\text{g/mL}$) and negative (30 $\mu\text{g/mL}$) inotropic activity (28). The inotropic activity was reported to be due to the modulation of Na^+/K^+ ATPase in a manner similar to strophanthin K. Antioxidant (29), calcium channel blocking (30), and fibrinolytic activity (31) have been reported in in vitro studies. Astragaloside IV was studied for potential cardioactivity. Various effects have been reported. Intravenous administration of astragaloside IV reduced the area of myocardial infarct and reduced plasma creatine phosphokinase release in dogs subjected to 3-hour ligation and increased coronary blood flow in anesthetized dogs. In isolated rat heart perfusion investigations, astragaloside IV significantly improved ($P < 0.01$) postischemic heart function and reduced creatine phosphokinase release from the myocardium. In addition, coronary blood flow during baseline perfusion and reperfusion in ischemic rat hearts was increased, while reperfusion damage was decreased. This activity was shown to be at least partially attributable to coronary dilation via an increase in endothelium-derived nitric oxide. Antioxidant activity via an increase in superoxide dismutase (SOD) activity has also been reported for astragalus and is considered to contribute to its cardioprotective effects (32). Astragaloside IV was also shown to significantly attenuate blood–brain barrier permeability in a rat ischemia/reperfusion model (33).

Hepatoprotective Effects

Hepatoprotective effects against numerous hepatotoxic agents (e.g., acetaminophen, carbon tetrachloride, and *Escherichia coli* endotoxin) have been reported in both animal and in vitro studies. In these experiments, improvement in histological changes in hepatic tissue, including fatty infiltration, vacuolar degeneration, and hepatocellular necrosis, was reported. These effects may be associated with saponin fractions (34). In one clinical study of hepatitis B patients, concomitant use of astragalus with lamivudine and α -2b interferon showed greater efficacy than with lamivudine alone (35).

Systemic Lupus Erythematosus

Astragalus was also studied for its ability to affect natural killer (NK) cell activity, using an enzyme-release assay. The NK cell activity of PBMCs from 28 patients with systemic lupus erythematosus (SLE) was increased after in vitro incubation with an undefined astragalus preparation. Low levels of NK cell activity were correlated with disease activity. PBMCs from patients with SLE had significantly decreased NK cell activity as compared with those from healthy donors. The extent of stimulation by the astragalus preparation was related to the dose and length of the preincubation period (36). Despite its use as an immune-enhancing agent, which would normally be considered contraindicated in autoimmune disorders, investigation of astragalus may be warranted as evidence

suggests that it elicits significant anti-inflammatory activity and improves ratios and function of T lymphocytes in SLE (37).

Viral Infections

Prophylaxis against flu and modulation of endogenously produced interferon have been reported in several animal studies utilizing astragalus alone (6).

Other Effects

In a new line of investigation for astragalus, two triterpenes (astragaloside II and isoastragaloside I) were shown to alleviate insulin resistance and glucose intolerance in mice. The two compounds selectively increased adiponectin secretion on primary adipocytes and potentiated the effects of the insulin-sensitizer rosiglitazone. Chronic administration of the compounds (specific details lacking) to both dietary and genetically obese mice resulted in a significant increase in serum adiponectin, resulting in an alleviation of hyperglycemia, glucose intolerance, and insulin resistance. These effects were diminished in mice lacking adiponectin (38).

One study showed that a liquid extract of astragalus (2 g/mL/intravenous) retarded the progression of renal fibrosis in a manner similar to the angiotensin-II-receptor antagonist losartan. The study reported that like losartan, astragalus decreased deposition of fibronectin and type-I collagen by significantly reducing the expression of transforming growth factor- β_1 and α -smooth muscle actin ($P < 0.05$) (39).

Astragalus was investigated for its potential effect of reducing atopic dermatitis in mice. Using prednisolone (3 mg/kg/day) as a comparator, an astragalus water extract was administered orally at 100 mg/kg. Astragalus significantly reduced the severity of chemically induced inflammation (2,4-dinitrofluorobenzene) to a degree similar to the comparator but, unlike prednisolone, did not inhibit interleukin-4 production (40).

CLINICAL STUDIES

There are both English and Chinese language studies on astragalus. As with much of the literature regarding Chinese herbs, there are few clinical data of high methodological quality. In addition, a positive publication bias regarding Chinese literature has been reported (41), while in primary American medical literature, a negative publication bias against dietary supplement studies has been reported (42).

Immunomodulatory Effects

Cancer

Among modern herbal practitioners, astragalus is recommended as an immune supportive botanical in conjunction with conventional chemo and radiation therapies for cancer. There is a common belief and some clinical and preclinical evidence that astragalus both reduces side effects associated with conventional cancer therapies and can potentiate the effects of certain therapies. The available evidence is not strong enough to recommend astragalus as a standard part of conventional cancer care. However,

its demonstrated safety, lack of negative interaction with conventional therapies, and its putative benefit in building, preserving, and restoring immunocompetency before and after conventional therapies warrant specific study. There is also potential for use of both oral and injectable preparations, the latter of which are not approved in North America but are widely used throughout Asia.

In one clinical study, an astragalus drip (20 mL in 250 mL saline solution daily for 84 days) was administered to cancer patients ($n = 60$). Compared with the control group (no astragalus), those in the astragalus group showed a slower rate of tumor progression, a lower rate of reduction in peripheral leukocytes and platelets, reduction in suppressor CD8s, improved CD4/CD8 ratios, increased IgG and IgM, and better Karnofsky scores (43).

In addition to its use alone, both as a primary treatment and as an adjunct to conventional cancer therapies, astragalus is most often combined with other similar acting immune-enhancing plants. A number of randomized prospective clinical studies of cancer patients were conducted using a combination of astragalus and *ligustrum* (*Ligustrum lucidum*) (undisclosed quantities) with positive results, such as mortality reduction in breast and lung cancer patients (44). These effects, of course, must be considered to be due to the cumulative effects of the two botanicals and cannot be presumed to occur with astragalus alone but are more consistent with the manner in which astragalus is used in TCM.

An early clinical trial reported that 53 cases of chronic leukopenia responded favorably to an astragalus extract (1:1; 2 mL daily intramuscularly for 1–2 weeks). Improvements in symptoms and white blood cell counts were observed, but specific data were lacking (34).

Cardiovascular Effects

Various cardioactive properties have been reported for astragalus, and astragalus is widely used in the treatment of both chronic and acute cardiovascular disease in China. In one study, 92 patients with ischemic heart disease were given an unidentified preparation of astragalus. Marked relief from angina pectoris and other improvements as measured by electrocardiogram (ECG) and impedance cardiogram were reported. Improvement in the ECG index was reported as 82.6%. Overall improvement was significant as compared with the control group ($P < 0.05$) (45). A similar result in cardiac performance was reported by other groups of researchers. In one study, 43 patients were hospitalized within 36 hours of acute myocardial infarct. After administration of an astragalus preparation (undefined profile), the ratio of preejection period/left ventricular ejection time was decreased, the antioxidant activity of SOD of red blood cells was increased, and the lipid peroxidation content of plasma was reduced (46). In another experiment, 20 patients with angina pectoris were given an undefined astragalus preparation. Cardiac output, as measured by Doppler echocardiogram, increased from 5.09 ± 0.21 to 5.95 ± 0.18 L/min 2 weeks after administration of astragalus ($P < 0.01$). In this study, neither improvement in left ventricular diastolic function nor inhibition of adenosine triphosphate was observed (47). Intravenous administration of astragalus (undefined preparation) was reported to significantly shorten the duration of ventric-

ular late potentials in cardiac patients (39.8 ± 3.3 ms vs. 44.5 ± 5.9 ms; $P < 0.01$) (48).

In another investigation, astragaloside IV (intravenous; unspecified amount) was given to patients with congestive heart failure for 2 weeks. Improvement in symptoms such as tightness in the chest, difficult breathing, and reduced exercise capacity were reported. Radionuclide ventriculography showed that left ventricular modeling improved and left ventricular end-diastolic and left ventricular end-systolic volume diminished significantly. The authors concluded that astragaloside IV is an effective positive inotropic agent (49), an action supported by others (27).

Hepatoprotective Effects

In China, astragalus is widely used in the treatment of chronic hepatitis where reductions in elevated liver enzymes and improvements in symptoms in humans have been reported. This activity is stated to be associated with polysaccharides that increase interferon production (35).

Viral Infections

According to one English language review of the Chinese literature, a prophylactic effect against the common cold was reported in an epidemiological study in China involving 1000 subjects. Administration of astragalus, given either orally or as a nasal spray, reportedly decreased the incidence of disease and shortened cold duration. Studies exploring this protective effect found that oral administration of the preparation to subjects for 2 weeks enhanced the induction of interferon by peripheral white blood cells. Levels of IgA and IgG antibodies in nasal secretions were reported to be increased following 2 months of treatment (34). The effect of astragalus on the induction of interferon was studied in a placebo-controlled study involving 28 people. Fourteen volunteers were given an extract equivalent to 8 g of dried root per day and the rest were given placebos. Blood samples were drawn before treatment, then 2 weeks and 2 months after treatment. Interferon production by leukocytes was statistically increased after both time periods ($P < 0.01$) in the astragalus group but not the control group (50). In another study, astragalus was shown to potentiate the effects of interferon (α IFN-1) in patients with chronic cervicitis (51).

Dosages

- Crude root: 9–30 g daily as a decoction (52).
- Decoction: 0.5–1 L daily.

SAFETY PROFILE

Side Effects

None cited in the literature.

Contraindications

None cited in the literature.

Precautions

There is some evidence to suggest that astragalus and its putative anti-inflammatory effects are beneficial in those with autoimmune conditions such as lupus. However, astragalus should be used cautiously for the treatment of

autoimmune diseases or in conjunction with immunosuppressive therapies. Because immunostimulating polysaccharides may stimulate histamine release, allergic symptoms may be aggravated by the use of astragalus. This, however, has not been reported in the literature or from clinical use. According to the principles of TCM, astragalus should not be used during acute infectious conditions unless under the care of a qualified TCM practitioner.

Interactions

Both positive and negative interactions may occur. Astragalus potentiates the effects of acyclovir (53); IL-2, -20, -21; and rIFN-1 and -2 therapies (50,51). Because of its immunopromoting effects, astragalus may be incompatible with immunosuppressive agents in general.

Pregnancy, Mutagenicity, and Reproductive Toxicity

According to one review, astragalus is reported to have no mutagenic effects (54).

Lactation

Based on an authoritative review of the available pharmacologic and toxicologic literature, no limitation is to be expected (6,34,54).

Carcinogenicity

Studies suggest an anticarcinogenic activity.

Influence on Driving

Based on the available pharmacologic and toxicologic literature, no limitation is to be expected (6,34,54).

Overdose and Treatment

Specific data are lacking.

Toxicology

Based on a review of the available data and the experience of modern practitioners, astragalus can be considered a very safe herb even when taken within its large dosage range. Investigations of specific fractions including flavonoids, polysaccharide, and triterpene similarly show little toxicity (14,34,54).

Regulatory Status

In the United States, astragalus is regulated as a dietary supplement.

CONCLUSIONS

Astragalus is one of the most frequently used herbal medicines throughout Asia and is a very popular botanical used in western herbal supplements. In China, astragalus is used for a myriad of purposes relating to its high regard as a strengthening tonifier, immune modulator, anti-inflammatory, and anti-hepatotoxic. In the West, astragalus figures prominently in immune supportive formulas. Despite its popularity, there are few clinical trials regarding its use. There is some evidence to support the oral administration of astragalus for the prevention of colds and upper respiratory infections, and as an adjunct to conventional cancer therapies. These are very common

indications for which astragalus is applied by herbal practitioners. For its use in cancer therapies, there are no definitive guidelines. The modern experience of practitioners together with the limited clinical and preclinical data pointing to an immunomodulatory effect suggests that there may be some value for these indications, including the concomitant use of astragalus to reduce doxorubicin-induced immune suppression. However, more specific investigation in this area is needed.

Regarding its putative immunomodulating effects, the following mechanisms of action have been proposed: restoration of immune function, increased stem cell generation of blood cells and platelets, lymphocyte proliferation, rise in numbers of antibody-producing spleen cells, potentiation of rIL-2 and rIFN-1 and -2 immunotherapy, enhancement of phagocytic activity by macrophages and leukocytes, and increased cytotoxicity by NK cells.

Potential benefits to cardiovascular health, including relief from angina and congestive heart failure and improvement in clinical parameters following acute myocardial infarct, have been reported. Limited animal studies suggest that astragalus enhances coronary blood flow, may potentiate the release of nitric oxide, and potentiates the effects of endogenous antioxidant systems (e.g., SOD).

In Asia, astragalus is also used in conjunction with conventional medical treatments for hepatitis. Both animal and in vitro studies offer support for such treatment. As in the use of astragalus in cancer therapies, further clinical trials are required.

Though methodologically sound clinical trials for astragalus are generally lacking, natural health practitioners have a generally high regard for its use as a prophylactic against infectious disease and for its ability to build, maintain, and restore immunocompetency when used as a part of conventional cancer therapies. In addition to the very limited number of formal clinical studies that are available in English language sources, the published medical literature on astragalus has to be considered cautiously, as a number of the supporting studies utilize injectable preparations of isolated fractions that are not consistent with the oral use of astragalus supplements. Still, the existing data do support many of the traditional uses for which astragalus has been employed for centuries.

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Bilberry

Marilyn Barrett

INTRODUCTION

Bilberry, *Vaccinium myrtillus* L., is a shrub with edible fruits that is native to Circumboreal regions from Europe to Asia as well as the Rocky Mountains in North America. Bilberries are related to other edible berries including blueberry, cranberry, huckleberry, and lingonberry. Bilberry fruits contain anthocyanins, which are natural pigments, responsible for the dark blue color of the fruits and for many of the health benefits. In vitro studies have shown that bilberry extracts have antioxidant activity, inhibit platelet aggregation, prevent degradation of collagen in the extravascular matrix surrounding blood vessels and joints, and have a relaxing effect on arterial smooth muscle. Bilberry extracts have also demonstrated anticancer and antibacterial actions, in vitro. Pharmacokinetic studies in animals and humans show that a small percentage of the anthocyanins is absorbed into the body and widely distributed. Human clinical studies have been conducted evaluating the potential benefits of bilberry preparations in treating venous insufficiency and visual disorders ranging from night vision to diabetic retinopathy as well as cancer prevention. No serious toxicities have been associated with preparations of the fruits in animal screens and no serious side effects have been identified in humans.

BACKGROUND

Bilberries are edible fruits from *V. myrtillus* L. of the family Ericaceae. Bilberry is the standardized common name for the fruit in the United States, but the fruit is also known as European blueberry, huckleberry, and whortleberry (1). Related to bilberry, and in the same genus of *Vaccinium*, are other edible berries including blueberry, cranberry, huckleberry, and lingonberry.

Bilberry is a shrub, 1–6 dm high, found in heaths, meadows, and moist coniferous forests in Circumboreal regions from Europe to Asia, with populations in the American and Canadian Rocky Mountains (2).

The blue-black berries are harvested when ripe, usually during the months of July through September. The berries are oblate-globose, 5–9 mm diameter, with 4–5 locules containing many seeds. The seeds are approximately 1 mm long with a yellow/brown-dimpled surface (2).

Both the leaves and fruits of bilberry have been used medicinally since the Middle ages.

The leaves were used topically for inflammation, infections, and burns, as well as internally as a treatment for diabetes. According to the herbalist Grieve, the fruits were

used to treat dysentery, diarrhea, gastrointestinal inflammation, hemorrhoids, vaginal discharges, scurvy, urinary complaints, and to dry up breast milk. More recently, it was found that bilberry was used by World War II pilots to improve their night vision (2,3).

Bilberry fruit preparations are still used to improve vision as well as for their benefits to the circulatory system: treating fragility and altered permeability of blood vessels that is either primary or secondary to arterial hypertension, arteriosclerosis, or diabetes (3).

CHEMISTRY AND PREPARATION

Bilberry fruits contain anthocyanins that are natural pigments in the chemical class known as flavonoids. Anthocyanins are glycosides or compounds with sugars attached at the 3 position, while anthocyanidins are aglycones (the same basic structure without the sugars attached) (see chapter 74, "Polyphenol Overview"). The majority (64%) of anthocyanins in the fruit are glycosides of cyanidin and delphinidin (Fig. 1). The quantity of anthocyanin in the fruit ranges from 300 to 700 mg per 100 g. Bilberry fruits also contain flavonols, tannins, phenolic acids, organic acids, sugars, vitamins, and volatile compounds (2).

The primary commercial source of bilberry fruits is "wild harvest" from regions in Europe and Scandinavia. The fruits are sold fresh, frozen, or dried. Besides the whole fruit, commercial products include cold macerates, decoctions, and dry extracts. The dry extracts are commonly prepared using alcohol, methanol or ethanol (2).

Until recently, a single-wavelength spectrophotometric technique (UV) was commonly used to standardize the anthocyanin content of bilberry products. However, this technique did not detect adulteration of bilberry preparations with substances of similar color (4). A high-performance liquid chromatographic technique that can detect and quantitate both anthocyanins and anthocyanidins has recently been developed enabling a better assurance of product identity and quality (5).

Most studies on bilberry have been conducted using extracts characterized as containing 36% anthocyanins or 25% anthocyanidins.

PRECLINICAL STUDIES

In vitro studies have shown that bilberry extracts have antioxidant activity, inhibit platelet aggregation, prevent

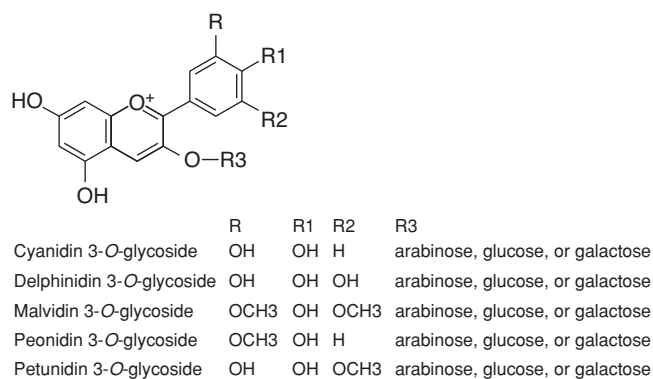


Figure 1 Chemical structures of anthocyanins.

degradation of collagen in the extravascular matrix surrounding blood vessels and joints, and have a relaxing effect on arterial smooth muscle. These actions are vaso-protective, increasing capillary resistance and reducing capillary permeability (3). Bilberry extracts have also demonstrated anticancer and antibacterial actions *in vitro*. There is no evidence of toxicity in animals at the effective doses.

Antioxidant Activity

Bilberry fruits have demonstrated antioxidant activity *in vitro* and in animal models. In the oxygen radical absorbance capacity (ORAC) assay, an *in vitro* test measuring free radical quenching, bilberry fruits had potent activity compared with other fresh fruits and vegetables ($44.6 \pm 2.3 \mu\text{mol Trolox equivalents (TE)/g}$) (6). In another assay, a bilberry extract (25% anthocyanins) exhibited antioxidant activity in protecting keratinocytes in culture from damage due to UVA and UVB light (7,8). The bilberry extract attenuated UVA-induced reactive oxygen species formation, peroxidation of membrane lipids, and depletion of intracellular glutathione in concentrations of 10–50 mg/L (7). In the same concentration range, the extract inhibited UVB-induced generation of reactive oxygen and nitrogen species, DNA strand breaks, as well as caspase-3 and capase-9 activity (mediators that execute apoptotic cell death) (8).

In an animal study, a bilberry extract characterized as containing 38% anthocyanins reduced oxidative stress caused in mice by removal of the animal's whiskers. The extract administered orally at a dose of 100 mg/kg for 7 days ameliorated the increase in oxygen radicals (thio-barbituric acid reactive substances), protein carbonyl formation, and lipid peroxidation in the brain, heart, kidney, and liver. The extract also suppressed the stress-induced changes in dopamine levels (9).

A bilberry extract (42% anthocyanins) reduced oxidative stress to the liver in a restraint-stress mouse model. The extract, administered at a dose of 200 mg/kg for 5 days, ameliorated the increase in plasma levels of alanine aminotransferase, a liver enzyme. The extract also reduced plasma and liver ORAC levels and increased plasma glutathione and vitamin C levels in the liver (10). A similar study was conducted in mice with kidney damage in-

duced by potassium bromate. Oral administration of the same extract ameliorated the increase in blood urea nitrogen levels and the decreases in kidney malondialdehyde, nitric oxide, and xanthine oxidase levels. The bilberry extract also improved the kidney ORAC levels (11).

MyrtoSelectTM, an extract containing approximately 40% anthocyanins, was tested for its effects on gene expression (DNA microarray) in a macrophage cell line stimulated with lipopolysaccharide (LPS). The extract, at a concentration of 75 $\mu\text{g/mL}$, appeared to mitigate the effect of LPS, targeting genes involved in inflammation and immune defense. Pretreatment with the bilberry extract affected 45% of the genes downregulated by LPS and 36% of genes upregulated by LPS (12).

Circulation

Bilberry extracts have demonstrated beneficial effects on the circulation, including inhibiting platelet aggregation, reducing capillary permeability, facilitating vasodilation, and inhibiting the development of atherosclerosis and angiogenesis.

Myrtocyan[®] (also known as MirtoSelectTM, containing 36% anthocyanins) inhibited platelet aggregation *in vitro* induced by adenosine diphosphate (ADP), collagen, and sodium arachidonate in rabbit platelet-rich plasma with IC_{50} values ranging from 0.36 to 0.81 mg/mL. Myrtocyan administered orally to rats (400 mg/kg) prolonged the bleeding time in the animals, without affecting coagulation pathways. The same dose administered to mice reduced the adhesiveness of platelets to glass (3). Myrtocyan administered to healthy human subjects, 480 mg/day (173 mg anthocyanins/day) for 30–60 days, reduced the aggregation response *ex vivo* to ADP and collagen (13).

In a rabbit skin model, oral treatment with 400 mg anthocyanins per kilogram body weight 30 minutes before topical application of chloroform reduced the capillary permeability caused by the irritant by 66%. In rats, administration of bilberry anthocyanins, 200 mg/kg orally, decreased bradykinin-induced capillary permeability by 39%. The same dose reduced carrageenin-induced rat paw edema by 45% (14). In a rat model of experimentally induced hypertension, 500 mg anthocyanins per kilogram body weight given orally for 12 days completely ameliorated the increase in blood–brain barrier permeability and reduced the increase in aortic vascular permeability by 40% (15). In a hamster cheek pouch model, 100 mg bilberry extract per kilogram daily for 4 weeks reduced the circulatory damage due to ischemic reperfusion (16). A rat model suggested that bilberry anthocyanins (50 mg/kg IP) inhibited the enzymatic degradation of collagen, decreasing the permeability of the blood–brain barrier caused by proteases (17).

Bilberry preparations are reported to relax arterial tissues *in vitro*. Preliminary experiments pointed to a mechanism involving prostaglandins. However, a more recent study using porcine coronary arteries demonstrated a mechanism involving nitric oxide (endothelial-derived relaxing factor) (18).

A bilberry extract was reported to inhibit the development of atherosclerosis in apolipoprotein E-deficient mice. The mice received diets supplemented with 0.02% of a bilberry extract (52% anthocyanins) for 16 weeks. The

extract reduced lipid deposits and the development of lesions. It did not affect plasma antioxidant capacity or plasma lipid levels (19).

A bilberry extract (25% anthocyanins) was tested for its effect on angiogenesis both in vitro and in vivo. The extract at concentrations of 0.3–30 µg/mL inhibited tube formation and the migration of human umbilical vein endothelial cells induced by vascular endothelial growth factor A. The extract also inhibited the induction of retinopathy in newborn mice, which was induced with oxygen. Intravitreal administration of 300 ng extract per eye significantly inhibited the area of neovascular tufts (20).

Anticancer

Anthocyanins have been reported to mediate several physiological functions that ultimately may result in cancer suppression. Anthocyanins suppress the growth of cancer cell lines in vitro, including HL60 human leukemia cells and HCT116 human colon cancer cells. A bilberry extract induced apoptotic cell bodies and nucleosomal DNA fragmentation in HL60 cells (21). A bilberry extract (Mirtocyan) has also been shown to suppress the activity of receptor tyrosine kinases, which are thought to play a crucial role in carcinogenesis and tumor progression. When tested over a number of tyrosine kinases, the activity was consistent but not specific (22).

Antibacterial

Phenolic compounds in bilberry have demonstrated in vitro antimicrobial effects against strains of *Salmonella* and *Staphylococcus* possibly through interfering with adhesion of the bacteria. Treatment of bilberry preparations with pectinase released phenolics from the cell wall matrix and increased the antibacterial activity (23). In experiments with *Neisseria meningitidis*, the bacteria that causes meningitis and septicemia, a bilberry juice fraction was reported to inhibit the binding of the bacteria to epithelial cells in culture. Fractions of the juice also bound to the bacterial pili. The authors concluded that anthocyanins were partly responsible for the activity but that there appeared to be other compounds in bilberry that may also interact directly with the pili or act synergistically with the anthocyanins (24).

Safety Studies (Animal Toxicology)

Myrtocyan (25% anthocyanins) has been tested for acute and chronic toxicity in animal studies. There were no deaths with an acute dose in rats up to 20 g/kg orally and in mice up to 25 g/kg. Six months treatment with doses of 125–500 mg/kg in rats and 80–320 mg/kg in dogs found no evidence of toxicity. The preparation was tested in guinea pigs for 2 weeks and in rats for 6 weeks with doses up to 43 mg/kg without incident (2,3).

PHARMACOKINETICS

Animal studies show that bilberry anthocyanins are absorbed intact, or after methylation. This is unlike other flavonoid glycosides which are hydrolyzed to their aglycones and metabolized to glucuronidated or sulfated

derivatives (25). Following administration of 400 mg/kg orally to rats, peak blood levels of anthocyanins were detected within 15 minutes and afterwards declined rapidly. Only 1% of the anthocyanins was eliminated in the urine and 4% in the bile. The absolute bioavailability of bilberry anthocyanins was estimated to be 1.2–5% (26). A study in mice reported that malvidin 3-glucoside and malvidin 3-galactoside were the principal anthocyanins in the plasma 60 minutes after oral administration of 100 mg/kg. When the mice were maintained on a diet containing 0.5% bilberry extract, plasma levels of anthocyanins reached 0.26 µM. Anthocyanidins were detected in the liver, kidney, and lung. They were not detected in the spleen, thymus, heart, muscle, brain, white fat, or eyes (25).

A pharmacokinetic study with six human subjects detected anthocyanins in the plasma 1.5–6 hours following intake of a bilberry–lingonberry puree. The study examined the production of urinary phenolic acids and found the greatest increase in methylated compounds. The amount of urinary phenolic acids was low, and the authors suggested that the fragmentation of anthocyanins to phenolic acids was not a major metabolic pathway (27). Another pharmacokinetic study with 20 subjects that consumed 100 g/day of berries, including black currant, lingonberries, and bilberries, for 8 weeks reported an increase in serum quercetin (up to 51% higher) compared with control subjects who did not consume berries (28). A study with 25 subjects administered 1.4–5.6 g Mirtocyan (25% anthocyanins) daily for 7 days reported detection of anthocyanins as well as methyl and glucuronide metabolites in the plasma and urine but not in the liver (29).

CLINICAL STUDIES

Human clinical studies have been conducted evaluating the potential benefits of bilberry preparations in treating venous insufficiency and visual disorders ranging from night vision to diabetic retinopathy as well as cancer prevention.

Vascular Health

Clinical studies have been conducted evaluating the potential benefits of bilberry preparations in treating venous insufficiency. A review of studies conducted between 1970 and 1985 included 568 patients with venous insufficiency of the lower limbs who were treated with bilberry preparations (30). The studies reported an improvement in circulation and in lymph drainage resulting in a reduction in edema. A more recent placebo-controlled study which included 60 participants with varicose veins reported improvement in edema in the legs and ankles, sensation of pressure, cramps, and tingling or “pins and needles” sensations with a dose of 160 mg Tegens®, three times daily for 1 month (31). Tegens (Inverni della Beffa, Italy) contains a bilberry extract named Myrtocyan or MirtoSelect (25% anthocyanins), manufactured by Indena SpA, Italy.

Visual Health

A systematic review was conducted on placebo-controlled studies on the effects of bilberry preparations on night

vision. Literature searches identified 30 clinical studies, and 12 of those met the inclusion criteria of being placebo controlled. Of the 12 studies, 5 were randomized. Healthy subjects with normal or above average eyesight were tested in 11 out of the 12 studies. Many of the studies were acute, using a single dose, and the longest treatment period was 28 days. Full characterization of the products used in the studies was not available, but assuming 25% anthocyanin content, the doses of anthocyanin ranged from 12 to 2880 mg. The techniques used to measure the extent and rate of dark adaptation ranged from visual acuity, contrast sensitivity, and critical flicker fusion to electroretinographic monitoring of response to light flashes. The four most recent randomized controlled studies with rigorous methodology reported negative results. One randomized controlled study and all seven of the non-randomized studies reported positive effects. The authors concluded that the present studies do not support the use of bilberry by those who are healthy with normal vision to improve their night vision. However, uncontrolled studies report a benefit for those with eye disorders, including retinal degeneration, myopia, simple glaucoma, and pathological fundus. Furthermore, studies with synthetic anthocyanins suggest a positive benefit for those with myopia, central retinal lesions, and night blindness (32).

Two studies on diabetic retinopathy, using a dose of 160 mg Tegens twice daily, demonstrated a trend toward improvement in mild cases of the disease. The first study was a 1-month, placebo-controlled study that included 36 subjects. At the end of the month, 10 of 13 patients in the Tegens group with ophthalmoscopically detectable retinal abnormalities (microaneurisms, hemorrhagic foci, exudates) were improved, while all 15 patients with these abnormalities in the placebo group remained unchanged. A similar trend was observed among those patients with fluoroangiographic abnormalities (33). The second study lasted 1 year and included 40 subjects who were given Tegens or placebo in addition to the usual therapy for retinopathy. As a result, in 50% of patients given bilberry, the retinal lesions and associated edema were improved compared with 20% in the control group (34).

A mixture of vitamin E and bilberry (FAR-1, Ditta Farmigea SpA, Italy) showed a trend toward prevention of senile cataracts after 4 months of 180 mg bilberry anthocyanins (25% anthocyanidins) and 100 mg DL-tocopheryl acetate twice daily. When the placebo group was changed from placebo to the bilberry preparation, and the trial continued for an additional 4 months, there was no statistical difference between the two groups. The rationale for this study was previous indications that antioxidants might prevent the development of senile cataracts (35).

A mechanistic study using Myrtocyan examined changes in pupillary reflexes to light following a single high dose of 240 mg anthocyanosides or placebo in 40 healthy volunteers. The study was conducted to explore the use of bilberry in work situations where exposure to high light intensities dampens pupillary reflexes and leads to vision fatigue. The authors of the study suggested that the pigments in bilberry might increase sensitivity to light and improve blood flow in the capillaries of the eye. Improvement in pupillary reflexes was observed

in both groups, with the improvement in the treatment group being only slightly better than that in the placebo group (36).

Cancer Prevention

In an open label study, 25 colorectal cancer patients scheduled to undergo surgery were given 1.4, 3.8, or 5.6 g bilberry extract (Mirtocyan) containing 0.2–2.0 g anthocyanins for 7 days before surgery. Availability of anthocyanins was determined by detection in the plasma, colorectal tissue, and urine but not in the liver. Anthocyanins detected in the body were unaltered, or products of metabolic glucuronidation and *O*-methylation. Proliferation of cells in the tumor tissue was decreased by 7% compared with before the bilberry intervention (29).

Side Effects and Adverse Effects

No side effects were reported in the clinical studies mentioned earlier. In a 1987 postmarketing surveillance study with 2295 subjects, only 94 (4.1%) complained of minor side effects, most of which involved the gastrointestinal track. Most of the participants took 160 mg Tegens twice daily for 1–2 months (3).

Observed Drug Interactions and Contraindications

No drug interactions or contraindications have been reported in the literature for bilberry.

CONCLUSIONS

Bilberry fruit extracts and anthocyanins have been the subject of pharmacological studies and human clinical trials. In vitro and in vivo studies demonstrate good evidence for the antioxidant activity of bilberry extracts along with strong indications of benefit to the cardiovascular system. Animal and human pharmacokinetic studies demonstrate bioavailability of anthocyanins, but absorption appears to be limited. Human clinical studies on the effects of bilberry extracts on eyesight and vascular diseases suffer from poor methodology, including small sample sizes and short-term exposures. While it appears doubtful that bilberry preparations benefit the night vision of healthy subjects, the benefit for those with diabetic retinopathy and other eye disorders merits exploration. Another area that appears promising is that of benefits to the cardiovascular system, specifically vasculitis or venous insufficiency. Bilberry products have been safely consumed, without significant adverse events or side effects.

REGULATORY STATUS

Bilberry is a food and preparations are also used medicinally. In the United States, preparations of bilberry are sold as foods and dietary supplements. The U.S. Pharmacopoeia has published a standard monograph for powdered bilberry extract (37). The German Commission E completed a monograph for bilberry fruits in which preparations of the ripe fruit are indicated orally for

nonspecific, acute diarrhea and topically for mild inflammation for the oral and pharyngeal mucosa (38). The European Scientific Cooperative on Phytotherapy (ESCOP) monograph lists the internal use of bilberry fruit preparations (enriched in anthocyanins) for symptomatic treatment of problems related to varicose veins, such as painful and heavy legs. The ESCOP monograph also lists the dried fruit as supportive treatment of acute, nonspecific diarrhea (39). In Canada, bilberry products are approved as natural health products for traditional use orally as an astringent and as a source of antioxidants as well as for use as a gargle to relieve mild inflammation of the mouth and/or throat (40).

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Biotin

Donald M. Mock

INTRODUCTION

Biotin is usually classified as a B-complex vitamin. "Biotin" is by far the most widely used term for this vitamin. However, discovery of biotin by different approaches has also led to names such as Bios IIB, protective factor X, vitamin H, coenzyme R, factor S, factor W, and vitamin B_W. This entry reviews the biochemistry of biotin and summarizes the clinical findings of deficiency. Readers are encouraged to use the references for further information.

SCIENTIFIC NAMES AND STRUCTURE

The molecular weight of biotin is 244.31 Da. The structure of biotin was elucidated independently by Kogl and du Vigneaud in the early 1940s and is shown in Figure 1 (1). Biotin is a bicyclic compound. The imidazolidone contains an ureido group (–N–CO–N–). The tetrahydrothiophene ring contains sulfur and has a valeric acid side chain attached to the C2 carbon of the sulfur-containing ring. This chain has a *cis* configuration with respect to the ring that contains the nitrogen atoms. The two rings are fused in the *cis* configuration, producing a boat-like structure. With three asymmetric carbons, eight stereoisomers exist; only one [designated D-(+)-biotin or, simply, biotin] is found in nature and is active when covalently joined via an amide bond between the carboxyl group of the valeric acid side chain of biotin and the ε-amino group of a lysine residue of an apocarboxylase. Biocytin (ε-*N*-biotinyl-L-lysine) is the product of digestion of protein-bound dietary biotin and cellular turnover of biotin-containing carboxylases and histones; biocytin is as active as biotin on a molar basis in mammalian growth studies.

Goldberg/Sternbach synthesis or a modification thereof is the method by which biotin is synthesized commercially (1). Additional stereospecific methods have been published (2,3).

HISTORY

Biotin was discovered in nutritional experiments that demonstrated a factor present in many foodstuffs that was capable of curing the scaly dermatitis, hair loss, and neurologic signs induced in rats fed dried egg white. Avidin, a glycoprotein found in egg white, binds biotin very specifically and tightly. From an evolutionary standpoint, avidin probably serves as a bacteriostat in egg white. Consistent with this hypothesis is the observation that avidin is resistant to a broad range of bacterial proteases in both free and biotin-bound form. Because avidin is also resistant to

pancreatic proteases, dietary avidin binds to dietary biotin (and probably any biotin from intestinal microbes) and prevents absorption, carrying the biotin on through the gastrointestinal tract.

Biotin is definitely synthesized by intestinal microbes; however, the contribution of microbial biotin to absorbed biotin, if any, remains unknown. Cooking denatures avidin, rendering this protein susceptible to pancreatic proteases and unable to interfere with the absorption of biotin.

BIOCHEMISTRY

Biotin acts as an essential cofactor for five mammalian carboxylases. Each has the vitamin covalently bound to a polypeptide. For monomeric carboxylases, this polypeptide is the apocarboxylase. For the dimeric carboxylases, this monomer with a biotinylation site is designated the chain. The covalent attachment of biotin to the apocarboxylase protein is a condensation reaction catalyzed by holocarboxylase synthetase (EC 6.3.4.10). These apocarboxylase regions contain the biotin motif (methionine-lysine-methionine), a specific sequence of amino acids present in each of the individual carboxylases; this sequence tends to be highly conserved within and between species. One interpretation concerning conservation of this amino acid sequence is that these residues allow the biotinylated peptide to swing the carboxyl (or acetyl) group from the site of activation to the receiving substrate.

All five of the mammalian carboxylases catalyze the incorporation of bicarbonate as a carboxyl group into a substrate and employ a similar catalytic mechanism. In the carboxylase reaction, the carboxyl moiety is first attached to biotin at the ureido nitrogen opposite the side chain. Then the carboxyl group is transferred to the substrate. The reaction is driven by the hydrolysis of ATP to ADP and inorganic phosphate. Subsequent reactions in the pathways of the five mammalian carboxylases release CO₂ from the product of the enzymatic reaction. Thus, these reaction sequences rearrange the substrates into more useful intermediates but do not violate the classic observation that mammalian metabolism does not result in the net fixation of carbon dioxide (4).

The five carboxylases are pyruvate carboxylase (EC 6.4.1.1), methylcrotonyl-CoA carboxylase (EC 6.4.1.4), propionyl-CoA carboxylase (EC 6.4.1.3), and two isoforms of acetyl-CoA carboxylase (EC 6.4.1.2), denoted I and II, which are also known as ACC and β ACC. Each

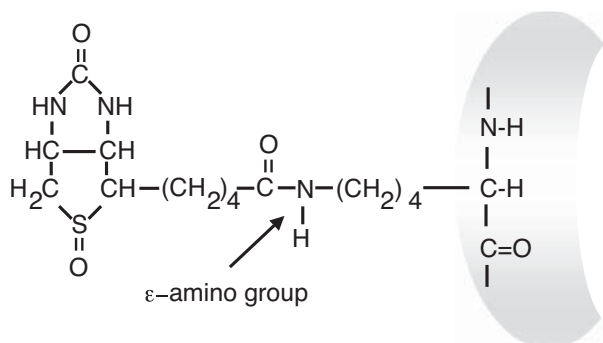


Figure 1 Protein-bound biotin with arrow showing the amide bond to the ϵ -amino acid.

carboxylase catalyzes an essential step in intermediary metabolism (Fig. 2).

Pyruvate carboxylase mediates in the incorporation of bicarbonate into pyruvate to form oxaloacetate, an intermediate in the Krebs tricarboxylic acid cycle. Thus, pyruvate carboxylase catalyzes an anaplerotic reaction. In gluconeogenic tissues (i.e., liver and kidney), the oxaloacetate can be converted to glucose. Deficiency of this enzyme (denoted by a block in the metabolic pathway) is likely the cause of the lactic acidosis and hypoglycemia observed in biotin-deficient animals and humans.

Methylcrotonyl-CoA carboxylase catalyzes an essential step in the degradation of the branch-chained

amino acid leucine. Deficient activity of this enzyme leads to metabolism of 3-methylcrotonyl CoA to 3-hydroxyisovaleric acid and 3-methylcrotonylglycine by an alternate pathway. Thus, increased urinary excretion of these abnormal metabolites reflects deficient activity of this carboxylase.

Propionyl-CoA carboxylase catalyzes the incorporation of bicarbonate into propionyl CoA to form methylmalonyl CoA, which undergoes isomerization to succinyl CoA and enters the tricarboxylic acid cycle. In a fashion analogous to methylcrotonyl-CoA carboxylase deficiency, inadequacy of this enzyme leads to increased urinary excretion of 3-hydroxypropionic acid and 3-methylcitric acid and enhanced accumulation of odd-chain fatty acids C15:0 and C17:0. The mechanism is likely the substitution of propionyl CoA for acetyl CoA during fatty acid elongation. Although the proportional increase is large (e.g., 2- to 10-fold), the absolute composition relative to other fatty acids is quite small (<1%) and likely produces little or no functional consequences.

Acetyl-CoA carboxylases, I and II both, catalyze the incorporation of bicarbonate into acetyl CoA to form malonyl CoA. Acetyl-CoA carboxylase I is located in the cytosol and produces cytosolic malonyl CoA, which is rate limiting in fatty acid synthesis (elongation). Acetyl-CoA carboxylase II is present on the outer mitochondrial membrane. As demonstrated by the pioneering work of Wakil and colleagues, acetyl-CoA carboxylase II controls a separate mitochondrial pool of malonyl CoA that, in turn, controls fatty acid oxidation in mitochondria through the inhibitory effect of malonyl CoA on fatty acid transport into mitochondria.

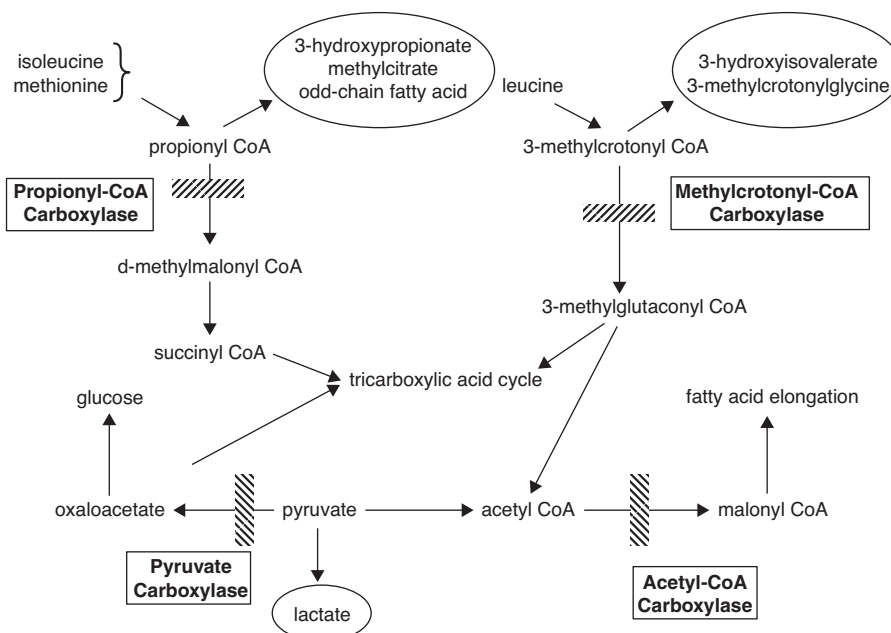


Figure 2 Pathways involving biotin-dependent carboxylases. Deficiencies (hatched bar) of pyruvate carboxylase, propionyl-CoA carboxylase, methylcrotonyl-CoA carboxylase, and acetyl-CoA carboxylase lead to increased blood concentrations and urinary excretion of characteristic organic acids denoted by ovals.

In the normal turnover of cellular proteins, holocarboxylases are degraded to biocytin or biotin linked to an oligopeptide containing at most a few amino acid residues. Because the amide bond between biotin and lysine (Fig. 1) is not hydrolyzed by cellular proteases, the specific hydrolase biotinidase [biotin amide hydrolase (EC 3.5.1.12)] is required to release biotin for recycling.

Biotin exists in free and bound pools within the cell that are responsive to changes in its status (5). The pool size is likely determined by a balance between cellular uptake and cellular release, incorporation into apocarboxylases and histones, release from these biotinylated proteins during turnover, and catabolism to inactive metabolites. Regulation of intracellular mammalian carboxylase activity by biotin remains to be elucidated.

Genetic deficiencies of holocarboxylase synthetase and biotinidase cause the two distinct types of multiple carboxylase deficiency that were previously designated the neonatal and juvenile forms. The genes for holocarboxylase synthetase and human biotinidase have been cloned, sequenced, and characterized (6). The gene coding for holocarboxylase synthetase is located on chromosome 21q22.1 and consists of 14 exons and 13 introns in a span of 240 kilobase (kb). Studies of human mutant holocarboxylase synthetase indicate that all forms of holocarboxylase synthetase are likely encoded by one gene. Biotinidase deficiency is particularly relevant to understanding biotin inadequacy because the clinical manifestations appear to result largely from secondary biotin depletion.

PHYSIOLOGY

Digestion of Protein-Bound Biotin

The content of free and protein-bound forms of biotin in foods is variable, but the majority in meats and cereals appear to be protein bound via an amide bond between biotin and lysine. Neither the mechanisms of intestinal hydrolysis of protein-bound biotin nor the determinants of bioavailability have been clearly delineated. Wolf et al. (7) have postulated that biotinidase plays a critical role in the release of biotin from covalent binding to protein. Doses of free biotin that do not greatly exceed the estimated dietary intake (e.g., 50–150 µg/day) appear adequate to prevent the symptoms of biotinidase deficiency. This suggests that biotinidase inadequacy in patients causes biotin deficiency, at least in part, through impaired intestinal digestion of protein-bound biotin.

Intestinal Absorption

At physiologic pH, the carboxylate group of biotin is negatively charged. Thus, the vitamin is at least modestly water soluble and requires a transporter to cross the membranes of enterocytes for intestinal absorption, of somatic cells for utilization, and of renal tubule cells for reclamation from the glomerular filtrate.

An excellent in-depth review of intestinal uptake of biotin has been published recently (8). Two biotin transporters have been described: (i) a multivitamin transporter present in many tissues including the intestine and (ii) a biotin transporter identified in human lymphocytes.

The transporter responsible for absorption of free biotin in the small and large intestine is saturable and Na⁺

dependent. The transporter also transports pantothenic acid and lipoate and is deemed the sodium-dependent multivitamin transporter (SMVT). SMVT was discovered in 1997 by Prasad et al. (9) in human placental choriocarcinoma cells. This transporter is widely expressed in human tissues (10). SMVT system has been cloned and demonstrated to be exclusively expressed at the apical membrane of enterocytes. SMVT is the main biotin uptake system that operates in human intestinal epithelial cells. The 5'-regulatory region of the SMVT gene has also been cloned and characterized both in vitro and in vivo (8). Intestinal biotin uptake is adaptively upregulated in biotin deficiency via a transcriptionally mediated mechanism that involves KLF4 sites. The cytoplasmic C-terminal domain of the polypeptide is essential for its targeting to the apical membrane domain of epithelial cells (8).

In rats, biotin transport is upregulated during maturation after weaning and by biotin deficiency (11). Carrier-mediated transport of the vitamin is most active in the proximal small bowel of the rat and humans (8). However, absorption from the proximal colon is still significant, supporting the potential nutritional significance of biotin synthesized and released by enteric flora (11). Clinical studies have provided some evidence that biotin is absorbed from the human colon (12). In contrast, more rigorous studies in swine indicate that biotin absorption from the hindgut is much less efficient than that from the upper intestine; furthermore, biotin synthesized by enteric flora may not present at a location or in a form in which bacterial biotin contributes importantly to absorbed biotin.

Exit of biotin from the enterocyte (i.e., transport across the basolateral membrane) is also carrier mediated (11). However, basolateral transport is independent of Na⁺, is electrogenic, and does not accumulate biotin against a concentration gradient.

Transport in Blood

Biotin dissolved in blood is carried from the site of absorption in the intestine to the peripheral tissues and the liver (1). Wolf et al. (13) originally hypothesized that biotinidase might serve as a biotin-binding protein in plasma or perhaps even as a carrier protein for the movement of biotin into the cell. Based on protein precipitation and equilibrium dialysis using ³H-biotin, Chauhan and Dakshinamurti (14) concluded that biotinidase is the only protein in human serum that specifically binds biotin. However, using ³H-biotin, centrifugal ultrafiltration, and dialysis to assess reversible binding in plasma from the rabbit, pig, and human, Mock and Lankford (15) found that less than 10% of the total pool of free plus reversibly bound biotin is reversibly bound to plasma protein; the biotin binding observed could be explained by binding to human serum albumin. Using acid hydrolysis and ³H-biotinyl-albumin, Mock and Malik (16) found additional biotin covalently bound to plasma protein. The percentages of free, reversibly bound, and covalently bound biotin in human serum are approximately 81%, 7%, and 12%. A biotin-binding immunoglobulin has been identified in human serum. An approximately fivefold higher concentration of this biotin-binding immunoglobulin was reported in patients with Graves disease than in normal and healthy controls (17). The role of plasma

proteins in the transport of biotin remains to be definitively established.

Biotin concentrations in erythrocytes are equal to those in plasma (D.M. Mock, unpublished observation). However, transport into erythrocytes is very slow, consistent with passive diffusion (18).

Uptake by the Liver

Studies in a variety of hepatic cell lines indicate that uptake of free biotin by the liver is similar to intestinal uptake and is mediated by SMVT (19–21). Transport is mediated by a specialized carrier system that is Na^+ dependent, electroneutral, and structurally specific for a free carboxyl group. At large concentrations, movement is carried out by diffusion. Metabolic trapping, for example, biotin bound covalently to intracellular proteins, is also important. After entering the hepatocyte, biotin diffuses into the mitochondria via a pH-dependent process.

The biotin transporter identified in lymphocytes is also Na^+ coupled, saturable, and structurally specific (22). Recent studies by Daberkow and coworkers provide evidence in favor of monocarboxylate transporter 1 as the lymphocyte biotin transporter (23).

A child with biotin dependence due to a defect in the lymphocyte biotin transporter has been reported (18). The child became acutely encephalopathic at the age of 18 months. Urinary organic acids indicated deficiency of several biotin-dependent carboxylases. Symptoms improved rapidly following biotin supplementation. Serum biotinidase activity and biotinidase gene sequence were normal. Activities of biotin-dependent carboxylases in lymphocytes and cultured skin fibroblasts were normal, excluding biotin holocarboxylase synthetase deficiency as the cause. Despite extracellular biotin sufficiency, biotin withdrawal caused recurrence of abnormal organic aciduria, indicating intracellular biotin deficiency. Biotin uptake rates into fresh lymphocytes from the child and into his or her lymphocytes transformed with Epstein-Barr virus were about 10% of normal fresh and transformed control cells, respectively. For fresh and transformed lymphocytes from his or her parents, biotin uptake rates were consistent with heterozygosity for an autosomal recessive genetic defect. *SMVT* gene sequence was normal; regulatory regions of the *SMVT* gene have not been characterized. These investigators speculated that lymphocyte biotin transporter is expressed in additional tissues such as the kidney and may mediate some critical aspect of biotin homeostasis, but the complete molecular etiology of this child's biotin transporter deficiency remains to be elucidated.

Ozand et al. (24) recently described several patients in Saudi Arabia with biotin-responsive basal ganglia disease. Symptoms include confusion, lethargy, vomiting, seizures, dystonia, dysarthria, dysphagia, seventh nerve paralysis, quadriparesis, ataxia, hypertension, chorea, and coma. A mutation in *SLC19A3* was identified, and defect in the biotin transporter system across the blood-brain barrier was postulated (25). However, in an elegant set of experiments, Said and coworkers demonstrated that *SLC19A3* is the apical thiamine transporter and renamed *SLC19A3* appropriately as *THTR2* (26), in contrast to the basolateral thiamine transporter *THTR1*. The explanation

for the documented biotin responsiveness of these patients remains unknown.

Renal Handling

Specific systems for the reabsorption of water-soluble vitamins from the glomerular filtrate contribute importantly to conservation of these vitamins (27). Animal studies using brush border membrane vesicles from human kidney cortex indicate that biotin is reclaimed from the glomerular filtrate against a concentration gradient by a saturable, Na^+ -dependent, structurally specific system (28). Using human-derived proximal tubular epithelial HK-2 cells as a model, Said and coworkers demonstrated that biotin uptake by human renal epithelial cells occurs via the SMVT system and that the process is regulated by intracellular protein kinase C and Ca^{++} /calmodulin-mediated pathways (29). The uptake process is adaptively regulated by extracellular biotin concentrations via transcriptional regulatory mechanisms (29) consistent with previous studies demonstrating reduced biotin excretion early in experimentally induced biotin deficiency in human subjects (30,31).

Subsequent egress of biotin from the tubular cells occurs via a basolateral membrane transport system that is not dependent on Na^+ . Biocytin does not inhibit tubular reabsorption of biotin (28). Studies in patients with biotinidase deficiency suggest that there may be a role for biotinidase in the renal handling of biotin (32,33).

Transport into the Central Nervous System

A variety of animal and human studies suggest that biotin is transported across the blood-brain barrier (1,34,35). The transporter is saturable and structurally specific for the free carboxylate group on the valeric acid side chain. Transport into the neuron also appears to involve a specific transport system as well as subsequent trapping of biotin by covalent binding to brain proteins, presumably the biotin-dependent carboxylases and histones.

Recently, concentrations of biotin were determined initially as total avidin-binding substances in cerebrospinal fluid (CSF) from 55 children, and biotin, biotin sulfoxide, and bisnorbiotin were quantitated by high-performance liquid chromatography (HPLC) and avidin-binding assay in CSF samples from a subset of 11 children (36). Concentrations of total avidin-binding substances averaged 1.6 nmol/L with substantial variability, $\text{SD} = 1.3$ nmol/L. CSF concentrations of biotin and biotin analogs varied widely, but substantial amounts of biotin sulfoxide were detected in every sample. Of the total, biotin accounted for $42\% \pm 16\%$, biotin sulfoxide for $41\% \pm 12\%$, and bisnorbiotin for $8\% \pm 14\%$. Surprisingly, the molar sum of biotin plus biotin sulfoxide and bisnorbiotin on average exceeded the total avidin-binding substances concentrations from the same CSF sample by >200 -fold. These investigators found no masking of detection or degradation of biotin or biotin sulfoxide. Gel electrophoresis and streptavidin Western blot detected several biotinylated proteins in CSF leading to the conclusion that biotin is bound to protein covalently, reversibly, or both; they speculated that biotin bound to protein likely accounts for the increase in detectable biotin after HPLC and that

protein-bound biotin plays an important role in biotin nutrition of the brain.

Placental Transport

Biotin concentrations are 3- to 17-fold greater in plasma from human fetuses compared with their mothers in the second trimester, consistent with active placental transport (37). Specific systems for transport of biotin from the mother to the fetus have been reported recently (10,38–40). The microvillus membrane of the placenta contains a saturable transport system for biotin that is Na⁺ dependent and actively accumulates biotin within the placenta, consistent with SMVT (10,38–40).

Transport into Human Milk

More than 95% of the biotin is free in the skim fraction of human milk (41). The concentration of biotin varies substantially in some women (42) and exceeds that in serum by one to two orders of magnitude, suggesting that there is a transport system into milk. The biotin metabolite bisnorbiotin (see discussion of metabolism under pharmacology section) accounts for approximately 50%. In early and transitional human milk, the biotin metabolite and biotin sulfoxide accounts for about 10% of the total biotin plus metabolites (43). With postpartum maturation, the biotin concentration increases, but the bisnorbiotin and biotin sulfoxide concentrations still account for 25% and 8% at 5 weeks postpartum. The concentration of biotin in human milk exceeds the plasma concentration by 10- to 100-fold, implying that a transport system exists. Current studies provide no evidence for a soluble biotin-binding protein or any other mechanism that traps biotin in human milk. The location and the nature of the biotin transport system for human milk have yet to be elucidated.

PHARMACOLOGY

Studies in which pharmacologic amounts of biotin were administered orally and intravenously to experimental subjects and tracer amounts of radioactive biotin were administered intravenously to animals show that biotin in pure form is 100% bioavailable when administered orally. The preponderance of dietary biotin detectable by bioassays is bound to carboxylases and perhaps to histones. The bioavailability of biotin from foodstuffs is not known, whereas that from animal feeds varies but can be well below 50%. After intravenous administration, the vitamin disappears rapidly from plasma; the fastest phase of the three-phase disappearance curve has a half-life of less than 10 minutes.

An alternate fate to being covalently bound to protein (e.g., carboxylases) or excretion unchanged in urine is catabolism to an inactive metabolite before excretion in urine (4). About half of biotin undergoes metabolism before excretion. Two principal pathways of biotin catabolism have been identified in mammals. In the first pathway, the valeric acid side chain of biotin is degraded by β -oxidation. This leads to the formation of bisnorbiotin, tetranorbiotin, and related intermediates

Table 1 Normal Range of Urinary Excretion of Biotin and Major Metabolites (nmol/24 hr; $n = 31$ Males and Females)

Biotin	Bisnorbiotin	Biotin sulfoxide
18–77	11–39	8–19

that are known to result from β -oxidation of fatty acids. The cellular site of this β -oxidation of biotin is uncertain. Nonenzymatic decarboxylation of the unstable β -keto-biotin and β -keto-bisnorbiotin leads to formation of bisnorbiotin methylketone and tetranorbiotin methylketone, which appear in urine. In the second pathway, the sulfur in the thiophene ring of biotin is oxidized, leading to the formation of biotin L-sulfoxide, biotin D-sulfoxide, and biotin sulfone. Combined oxidation of the ring sulfur and β -oxidation of the side chain lead to metabolites such as bisnorbiotin sulfone. In mammals, degradation of the biotin ring to release carbon dioxide and urea is quantitatively minor.

On a molar basis, biotin accounts for approximately half of the total avidin-binding substances in human serum and urine (Table 1). Biocytin, bisnorbiotin, bisnorbiotin methylketone, biotin sulfoxide, and biotin sulfone form most of the balance. Biotin metabolism is accelerated in some individuals by anticonvulsant therapy and during pregnancy, thereby increasing the ratio of biotin metabolites to biotin excreted in urine.

OCCURRENCE AND DIAGNOSIS OF BIOTIN DEFICIENCY

The fact that normal humans have a requirement for biotin has been clearly documented in two situations: prolonged consumption of raw egg white and parenteral nutrition without biotin supplementation in patients with short-gut syndrome and other causes of malabsorption (1). Deficiency of this member of the vitamin B group also has been clearly demonstrated in biotinidase deficiency (6).

The clinical findings and biochemical abnormalities in cases of biotin deficiency include dermatitis around body orifices, conjunctivitis, alopecia, ataxia, and developmental delay (1). The progression of clinical findings in adults, older children, and infants is similar. Typically, the symptoms appear gradually after weeks to several years of egg white feeding or parenteral nutrition. Thinning of hair progresses to loss of all hair, including eyebrows and lashes. A scaly (seborrheic), red (eczematous) skin rash was present in the majority of reports. In several reports, the rash was distributed around the eyes, nose, mouth, and perineal orifices. The appearance of the rash was similar to that of cutaneous candidiasis; *Candida albicans* could often be cultured from the lesions. These manifestations on skin, in conjunction with an unusual distribution of facial fat, have been dubbed "biotin deficiency facies." Depression, lethargy, hallucinations, and paresthesias of the extremities were prominent neurologic symptoms in the majority of adults, while infants showed hypotonia, lethargy, and developmental delay.

In cases severe enough to produce the classic cutaneous and behavioral manifestations of biotin deficiency, urinary excretion rates and plasma concentrations

of biotin are frankly decreased. Urinary excretion of the organic acids discussed in biochemistry section and shown in Figure 2 is frankly increased. The increase is typically 5- to 20-fold or more. However, such a severe degree of biotin deficiency has never been documented to occur spontaneously in a normal individual consuming a mixed general diet.

Of greater current interest and debate are the health consequences, if any, of marginal biotin deficiency. Concerns about the teratogenic effects have led to studies of biotin status during human gestation (44–48). These studies provide evidence that a marginal degree of deficiency develops in at least one-third of women during normal pregnancy. Although the degree of biotin deficiency is not severe enough to produce overt manifestations, the deficiency is severe enough to produce metabolic derangements. A similar marginal degree of biotin deficiency causes high rates of fetal malformations in some mammals (30,49,50). Moreover, data from a multivitamin supplementation study provide significant, albeit indirect, evidence that the marginal degree of deficiency that occurs spontaneously in normal human gestation is teratogenic (44).

Valid indicators of marginal biotin deficiency have been reported. Asymptomatic biotin shortage was induced in normal adults housed in a general clinical research center by egg white feeding. Decreased urinary excretion of biotin, increased urinary excretion of 3-hydroxyisovaleric acid, and decreased activity of propionyl-CoA carboxylase in lymphocytes from peripheral blood are early and sensitive indicators of biotin deficiency (30,31,51). On the basis of a study of only five subjects, 3-hydroxyisovaleric acid excretion in response to a leucine challenge appears to be an even more sensitive indicator of marginal biotin status (31). The plasma concentration of biotin and the urinary excretion of methylglycine, 3-hydroxypropionic acid, and 3-methylcitric acid do not appear to be good indicators of marginal biotin deficiency (52). In a biotin repletion study, the resumption of a mixed general diet produced a trend toward normalization of biotin status within 7 days. This was achieved when the supplement was started immediately at the time of resuming a normal diet. However, supplementation of biotin at 10 times the dietary reference intake (DRI) (300 $\mu\text{g}/\text{day}$) for 14 days reduced 3-hydroxyisovaleric acid excretion completely to normal in only about half of pregnant women who were marginally biotin deficient (47) suggesting a substantial depletion of total body biotin, a substantially increased biotin requirement, or both.

On the basis of decreased lymphocyte carboxylase activities and plasma biotin levels, Velazquez et al. (53) have reported that biotin deficiency occurs in children with severe protein-energy malnutrition. These investigators have speculated that the effects of biotin inadequacy may be responsible for part of the clinical syndrome of protein-energy malnutrition.

Long-term treatment with a variety of anticonvulsants appears to be associated with marginal biotin deficiency severe enough to interfere with amino acid metabolism (54–56). The mechanism may involve both accelerated biotin breakdown (56–58) and impairment of biotin absorption caused by the anticonvulsants (59).

Biotin deficiency has also been reported or inferred in several other circumstances including Leiner disease (60–62), sudden infant death syndrome (63,64), hemodialysis (65–69), gastrointestinal diseases and alcoholism (1), and brittle nails (70). Additional studies are needed to confirm or refute an etiologic link of these conditions to the vitamin's deficiency.

The mechanisms by which biotin deficiency produces specific signs and symptoms remain to be completely delineated. However, several studies have given new insights on this subject. The classic assumption for most water-soluble vitamins is that the clinical findings of deficiency result directly or indirectly from deficient activities of the vitamin-dependent enzymes. On the basis of human studies on deficiency of biotinidase and isolated pyruvate carboxylase, as well as animal experiments regarding biotin deficiency, it is hypothesized that the central nervous system effects of biotin deficiency (hypotonia, seizures, ataxia, and delayed development) are likely mediated through deficiency of brain pyruvate carboxylase and the attendant central nervous system lactic acidosis rather than by disturbances in brain fatty acid composition (71–73). Abnormalities in metabolism of fatty acids are likely important in the pathogenesis of the skin rash and hair loss (74).

Exciting new work has provided evidence for a potential role for biotin in gene expression. These findings will likely provide new insights into the pathogenesis of biotin deficiency (75,76). In 1995, Hymes and Wolf discovered that biotinidase can act as a biotinyl transferase; biocytin serves as the source of biotin, and histones are specifically biotinylated (6). Approximately 25% of total cellular biotinidase activity is located in the nucleus. Zemleni and coworkers have demonstrated that the abundance of biotinylated histones varies with the cell cycle, that these histones are increased approximately twofold compared with quiescent lymphocytes, and that these are debiotinylated enzymatically in a process that is at least partially catalyzed by biotinidase (77–79). These observations suggest that biotin plays a role in regulating DNA transcription and regulation.

Biotinylation of histones is emerging as an important histone modification. Recent studies from Hassan and Zemleni provide evidence that biotinylation likely interacts with other covalent modification of histones to suppress gene expression and gene transposition (80). Although the relative importance in biotinidase and holocarboxylase synthetase in the biotinylation and debiotinylation of histones has yet to be fully elucidated, Gravel and Narang have produced evidence that holocarboxylase synthetase is present in the nucleus in greater quantities than in the cytosol or the mitochondria and that holocarboxylase synthetase likely acts in the nucleus to catalyze the biotinylation of histones (81). Moreover, fibroblasts from patients with HCLS deficiency are severely deficient in histone biotinylation (82). Zemleni and coworkers have shown that biotinylation of lysine-12 in histone H4 (K12BioH4) causes gene repression and have proposed a novel role for HCS in sensing and regulating levels of biotin in eukaryotic cells (83). They have hypothesized that holocarboxylase synthetase senses biotin and that biotin regulates its own cellular uptake by participating in holocarboxylase synthetase-dependent chromatin

remodeling events at an SMVT promoter locus. Specifically, they hypothesize that nuclear translocation of HCS increases in response to biotin supplementation and then biotinylates histone H4 at SMVT promoters, silencing biotin transporter genes. This group has shown that nuclear translocation of HCS is a biotin-dependent process potentially involving tyrosine kinases, histone deacetylases, and histone methyltransferases. The nuclear translocation of holocarboxylase synthetase correlates with biotin concentrations in cell culture media and is inversely linked to SMVT expression. Moreover, biotin homeostasis by holocarboxylase synthetase-dependent chromatin remodeling at an SMVT promoter locus is disrupted in holocarboxylase synthetase knockdown cells.

Transposable elements such as retrotransposons containing long-terminal repeats constitute about half of the human genome, and the transposition events associated with these elements impair genome stability. Epigenetic mechanisms are important for transcriptional repression of retrotransposons, preventing transposition events, and abnormal regulation of genes. Zemleni and coworkers have provided evidence that the covalent binding of biotin to lysine-12 in histone H4 and lysine-9 in histone H2A mediated by holocarboxylase synthetase is an epigenetic mechanism to repress retrotransposon transcription in human and mouse cell lines and in primary cells from a human supplementation study. Abundance of biotinylation at those sites depended on biotin supply and on holocarboxylase synthetase activity and was inversely linked with the abundance of long terminal repeat transcripts. Knockdown of holocarboxylase synthetase in *Drosophila* enhanced retrotransposition. Depletion of biotinylation at those sites in biotin-deficient cells correlated with increased production of transposition events and decreased chromosomal stability.

Recently, controversy has arisen concerning the role of biotin as an *in vivo* covalent modifier of histones. Bailey and coworkers have reported that streptavidin binds to histones independently of biotinylation (84). To further investigate this phenomenon, 293T cells were grown in ^{14}C -biotin; in contrast to the ready detectability of ^{14}C -biotin in carboxylases, ^{14}C -biotin was undetectable in histones (i.e., represented no more than 0.03% of histones) (84). In a subsequent study, Healy and coworkers demonstrated that histone H2A is nonenzymatically biotinylated by biotinyl-5'-AMP and provided evidence that these enzymes promotes biotinylation of histone H2A by releasing biotinyl-5'-AMP, which then biotinylates lysines in histone H2A somewhat nonspecifically (85). Recently, this group has proposed that biotin is not a natural histone modifier at all. On the basis of studies that fail to find *in vivo* biotin incorporation into histones using ^3H -biotin uptake, Western blot analysis of histones, or mass spectrometry of affinity purified histone fragments, these investigators concluded that biotin is absent in native histones to a sensitivity of 1 part per 100,000 and that the regulatory impact on gene expression must occur through a mechanism other than histone modification (86). These conclusions are likely to generate a lively debate until definitive evidence is provided using mass spectrometric analysis of *in vivo* histones harvested at various phases of the cell cycle and at specific locations within particular histones.

Table 2 Adequate Intake for Biotin Consumption

Age	Amount ($\mu\text{g}/\text{day}$)
0–6 mo	5
7–12 mo	6
1–3 yr	8
4–8 yr	12
9–13 yr	20
14–18 yr	25
19– >70 yr	30
Pregnancy	30
Lactation	35

Note: Values for males and females in all age groups were combined because they do not differ.

Source: From Ref. 88.

INDICATIONS AND USAGE

In 1998, the United States Food and Nutrition Board of the National Academy of Sciences reviewed the recommendations for biotin intake (87). The committee concluded that the data were inadequate to justify setting an estimated average requirement. However, adequate intake (AI) was formulated (Table 2). The AI for infants was based on an empirical determination of the biotin content of human milk. Using the value for free biotin determined microbiologically ($6 \mu\text{g}/\text{L}$) and an average consumption of $0.78 \text{ L}/\text{day}$ by infants of age 0–6 months, an AI of $5 \mu\text{g}/\text{day}$ was calculated. The AI for lactating women has been increased by $5 \mu\text{g}/\text{day}$ to allow for the amount of biotin secreted in human milk. Using the AI for 0–6-month-old infants, the reference body weight ratio method was used to extrapolate AIs for other age groups (see Table 2).

TREATMENT OF BIOTIN DEFICIENCY

If biotin deficiency is confirmed, biotin supplementation should be undertaken and effectiveness should be documented. Doses between $100 \mu\text{g}$ and 1 mg are likely to be both effective and safe on the basis of studies supplementing biotin deficiency during pregnancy, chronic anticonvulsant therapy, and biotinidase deficiency.

TOXICITY

Daily doses of up to 200 mg orally and up to 20 mg intravenously have been given to treat biotin-responsive inborn errors of metabolism and acquired biotin deficiency. Toxicity has not been reported.

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Bitter Orange

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INTRODUCTION

Citrus aurantium (*C. aurantium*) is the Latin name for a plant commonly referred to as bitter orange, sour orange, Neroli, Chongcao, or Seville orange. It is a source of synephrine and several other biogenic amines, as well as other bioactive phytochemicals and has been used in dietary supplements for weight loss. In this entry, we discuss the available evidence pertaining to safety and efficacy of *C. aurantium* for weight loss, as examined in animal studies, clinical trials, and case reports.

GENERAL DESCRIPTION

Bitter orange is a member of the Rutaceae family, a hybrid between Pummelo, *Citrus grandis*, and Mandarin, *Citrus reticulata*. Native to Asia, various parts of the plant are used throughout the world for a variety of indications. Bitter orange and its components are commercially available in herbal weight loss supplements, ostensibly for their adrenergic agonistic properties (1), often in combination with other ingredients hypothesized to promote weight loss. Its constituent *p*-octopamine and synephrine alkaloids (SAs) are usually cited as the active ingredients in such products (2). With the banning of ephedra in the United States in 2004, bitter orange has been increasingly included in weight loss supplement formulations. Because of similarities in their constituents and possible mechanisms (both sources of natural alkaloids with sympathomimetic activity), concerns have been raised that bitter orange may carry risks similar to those hypothesized to exist for ephedra (3).

HISTORICAL USE

C. aurantium's origin is in China and appears in writing as far back as 300 BC. Its ancient use has also been documented in Japan and Rome (4). It is native to eastern Africa, Arabia, and Syria and is cultivated in various European, North American, and South American regions. The leaf was historically used as a tonic, laxative, or sedative in Mexico and South America and for insomnia, palpitations, or stomachaches by the European Basque people (5,6). The fruit and peel are also used for stomachaches, as well as high blood pressure (BP), spasm, and a variety of gastrointestinal conditions by both the Basque and practitioners of traditional Chinese medicine (7). While the practice arose in Ancient Egypt, neroli oil is still currently used for aromatherapy and bergamot, a subspecies of *C. aurantium*, is used for flavoring and aroma in Earl Grey teas (8). Modern uses for *C. aurantium* include digestive,

cardiovascular, neuromuscular, and antiseptic indications in countries such as China, Curacao, Haiti, India, Mexico, Trinidad, Turkey, and the United States (9). The most common current western use, however, is as a dietary supplement for weight loss.

POTENTIAL CONSTITUENTS

Some authors (10) state that *C. aurantium* contains *meta*-synephrine (*m*-synephrine, *m*-s), whereas others (11) state that it contains only *para*-synephrine (*p*-synephrine, *p*-s). However, research (I.A. Khan, oral communication, 2005) has shown that *C. aurantium* naturally contains *p*-synephrine and does not contain *m*-synephrine. Allison and colleagues reported that at least one over-the-counter (OTC) product purportedly containing SAs from *C. aurantium* contains both *p*-synephrine and *m*-synephrine (12), raising concerns about possible adulteration and mislabeling. There is also an *ortho* isomer of synephrine (*o*-synephrine), whose content in *C. aurantium* is unknown. *p*-, *m*-, and *o*-synephrine can each exist in D or L forms.

p-Synephrine, an undisputed component of *C. aurantium*, is typically referred to simply as synephrine (13). It is an α -adrenergic agonist (14) that also has some β -adrenergic properties (15). *p*-Synephrine occurs naturally in the human body in small quantities and might act as a neurotransmitter (16). Under the name oxedrine, it has been used since 1927 (17) in eyedrops. *p*-Synephrine is thought to be the ingredient in *C. aurantium* primarily responsible for weight loss. However, neither this nor whether *C. aurantium* actually produces weight loss in humans is firmly established.

m-Synephrine, often referred to as phenylephrine, is an isomer of *p*-synephrine. To the best of our knowledge, *m*-synephrine is not contained naturally in *C. aurantium*. *m*-Synephrine is also an α -adrenergic agonist that has some β -adrenergic agonist properties. It has been studied more extensively than *p*-synephrine and is one of the two most widely used OTC decongestants today (Fig. 1) (13). *p*-Synephrine and *m*-synephrine have similar structure to ephedrine, as well as other substances that have some effects on reducing food intake and/or body weight such as epinephrine and norepinephrine (Fig. 1), supporting the conjecture that, to the extent that function follows structure, *p*-synephrine and *m*-synephrine, may also reduce food intake and or body weight.

The α -adrenergic sympathomimetic amine, *p*-octopamine, is also present in *C. aurantium*, though possibly at inappreciable levels (2). Like both forms of synephrine, it is an α -adrenergic agonist with some

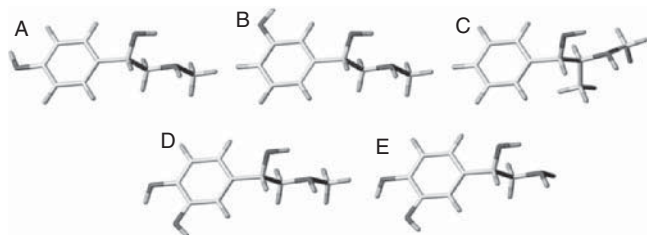


Figure 1 Chemical structures of (A) *p*-synephrine, (B) *m*-synephrine, (C) ephedrine, (D) epinephrine, and (E) norepinephrine.

β -adrenergic properties. It is used to treat hypertension and as a cardiostimulant (13) and has also been examined for its potential role in promoting weight loss (18).

Because of their similar properties and the overlap of their inclusion in supplements, we will refer to these substances collectively as synephrine alkaloids (SAs). SAs are used clinically as decongestants (1), during surgical procedures as a vasopressor (19), for acute treatment of priapism (20), and in ophthalmological examinations for pupil dilation (21). Products that contain *C. aurantium* or its derivatives, including OTC weight loss supplements, will be referred to as *C. aurantium* products (CAPs).

Regulatory oversight for dietary supplements is much less rigorous than for pharmaceuticals, and extensive evidence is not required prior to release of a product on the public market. While a phase of requirements for meeting good manufacturing practices is currently underway, this may help to explain why the quality and quantity of the evidence we have available to evaluate the safety and efficacy of *C. aurantium* is minimal.

POTENTIAL MECHANISMS

As sympathomimetic agents with both α - and β -adrenergic receptor agonist properties, SAs might increase energy expenditure and/or decrease food intake (22). In addition, there is some evidence that adrenergic agonists, including SAs, decrease gastric motility (23). Similar to compounds such as cholecystokinin and other gut peptides which both decrease gastric motility and food intake (24), one might conjecture that SAs may also decrease food intake via reducing gut motility. Activation of lipolysis is a known β -adrenergic activity (25) that may be fueled by these components of *C. aurantium*.

ANIMAL STUDIES

Weight Loss

SAs reduce food intake in rodents (26), and some studies indicate that SAs can reduce rodent body weight (13,26). SAs have also been shown to promote lipolysis in adipocytes through β -adrenergic stimulation (27) and to increase lipoprotein lipase activity in the parametrial fat pad of female hamsters (28). However, among monosodium glutamate-treated obese mice, SAs reduced weight gain but had no effect on body fat percent (29).

Toxicity and Mortality

Data suggest that *m*-synephrine (not present in bitter orange) may prolong life in rodents. A 2-year study by the National Toxicology Program (13) evaluated the effects of *m*-synephrine on spontaneous food intake of rats and mice. At 2 years, there were no significant differences in survival among mice or female rats. However, for male rats, there was a significant reduction in mortality rate, although there was increased mortality in the early phase of the study at the highest dose. It should be noted that too few deaths occurred during the 2-year trial to provide the degree of precision and power desired for a rigorous longevity study (30). Nonetheless, similar results have been reported for ephedrine, another sympathomimetic amine (31).

Arbo et al. (32) conducted a subchronic toxicity study in mice and the effects of *p*-synephrine and *C. aurantium* L. extract on oxidative stress biomarkers that are believed to be indicators of cell membrane injury (malondialdehyde) and (glutathione and the enzyme glutathione peroxidase) indicative of amphetamine-induced toxicity. The study evaluated adult male CF1 mice treated with 400, 2000, or 4000 mg/kg *C. aurantium* dried extract and *p*-synephrine 30 or 300 mg/kg over the course of 28 days. Results showed a reduction in glutathione in mice treated with *C. aurantium* 400 mg/kg and *p*-synephrine 30 and 300 mg/kg. Inhibition of glutathione peroxidase activity occurred within mice treated with *C. aurantium* 400 and 2000 mg/kg and *p*-synephrine 30 and 300 mg/kg; however, no change occurred within malondialdehyde levels. These two findings suggest the possibility of subchronic toxicity. No significant change in weight occurred in any of the groups, suggesting on the positive side a lack of severe toxicity, and on the negative side a lack of efficacy in producing weight loss.

With regard to adverse effects, a study (33) of male Sprague-Dawley rats reported what was believed to be evidence of cardiotoxicity when *C. aurantium* fruit extracts standardized to 4% and 6% SAs were administered. Increased mortality has been observed among CAPs-treated rats (33) as well as a strain of mice selected to be uniquely susceptible to the effects of adrenergic stimulation (34).

CLINICAL TRIALS

Weight Loss

Few clinical trials have examined the effects of CAPs alone or in combination with other ingredients on body weight and/or body composition (Table 1). It should be kept in mind that these trials are of short duration and the sample sizes are frequently quite small. Nonetheless, these trials suggest that body weight and/or fat loss may be enhanced by CAPs or SAs. The mechanisms involved are unclear but may be partially due to a suppressing effect of appetite and/or a moderate increase in resting energy expenditure.

Armstrong et al. (37) evaluated exercise and herbal preparation containing Ma Huang, bitter orange (5 mg SAs), and guarana over 6 weeks in a randomized, controlled trial. Compared with controls, the intervention group obtained significant reductions in fat mass and a nearly significant reduction in body mass index (kg/m^2).

Table 1 Summary of Clinical Weight Loss Trials

Reference	Treatment	Design	Sample size	Duration	Results	Comments
Colker et al. (10)	975 mg <i>Citrus aurantium</i> , +528 caffeine and 900 mg St. John's wort; placebo (with pill) and control (no pill)	Blinded parallel groups RCT	Supplement $n = 9$; placebo, $n = 7$; control group (no pills), $n = 4$	6 wk	Supplement group lost more fat (3.1 kg; $P < 0.05$) than other groups and increased RMR (2–3%)	<i>Citrus aurantium</i> may assist individuals in losing body fat, due to increased energy and reduced energy intake expenditure. No adverse events were reported
Kalman et al. (36)	Ephedrine and synephrine alkaloids (SAs) (5 mg twice daily) based product vs. placebo with exercise and diet	Prospective, randomized, double blind	30 overweight subjects; BMI > 27	8 wk	3.4 kg weight loss in experimental group vs. 2.05 kg in placebo ($P < 0.05$)	No adverse events; findings indicate apparent short-term safety and efficacy of ephedrine and synephrine-based compound
Armstrong et al. (37)	Exercise program with assignment to drug (Ma Huang, bitter orange, and guarana) or placebo. Bitter orange standardized for 5 mg synephrine	Randomized trial—unclear if study is blinded	Five overweight males/14 females	44 days	Supplement increased fat loss (2.5 kg; $P = 0.033$) more than placebo (0.5 kg)	Low statistical power, no marked side effects
Greenway et al. (38): Pilot 1	Two capsules containing pantothenic acid, 40 mg; green tea leaf extract, 200 mg; guarana extract, 550 mg; bitter orange, 150 mg; white willow bark extract, 50 mg; ginger root, 10 mg; proprietary charge thermoblend (L-tyrosine, L-carnitine, naringin), 375 mg	Prospective, randomized, double blind	Eight subjects (1:1 ratio) between supplement group and placebo group	8 wk	Supplement group gained more weight (1.04 ± 0.27 kg; $P < 0.04$) than placebo and increased RMR (but not at 8 wk)	CAP was not efficacious for weight loss
Greenway et al. (38): Pilot 2	<i>m</i> -Synephrine 20 mg	Prospective, randomized, double blind	Twenty subjects (1:1 ratio) between supplement group and placebo group	8 wk	Supplement group lost weight (0.8 ± 3.4 kg; <i>not significant</i>) in 8 wk, and increased RMR in 8 wk. No control group was used (Greenway, written communication, November 1, 2009)	<i>m</i> -Synephrine was not efficacious for weight loss

Abbreviations: BMI, body mass index; CAP, *Citrus aurantium* product; RCT, randomized, controlled trial; RMR, resting metabolic rate.

and fat percentage. No significant changes were noted in resting energy expenditure, blood chemistries, or dietary intake between the placebo and experimental groups.

In a double-blind, placebo-controlled, randomized trial, Colker et al. found that subjects receiving a combination of *C. aurantium*, caffeine, and St. John's wort, along with diet and exercise protocols, lost a statistically significant amount of body weight. Analysis comparing changes in this group with those in placebo or control groups on the same diet and exercise regimen did not show significant differences, though loss of fat mass was significantly greater in the experimental group (35). BP, heart rate, electrocardiographic, blood, and urine analyses were not significantly different between the groups.

Another randomized trial (36) of 30 overweight adults investigated the effects of supplementation, along with a cross-training exercise regimen and dietary education program compared with exercise and dietary education alone on body composition. Supplementation included ephedrine, SAs, caffeine, and calicine. Greater weight and fat loss occurred for the supplement group compared with the exercise–diet only group.

Overall, studies indicate a weight loss of 2.4–3.4 kg among participants using SAs, while placebo groups lost 0.94–2.05 kg, suggesting the plausibility of some weight loss benefit from SA supplementation, beyond diet and exercise alone. However, these studies do not separate

the effects of *C. aurantium* or SAs from other ingredients, particularly ephedrine and caffeine.

Metabolic Rate and Cardiovascular Effects

Several studies have evaluated the effects of acute administration of SAs on cardiovascular indicators. Kalman et al. (39) tested a product containing 335 mg Ma Huang standardized for 20 mg ephedrine alkaloids, 910 mg guarana standardized for 200 mg caffeine, and 85 mg bitter orange standardized for 5 mg SAs per two capsules. Twenty-seven overweight adults were randomized to treatment or placebo for 14 days. BP, heart rate, electrocardiogram, and Doppler echocardiograms were evaluated before and after treatment. Ingestion of this commercial weight loss supplement did not produce any detectable cardiovascular side effects.

Penzak et al. (10) examined cardiovascular outcomes in 12 normotensive individuals who were administered 8 oz of Seville orange juice (containing 13–14 mg SAs) and water in a crossover fashion, followed by a repeat ingestion 8 hours later. No changes in cardiovascular indices (BP, maximal arterial pressure, and heart rate) were detected.

Thomas et al. (40) evaluated the cardiovascular effects of 10 mg oral SAs in healthy volunteers over a 4-hour period on impedance cardiography and forearm plethysmography. Elevation in total peripheral resistance was observed 30–60 minutes after dosing, although other hemodynamic indexes were not affected.

Hemodynamic effects were observed in a crossover design, placebo-controlled study (41) with the administration of Xenadrine, a CAP that contains a variety of other potentially bioactive substances, including green tea extract, cocoa extract, yerba mate, ginger root, grape seed extract, and others. However, these increases in heart rate, and systolic and diastolic BP were not observed with administration of Advantra Z, which contains *C. aurantium* alone, even at an eightfold higher dose.

Haller et al. (42) evaluated a dietary supplement [Ripped Fuel Extreme Cut, containing synephrine from *C. aurantium* (presumably *p*-synephrine) and caffeine] in 10 healthy adults (three women) aged 20–31 years. Each subject was given one dose of the dietary supplement under three conditions: (i) resting conditions (without placebo); (ii) moderately intense exercise; and (iii) placebo plus moderately intense exercise in a three-arm, randomized, crossover study. Greater postexercise diastolic BP was seen with the dietary supplement plus exercise than with placebo plus exercise. There were no obvious supplement effects on postexercise HR, systolic BP, or body temperature.

Bui et al. (43) reported the effect on BP (systolic and diastolic) and heart rate over 6 hours after one dose of a CAP (Nature's Way Bitter Orange) on 15 young, healthy adults in this prospective, randomized, double-blind, placebo-controlled, crossover study. Systolic and diastolic BP increased significantly within the 1–5 hours time period in comparison with the placebo group with the peak being 7.3 ± 4.6 mm Hg, while the 4–5 hours time period increase was 2.6 ± 3.8 mm Hg after consumption in comparison with the placebo group with the peak being 4.2 ± 4.5 beats/minute, while

heart rate was significantly elevated 2–5 hours after ingestion.

In one study of obese adults, increases in resting metabolic rate (RMR) were observed with *C. aurantium*, both alone and with food, beyond the thermic effect of food (TEF) alone (44). (RMR is a measure of the energy required to maintain basic physiological function while the body is at rest.) However, another recent investigation (45) found that the thermic response to CAPs increased in women only, who had lower TEF than men at baseline. After the intervention, TEF did not differ by gender. BP and pulse rate were not affected, but epinephrine secretion increased. In normal weight adults, an increase in RMR was also found when the extract was taken with a meal (46). No adverse changes in pulse rate or BP were reported.

Finally, the effects of two dietary supplement formulas on RMR and other metabolic indicators were evaluated (47). When compared with placebo, Formula A (containing ephedra, guarana, green tea, yohimbe, and quercetin) and Formula B (containing *C. aurantium*, jing jie, fang feng, guarana, green tea, yohimbe, and quercetin) resulted in increased total RMR, decreased respiratory exchange ratio toward fat burning, and increased body core temperature. Heart rate and RMR increased at each 15-minute interval with Formula A only. BP increased with both, but to a greater extent with Formula A.

CASE REPORTS OF ADVERSE EVENTS

Nykamp et al. (48) describe a case of acute lateral-wall myocardial infarction co-occurring with consumption of CAPs in a 55-year-old woman with undetected coronary vascular disease. She reported taking a multicomponent dietary weight loss supplement containing 300 mg of bitter orange over the preceding year.

A Consumer Reports article (49) describes a 21-year-old woman who took ephedra-free *Xenadrine EFX* (which contains *C. aurantium*). After 3 weeks on the supplement, she suffered a seizure. Her neurologist believes the bitter orange in the supplement was the most likely the cause, though the basis for this conclusion is unknown.

Nasir et al. (50) described exercise-induced syncope in a healthy 22-year-old woman that occurred 1 hour after a second dose of *Xenadrine EFX*, a weight loss supplement that contains, among other compounds, ephedrine and synephrine. The electrocardiography revealed prolongation of the QT interval, which resolved in 24 hours.

Bouchard et al. (51) report a case of a 38-year-old male patient with ischemic stroke that occurred after taking a CAP for 1 week. The patient reportedly had no relevant medical history or major atherosclerotic risk factors and took no other medications.

Gray and Woolf (52) reported a case of CAPs use by an adolescent with anorexia nervosa and raised concerns that the SAs may have masked bradycardia and hypotension while exacerbating her weight loss. Firenzuoli et al. (53) report a case of a 52-year-old woman that had an allergic reaction after taking a CAPs product.

Sultan et al. (54) reported a case of a 52-year-old woman with ischemic colitis that occurred 1 week after consumption of a CAP (Natural Max Skinny Fast, containing bitter orange). She reported no known drug

Table 2 Summary of Effects, Safety, and Efficacy of *Citrus aurantium*

Physiological effects	Effects on weight	Effects on body composition	Safety
Variable changes in BP in animals; generally stable BP, heart rate, pulse rate, blood and urine measures in humans; inconsistent changes to resting metabolic rate	Weight loss documented in rodents; weakly supported in humans, as studies used multiple supplements or did not find significant difference from controls	Limited support for loss of fat mass in human studies, noting a trend or using multiple supplements; for animals, some increased lipase activity	Inconsistent mortality data in rodents; some evidence of elevated BP. Results not consistent from study to study, but this may be a function of small sample sizes used in most studies. Several case reports of serious adverse events

allergies and took no other medications. Symptoms resolved over 24–48 hours with conservative management after the supplement was discontinued.

Health Canada reported that from January 1, 1998, to February 28, 2004, it received 16 reports in which products containing bitter orange or synephrine were suspected of being associated with cardiovascular events, including tachycardia, cardiac arrest, ventricular fibrillation, transient collapse, and blackout. All cases were considered serious (55).

Adverse events from CAPs are currently fairly rare in scientific literature. As CAPs are used more widely in place of ephedrine-containing products, any potentially harmful effects may be clarified over time.

DISCUSSION

The Safety of CAPs

Some have hailed the potential therapeutic value of CAPs (1), while others have warned about possible safety concerns (33). The safety concerns pertain primarily to adverse cardiovascular and cerebrovascular effects. Information on the safety of CAPs comes from the three sources described above: animal studies, clinical trials, and case reports. To date, no large epidemiologic (case control or cohort) studies have evaluated the safety of CAPs.

Of course, one cannot extrapolate the safety of CAPs from short-term studies used for one indication (e.g., several days for relief of nasal congestion among the general population) to long-term studies use for another indication (e.g., several months or years for weight loss among obese individuals). Although substantial safety-related data exist for CAPs (13,56), there is no published human weight loss trial of CAPs with more than 20 participants or for a duration of more than 7 weeks.

It is important to note that the majority of studies evaluating the safety of CAPs are performed with normotensive subjects. However, because hypertension is a common comorbidity associated with overweight/obesity, studies that evaluate the effects of CAPs on BP should also be conducted with obese hypertensive adults.

While *C. aurantium* extracts have been used in a variety of cultures for thousands of years, they have not been traditionally utilized for long periods of time, or specifically for weight loss (1). As such, there is little, if any, basis for making definitive statements about the intermediate or long-term safety/risk of CAPs used for weight loss. Table 2 summarizes the physiological effects, safety, and weight loss efficacy of *C. aurantium*.

DOSE CONSIDERATIONS

Given the dearth of weight loss trials, the optimal dose (if one exists) of *C. aurantium* or its SA constituents for weight loss is unknown. Table 3 highlights some relevant dosage information. Although generalizing across species and compounds is difficult and can only provide a limited basis for conjecture, the following comparisons with ephedrine can be made. We analyzed data (12) in which ephedrine or SAs was given to mice. Regression of weight and food intake on dose of ephedrine or SAs yielded slopes (in absolute value) that were approximately four to six times greater for ephedrine than for SAs. Based on linear projections, it would take four to six times the dose of SAs (in these mice) to achieve equivalent reduction in intake and body weight as for ephedrine. In human studies of ephedrine, doses of about 50 mg per day begin to be effective (57). Although an extrapolation, this might suggest a useful clinical dose for SAs as high as 240–360 mg

Table 3 Dosage Information on *Citrus aurantium* or Synephrine Alkaloids (SAs)

Dose	
5–14 mg/day	<i>Citrus aurantium</i> extract with SAs has been used (34–36) and no serious adverse events were reported. These doses <i>purportedly</i> showed efficacy, but products tested included substances beyond <i>C. aurantium</i> , notably ephedrine which we know to be effective for weight loss. We believe that these doses of SAs are very unlikely to be effective when used without ephedrine
32 mg/day	The nasal decongestant Endal (60) contains 20 mg of <i>m-s</i> per tablet and two tablets per dose twice per day are recommended
120 mg/day	Via <i>C. aurantium</i> extract, SAs are marketed in over-the-counter (OTC) products for weight loss. In products, such as Nutres Lipo 6 (61), the directions suggest that for “extreme fat loss” a recommended dosage is two capsules three times per day. The SA content per capsule is 20 mg; this provided a maximal recommended dose of 120 mg/day
300 mg/day	According to <i>Clarke’s Analysis of Drugs and Poisons</i> (62), oxedrine (<i>p</i> -synephrine) is used clinically at ~300 mg/day
1000 mg/day	Minimum adult lethal dose of <i>m-s</i> (63)

per day. From a safety point of view, SAs (per equal weight) have lower potential to raise BP than ephedrine; however, nearly all commercial preparations of SAs also contain caffeine, which might compound any cardiovascular effects. In the absence of caffeine, human studies suggest that 15–30 times the dose of SAs are required to elevate BP to the same degree as ephedrine (58,59). This suggests that such high doses might be well tolerated, but clearly more data are needed, particularly regarding potential synergistic effects of CAPs components.

SAs appear to be readily absorbed after oral administration (63). About 80% of oral doses are excreted in the urine within 24 hours. After single oral doses, peak plasma concentrations are typically reached in 1–2 hours. Plasma half-life is ~2–3 hours. Sympathomimetic drugs for weight loss are typically given TID before meals (64) reducing the evening dose if sleep problems arise.

CONTRAINDICATIONS

Topical application (as with aromatherapy or antifungal uses) of CAPs may result in photosensitivity for fair-skinned individuals (65) (possibly due to photosensitizing furanocoumarins that occur in the rinds of certain citrus species, especially immature fruits). Although rare, this has also occurred after oral ingestion. To reduce this risk, exposure to ultraviolet light can be minimized. Caution is recommended for use in children, as it may conceivably produce toxic effects (66). Some sources advise that CAPs should be avoided by women who are pregnant or breast-feeding (7,67), while others claim that CAPs can be used safely during pregnancy (66). While effects on BP are unclear, those with hypertension, tachyarrhythmia, or narrow-angled glaucoma may consider refraining from use of CAPs until further evidence confirms their safety (67). CAPs could also possibly exacerbate symptoms for those with stomach or intestinal ulcers (68).

DRUG INTERACTIONS

Because CAPs may increase stomach acid, they could potentially reduce the efficacy of acid-lowering drugs, such as antacids and ulcer medications (69). Although a speculative precaution, those taking medications containing SAs, including some cold medications and monoamine oxidase inhibitors (MAOIs), should consider the combined dose of these products with the SAs present in CAPs formulations and possible multiplicative effects (68,69). It has been suggested that CAPs could interfere with the activity of drugs that are metabolized by the liver enzyme cytochrome P450-3A, CYP3A (70,71). A recent comment in *Experimental Biology and Medicine* noted that some research on drug effects have utilized parts of the plant or methods of administration that may not be applicable to oral consumption of currently marketed dietary supplements (72).

FUTURE RESEARCH

The safety and efficacy of CAPs and SAs for weight loss are not well established. While existing literature

demonstrates plausibility for reducing weight, previous trials were not designed to rigorously evaluate safety and efficacy. Doing so will require better-designed randomized clinical trials with large sample sizes, reliable well-established outcome measures, and active surveillance of side effects and adverse events. To better understand the effects of CAPs or SAs specifically, studies will need to test these components without combining them with other ingredients postulated to have antiobesity effects. It would also be worthwhile to examine differences between the types of synephrine-containing compounds that are derived from various sources and how this influences the consistency and potency of supplements.

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Black Cohosh

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INTRODUCTION

Black cohosh is a native eastern North American plant that was used as traditional medicine by Native Americans. Extracts of the roots and rhizomes were used for analgesic, sedative, and anti-inflammatory properties. More recently, root and rhizome black cohosh preparations have had a rich clinical history, spanning almost 60 years of study. These studies have primarily focused on relieving climacteric symptoms associated with menopause as a possible alternative to classical hormone or estrogen replacement therapy.

BACKGROUND

The common name for black cohosh [*Actaea racemosa* L. syn., *Cimicifuga racemosa* (L.) Nutt. (Ranunculaceae, Buttercup Family)] originated with North American Indians. The term cohosh is thought to be an Algonquian word meaning "rough," with reference to the texture of the thick, knotted roots and underground stems (rhizomes). A New World plant used by Native Americans, it was most abundant in the Ohio River Valley, but it could also be found from Maine to Wisconsin, south along the Allegheny Mountains to Georgia, and west to Missouri.

Various common names have been used to refer to black cohosh, including black snakeroot, bugbane, rattleroot, squawroot, and macrotys. It is a member of the Ranunculaceae or Buttercup family, which includes other medicinal plants such as aconite, goldenseal, and pulsatilla. It has been known by the scientific name *C. racemosa* and recently has been reassigned to *A. racemosa*. The generic name *Cimicifuga* derives from the Latin *cimex* (a kind of bug) and *fugare* (to put to flight), which is perhaps indicative of the use of some strongly smelling close relatives to repel insects. The specific epithet *racemosa* refers to the flowering stalk, termed a raceme. The name rattleroot is indicative of the rattling sound made by the dry seeds in their pods. This plant prefers the shade of rich open hardwood forests, but it will tolerate some sunny spots.

Black cohosh has been used clinically for relief of climacteric symptoms for more than 60 years, and its popularity in the United States as a botanical dietary supplement has increased due to the recently recognized potential risks associated with classical estrogen replacement therapy or hormone replacement therapy (1,2). The part of the black cohosh plant used in medicinal preparations is the root and rhizome. It was officially recognized in the

United States Pharmacopeia (USP) from the first edition in 1820 to 1936 and in the National Formulary from 1936 to 1950. The eclectic physicians used a preparation of black cohosh called macrotys. It was considered one of the best-known, specific medicines for heavy, tensive, and aching pains as it was noted to have a direct influence on the female reproductive organs.

While the mechanism of action has not been completely elucidated, recent literature suggests that alleviation of climacteric symptoms is mediated through neurotransmitter regulation and not through classical estrogen receptor (ER) endocrine pathways (3,4).

CHEMISTRY

More than 60 triterpene glycosides, most with a 9,19 cycloartane skeleton, and unique to *Actaea* spp., have been reported from the roots and rhizomes of *A. racemosa* (5,6). The compound 23-*epi*-26-deoxyactein (formerly 27-deoxyactein) is the constituent usually selected for standardization of commercial products based on its abundance in the roots and rhizomes (7–12). The pharmacokinetics of 23-*epi*-26-deoxyactein in serum and urine has recently been reported (13). While triterpenes are structurally similar to steroids and possess a broad range of biological activity (14–17), no significant ligand binding affinity was found toward ER- β in the evaluation of 23-*epi*-26-deoxyactein, cimicifugoside F and cimicifugoside, and their respective aglycones (18). This, coupled with the lack of demonstrated estrogenic activity in *A. racemosa* extracts, has called into question the notion that black cohosh acts through direct ER binding by the triterpenes, as has been hypothesized (19–23).

In addition to the triterpene saponins, the roots and rhizomes of black cohosh also contain a number of aromatic acids/polyphenols that possess a wide array of biological activities (5,24–26). Caffeic acid, which is found widely across all species of flowering plants, has shown pregnant mare antigonadotropin activity (27–29), rat uterine antispasmodic activity (30), and smooth muscle relaxant/antispasmodic activity in rats (31) and guinea pig ileum (32). Ferulic acid, also more or less ubiquitous among flowering plants, has demonstrated luteinizing hormone (LH) release inhibition (33), follicle-stimulating hormone (FSH) release stimulation (33), antiestrogenic activity (34), prolactin stimulation in cows (35) and inhibition in rats (33), and uterine relaxant/antispasmodic activity in rats (36). Fukinolic acid produced an estrogenic effect on MCF-7 cells with reference to estradiol

(37). A more recent study refuted this effect and demonstrated a lack of estrogenic effect for 10 other phenolic esters, many of which are unique to *Actaea* spp. (caffeoylglycolic acid; 2-caffeoylpiscidic acid (cimicifugic acid D); 3,4-dihydroxyphenacyl caffeate (petasiphenone); 3,4-dihydroxyphenyl-2-oxopropyl isoferulate (cimiciphenol); 3,4-dihydroxyphenacyl isoferulate (cimiciphenone); cimicifugic acids A, B, E, F; and fukiic acid) from black cohosh (38).

Studies on the phenolic acid constituents of black cohosh have shown antioxidant activity (24,39) that may correlate with or prove useful in the determination of the mechanism of action of black cohosh. In addition, a number of plant sterols and fatty acids, generally regarded as ubiquitous in the plant kingdom, are contained in the roots and rhizomes for which the biological activities probably do not relate to the mechanism of action of black cohosh (5). In the past 5 years, novel guanidine alkaloids have been isolated from *A. racemosa* underground parts (40,41). New phytochemical methodology called pH zone refinement gradient centrifugal partitioning chromatography coupled with a sensitive liquid chromatography–mass spectral dereplication method led to the identification of *N*-(omega)-methylserotonin as a potential active principle with serotonergic properties (41). Alkaloids have also been reported from other *Actaea* spp. roots and rhizomes (42,43).

There has been some debate over the occurrence of the weakly estrogenic compound formononetin in the plant (44–49). Although there has been at least one report of its occurrence in *A. racemosa* (46), prior studies using plant material collected from different sites in the Eastern United States at different times of the year failed to find formononetin (47,48). More recent studies on both commercial black cohosh products and wild-crafted material, incorporating both high-performance liquid chromatography with mass spectral and photodiode array detection, confirmed the prior findings of no detectable formononetin in black cohosh (8,49).

BOTANICAL DESCRIPTION

A. racemosa syn. *C. racemosa* is an erect, smooth-stemmed perennial 1–2.5 m in height. Large compound leaves are alternately arranged and triternate on short clasping petioles. Basal leaf petioles are grooved in young specimens. This shallow, narrow sulcus in *A. racemosa* disappears as the petiole enlarges, whereas it remains present throughout the life of the two related eastern North American species, *A. cordifolia* DC syn. *C. rubifolia* Kearney and *A. podocarpa* DC syn. *C. americana* Michx (50). Terminal leaflets of *A. racemosa* are acute and glabrous with sharp serrated margins, often trilobate, occasionally bilobed. Fruits are ovoid follicles occurring sessile on the pedicel. The flowering portion, the raceme, is a long wand-like structure with showy white flowers. The flowers possess numerous characteristic stamen and slender filaments with distinctive white anthers (51). The roots and rhizomes are branched and knotted structures with a dark brown exterior and are internally white and mealy or brown and waxy. The upper rhizome surface has several buds and numerous large stem bases terminated frequently by deep, cup-shaped, radiating scars, each of which show a radiate

structure or less frequently fibrous strands. Lower and lateral surfaces exhibit numerous root scars and a few short roots. The fracture is horny, the odor slight, and the taste bitter and acrid (52).

EFFECTS ON CLIMACTERIC SYMPTOMS RELATED TO MENOPAUSE

With a history of clinical study spanning almost 60 years, mainly in Europe (53), black cohosh is one of the more popular alternatives to hormone replacement therapy. Most of the clinical research over this span has been performed on the product known as Remifemin[®], whose formula has changed over the years. However, a number of other commercial formulations are also available. In 2007, black cohosh was the 50th best-selling dietary supplement in the United States with sales of approximately \$52 million (USD), according to the Nutrition Business Journal (54).

Black cohosh clinical study outcomes have been evaluated using a variety of tools, including self- or physician assessments of symptom scores and physiological parameters. Typical measurements include psychological, neurovegetative, somatic, and physiological markers of menopause or relief from the climacteric symptoms of menopause. As in all clinical trials, study design is vital, so studies that are adequately powered, incorporate proper controls, and are designed to address confounders relevant to climacteric symptoms such as the placebo effect and botanical product quality should be given more weight than studies that are not as well designed (55–59).

Placebo effects in menopausal trials are generally large (60) and reflect underlying fluctuations of symptoms. Therefore, any well-designed study must adjust the appropriate variables (i.e., study duration, number of subjects (*n*), and/or dosage) to account for such an effect. In the evidence-based medicine model, the gold standard in terms of efficacy involves randomized, controlled trials (RCTs). Many RCTs on black cohosh exist. When high-quality studies are combined, more than 3000 subjects have been randomized, with the more recent studies adding layers of design sophistication. For example, double-blind, multicenter, placebo-controlled trials that provide details regarding clinical material specifications are becoming more prevalent (55–60).

A recent phase III, double-blind, randomized, placebo-controlled crossover trial of the effectiveness of black cohosh for the management of hot flashes was conducted over two 4-week periods (one capsule, 20 mg bid) (61). The study used a daily hot flash diary and found that subjects receiving the black cohosh material reported a mean 20% decrease in hot flash score (comparing the fourth treatment week to the baseline week) versus a 27% decrease for patients on placebo ($P = 0.53$), mean hot flash frequency was reduced 17% in the black cohosh group and 26% on placebo ($P = 0.36$). Thus, the authors concluded that the study did not provide any evidence that black cohosh reduced hot flashes more than the placebo. Critics of the study point to the short duration and low dose as potential confounders of the results.

The Herbal Alternatives for Menopause trial or HALT trial compared the efficacy of 160 mg daily black

cohosh against several other interventions (200 mg daily multibotanical with black cohosh and nine other ingredients; 200 mg daily multibotanical plus dietary soy counseling; 0.625 mg daily conjugated equine estrogen with or without 2.5 mg medroxyprogesterone acetate daily; and placebo) in 351 menopausal and postmenopausal women of ages 45–55 years with two or more vasomotor symptoms per day. Results did not suggest efficacy for any of the herbal interventions when compared with placebo at any time point over the 1-year course of the study (62).

The Jacobson study (63), spanning only 60 days of treatment, suggests that the short study duration may have limited the findings (60). In addition, all the study participants had a history of breast cancer. The authors reported that the median number of hot flashes decreased 27% in both the placebo and black cohosh groups. No significant differences were observed between groups. Thus, black cohosh, on the basis of this study, was no more effective than placebo in the treatment of hot flashes. The source and formulation of the extract used in this study was not specified. A more recent open-label study that treated breast cancer survivors with either Tamoxifen[®] or a combination of BNO 1055, a proprietary black cohosh extract, with Tamoxifen suggested a reduction in the number and severity of hot flashes in the combination treatment group (64).

In another randomized, double-blind, placebo-controlled study that lasted 12 weeks, black cohosh was compared with standard conjugated estrogen (CE) therapy (0.625 mg/daily). Patients' physical and psychological symptoms were measured every 4 weeks. The end result of the study was that the patients treated with black cohosh had significantly lower index scores on both the Kupperman menopausal (KM) and the Hamilton menopausal (HAM-A) scales compared with placebo, indicating a decrease in severity and frequency of hot flashes. In addition, this study showed an increase in the number of estrogenized cells in the vaginal epithelium in the black cohosh treatment arm, which could indicate an estrogenic action in this tissue (65).

In 2003, a similar study compared effects of two different preparations of BNO 1055 extract and CE therapy on climacteric symptoms and serum markers of bone metabolism (66). The study outcomes were evaluated using patient self-assessment (diary and menopause rating scale), CrossLaps (to measure bone resorption), bone specific alkaline phosphatase (marker of bone formation), and endometrial thickness (measured by ultrasound). Both BNO 1055 extracts were equipotent to CE therapy and significantly greater than placebo at reducing climacteric complaints. In addition, the study showed that both BNO 1055 preparations had beneficial effects on bone metabolism in serum. Specifically, an increase in bone-specific alkaline phosphatase and no reduction in bone resorption were noted indicating an increase in bone turnover formation. No change in endometrial thickness was observed in either BNO 1055 treatment groups, but it was significantly increased with CE therapy. An increase in superficial vaginal cells was observed in the CE and both BNO 1055 treatment groups. The authors of the study hypothesized that the activity of both BNO 1055 preparations was similar to the effects of selective estrogen receptor modulating (SERM), that is, Raloxifene[®] therapy on

bone and neurovegetative climacteric symptoms, without any uterotrophic effects (66).

A recent high-quality, double-blind, randomized study evaluated the effects of two dosages (low, 39 mg; high, 127 mg) of a Remifemin extract on menopausal symptoms. Effectiveness was measured using the KM index, self-assessment depression scale (SDS), clinical global impression scale (CGI), serum levels of LH and FSH, sex hormone-binding globulin, prolactin, 17- β -estradiol, and vaginal cytology. Reductions in the KM and SDS indices were significant. Global efficacy (CGI) was scored at good to very good in 80% (low dosage) and 90% (high dosage) of the patients in the treatment groups (67). No effect on serum hormone levels or vaginal cytology was shown, prompting the authors of the study to suggest that black cohosh does not have a direct estrogenic effect on the serum hormone levels or vaginal epithelium (68). Two recent open-label studies using unspecified types of extracts reported reduced KM index scores. One study reported a significant reduction in 1 month (69), while the other, which also used the HAM-A scale, recorded a 90% improvement in climacteric symptoms in menopausal women after 3 months of black cohosh administration (70).

Chung and colleagues (71) examined a combination of black cohosh and St. John's wort (Gynoplus[®]) in a multicenter RCT in 89 peri- or postmenopausal women with climacteric symptoms. Subjects were treated for 12 weeks with either the Gynoplus extract or placebo. In addition to climacteric complaints, investigators also examined effects on vaginal atrophy, serum hormone levels (FSH, LH), and lipid profiles [total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein cholesterol, and triglyceride]. Significant improvements in climacteric symptoms and hot flashes, as well as an increase in HDL, were observed in the Gynoplus group by 4 weeks and maintained after 12 weeks, but there was no significant impact on vaginal atrophy.

In a 12-month, randomized, four-arm, double-blind clinical trial of standardized black cohosh, red clover, placebo, and 0.625 mg conjugated equine estrogens plus 2.5 mg medroxyprogesterone acetate (conjugated equine estrogens (CEE) and medroxyprogesterone acetate (MPA); $n = 89$), black cohosh did not significantly reduce the frequency of vasomotor symptoms as compared with placebo. The primary outcome measures were reduction in vasomotor symptoms (hot flashes and night sweats) by black cohosh and red clover compared with placebo; secondary outcomes included safety evaluation, reduction in somatic symptoms, relief of sexual dysfunction, and overall improvement in quality of life. Reductions in number of vasomotor symptoms after a 12-month intervention were as follows: black cohosh (34%), red clover (57%), placebo (63%), and CEE/MPA (94%), with only CEE/MPA differing significantly from placebo. Secondary measures indicated that both botanicals were safe as administered. In general, there were no improvements in other menopausal symptoms (72).

A 12-week trial investigating the effects of black cohosh on menopause-related anxiety disorder found no statistically significant anxiolytic effect of black cohosh versus placebo. However, small sample size, choice of black cohosh preparation, and dosage used may have

contributed to the negative results according to the study's authors (73).

More details of the human studies discussed here, as well as others, are presented in Table 1.

BIOCHEMISTRY AND FUNCTIONS

Despite the extensive clinical research, the mechanism of action of black cohosh on menopausal and other symptoms remains unclear, which is consistent with the varied results from clinical trials. A majority of the older literature suggest a direct estrogenic effect. More recent hypotheses have proposed an effect on the limbic system (hypothalamus) or an effect on the neurotransmitters involved in regulation of this system as being responsible for the activity of black cohosh. Data fall into the following categories.

Estrogen Receptor Competitive Binding

The first report of ER-binding activity of black cohosh indicated this as a possible mechanism of action (74). Additional studies were carried out to substantiate this purported endocrine activity (75,76). However, a factor frequently overlooked regarding black cohosh receptor binding studies is the lipophilic nature of the extracts tested. Chemically, lipophilic extracts and fractions that display ER-binding activity are significantly different from the typical hydroalcoholic extracts used to make products for human consumption. A lipophilic extract of the plant showed relatively weak (35 $\mu\text{g/mL}$) ER binding on rat uteri (75). Another study also confirmed the ER-binding activity of an unspecified lipophilic subfraction on ovariectomized (ovx) rat uterine cells, with no binding activity seen with a hydroalcoholic extract (76).

Recent reports have contradicted the ER-binding affinity of black cohosh extracts (4,20,22,77,78). A root extract tested in an in vitro competitive cytosolic ER (from livers of ovx rat) binding assay with diethylstilbesterol (50), an inhibitor of estrogen binding, showed a significant inhibition of estradiol binding in the presence of diethylstilbesterol (77). However, no binding was demonstrated for the black cohosh extract. A hydroalcoholic *A. racemosa* rhizome extract (50% aqueous ethanol) was assayed for ER binding in intact human breast cancer cell lines MCF-7 and T-47-D. Again no binding affinity was shown for the black cohosh extract. However, binding activity was evident for other hydroalcoholic plant extracts, such as red clover (78). In another study, a high concentration (200 $\mu\text{g/mL}$) methanol extract of black cohosh displayed no binding affinity for recombinant diluted ER- α and ER- β (20). A study using BNO 1055 showed contrasting results (79). The extract displayed dose-dependent competition with radio-labeled estradiol in both a porcine and human endometrial cytosolic ER ligand-binding assay system. However, the extract did not displace human recombinant ER- α and ER- β . These contradictory findings prompted the authors to suggest that their product contains estrogenic compounds that have binding affinity for a putative ER- γ . The absence of a direct estrogenic effect was again confirmed in a human study (21). Postmenopausal women took black cohosh extract for 12 weeks followed by a 12-week washout. Black cohosh demonstrated no effect on

estrogenic markers in serum and no effect on pS2 or cellular morphology in nipple aspirate fluid (21).

Receptor Expression

As with the receptor-binding assays, the nature of the extract or fraction is a decisive factor in the expression of ERs. A lipophilic and hydrophilic black cohosh extract was studied for luciferase expression in a MCF-7 α - and β -ER expressing subclone (80). The lipophilic extract at 35 $\mu\text{g/mL}$ activated transcription of the estrogen-regulated genes, while the hydrophilic extract showed no activity. A recent study measuring an extract at a low concentration (4.75 $\mu\text{g/L}$) increased ER levels in human MCF-7 cells as did estradiol (81). An unspecified black cohosh extract tested in a transient gene expression assay using HeLa cells co-transfected with an estrogen-dependent reporter plasmid in the presence of human ER- α or ER- β cDNA failed to show transactivation of the gene (82).

Plasma Hormone Levels

The effect of black cohosh on serum concentrations of FSH and LH has been studied extensively. Crude alcoholic extracts suppressed plasma LH with no effect on FSH in ovx rats (75,77). Further fractionation of the crude extract resulted in activity of the lipophilic fraction while the hydrophilic fractions were devoid of this activity (74). A later study in rats using lipophilic and hydrophilic extracts at high doses (140 and 216 mg/rat, IP) resulted in LH suppression with a single injection administration of the lipophilic but not the hydrophilic extract (75). Another study reported LH suppression in ovx rats with an unspecified dose of black cohosh extract (83). A recent study compared the effect of BNO 1055 with that of estradiol on LH levels (79). Extract administered subcutaneously at a dosage of 60 mg/day for 7 days was reported to reduce LH levels in the treated animals. However, another study reported no estrogen agonistic effects on FSH, LH, or prolactin levels in ovx rats using the 7,12-Dimethylbenz(a)anthracene model following 7 weeks of daily administration of a 40% isopropanolic extract of the plant (Remifemin) (84).

Hormonal Secretion

The effect of black cohosh on prolactin secretion in pituitary cell cultures was measured using an unspecified extract (85). Basal and Thyrotropin-releasing hormone (TRH)-stimulated prolactin levels were significantly reduced at doses of 10 and 100 $\mu\text{g/mL}$. This effect was reversed by the addition of haloperidol (D₂-antagonist) to the cell cultures, suggesting dopaminergic regulation of hormone secretion by black cohosh.

Osteopenia Inhibition

The black cohosh extract BNO 1055 (60 mg/rat, SC) has been shown to increase the expression of collagen I and osteocalcin in rats in a manner similar to that produced by 8 μg of estradiol in ovx rats (79). An additional study using BNO 1055 demonstrated an osteoprotective effect as shown by a reduced loss of bone mineral density in rat tibia after 3 months of administration (81). A study using an unspecified isopropanol extract of black cohosh showed reduced urinary markers of bone loss. The authors

Table 1 Selected Black Cohosh Clinical Studies

Author (reference no.)	Year	Extract/formulation/dosage	Study length	N	Outcome measure/result	Study design
Kesselkaul (110)	1957	Remifemin [®] 60 drops	2 wk	63	Alleviation of climacteric complaints in 95% of patients	Case series
Schotten (111)	1958	Remifemin 20 drops	3–4 wk	22	Alleviation of neurovegetative and psychic complaints associated with menopause and premenopause	Case series
Foldes (53)	1959	Remifemin, 3 tablets/day	Unknown	41	31 patients of the <i>verum</i> group responded to the treatment with a decrease in menopausal complaints	Placebo, controlled, open, crossover, patient self-assessment
Starfinger (112)	1960	Remifemin, 3–20 drops/day	1 yr	105	Decreased climacteric complaints without incidence of side effects or resulting in nonphysiological bleeding	Case series
Brucker (113)	1960	Remifemin, tablets, variable dose	Variable	87 (517)	Alleviation of menopausal complaints	Case series
Heizer (114)	1960	Remifemin, tablets 3–6/day	2–18 mo	66	Alleviation of menopausal (neurovegetative and psychic) complaints in 47% of patients with intact uteri and 35% with hysterectomies	Case series
Gorlich (115)	1962	Remifemin, tablets, variable dose	Variable	41 (258)	Alleviation of climacteric and vascular symptoms in 85% of patients	Case series
Schildge (116)	1964	Remifemin, fluid extract 60 drops/day	Variable	135	Euphoric and mild sedative-calming effects in all pts	Case series
Stolze (117)	1982	Remifemin, fluid extract 80 drops/day	6–8 wk	629	Alleviation of neurovegetative and psychological menopausal symptoms in 80% of patients	Open, physician and patient self-assessment
Daiber (118)	1983	Remifemin, fluid extract 80 drops/day	12 wk	36	Alleviation of climacteric complaints (hot flashes, insomnia, sweating, and restlessness)	Open, KMI, CGI
Vorberg (119)	1984	Remifemin, fluid extract 80 drops/day	12 wk	50	Significant or highly significant alleviation of menopausal (neurovegetative and psychic) complaints; study included subjects contraindicated to hormone therapy	Randomized, open, KMI, CGI, POMS
Warnecke (120)	1985	Remifemin, fluid extract 80 drops/day	12 wk	20	Significant alleviation of symptoms (psychic and neurovegetative) in the black cohosh, conjugated estrogen, and diazepam groups. Vaginal cytology of treatment group was comparable to estrogenic stimulation	Randomized, open, KMI, HAM-A, SDS, CGI, karyopyknosis index, eosinophil index
Stoll (121)	1987	Remifemin, tablets equivalent to 8 mg extract/day	12 wk	26	Significant alleviation of climacteric symptoms (vaginal atrophy, neurovegetative and psychic complaints) in comparison with estrogen and placebo groups	Double-blinded, randomized, placebo-controlled, KMI, HAM-A, VMI (vaginal epithelium)
Petho (122)	1987	Remifemin, tablets, unspecified dose	6 mo	50	KMI decreased significantly from 17.6 to 9.2, correlates with a significant reduction in neurovegetative symptoms. Severity of subjective self-assessments of subjects physical and psychological symptoms decreased	Open, KMI, patient self-assessment
Lehman-Willenbrock and Riedel (123)	1988	Remifemin, tablets equivalent to 8 mg extract/day	6 mo	15	Significant alleviation of climacteric symptoms in black cohosh and drug treatment groups. No significant change in gonadotropin (FSH, LH) levels	Randomized, open, KMI
Duker et al. (75)	1991	Remifemin, tablets equivalent to 40 mg dried herb/day	2 mo	110	LH suppression	In vitro study using blood from menopausal women taking black cohosh

Table 1 Selected Black Cohosh Clinical Studies (Continued)

Author (reference no.)	Year	Extract/formulation/dosage	Study length	N	Outcome measure/result	Study design
Baier-Jagodinski (124)	1995	Cimisan [®] T Tropfen, variable dose	4–8 wk	157	89% of patients showed symptom improvement after 4 wk. At final visit, the efficacy was assessed as very good, 40%; good, 41%; sufficient, 12%; inadequate, 7%	Open, uncontrolled
Mielnik (69)	1997	Uncharacterized extract, 4 mg daily	6 mo	34	Alleviation of climacteric (neurovegetative) symptoms in 76% of patients after 1 mo	Open, KMI
Georgiev and Iordanova (70)	1997	Uncharacterized extract, unspecified dose	3 mo	50	Alleviation of climacteric symptoms in 90% of patients. Increase in vaginal cell proliferation (VMI) in 40% of treated women	Open, KMI, HAM-A, VMI
Nesselhut and Liske (125)	1999	Remifemin, tablets, equivalent to 136 mg dried herb/day	3 mo	28	Good to very good alleviation of 10 menopausal symptoms in 80% of study participants	Open, postmarket surveillance
Jacobson, et al. (63)	2001	Remifemin, tablets equivalent to 40 mg dried herb/day	60 days	42 ^a	No change in median number or intensity of hot flashes	Double blinded, randomized, placebo controlled, patient self-assessment, VAS, MSS
Liske et al. (67)	2002	Unique <i>Cimicifuga racemosa</i> preparation, equivalent to 39 or 127.3 mg/day	6 mo	152	No direct systemic estrogenic effect on serum levels of FSH, LH, SHBG, prolactin, and 17- β estradiol. No change in vaginal cytology. Higher dose had a more significant reduction in KM index after 6 mo. Significant reduction with both doses in neurovegetative and psychic complaints	Drug equivalence trial, KMI, SDS, CGI
Hernandez Munoz and Pluchino (66)	2003	BNO 1055	12 mo	136	Combination therapy with tamoxifen (20 mg) reduced severity and incidence of hot flashes	Open, randomized, patient self-assessment
Wuttke et al. (64)	2003	Klimadynon [®] /BNO 1055	3 mo	62	Equipotent to 0.6 CE for relief of climacteric complaints and for bone resorption. No effect on endometrial thickness	Randomized, double blinded, placebo controlled, multicenter, MRS
Verhoeven et al. (126)	2005	125 mg soy extract daily (providing 50 mg isoflavones including 24 mg genistein and 21.5 mg daidzein), 1500 mg evening primrose oil extract (providing 150 mg gamma linoleic acid), 100 mg <i>Actaea racemosa</i> L. extract (providing 8 mg deoxyacetein), 200 mg calcium, 1.25 mg vitamin D, and 10 IU vitamin E, placebo group received 2000 mg olive oil daily	12 wk	124	Subjects were experiencing at least five vasomotor symptoms every 24 hr at study entry. At weeks 6 and 12, all scores in both groups had improved compared with baseline, though the overall difference in scores between the groups was not statistically significant	Multicenter, randomized, placebo-controlled, double-blind study, Kupperman index and Greene Climacteric scale
Nappi et al. (127)	2005	Aqueous isopropanolic extract 40 mg/day	3 mo	64	Postmenopausal women were recruited. Both CR and low-dose TTSE2 significantly reduced the number of hot flushes per day ($P < 0.001$) and vasomotor symptoms ($P < 0.001$), starting at the first month of treatment. Such a positive effect was maintained throughout the 3 mo of observation, without any significant difference between the two treatments. An identical effect was evident also for both anxiety ($P < 0.001$) and depression ($P < 0.001$), which were significantly reduced following 3 mo of both CR and low-dose TTSE2. Total cholesterol was unchanged by CR treatment but significantly ($P < 0.033$) reduced by 3 mo of low-dose TTSE2. A slight but significant increase of HDL cholesterol	Randomized, controlled, clinical study

(continued)

Table 1 Selected Black Cohosh Clinical Studies (Continued)

Author (reference no.)	Year	Extract/formulation/dosage	Study length	N	Outcome measure/result	Study design
					($P < 0.04$) was found only in women treated with CR, while LDL-cholesterol levels were significantly lowered by 3 mo of both CR ($P < 0.003$) and low-dose TTSE2 ($P < 0.002$). Triglyceride levels were not affected by both treatments nor was liver function. FSH, LH, and cortisol were not significantly affected after the 3-mo treatment, while PRL ($P < 0.005$) and 17- β -E2 ($P < 0.001$) were increased slightly only by low-dose TTSE2. Endometrial thickness was not affected by either CR or low-dose TTSE2	
Frei-Kleiner et al. (128)	2005	6.5 mg dry rhizome extract; 60% ethanol extraction solvent. Dose = 1 cap daily	12 wk	122	Menopausal women were recruited. The primary efficacy analysis showed no superiority of the tested black cohosh extract compared with placebo. However, in the subgroup of patients with a Kupperman index $> \text{or} = 20$ a significant superiority regarding this index could be demonstrated ($P < 0.018$). A decrease of 47% and 21% was observed in the black cohosh and placebo group, respectively. The weekly weighted scores of hot flashes ($P < 0.052$) and the Menopause Rating Scale ($P < 0.009$) showed similar results. Prevalence and intensity of the adverse events did not differ in the two treatment groups	Multicenter, randomized, placebo-controlled, double-blind, parallel group study
Pockaj et al. (61)	2006	20 mg <i>C. racemosa</i> and rhizome extract standardized to contain 1 mg of triterpene glycosides as calculated by 27-deoxyacetin, placebo	Two 4-wk crossover treatment periods	132	Toxicity was minimal and not different by treatment group. Patients receiving black cohosh reported a mean decrease in hot flash score of 20% (comparing the fourth treatment week with the baseline week) compared with a 27% decrease for patients on placebo ($P = 0.53$). Mean hot flash frequency was reduced 17% on black cohosh and 26% on placebo ($P = 0.36$). Patient treatment preferences were measured after completion of both treatment periods by ascertaining which treatment period, if any, the patient preferred. Thirty-four percent of patients preferred the black cohosh treatment, 38% preferred the placebo, and 28% did not prefer either treatment	Double-blind, randomized, crossover clinical trial. Primary end point was the average inpatient hot flash score (a construct of average daily hot flash severity and frequency) difference between the baseline week and the last study week of the first treatment period. Green Climacteric scale
Newton et al. (HALT) (62)	2006	(i) Black cohosh, 160 mg daily; (ii) multibotanical with black cohosh, 200 mg daily, and 9 other ingredients; (iii) multibotanical plus dietary soy counseling; (iv) conjugated equine estrogen, 0.625 mg daily, with or without medroxyprogesterone acetate, 2.5 mg daily; or (v) placebo	1 yr	351	Women aged 45–55 yr with two or more vasomotor symptoms per day were recruited. Vasomotor symptoms per day, symptom intensity, Wiklund Vasomotor Symptom Subscale score did not differ between the herbal interventions and placebo at 3, 6, or 12 mo or for the average over all the follow-up time points ($P > 0.05$ for all comparisons) with 1 exception: At 12 mo, symptom intensity was significantly worse with the multibotanical plus soy intervention than with placebo ($P > 0.016$). The difference in vasomotor symptoms per day between placebo and any of the herbal treatments at any time point was less than one symptom per day; for the average over all the follow-up time points, the difference was less than 0.55 symptom per day. The difference for hormone therapy versus placebo was -4.06 vasomotor symptoms per day for the average over all the follow-up time points (95% CI, -5.93 to -2.19 symptoms per day;	Randomized, double-blind, placebo-controlled trial. Wiklund Vasomotor Symptom scale

Table 1 Selected Black Cohosh Clinical Studies (Continued)

Author (reference no.)	Year	Extract/formulation/dosage	Study length	N	Outcome measure/result	Study design
					$P > 0.001$). Differences between treatment groups smaller than 1.5 vasomotor symptoms per day cannot be ruled out. Black cohosh containing therapies had no demonstrable effects on lipids, glucose, insulin, or fibrinogen (124)	
Raus et al. (129)	2006	Dried aqueous/ethanolic (58% vol/vol) extract CR BNO 1055 of the rhizome of <i>Actaea</i> or CR (black cohosh)	1 yr	400	Postmenopausal women with symptoms related to estrogen deficiency were recruited. The lack of endometrial proliferation and improvement of climacteric complaints as well as only a few gynecologic organ-related adverse events are reported for the first time after a treatment period of 1 yr	Prospective, open-label, multinational, multicenter study. Endovaginal ultrasonography
Sammartino et al. (130)	2006	Group A ($n = 40$) was treated with 1 tablet/day per os containing a combination of isoflavones [soy germ extracts, Glycine max, no OGM-SoyLife: 150 mg, titrated in isoflavones (40%) = 60 mg], lignans [flaxseed extracts, <i>Linum usitatissimum</i> , no OGM-LinumLife: 100 mg, titrated in lignans (20%) = 20 mg] and <i>C. racemosa</i> [50 mg, titrated in triterpene (2.5%) = 1.25 mg] (Euclim [®] ; Alfa Wassermann, Italy); group B ($n = 40$) was treated with calcium supplements (Metocal, Rottapharm, Monza, Italy)	Three cycles of 28 days	80	Healthy postmenopausal women were recruited. At baseline no significant difference was detected in KI between groups A and B; however, after three cycles of treatment, KI was significantly ($P > 0.05$) lower in group A compared with baseline and with group B	Double-blind, randomized, placebo-controlled trial, Kupperman index
Gurley et al. (131)	2006	Milk thistle (300 mg, three times daily, standardized to contain 80% silymarin), black cohosh extract (20 mg, twice daily, standardized to 2.5% triterpene glycosides), rifampin (300 mg, twice daily), and clarithromycin (500 mg, twice daily)	14 days	16	Young adults (8 females) (age, mean \pm SD = 26 ± 5 yr; weight, 75 ± 13 kg) compared with the effects of rifampin and clarithromycin, the botanical supplements milk thistle and black cohosh produced no significant changes in the disposition of digoxin, a clinically recognized P-gp substrate with a narrow therapeutic index. Accordingly, these two supplements appear to pose no clinically significant risk for P-gp-mediated herb-drug interactions	Randomized controlled, clinical pharmacokinetic trial
Rebbeck et al. (132)	2007	Varied	Case-control design	949 breast cancer cases; 1524 controls	HRS varied significantly by race, with African American women being more likely than European American women to use any herbal preparation (19.2% vs. 14.7%, $P = 0.003$) as well as specific preparations including black cohosh (5.4% vs. 2.0%, $P > 0.003$), ginseng (12.5% vs. 7.9%, $P < 0.001$) and red clover (4.7% vs. 0.6%, $P < 0.001$). Use of black cohosh had a significant breast cancer protective effect (adjusted odds ratio 0.39, 95% CI: 0.22–0.70). This association was similar among women who reported use of either black cohosh or Remifemin (a herbal preparation derived from black cohosh; adjusted odds ratio 0.47, 95% CI: 0.27–0.82)	Population-based case-control study

(continued)

Table 1 Selected Black Cohosh Clinical Studies (Continued)

Author (reference no.)	Year	Extract/formulation/dosage	Study length	N	Outcome measure/result	Study design
Hirschberg et al. (133)	2007	Remifemin (batch no. 229690), one tablet twice daily. Each tablet contains 0.018–0.026 mL liquid extract of black cohosh rootstock (0.78–1.14:1) corresponding to 20 mg herbal drug [i.e., 2.5 mg dry extract, extraction agent isopropanol 40% (vol/vol)], 40 mg/day	6 mo	74	None of the women showed any increase in mammographic breast density. Furthermore, there was no increase in breast cell proliferation. The mean change \pm SD in proportion of Ki-67-positive cells was $0.5\% \pm 2.4\%$ (median, 0.0; 95% CI = -1.32 – 0.34) for paired samples. The mean change in endometrial thickness \pm SD was 0.0 ± 0.9 mm (median, 0.0). A modest number of adverse events were possibly related to treatment, but none of these were serious. Laboratory findings and vital signs were normal	Prospective, open, uncontrolled drug safety study
Chung et al. (71)	2007	Gynoplus (264 mg tablet with 0.0364 mL <i>Cimicifuga racemosa</i> rhizome, equivalent to 1 mg terpene glycosides; 84 mg dried <i>Hypericum perforatum</i> extract, equivalent to 0.25 mg hypericin, with 80% methanol)	12 wk	89	Kupperman index (KI) for climacteric complaints. Vaginal maturation indices, serum estradiol, FSH, LH, total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride levels. Significant improvements in climacteric symptoms and hot flashes, as well as an increase in HDL (from 58.32 ± 11.64 to 59.74 ± 10.54) were observed in the Gynoplus group by 4 wk and maintained after 12 wk, compared with the placebo group. There was no significant impact on superficial cell proportion	Randomized, double-blind, placebo-controlled trial
Ruhlen et al. (22)	2007	Remifemin R and CimiPure (2.5% triterpenes; 40 mg capsule contains 1 mg 23- <i>epi</i> -26-deoxyactein)	12 wk followed by 12 wk washout	61	Subjects experienced relief of menopausal symptoms, with reversion to baseline after washout. No effect on serum estrogenic markers. No effect on pS2 or cell morphology in nipple aspirate	Open study
Gurley et al. (134)	2008	Milk thistle (300 mg, three times daily, standardized to contain 80% silymarin), black cohosh extract (40 mg, twice daily, standardized to 2.5% triterpene glycosides), rifampin (300 mg, twice daily), and clarithromycin (500 mg, twice daily)	14 days	19	Young adults [9 women; age (mean \pm SD) = 28 ± 6 yr; weight = 76.5 ± 16.4 kg]. Milk thistle and black cohosh appear to have no clinically relevant effect on CYP3A activity in vivo. Neither spontaneous reports from study participants nor their responses to questions asked by study nurses regarding supplement/medication usage revealed any serious adverse events	Randomized controlled, clinical pharmacokinetic trial
Amsterdam et al. (73)	2009		12 wk	28 (15 treatment/13 placebo)	The primary outcome measure was changed over time in total HAM-A scores. Secondary outcomes included a change in scores on the Beck Anxiety Inventory, Green Climacteric Scale (GCS), and Psychological General Well-Being Index (PGWBI) and the proportion of patients with a change of 50% or higher in baseline HAM-A scores. There was neither a significant group difference in change over time in total HAM-A scores ($P = 0.294$) nor a group difference in the proportion of subjects with a reduction of 50% or higher in baseline HAM-A scores at study end point ($P = 0.79$). There was a significantly greater reduction in the total GCS scores during placebo (vs. black cohosh; $P = 0.035$) but no group difference in change over time in the GCS subscale scores or in the PGWBI ($P = 0.140$). One subject (3.6%) taking black cohosh discontinued treatment because of adverse events	Randomized, double-blind, placebo-controlled trial

Table 1 Selected Black Cohosh Clinical Studies (Continued)

Author (reference no.)	Year	Extract/formulation/dosage	Study length	N	Outcome measure/result	Study design
Geller et al. (72)	2009		12 mo	89	Primary outcome measures were reduction in vasomotor symptoms (hot flashes and night sweats) by black cohosh and red clover compared with placebo; secondary outcomes included safety evaluation, reduction of somatic symptoms, relief of sexual dysfunction, and overall improvement in quality of life. Reductions in number of vasomotor symptoms after a 12-mo intervention were as follows: black cohosh (34%), red clover (57%), placebo (63%), and CEE/MPA (94%), with only CEE/MPA differing significantly from placebo. Black cohosh and red clover did not significantly reduce the frequency of vasomotor symptoms as compared with placebo. Secondary measures indicated that both botanicals were safe as administered. In general, there were no improvements in other menopausal symptoms	Randomized, double-blind, placebo-controlled trial

Studies listed by year of publication.

^aAll with breast cancer history.

Abbreviations: CGI, Clinician's Global Impression scale; HAM-A, Hamilton Anxiety scale; KMI, Kupperman Menopausal Index; MSS, unspecified menopausal index using the Likert scale; Open, open-labeled; POMS, Profile of Mood States Scale; SDS, Self-Assessment Depression scale; VAS, Visual Analog Scale; VMI, Vaginal Maturity Index.

of this study suggested the action was similar to that of the SERM Raloxifene (86). A follow-up study using BNO 1055 versus CE therapy showed beneficial effects of the extract on bone metabolism in humans, specifically an increase in bone-specific alkaline phosphatase in serum (64). While no direct correlation between species has been established, it is of note that studies of Asian *Cimicifuga* species have demonstrated similar activity and may be of importance for further investigation of this biological activity (87,88).

Uterine Weight/Estrous Induction

Uterine and ovarian weight increase, cell cornification, and an increased duration of estrous are generally considered evidence of endometrial estrogenic activity. However, it has recently been proposed that uterine weight is a poor marker for endometrial effects (89). Three studies demonstrating that black cohosh extracts increased the uterine weight of ovx rats have been reported (50,77,90) with two of the studies using an undescribed root extract (77,90). One study on immature mice reported similar findings (50). By contrast, two studies on ovx rats (79,91), as well as four studies on immature mice, reported the converse (79,81,83,92). One of these studies found that although there was no increase in uterine or ovarian weight, the duration of estrous was significantly increased by black cohosh (92). A subsequent study by the authors and collaborators demonstrated no attenuation in uterine weight at variable doses (4, 40, and 400 mg/kg/day) of a 40% isopropanol extract in ovx rats (4).

Cell Proliferation

An unspecified black cohosh extract failed to significantly induce growth of MCF-7 cells when compared with untreated control cells (81). A study using isopropanolic and

ethanolic extracts also failed to induce growth of MCF-7 cells (93).

CNS Effects and Neurotransmitter Binding

A murine study using an unspecified extract (25–100 mg/kg, orally) measured effects on body temperature and ketamine-induced sleep time using bromocriptine (D₂-agonist) as a positive control. Pretreatment with sulpiride (D₂ blocker) suggested a receptor-mediated dopaminergic effect (84). An additional mouse study was carried out to characterize neurotransmitter levels in the striatum and hippocampus after pretreatment with the extract for 21 days (94). Serotonin and dopamine metabolic levels in the striatum were substantially lower in comparison with the control group. These studies have led to the hypothesis that dopaminergic, rather than estrogenic, activity is responsible for the reported success of black cohosh in reducing climacteric symptoms (95,96). A study by the authors and collaborators has pointed to the effects of black cohosh being mediated by serotonin (5-HT) receptors (4). Three different extracts (100% methanol, 40% isopropanol, 75% ethanol) were found to bind to the 5-HT₇-receptor subtype at IC₅₀ ≤ 3.12 µg/mL. The 40% isopropanol extract inhibited (³H)-lysergic acid diethylamide binding to the 5-HT₇ receptor with greater potency than (³H)-8-hydroxy-2-(di-*N*-propylamino)tetralin to the rat 5-HT_{1A}. Analysis of ligand-binding data suggests that the methanol extract functioned as a mixed competitive ligand of the 5HT₇ receptor. Further testing of the methanol extract in 293T-5-HT₇ transfected HEK cells raised cAMP levels; these raised levels were reversed in the presence of the 5-HT antagonist methiothepin, indicating a receptor-mediated process and possible agonist activity local to the receptor (4).

Antioxidant

A black cohosh methanol extract protected S30 breast cancer cells against menadione-induced DNA damage at variable concentrations and scavenged DPPH free radicals at a concentration of 99 μ M (38).

USE IN PREGNANT/LACTATING WOMEN

Despite an absence of mutagenic effects reported to date, the use of black cohosh during pregnancy is contraindicated according to WHO suggestions (97). Data are inconclusive regarding the effects on lactation.

DOSAGE (97,98)

Recommended doses for black cohosh are as follows:

1. Dried rhizome and root: 1 g up to three times daily.
2. Tincture (1:10): 0.4 mL daily (40–60% alcohol vol/vol).
3. Fluid extract (1:1): 20 drops twice daily (60% ethanol vol/vol, equivalent to 40 mg dried herb).
4. Tablet equivalence: two tablets a day (equivalent to 40 mg dried extract).

The Commission E monograph also recommends that usage not be extended for more than 6 months due to a lack of long-term safety data. Experimental data are not available to suggest this 6-month limit.

ADVERSE EFFECTS/SAFETY

A majority of adverse event reports (AERs) for black cohosh have been associated with Remifemin products, probably due to its widespread use. Thus, the AER data may speak more to the safety of this particular product rather than black cohosh extracts in general. In clinical trials, minor cases of nausea, vomiting, dizziness, and headaches have been reported (61–73). An analysis of the safety data from published clinical trials, case studies, postmarketing surveillance studies, spontaneous report programs, and phase I studies was carried out (99). The data obtained from more than 20 studies, including more than 2000 patients, suggest that adverse event occurrence with black cohosh is rare, and that such events are mild and reversible, the most common being gastrointestinal upset and rashes. The same review investigated black cohosh preparation and AERs and concluded that adverse events are rare, mild, and reversible (99).

That said, black cohosh has garnered a great deal of attention with respect to its safety over the past 5 years, with the emergence of a few case reports citing acute hepatitis, convulsions, cardiovascular, and circulatory insult (100–104). It is important to note that in a number of these reports, no effort was made to positively identify the botanical associated with the event as black cohosh. In one case, depositions taken during a legal proceeding revealed that the lack of alcohol consumption and concomitant medications reported in a published case report (101) was inaccurate (105). Underreporting of adverse effects may also be a common problem with botanical supplement (100–104). However, these case reports have gen-

erated much interest within the research community, so much so that two workshops have been convened by the National Institutes of Health (NIH) on the specific issue of the safety of black cohosh preparations: one workshop sponsored by the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS) in November 2004 and a more recent workshop sponsored by the ODS held in June 2007. The report from the 2004 workshop indicated that there is “no plausible mechanism of liver toxicity.” The 2007 workshop offered no conclusions on safety to contradict those of the 2004 meeting regarding hepatotoxicity of black cohosh preparations. The 2007 workshop did recommend that active steps be taken to monitor liver health in human clinical trials of black cohosh (106).

It is also noteworthy that in the 2004 workshop, it was agreed that “suspected hepatotoxicity should not be broadcast when toxicity has not been demonstrated.” Despite concerns by some scientists, a warning statement on commercial black cohosh product labels was mandated in Australia by the Therapeutic Goods Administration (TGA), and the European Medicines Agency (EMA) released a press statement on July 18, 2006, urging patients to stop taking black cohosh if they develop signs suggestive of liver injury. It is noteworthy that it is not clear and has never been fully disclosed as to how these agencies reached their decision and what the scientific data were that led to these warning statements.

While the notion of idiosyncratic hepatotoxicity was raised in the June 2007 workshop by toxicologists from the Food and Drug Administration (FDA), it was acknowledged by these toxicologists that without data from a mandatory adverse event reporting system, no real conclusion on causality regarding idiosyncratic hepatotoxicity can be drawn from case reports.

In the September–October 2007 edition of USP’s Pharmacopeial Forum (100), the USP proposed the addition of a cautionary statement for USP quality black cohosh products with regard to liver toxicity. The American Botanical Council (ABC) responded that given the long history of safe black cohosh use and the lack of clear scientific evidence for toxicity, there is not enough information for such a warning. The ABC noted that of the 42 case reports of toxicity cited by the USP, only 18 met criteria for assessment based on a standard-rating scale, and of these, 3 met criteria for “possible” toxicity, and 2 for “probable” toxicity. Many case reports were also said to lack adequate documentation regarding the actual identity of the black cohosh used and possible confounding factors (107).

COMPENDIAL/REGULATORY STATUS

Black cohosh products are regulated and marketed in the United States as dietary supplements under the provisions of the Dietary Supplement Health and Education Act (DSHEA) of 1994 (U.S.C. § 321). Dried black cohosh rhizome and roots, powdered black cohosh, black cohosh fluid extract, powdered black cohosh extract, and black cohosh tablets now have official standing in dietary supplement monographs in the *United States Pharmacopoeia—National Formulary* (108). In the European Union nations,

black cohosh products are approved as nonprescription phytomedicines when administered orally in compliance with the German Commission E monographs (109).

CONCLUSIONS

With the elevated concern surrounding side effects related to classical hormone/estrogen therapy for menopause, modulation of certain climacteric symptoms of menopause by both dopaminergic and serotonergic drugs is becoming a more viable and frequent treatment option. A review of the clinical trials associated with black cohosh leads to the conclusion that women using hydroalcoholic extracts of the rhizomes and roots of this plant may gain relief from climacteric symptoms (i.e., hot flashes) in comparison with placebo over the short term, whereas longer studies have not shown the same degree of efficacy. Further clouding the review of these clinical trials is the wide variety and different types of extracts administered in published studies. Early in vitro studies reported that black cohosh extracts acted on ERs or had a sort direct effect on ERs. Now it is becoming clear that the beneficial effect of reducing hot flashes is related, at least in part, to serotonergic or dopaminergic mechanisms that regulate hypothalamic control and possibly mediate estrogenic mechanisms. As mentioned earlier, the controversy surrounding a purported direct estrogenic mechanism of action may also be due to variance in the extracts assayed. Overall, given variation in trial length, extract types, and other potential confounders, the efficacy of black cohosh as a treatment for menopausal symptoms is uncertain and further rigorous trials seem warranted.

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Blue-Green Algae (Cyanobacteria)

Wayne W. Carmichael and Mary Stukenberg with Joseph M. Betz

INTRODUCTION

In Asia, Africa, and parts of Central/South America, naturally occurring green and blue-green algae have been harvested and consumed for their nutritive properties for centuries. In western cultures, for approximately 30 years, certain fresh water blue-green algae (cyanobacteria) have been accepted as a source of food, in particular *Spirulina* (*Arthrospira*) *platensis* and *Spirulina maxima*. Beginning in the early 1980s, another blue-green species, *Aphanizomenon flos-aquae* (AFA), was adopted for similar uses. Both are rich in proteins, vitamins, essential amino acids, minerals, and essential fatty acids. Consumers of blue-green algae report a wide variety of putative effects, such as mental clarity, increased energy, blood and colon cleansing, increased focus, particularly in children with attention deficit disorder, improved digestion, increased eye health, healthier joints, and tissues. In the past 10 years, owing largely to the strong anecdotal consumer testimony about them, studies have been conducted to verify not only their nutritional efficacy but also their potential pharmaceutical benefits as well.

BACKGROUND

Worldwide, algae, for thousands of years, have been a food source and treatment for various physical ailments. In coastal regions of the Far East, recorded use of macroalgae (sea weed) as a food source began approximately 6000 BC, with evidence that many species were used for food and medical treatment by around AD 900. The Spanish recorded the use of microalgae as a food source when they reported that the natives of Lake Texcoco collected cyanobacteria from the waters of the lake to make sun-dried cakes. In present day Africa, local tribes harvest cyanobacteria in the Lake Chad region, primarily *Spirulina*, and also use it to make hard cakes, called *dihe*. In some regions of Chad, people consume from 9 to 13 g/meal, constituting 10% to 60% of the meal. However, the longest recorded use of cyanobacteria as food is the consumption of *Nostoc flagelliforme* in China, where there are records of its use for some 2000 years and where it is still harvested on a large scale. Use of microalgae in the western culture began in the 1970s. Most commercial producers of microalgae are located in the Asia-Pacific rim, where approximately 110 commercial producers of microalgae have an annual production capacity from 3 to 500 tons. These cultivated microalgae include *Chlorella*, *Spirulina*, *Dunaliella*, *Nannochloropsis*, *Nitzschia*,

Crypthecodinium, *Tetraselmis*, *Skeletonema*, *Isochrysis*, and *Chaetoceros*.

Within the cyanobacteria, *Spirulina* (*Arthrospira*) *platensis* and *S. maxima* have been commercially produced as a human and animal food supplement and food coloring for approximately 30 years. *Spirulina* is cultured in constructed outdoor ponds in Africa, California, Hawaii, Thailand, China, Taiwan, and India. World production in 1995 was approximately 2×10^6 kg.

The newest cyanobacterium to be used as a food supplement is AFA, the production of which differs significantly from *Spirulina*, because it is harvested from a natural lake rather than constructed ponds. Since the early 1980s, this alga has been harvested from Upper Klamath Lake, Oregon, and sold as a food and health food supplement. The popularity of both *Spirulina* and AFA blue-green algae products over other seaweeds and green algae may be attributable to the convenience of its packaging and consumption, as well as to its highly directed marketing to the health-conscious consumer. In 1998, the market for AFA as a health food supplement was approximately US \$100 million with an annual production greater than 1×10^6 kg (dry weight) (1–18).

Chemistry and Preparation

Edible blue-green algae are nutrient dense food. The features common to all blue-green algae include a high content of bioavailable amino acids and minerals, such as zinc, selenium, and magnesium. The nutrient profile is subject to variation by habitat, harvest procedure, quality control for contaminating species, proper processing to preserve nutrients, and storage conditions, all of which influence the vitamin content and antioxidant properties delivered by the final product. However, the appeal of blue-green algae is their raw, unprocessed nature and their abundance of carotenoids, chlorophyll, phycocyanins, phytosterols, glycolipids, γ -linolenic acid, and other bioactive components (19–21).

Approximately, 40 cyanobacteria species and genera produce potent toxins. *Spirulina* products have not been associated with toxicity reports in humans, largely owing to its being grown under cultured conditions (22). Natural samples and cultured strains of AFA have been reported to produce neurotoxins including paralytic shellfish poisons (neosaxitoxin and saxitoxins) and anatoxin-a. Recent work seems to indicate that a different *Aphanizomenon* species is the toxin producer. *A. flos-aquae* has been reported to be dominant or codominant in water blooms containing *Microcystis* and *Anabaena* and is found in many eutrophic water bodies. Species of *Microcystis* can produce a family

of potent liver toxins called microcystins. *Cylindrospermopsis* is a hepatotoxic and nephrotoxic compound produced by several freshwater cyanobacteria, including *Cylindrospermopsis raciborskii* and *Anabaena* spp (23). Several species of marine and freshwater cyanobacteria (including a number of *Nostoc*, *Anabaena*, and *Microcystis* species) produce the neurotoxic amino acid BMAA (β -N-methylamino-L-alanine) (24). Although these toxic substances are probably not naturally present in the target species discussed below, the possibility that they might be present as contaminants in commercial products highlights the need for rigorous quality-control measures.

Blue-green algae products most often come in a tablet form as algal material directly compressed. The tablets can contain fillers such as sugars or starches called binders, which give shape and stability to the tablets. Algae supplements also come in a capsule form to neutralize the taste and make the product easier to swallow, or can be bought by the pound in powder form or in liquid extract forms. Some companies combine the algae in "green supplements" that contain other health-enhancing ingredients such as alfalfa sprouts. Supplements come in kosher or vegetarian forms, and can be combined with digestive aids. Recommended dosages of blue-green algae products vary widely, but can be as much as 20 g/day. On the average, companies that produce algal products for consumption as nutritional supplements recommend 500 mg to 1 g/day to start, with a build up over time to several grams a day, often without an upper limit on consumption (25,26).

Efficacy

Two types of blue-green algae form the major nutritional supplement groups, *Spirulina* and AFA. As the traits of each vary slightly, they are addressed separately below.

Spirulina

The blue-green alga *Spirulina* was so named for its helically coiled trichomes or rows of cells. Until recently, *Spirulina* and *Arthrospira* were thought to belong to separate genera, and the distinction was thought to be especially important as only the strains of *Arthrospira* had been proven to be safe for human consumption. These two are now referred to as *Arthrospira* in scientific circles. Although the name *Spirulina* has been persisted for commercial labeling, the two are synonymous (27).

Spirulina is generally produced in large outdoor ponds under controlled conditions. The safety of *Spirulina* for human food has been established through long use, and through various toxicological studies done under the auspices of the United Nations Industrial Development Organization (28). *Spirulina* is 60% to 70% protein by weight and contains many vitamins, especially vitamin B₁₂ and β -carotene, and minerals, especially iron and γ -linolenic acid (Table 1). Recent reports suggest that a number of therapeutic effects and pharmaceutical uses are potential benefits of *Spirulina* as well (18).

Most studies of the effects of *Spirulina* on enhanced body function have been performed on animals, and therapeutic effects have been demonstrated in some cases. Conclusive human studies are rare, but those that carry substantive results are cited below.

Table 1 Nutritional Profile of a Commercial *Spirulina* Product

Composition	<i>Spirulina</i> powder
Per 100 g	
Macronutrients ^a	
Calories	382
Total fat	7.1 g
Total carbohydrate	15.5 g
Dietary fiber	6.8 g
Protein	55 g
Essential amino acids (mg)	
Histidine	900
Isoleucine	3170
Leucine	5030
Lysine	2960
Methionine	1290
Phenylalanine	2510
Threonine	2770
Tryptophan	740
Valine	3500
Nonessential amino acids (mg)	
Alanine	4110
Arginine	4130
Aspartic acid	5670
Cystine	580
Glutamic acid	9180
Glycine	2860
Proline	2170
Serine	2670
Tyrosine	2300
Vitamins ^b	
Vitamin A (as 100% β -carotene)	$\geq 200,000$ IU
Vitamin K	548 μ g
Thiamine HCl (Vitamin B-1)	0.13 mg
Riboflavin (Vitamin B-2)	2.55 mg
Niacin (Vitamin, B-3)	14.3 mg
Vitamin B-6 (Pyridox.HCl)	0.77 mg
Vitamin B-12	93 μ g
Minerals ^b	
Calcium	446 mg
Iron	56 mg
Phosphorus	1010 mg
Iodine	39.1 μ g
Magnesium	305 mg
Zinc	1.27 mg
Selenium	19.6 μ g
Copper	0.32 mg
Manganese	3.0 mg
Chromium	91.7 μ g
Potassium	1620 mg
Sodium	815 mg
Phytonutrients ^b	
Phycocyanin	10 g
Chlorophyll	0.9 g
Superoxide dismutase (SOD)	531,000 IU
γ -linolenic acid (GLA)	1180 mg
Total carotenoids	≥ 370 mg
β -Carotene	≥ 120 mg
Zeaxanthin	≥ 95 mg
Other carotenoids	~ 155 mg

This is a natural product and nutrient data may vary from one lot to another. One example of a nutrient profile for Earthrise[®] *Spirulina* Powder, a commercial *Spirulina* product, is shown in the above table.

^aMacronutrient data are based on most recent proximate analysis.

^bThe data indicate minimum values observed over a four-year period except for sodium where the maximum observed value is used.

Nutritional Rehabilitation

A multicenter study of 182 malnourished children, aged 3 months to 3 years, reported that a 5 g/day dose of *Spirulina* (*Arthrospira*) *platensis* had no added benefit over 90 days when compared to traditional renutrition (29).

Four groups of undernourished children under the age of 5 (550 total) were provided with Misola (60% millet flour, 20% soy, 10% peanut, 9% sugar, 1% salt), Misola plus 5 g of *S. platensis*, traditional meals, or traditional meals plus 5 g of *Spirulina*. All diets contained about the same number of kilocalories/day. The authors concluded that Misola, *Spirulina* plus Misola, and *Spirulina* plus traditional diet are all good food supplements for undernourished children, but that Misola plus *Spirulina* were superior to the other combinations (30).

Cardiovascular

In ischemic heart disease patients, *Spirulina* supplementation was shown to significantly lower blood cholesterol, triglycerides, and LDL and very-low density lipoprotein cholesterol, and raise HDL (the so-called "good") cholesterol. A 4 g/day supplementation showed a higher effect in reducing total serum cholesterol and LDL levels than did 2 g/day (31). In a small two-month study of the effects of 1 g/day of *Spirulina* (species not specified) plus medication versus medication alone on lipid parameters in pediatric hyperlipidemic nephritic syndrome patients, Samuels et al. (32) reported that supplementation of medication with *Spirulina* helped reduce increased lipid levels in these patients.

Several studies in healthy populations have shown positive effects on cardiovascular endpoints. Consumption of *Spirulina* was found to reduce total lipids, free fatty acids, and triglyceride levels in a human study involving diabetic patients. A reduction in LDL/HDL ratio was also observed (33). A nonplacebo-controlled open label trial of 36 healthy adults administered 4.5 g/day of *S. maxima* for six weeks reported a hypolipidemic effect, especially on triacylglycerols and LDL = cholesterol, systolic, and diastolic blood pressure were also reduced (34). Effects of 8 g/day *Spirulina* (species not given) versus placebo on health-related endpoints in 78 healthy elderly Koreans were determined in a 16-week double-blinded trial. In the verum group, significant reductions in total plasma cholesterol and interleukin (IL)-6 concentrations were observed, along with increases in interleukin (IL)-2 concentrations and total antioxidant status (35). Juárez-Oropeza et al. (36) reported results of investigations of the effects of *S. maxima* on vascular reactivity in rats and lipid status and blood pressure in healthy humans. The authors suggest that the results of the rat portion of the study indicate that *Spirulina* induces a tone-related increase in endothelial synthesis/release of nitric oxide and of a vasodilating cyclooxygenase-dependent arachidonic acid metabolite (or a decrease in synthesis/release of an endothelial vasoconstricting eicosanoid). In the nonplacebo-controlled study of the effects of 4.5 g/day *Spirulina* on vascular and lipid parameters in 36 human volunteers, the authors reported reductions in blood pressure and plasma lipid concentrations (especially triacylglycerols and LDL-cholesterol).

Immune System Function

Spirulina was found to have a positive effect on the immune system. In a paper presented at a meeting of the Japanese Society for Immunology, volunteers consuming a *Spirulina* drink for two weeks experienced enhanced immune system function, which continued for up to six months after the extract administration was discontinued (37). A follow-up study reported that administration of 50 mL of a hot water extract of *S. platensis* augmented interferon production and natural killer cell (NK) cytotoxicity in more than 50% of 12 healthy human volunteers (38). Results of a study on immunoglobulin-A in human saliva showed a significant correlation between the immunoglobulin-A level in saliva and the amount of *Spirulina* consumed (39).

Much attention has been focused on the potential mitigation of allergies through *Spirulina* intake. A group of Russian researchers are pursuing a patent on their success with the normalization of immunoglobulin-E in children living in radioactive environments (40). In a more recent study of allergic rhinitis patients, the production of cytokines, critical in regulating immunoglobulin-E-mediated allergy, was measured. In a randomized double-blind crossover study versus placebo, allergic individuals were fed daily with either placebo or *Spirulina* at 1000 or 2000 mg for 12 weeks. Although *Spirulina* seemed to be ineffective at modulating the secretion of Th-1 cytokines (one type of the so-called "killer" cells), the study reported that at 2000 mg/day, *Spirulina* significantly reduced IL-4 levels by 32% (41). A six-month double-blind placebo-controlled trial of the effects of 2 g/day of *S. platensis* on allergic rhinitis in 150 otherwise healthy individuals aged 19 to 49 reported that the cyanobacterium treatment significantly improved symptoms and physical findings including nasal discharge, sneezing, nasal congestion, and itching (42).

Cancer

The sole human cancer intervention study involving *Spirulina* intake was done in India on a group of tobacco chewers afflicted with oral leukoplakia. In a study involving 44 subjects in the intervention group and 43 in a placebo group, it was found that supplementation with 1 g of *Spirulina* per day for one year resulted in complete regression of lesions in 45% of the intervention group and in only 7% of the control group. As supplementation with *Spirulina* did not result in an increase in retinal β -carotene, the authors concluded that other components in *Spirulina* may be responsible for the regression of lesions observed (43).

Other Endpoints

A series of four N-of-1 double-blind randomized trials were performed on four individuals who complained of idiopathic chronic fatigue. Each patient was his own control and received three pairs of treatments comprising four weeks of *S. platensis* and four weeks of placebo in doses of 3 g/day. Outcome measures were severity of fatigue measured on a 10-point scale. The score of fatigue was not significantly different between *Spirulina* and placebo (44). A small study compared the effects of *S. platensis* plus a normal diet against soy protein plus normal diet in the prevention of skeletal muscle damage in untrained

student volunteers. Sixteen subjects were divided into two equal groups (7.5 g/day *S. platensis* or soy protein). They were administered the Bruce incremental treadmill exercise prior to treatment, took the intervention for three weeks, and were then readministered the treadmill exercise. Results suggested that ingestion of *Spirulina* (but not soy protein) protected against skeletal muscle damage and may have led to postponement of the time to exhaustion during all-out exercise (45).

Most of the research on *Spirulina*'s efficacy for human nutrition and pharmaceutical use has been concerned with the areas of vitamin and mineral enrichment, immune system function, antioxidant effects, and anticancer and antiviral effects. Although the number of studies referenced by Amha Belay for his *Spirulina* research review article in 1993 contained 41 references, 18 his review in 2002 contained 98, 17 and this chapter has added additional information, few of the human studies in almost any area can be said to be conclusive. Studies are small or very small, and most are open label nonplacebo-controlled studies. A number of the publications do not provide adequate information (many fail to identify the test cyanobacteria to the species level). Interesting results in both the human studies and in vitro and animal studies show that further research is merited.

Adverse Effects

As previously noted, *Spirulina* products have not been associated with toxicity reports in humans, probably because commercial production is via large-scale culture rather than wild harvest (22).

AFA (*Aphanizomenon flos-aquae*)

In western cultures, certain cyanobacteria have been an accepted source of microalgal biomass for food for approximately 30 years, in particular, as discussed earlier, *Spirulina* (*Arthrospira*) *platensis* and *S. maxima*. Beginning in the early 1980s, another species, AFA, was adopted for similar uses. Members of this genus are free floating (planktonic) and occur either singly or form feathery or spindle-shaped bundles, are cylindrical in shape, much longer than they are wide, and contain abundant gas vesicles. They occur in temperate climates and are most abundant in summer and fall (46). The only known commercial harvesting of AFA is from Upper Klamath Lake, the largest freshwater lake system in Oregon. In 1998, the annual commercial production of AFA was approximately 1×10^6 kg. As this species is not cultured like *Spirulina* in outdoor ponds or raceways, it requires very different procedures for harvesting and processing. Other procedures, such as those for removal of detritus and mineral materials, and those for monitoring and reducing the amounts of certain contaminant cyanobacteria, which can produce cyanotoxins, have also become important in quality control and marketing (47). The nutrient profile for AFA is very similar to that for *Spirulina* (Table 2). Consumers of AFA nutritional supplements report a variety of benefits from enhanced energy to boosted immune system function. Cited below are the peer-reviewed human studies extant in the literature confirming certain of these nutritive and pharmaceutical attributes.

Table 2 Nutritional Profile of a Commercial AFA Product

Nutrient	Units	Amount
General composition		
Protein	%	55.1
Carbohydrate	%	29.1
Calories	%	3.7
Minerals (ash)	%	6.8
Fat calories	cal/g	0.3
Cholesterol	mg/g	0.3
Total dietary fiber	%	5.7
Sugar profile		
Dextrose (glucose)	mg/g	19.4
Fructose	mg/g	0.5
Maltose	mg/g	5.6
Sucrose	mg/g	0.8
Total sugars	mg/g	26.2
Minerals and trace metals		
Calcium	mg/g	8.5
Chloride	mg/g	2.0
Chromium	μg/g	1.2
Copper	μg/g	10.5
Iron	mg/g	0.7
Magnesium	mg/g	1.8
Manganese	μg/g	31.2
Molybdenum	μg/g	4.7
Phosphorus	mg/g	4.7
Potassium	mg/g	10.6
Selenium	μg/g	0.4
Sodium	mg/g	2.5
Zinc	μg/g	12.1
Vitamins		
Vitamin A (β-carotene)	IU/g	1523
Thiamin (B1)	μg/g	19.0
Riboflavin (B2)	μg/g	44.9
Pyridoxine (B6)	μg/g	14.6
Cobalamin (B12)	μg/g	3.7
Ascorbic acid (C)	mg/g	0.4
Niacin	mg/g	0.4
Folic acid	μg/g	0.6
Choline	mg/g	1.3
Pantothenic acid	μg/g	3.1
Biotin	μg/g	0.2
Vitamin D	IU/g	0.4
Vitamin E	IU/g	0.1
Vitamin K	μg/g	47.7
Amino acids		
Arginine	mg/g	29
Histidine	mg/g	9
Isoleucine	mg/g	25
Leucine	mg/g	43
Lysine	mg/g	29
Methionine	mg/g	9
Phenylalanine	mg/g	21
Threonine	mg/g	29
Tryptophan	mg/g	6
Valine	mg/g	29
Asparagine	mg/g	49
Alanine	mg/g	39
Glutamine	mg/g	78
Cystine	mg/g	3
Glycine	mg/g	23
Proline	mg/g	20
Serine	mg/g	25
Tyrosine	mg/g	16
Aspartic acid	mg/g	46
Glutamic acid	mg/g	49
Total amino acids	mg/g	579

Table 2 Nutritional Profile of a Commercial AFA Product (Continued)

Nutrient	Units	Amount
Lipid analysis		
Total lipid (fat) content	4.4%	44 mg/g
Total saturated fat	43%	19 mg/g
Total unsaturated fat	57%	25 mg/g
Total essential fatty acids	45%	20 mg/g
Total Omega-3 essential fatty acids	38%	17 mg/g
α -Linolenic acid (ALA)	37%	16 mg/g
Eicosapentanoic acid (EPA)	0.4%	0.2 mg/g
Total Omega-6 essential fatty acids	8%	3 mg/g
Linoleic acid (LA)	8%	3 mg/g
Arachidonic acid (AA)	0.1%	0.04 mg/g

One example of a nutrient profile for Cell Tech Super Blue-Green Algae, a commercial AFA product, as of 4-22-05, is shown in the above table.

Circulation and Immune Function

In a study examining the short-term effects of consumption of moderate amounts (1.5 g/day) of AFA on the immune system, it was discovered that AFA resulted in increased blood cell counts when compared to subjects taking a placebo. When the volunteers were grouped into long-term AFA consumers and naïve volunteers, the naïve volunteers exhibited a minor reduction in natural killer cells, and the long-term consumers exhibited a pronounced reduction. It was further determined that AFA does not activate lymphocytes directly, but that it does increase immune surveillance without directly stimulating the immune system. The authors of this study conclude that AFA has a mild but consistent effect on the immune system and could function as a positive nutritional support for preventing viral infections. They also recommend further research into AFA's potential role in cancer prevention (48). In a later report, an aqueous extract of AFA found to contain a novel ligand for CD62L (L-selectin). Consumption of the extract by 12 healthy subjects in a double-blind randomized crossover study caused mobilization of human CD34⁺CD133⁺ and CD34⁺CD133⁻ stem cells (49).

Eye Disease—Blepharospasm and Meige Syndrome

A study to determine whether blue-green algae could be helpful in improving the eyelid spasms associated with essential blepharospasm and Meige syndrome was undertaken by a group of physicians. Although a few patients exhibited a positive effect, for most patients, neither the severity nor the frequency of facial spasms was significantly reduced (50).

Adverse Effects

No cases of human intoxication by AFA were found in the literature. As noted, around 40 cyanobacterial species have been reported to produce potent natural toxins. Certain *A. flos-aquae* strains were long thought to produce neurotoxins including the paralytic shellfish poisons neosaxitoxin and saxitoxin and anatoxin-a. A reevaluation of the species using gene sequencing data led to the conclusion that a different *Aphanizomenon* species was the actual toxin producer (51). While it now seems clear that AFA is not a toxin-producing species, the toxigenic *Aphanizomenon* species seems to be distinguishable from AFA only by the presence or absence of toxins and by genetic sequencing.

No evidence exists to suggest that AFA is a toxin-producing strain (51), but water blooms of AFA may also contain *Microcystis* and *Anabaena*. Species of *Microcystis* can produce a family of potent liver toxins called microcystins, while *Anabaena* species can produce anatoxins and BMAA. The microcystins, especially, are of concern, since hepatic damage caused by this toxin is cumulative. This has led the State of Oregon to set a safe level of microcystin in AFA product from Klamath Lake. Currently this level is set at 1 μ g/g dry weight of product, and was set to correspond to an average daily adult intake for AFA of 2 g. Recently, the cyanobacterial toxin anatoxin-a was reported in 3 of 39 cyanobacterial dietary supplement product samples at concentrations of 2.5 to 33 μ g/g (52). Exposure guidelines have been summarized by Burch (23). This points to the need for rigorous quality-control measures in production of products. These measures may range from existing practices such as modifying the harvesting equipment to exclude *Microcystis*, harvesting the water bloom only at times when *Microcystis* is at a minimum, or developing and using toxin-detecting methods in an integrated testing scheme (47,53,54).

CONCLUSIONS

As a natural source of many vitamins and minerals, proteins, and chlorophyll, it is not surprising that blue-green algae have attracted attention among those interested in natural sources of nutrition. Thousands of people consume blue-green algae in its most popular forms (*Spirulina* and AFA), and as a result, a large body of anecdotal material has existed for many years concerning the positive health benefits of blue-green algae consumption. The volume of the testimony has contributed to a growing interest in recent years in verifying these benefits through scientific research. Beginning with animal and in vitro studies, and moving toward human studies, scientists have only recently begun to investigate some of the positive health effects attested to by long-term consumers of blue-green algae. These include cholesterol reduction, weight loss, enhanced immune system function, regression of cancer-related lesions, and enhancement of blood circulation, as well as many vitamin and mineral benefits. It must be stated, however, that there is an overall paucity of well-designed, controlled human trials using blue-green algal products as interventions.

As the industry relies on self-regulation, it is important to be aware of the quality-control issues involved in harvesting and packaging blue-green algae for consumption, particularly in the case of AFA, which is harvested from the wild. The World Health Organization has determined through current knowledge of microcystin toxins and what it calls a tolerable daily intake (TDI), an estimate of the intake over a lifetime that does not constitute an appreciable health risk. This TDI is derived through existing knowledge of toxin tolerance in mice combined with principles used in defining the health risks of other chemicals. It carries with it a degree of uncertainty owing to the lack of long-term data for the effect of microcystin on humans (55). To be sure that their risk has been minimized as much as possible, consumers of blue-green algae supplements would be wise to check to see that the product has been

tested for toxins, and it has been found to be below the WHO/Oregon Department of Health Regulatory Level of 1 µg/g of microcystin (56).

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Boron

Curtiss Hunt

INTRODUCTION

The element boron is essential for all higher plants in phylogenetic kingdom Viridiplantae (1) and at least some organisms in the phylogenetic kingdoms Eubacteria (2), Stramenopila (3), and Animalia (4,5). Specific species in the kingdom Fungi have a demonstrated physiological response to boron, an important finding because Fungi species are thought to share a common ancestor with animals exclusive of plants (6). Physiologic concentrations of the element are needed to support metabolic processes in several species in Animalia. For example, embryological development in fish and frogs does not proceed normally in the absence of boron. There is evidence that higher vertebrates, that is, chicks, rats, and pigs require physiological amounts of boron to assist normal biologic processes including immune function, bone development, and insulin regulation. In humans, boron is under apparent homeostatic control and is beneficial for immune function and calcium and steroid metabolism.

COMMON CHEMICAL FORMS

Boron is the fifth element in the periodic table with a molecular weight of 10.81 and is the only nonmetal in Group III. Organoboron compounds are those organic compounds that contain B–O bonds, and they also include B–N compounds, because B–N is isoelectronic with C–C (7). Organoboron compounds are apparently important in biological systems and are the result of interaction with OH or amine groups. Organoboron complexes occur in plants and are produced in vitro with biomolecules isolated from animal tissues (8).

BORON SPECIATION

Environmental Forms

Boron does not naturally occur free nor bind directly to any element other than oxygen except for trivial exceptions, for example, NaBF_4 (ferrucite) and $(\text{K,Cs})\text{BF}_4$ (avogadrite) (7). Its average concentration in the oceans is 4.6 mg/L and is the 10th most abundant element in oceanic salts (9). Weathering of clay-rich sedimentary rock is the major source of total boron mobilized into the aquatic environment (10). Undissociated boric acid (orthoboric acid) is the predominant species of boron in most natural freshwater systems (10) where most concentrations are below 0.4 mg/L and not lowered by typical treatments for drinking water. The most common commercial compounds of boron are anhydrous, pentahydrate, and decahydrate

(common name: tincal) forms of disodium tetraborate (borax, $\text{Na}_2\text{B}_4\text{O}_7$), colemanite ($2\text{CaO} \cdot 3\text{B}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$), ulexite ($\text{Na}_2\text{O} \cdot 2\text{CaO} \cdot 5\text{B}_2\text{O}_3 \cdot 16\text{H}_2\text{O}$), boric acid (H_3BO_3), and monohydrate and tetrahydrate forms of sodium perborate (NaBO_3) (11).

Inorganic boron, within the concentration range expected for human blood (2–61 μM B; 22–659 ng B/g wet blood) (12), is essentially present only as the monomeric species orthoboric acid (common name: boric acid) $\text{B}(\text{OH})_3$ and borate, that is, $\text{B}(\text{OH})_4^-$ (13). Boric acid is an exclusively monobasic acid and is not a proton donor, but rather accepts a hydroxyl ion (a Lewis acid) and leaves an excess of protons to form the tetrahedral anion $\text{B}(\text{OH})_4^-$ (14):



Within the normal pH range of the gut and kidney, $\text{B}(\text{OH})_3$ would prevail as the dominant species (pH 1: ~100% $\text{B}(\text{OH})_3$; pH 9.3: 50%; pH 11: ~0%) (15).

Biochemical Forms

Many biomolecules contain one or more hydroxy groups and those with suitable molecular structures can react with boron oxo compounds to form boroesters, an important class of biologically relevant boron species. Several types of boron esters exist. Boric acid reacts with suitable dihydroxy compounds to form corresponding boric acid monoesters (“partial” esterification) (Fig. 1) that retain the trigonal-planar configuration and no charge.

In turn, a boric acid monoester can form a complex with a ligand containing a suitable hydroxyl to create a borate monoester (“partial” esterification; monocyclic) (Fig. 2), but with a tetrahedral configuration and a negative charge. A compound of similar configuration and charge is also formed when borate forms a complex with a suitable dihydroxy compound. The two types of boromonoesters can react with another suitable dihydroxy compound to give a corresponding spirocyclic borodiester (“complete” esterification) that is a chelate

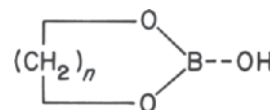


Figure 1 Boric acid may complex with a suitable dihydroxy ligand to form a boric acid monoester (“partial” esterification) that retains a trigonal-planar configuration and no charge.

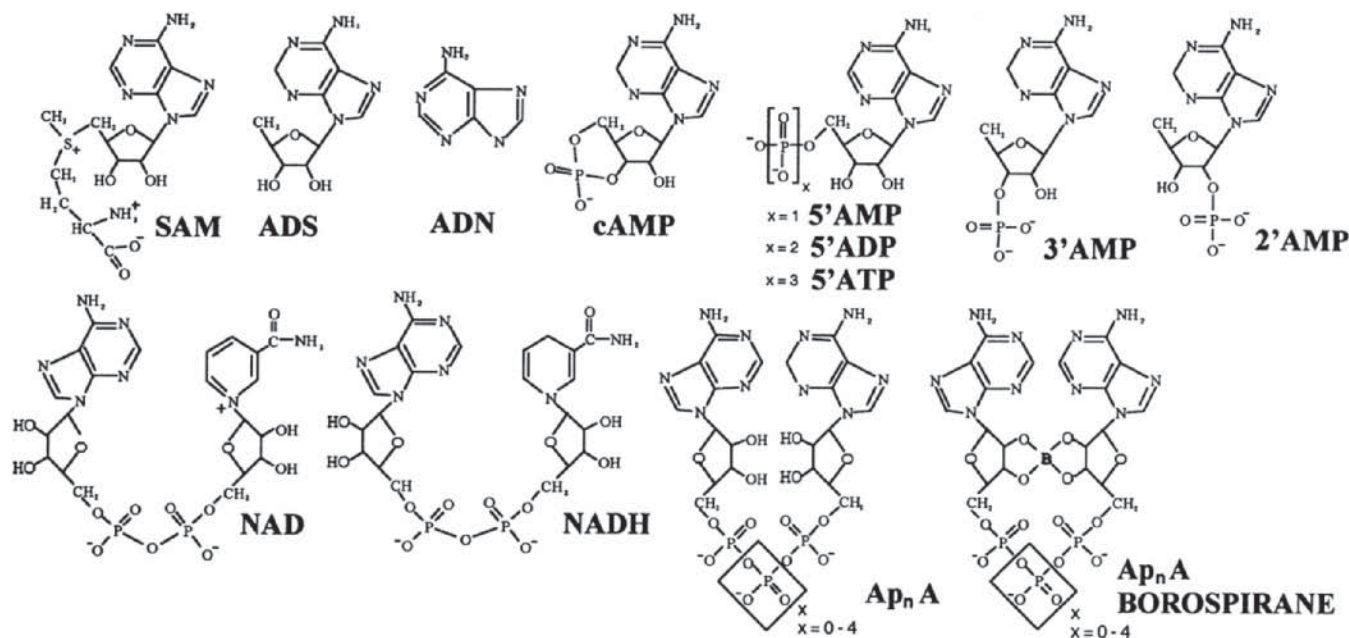


Figure 6 Experimental data indicate that biochemical species with vicinal *cis*-diols bind strongly to boron: (S)-adenosylmethionine (SAM) \equiv diadenosine hexophosphate (Ap₆A) \equiv Ap₅A > Ap₄A > Ap₃A \equiv NAD⁺ > Ap₂A > NADH \equiv 5'ATP > 5'ADP > 5'AMP > adenosine (ADS). Species without these moieties do not bind boron well: 3'AMP \equiv 2'AMP \equiv cAMP \equiv adenine (ADN).

boron; those without these moieties typically do not. Of these animal or human biocompounds examined, SAM has the highest known affinity for boron (8). It is the predominant methyl donor in biological methylations and is therefore a versatile cofactor in various physiologic processes. NAD⁺, an essential cofactor for five sub-classes of oxidoreductase enzymes, also has a strong affinity for boron (23). The di-adenosine-phosphates (Ap_nA) are structurally similar to NAD⁺. Boron binding by Ap₄A, Ap₅A, and Ap₆A is greatly enhanced compared with NAD⁺ but is still less than that of SAM (8). The Ap_nA molecules are present in all cells with active protein synthesis and reportedly regulate cell proliferation, stress response, and DNA repair (24). At physiologic pH, the adenine moieties of Ap_nA are driven together by hydrophobic forces and stack interfacially (25). Stacking of the terminal adenine moieties brings their adjacent ribose moieties into close proximity, a phenomenon that apparently potentiates cooperative boron binding between the opposing riboses (Fig. 6).

Plant-Based Foods

All higher plants require boron and contain organoboron complexes. There may have been considerable evolutionary pressure exerted to select for carbohydrate energy sources that do not interact with boron. Sugars often form intramolecular hemiacetals: those with five-membered rings are called furanoses and those with six-membered rings are called pyranoses. In cases where either five- or six-membered rings are possible, the six-membered ring usually predominates for unknown reasons (26). In general, compounds in a configuration in which there are *cis*-diols on a furanoid ring (e.g., ribose, apiose, and ery-

thritan) form stronger complexes with boron than do those configured to have *cis*-diols predominately on a pyranoid ring (e.g., the pyranoid form of α -D-glucose). D-Glucose reacts with boric acid (27) but the near absence (<0.5%) of an α -furanose form of D-glucose in aqueous solutions (26) suggests that glucose was selected as the aldose for general energy metabolism because of its lower reactivity with boric acid. On the other hand, ribose may have been selected as part of the chemistry of nucleic acid and nucleotide function and apiose for, rather than against, its extraordinary borate-complexing capability.

Recent evidence suggests that the predominant place of boron function in plants is in the primary cell walls where it cross-links rhamnogalacturonan II (RG-II) (Fig. 7), a small, structurally complex polysaccharide of the pectic fraction. RG-IIs have an atom of boron that cross-links two RG-II dimers at the site of the apiose residues to form a borodiester (28). However, this function is not adequate to explain all boron deficiency signs in plants. Twenty-six boron-binding membrane-associated proteins were identified recently in the higher plant, *Arabidopsis thaliana* (29), and boron oxo compounds also form stable ionic complexes with the polyol ligands mannitol, sorbitol, and fructose in liquid samples of celery phloem sap and vascular exudate and phloem-fed nectaries of peach (30).

Dietary Supplements

Boron speciation in dietary supplements varies widely (31) as does the relevant information provided by various dietary supplement manufacturers. It is sometimes listed only in a general manner (e.g., "borates" or "boron"), and occasionally in a more specific manner (e.g., "sodium

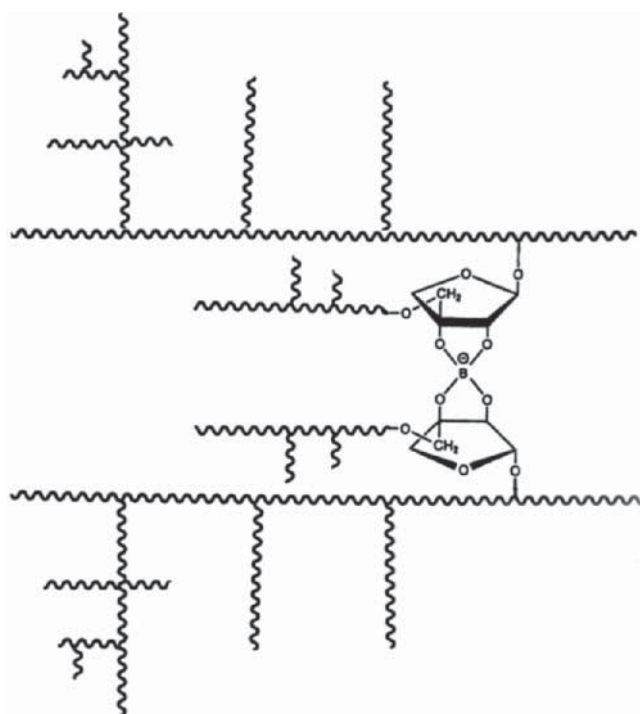


Figure 7 Schematic representation of two monomers of the pectic polysaccharide rhamnogalacturonan-II cross-linked by an atom of boron at the site of the apiose residues to form a borodiester. Multiple cross-links form a supramolecular network. Source: From Ref. 75.

borate" or "sodium tetraborate decahydrate"). Several commercially available forms (e.g., "boron amino acid chelate," "boron ascorbate," "boron aspartate," "boron chelate," "boron citrate," "boron gluconate," "boron glycerborate," "boron glycinate," "boron picolinate," "boron proteinate," "boron bonded with niacin," and "calcium fructoborate") are not well characterized in the scientific literature. Most often, dietary boron supplements are provided in conjunction with other nutrient supplements.

BIOAVAILABILITY, EXCRETION, AND HOMEOSTASIS

If plant and animal boron absorption mechanisms are analogous, the organic forms of the element *per se* are probably unavailable (32). However, the strong association between boron and polyhydroxyl ligands (described later) is easily and rapidly reversed by dialysis, change in pH, heat, or the excess addition of another low-molecular polyhydroxyl ligand (27). Thus, within the intestinal tract, most ingested boron is probably converted to orthoboric acid (common name: boric acid), $B(OH)_3$, the normal end product of hydrolysis of most boron compounds (7). Gastrointestinal absorption of inorganic boron and subsequent urinary excretion (33) is near 100%.

Several lines of evidence suggest regulation of boron in humans. For example, lack of boron accumulation and relatively small changes in blood boron values during a substantial increase in dietary boron support the concept of boron homeostasis (33). Boron contents in human milks

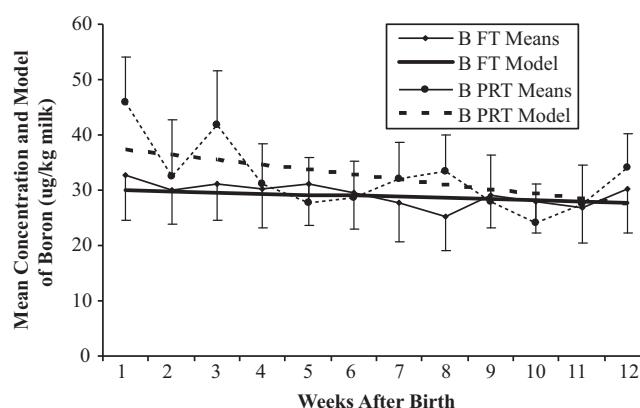


Figure 8 Model and mean (\pm SE) concentrations of boron in breast milk from mothers of full-term (FT) and premature (PRT) infants; $n = 9$ per group over 12 weeks after birth. During the first 12 weeks of lactation, prematurity affected the rate of change in concentrations ($P = 0.01$).

were similar and stable throughout lactation of full-term infants in two cohorts of women living in either Houston, TX (34), or St. John's, Newfoundland (Fig. 8) (35), have been interpreted as suggestive of regulatory mechanisms for the elements, which remain undefined. Evidence for the homeostatic control of boron is enhanced further by a report (36) of a specific borate transporter, NaBC1, in mammalian cell lines, a finding that has yet to be confirmed by another laboratory.

DIETARY BORON SOURCES AND INTAKES

Dietary Recommendations

The tolerable upper intake level (UIL) for boron varies by life stage (Table 1) (37). No estimated average requirement, recommended dietary allowance, or adequate intake has been established for boron for any age–sex group.

Dietary Supplements

For adults, the amount of boron commonly provided in a single dietary boron supplement is 0.15 mg (31). However, the reported amount of boron available per serving varies considerably among commercially available products as indicated in the relevant information provided by

Table 1 Upper Limits for Boron Set by the 2001 Food and Nutrition Board of the National Academy of Sciences (37)

Life stage	Age (yr)	Upper limit (mg/day)
Children	1–3	3
	4–8	6
	9–13	11
Adolescents	14–18	17
Adults	19–70	20
	70	20
Pregnancy	≤ 18	17
	19–50	20
Lactation	≤ 18	17
	19–50	20

various dietary supplement manufacturers. Some manufacturers publish reported values of 6.0 mg boron per serving of dietary supplement (38). The mean of usual intake of boron (mg/day) from dietary supplements for children (1–8 years), adolescents (9–15 years), males (19+ years), females (19+ years), and pregnant/lactating women is 0.269, 0.160, 0.174, 0.178, and 0.148, respectively. The median boron intake from supplements in the U.S. population is approximately 0.135 mg/day (37).

Nonfood Personal Care Products

Boron is a notable contaminant or ingredient of many nonfood personal care products. For example, an antacid was reported to have a high concentration of boron (34.7 µg/g) (39) such that the maximum recommended daily dose would provide 2.0 mg B/day, two times the estimated daily boron consumption for the overall adult U.S. population.

Dietary Sources and Intakes

Ten representative foods with the highest boron concentrations are distributed among several food categories (40): raw avocado (14.3 µg/g), creamy peanut butter (5.87 µg/g); salted dry roasted peanuts (5.83 µg/g), dry roasted pecans (2.64 µg/g), bottled prune juice (5.64 µg/g), canned grape juice (3.42 µg/g), sweetened chocolate powder (4.29 µg/g), table wine (12.2% alcohol) (3.64 µg/g), prunes with tapioca (3.59 µg/g), and granola with raisins (3.55 µg/g). Several fruit, bean, pea, and nut products contained more than 2 µg B/g. Foods derived from meat, poultry, or fish have relatively low concentrations of boron.

Infant foods supply 47% of boron (B) intake to infants. For toddlers, consumption from fruits and fruit juices, combined, is twice that from milk/cheese (38% vs. 19%). For adolescents, milk/cheese foods are the single largest source of boron (18–20%), and for adults and senior citizens, it is beverages (mainly represented by instant regular coffee) (21–26%). For all groups (except infants), 7% to 21% of boron intake is contributed by each of the vegetable, fruit, and fruit drink products. Infants, toddlers, adolescent girls and boys, adult women and men, and senior women and men are estimated to consume the following amounts of boron: 0.55, 0.54, 0.59, 0.85, 0.70, 0.91, 0.73, and 0.86 mg/day, respectively.

INDICATIONS AND USAGE

Boron and Calcium Metabolism and Bone Structure

There are several lines of evidence that dietary boron is important for normal bone growth and function. Boron deprivation induced abnormal limb development in frogs (41) and retarded maturation of the growth plate in chicks (42). Dietary boron deprivation decreases bone strength in pigs (43) and rats (44). The trabecular microarchitecture of vertebral bone was impaired in rats deprived of the element (44). Similarly, in mice, modeling and remodeling of alveolar bone (45), as well as alveolar bone healing after experimental tooth extraction (46), was impaired by dietary boron deprivation.

Findings from human studies suggest that boron influences calcium metabolism. For example, in post-

menopausal women, boron supplementation (3 mg/day) of a low-boron diet (0.36 mg B/day) resulted in a 5% increase in urinary calcium excretion (33). A similar phenomenon occurred in either free-living sedentary or athletic premenopausal women consuming self-selected typical Western diets: boron supplementation increased urinary calcium loss (47). These findings may reflect an increase in intestinal calcium absorption because increase in dietary calcium often result in increased urinary calcium excretion.

Dietary boron also alleviates the signs of marginal vitamin D deficiency relevant to bone structure and function. Marginal vitamin D deficiency impairs bone structure, elevates plasma alkaline phosphatase concentrations, and reduces body weight. In the growing rachitic chick, dietary boron substantially alleviated the perturbed histomorphometric indices of bone growth cartilage (42,48), reduced elevated serum concentrations of alkaline phosphatase (49,50), and improved body weight (50,51).

Boron and Insulin and Energy Substrate Metabolism

Circulating insulin concentrations respond to dietary boron in a manner that suggests the element may function to reduce the amount of insulin needed to maintain glucose levels. For example, in the rat model (with overnight fasting), boron deprivation increased plasma insulin with no concurrent change in glucose concentrations (52). In the chick model, boron deprivation increased *in situ* peak pancreatic insulin release (52). In older volunteers (men and women) fed a low-magnesium, marginal copper diet, dietary boron deprivation induced a modest but significant increase in fasting serum glucose concentrations (53).

Findings from several studies indicate that dietary boron may attenuate the deleterious effects of marginal vitamin D deficiency on insulin and energy substrate metabolism. In vitamin D–deprived rats, hyperinsulinemia was decreased by dietary boron (54). It has been demonstrated repeatedly in the chick model that physiological amounts of dietary boron can attenuate the rise in plasma glucose concentration induced by vitamin D deficiency (42,48,55). In addition, boron decreases the abnormally elevated plasma concentrations of pyruvate, β-hydroxybutyrate, and triglycerides that are typically associated with this inadequacy (42). It is not understood how boron deprivation perturbs energy substrate metabolism in humans and animal models, particularly when other nutrients are provided in suboptimal amounts.

Boron and Immune Function

Dietary boron may have a role in control of the normal inflammatory response, especially as it relates to production of various cytokines. Cytokines, including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), are produced and secreted by immune cells that regulate immune responses. Production of IFN-γ and TNF-α was increased in peripheral blood monocytes cultured in the presence of lipopolysaccharide (an inflammatory agent) after isolation from pigs fed supplemental dietary boron. In the same animals, boron caused a reduction in localized inflammation following a challenge with

the antigen phytohemagglutinin (PHA) (56). In cell culture studies with human fibroblasts (57) and chick embryo cartilage (58), the addition of boric acid also increased TNF- α release by the respective cells. Certain boron-containing RG-II s from *Panax ginseng* leaves enhanced the expression of IL-6-producing activity of mouse macrophages (59). Finally, perimenopausal women who excreted <1.0 mg B/day during the placebo period exhibited an increased percentage of polymorphonuclear leukocytes during the boron (as sodium borate) supplementation period (60). Dietary boron may serve as a signal suppressor that down-regulates specific enzymatic activities typically elevated during inflammation at the inflammation site. Suppression, but not elimination, of these enzyme activities by boron is hypothesized to reduce the incidence and severity of inflammatory disease.

Boron and Steroid Metabolism

There is a clear evidence that dietary boron affects steroid metabolism. In particular, circulating concentrations of vitamin D metabolites are sensitive to boron nutriture. Findings from animal models indicate that dietary boron enhances the efficacy of vitamin D but cannot substitute for the vitamin. In volunteers (men, and women on or not on estrogen therapy), boron supplementation after consumption of a low-boron diet increased serum 25-hydroxycholecalciferol concentrations (62.4 ± 7.5 vs. 44.9 ± 2.5 mmol/L; mean \pm SEM) (61,62), an effect that may be especially important during the winter months when these concentrations normally range between 35 and 105 mmol/L (63).

The circulating concentrations of 17β -estradiol also respond to boron nutriture. Perimenopausal women who excreted <1.0 mg B/day during the placebo period exhibited increased serum concentrations of estradiol after boron supplementation (2.5 mg B/day) of self-selected diets (60). In a separate study, postmenopausal women on estrogen therapy, but neither men nor postmenopausal women not ingesting estrogen, also exhibited increased serum concentrations of estradiol after boron supplementation (3 mg B/day) of a low-boron diet (0.25 mg B/2000 kcal) (62). However, plasma estradiol, but not testosterone, concentrations increased in young male volunteers when their self-selected diets were supplemented with ample amounts of boron (10 mg/day) (64).

Boron and Cancer

Indirect evidence from several epidemiological and cell culture studies indicate that dietary boron intake may affect cancer risk. For example, observations from epidemiologic human studies suggest that increased intakes of boron are associated with decreased risk of prostate (65) and lung (66) cancers and abnormal cervical cytopathology (67). In cultures of human prostatic epithelial cells (not tested for proliferative activity), physiological levels of boron reduced Ca^{2+} release from ryanodine receptor-sensitive stores in a dose-dependent manner, without affecting cytoplasmic Ca^{2+} concentrations (68). In immunocompromised mice fed physiological amounts of dietary boron, the element reduced the growth of transplanted human prostate adenocarcinoma tumors (69).

OVERDOSAGE

As with all other elements, boron produces toxicity in all tested biological organisms when excessive amounts are absorbed. The toxicity signs associated with boric acid when used as an antiseptic in lieu of antibiotics on abraded epithelium (i.e., surgical wounds and diaper rash) were overlooked for many years even though signs of poisoning were reported soon after its introduction into clinical use. Boron is more bacteriostatic than does bactericidal and, thus, may suppress bacterial growth.

Deaths can occur at doses between 5 and 20 g of boric acid for adults and below 5 g total for infants (70,71). Potential lethal doses are usually cited as 3 to 6 g total for infants and 15 to 20 g total for adults. However, an independent examination of 784 cases of boric acid ingestion found minimal or no toxicity at these intake levels or higher (72). Signs of acute boron toxicity, regardless of route of administration, include nausea, vomiting, headache, diarrhea, erythema, hypothermia, restlessness, weariness, desquamation, renal injury, and death from circulatory collapse and shock. Autopsy may reveal congestion and edema of brain, myocardium, lungs, and other organs, with fatty infiltration of the liver. Chronic heavy borax dust occupational exposure (average air concentration: 4.1 mg/m^3 ; range: $1.2\text{--}8.5 \text{ mg/m}^3$) may manifest as eye irritation, nosebleeds, chest tightness, sore throat, dry mouth, and productive cough (71). Chronic boron toxicity symptoms include poor appetite; nausea; weight loss; decreased sexual activity, seminal volume, sperm count, and motility and increased seminal fructose. At present, death from boron poisoning is exceptionally rare probably because of the emphasis placed on maintaining electrolytic balance and supporting kidney function during the worst part of the illness. Depending upon boron blood levels, treatment ranges from observation to gastric lavage to dialysis.

CONCLUSIONS

Boron is ubiquitous in the environment and daily dietary boron intakes of adult American males, for example, are slightly less than 1.0 mg. The evidence to date suggests that higher animals (43,73) and humans (33,62,74) probably require boron to support normal biological functions. Despite the progress made in studies of boron essentiality for plants, animals, and man, the biochemical mechanisms responsible for the beneficial physiologic effects of boron across the phylogenetic spectrum are poorly understood. However, the unique nature of boron biochemistry suggests specific lines of investigation. In particular, further characterization of the various cell signaling molecules that form complexes with boron under physiological conditions should provide insights into the specific biochemical function(s) of boron in humans.

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Caffeine

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INTRODUCTION

Caffeine is undoubtedly one of the most widely consumed and studied dietary supplements because it is found in many products, including numerous foods and drugs. Approximately 50% of the U.S. adult population regularly uses one or more dietary supplements, but 80% or more regularly consumes caffeine. Thousands of studies have investigated this substance, and a comprehensive discussion of all aspects of the literature on caffeine would require hundreds of pages of text. Substantial literature on caffeine can be found in multiple scientific fields including pharmacology, exercise and cardiovascular physiology, psychology, psychiatry, and epidemiology (1,2).

Caffeine occurs naturally in beverages and foods, including coffee, tea, the South American drink maté, and chocolate. Additional caffeine is added to beverages, including colas, which naturally contain caffeine, because manufacturers of these products have determined that optimal levels of caffeine should be greater than their naturally occurring concentration. Caffeine is behaviorally active in the doses present in foods (3) and is the most widely consumed psychoactive substance in the world. Caffeine is recognized in scientific and regulatory domains as both a naturally occurring food and a drug, a distinction that few, if any, other substances hold.

HISTORY OF CAFFEINE USE

The first written mention of a caffeine-containing food or beverage, tea, is in a Chinese dictionary from about AD 350 (4). However, it is likely that tea was in use long before then. Coffee was cultivated in Ethiopia as early as the sixth century AD, where it originated. Coffee beans were probably first eaten whole or mixed with food (4). Coffee came into use as a hot beverage around AD 1000 in the Middle East but did not spread to Europe until the 17th century. Coffee is currently second only to water as the beverage of choice around the world, with an estimated 400 billion cups of coffee consumed each year (5). The two most common species of the coffee plant are *Coffea arabica* and *Coffea canephora* Pierre ex. A. Froehner (commonly known as robusta). Approximately two-thirds of the world's coffee comes from arabica plants, whereas one-third comes from robusta (5). Arabica coffee has a smoother and superior taste but requires extensive care in growing. Arabica

beans contain approximately 1.5% or less of caffeine by dry weight (5). Robusta beans, grown in regions such as Brazil, have a higher caffeine content, 2.4% to 2.8%, which may explain their less-preferred flavor, because caffeine itself is quite bitter (5). At least 60 species of plants contain caffeine. The reason so many plants contain caffeine is not known, but caffeine protects plants from certain insects (6).

Tea [*Camellia sinensis* (L.) Kuntze] is the caffeinated beverage of choice in a large part of the world, although it contains less caffeine than coffee (Table 1). It appears that caffeine-containing beverages originated independently in at least four different locations throughout the world. In both North and South America, caffeine-containing beverages were made by the native inhabitants prior to contact with Europe. The sources of caffeine in North America were the cassina or Christmas berry tree (*Ilex vomitoria* Ait.) and in South America guarana (*Paullinia cupana* Kunth) and yoco (4). Caffeine is present in cocoa beans (*Theobroma cacao* L.), native to Central and South America. A compound chemically similar to caffeine, theobromine, is also found in cocoa but in substantially greater amounts than caffeine. Although the popularity of caffeine is widely recognized, the rationale for its unique status in the diets of humans is not known, but many have speculated its mild stimulant properties account for its popularity.

PHARMACOLOGY OF CAFFEINE

Caffeine is a methylated xanthine, 1,3,7-trimethylxanthine; theophylline and theobromine are two other methylated xanthines found in foods and/or drugs (Fig. 1). Theobromine, 3,7-dimethylxanthine, is not behaviorally active in doses found in foods (7). Theophylline, 1,3-dimethylxanthine, used to treat asthma, is not present in coffee but found in small quantities in tea (*C. sinensis*). The parent compound of these methylated compounds is xanthine, a dioxypurine structurally similar to uric acid (8).

When ingested, caffeine is rapidly absorbed into the systemic circulation and reaches peak levels in 45 minutes or less (9). Caffeine is distributed to all tissues and readily crosses the blood-brain barrier, which explains its behavioral activity. Caffeine is initially absorbed by the buccal membranes (in the mouth) and, when consumed in chewing gum, enters the circulatory system more rapidly than when ingested in pill form (10).

Table 1 Estimated Caffeine Content of Selected Beverages, Foods, and Dietary Supplements (32) (Energyfiend.com; Evitamins.com)

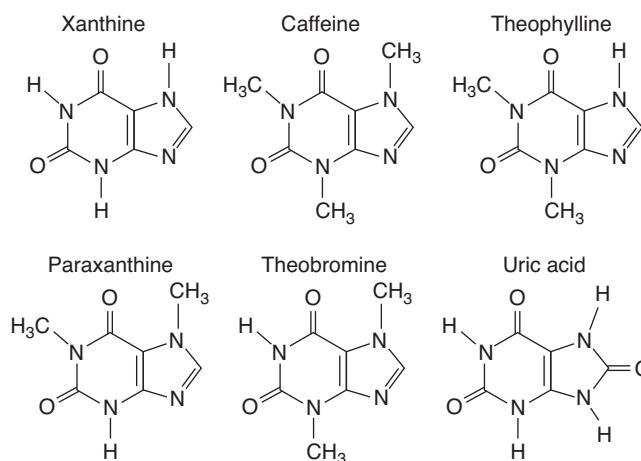
Item	Caffeine content (mg/serving)
Coffee (5 oz)	
Drip method	90–150
Instant	40–108
Decaffeinated	2–5
Tea, loose or bags	
1-minute brew (5 oz)	9–33
5-minute brew (5 oz)	20–50
Iced tea (12 oz)	22–36
Chocolate products	
Hot cocoa (6 oz)	2–8
Chocolate milk (8 oz)	2–7
Milk chocolate (1 oz)	1–15
Baking chocolate (1 oz)	35
Cola beverages (12 oz)	
Coca-Cola® Classic	35
Diet Coke®	47
Pepsi®	38
Diet Pepsi®	36
Other Soft drinks (12 oz)	
Dr Pepper®	40
Mountain Dew®	55
Pibb Xtra®	41
Barq's® Root Beer	23
Energy drinks and shots	
AMP™ (16 oz)	142
Monster Energy™ (16 oz)	160
Monster Energy™ Lo-Carb (16 oz)	135
Red Bull® (8.3 oz)	80
Rockstar® (16 oz)	160
5-Hour Energy® Shot (2 oz)	138
DynaPep™ Micro Shot (4 mL)	80
Extreme Energy™ 6-Hour Shot	220
Jolt® Endurance Shot	200
Dietary supplements	
Thermogenic Hydroxycut™ Advanced (2 pills)	200 ^a
Zantrex® 3 (2 pills)	320
Stacker 2® Ephedra Free (1 pill)	200
Metabolift™ (2 pills)	176
Slenderite™ (2 pills)	75
Skinny Fast® (3 pills)	0
Nature's Plus® Fat Busters (2 pills)	0

^aAs reported by Energyfiend.com.

CAFFEINE METABOLISM

The period of time caffeine remains in the circulatory system, measured as half-life, varies dramatically. Half-life of caffeine in a healthy adult is approximately four to five hours, but in women taking oral contraceptives, it can increase substantially. In cigarette smokers, caffeine is metabolized more rapidly and has a half-life of about three hours (11,12).

Caffeine is metabolized in the liver by a complex series of processes. The principal metabolic pathway, which accounts for approximately 95% of initial breakdown of caffeine, is catalyzed by the cytochrome P450 enzyme CYP1A2 (13). The process begins with removal of a methyl

**Figure 1** Chemical structures of caffeine, its demethylated derivatives (theobromine, theophylline, paraxanthine), its parent compound (xanthine), and uric acid.

group to form paraxanthine and, to a lesser extent, theobromine and theophylline (Fig. 2).

Various factors alter CYP1A2 activity. For example, both pregnancy and severe liver disease result in decreased caffeine clearance (13,14). Conversely, smoking induces CYP1A2 activity, thereby decreasing half-life of caffeine (14). Many pharmacologic substances also affect this enzyme: oral contraceptives and cimetidine inhibit the enzyme and slow caffeine clearance (14), whereas other drugs (e.g., phenytoin, carbamazepine) induce the enzyme, accelerating caffeine metabolism.

Dietary practices influence CYP1A2 activity. Caffeine intake itself induces this enzyme, so heavy consumers metabolize caffeine more rapidly (15), explaining, in part, why they are less sensitive to its behavioral and physiological effects. Cruciferous vegetables (e.g., broccoli, kale, turnip) increase CYP1A2 activity, whereas apicomplex vegetables (e.g., cilantro, parsnip, celery) inhibit it (16).

Genetic Differences in Caffeine Metabolism

Genetic variation is partly responsible for different phenotypes of caffeine metabolism (17). Several CYP1A2 single nucleotide polymorphisms (SNPs) have been characterized (18). One, CYP1A2*1F, is a substitution (A → C) at position 734 on the CYP1A2 gene. Approximately 10% to 16% of individuals have the CYP1A2 C/C (homozygous) genotype, whereas about half possess two A alleles (18). Individuals with the CYP1A2*1F A → C polymorphism are slower to metabolize caffeine and less likely to increase CYP1A2 activity following exposure to inducers. There may be physiological consequences of genetic differences in caffeine metabolism. For example, a recent study linked the slow-metabolizing CYP1A2*1F C/C genotype, with increased risk of heart disease associated with coffee consumption (19).

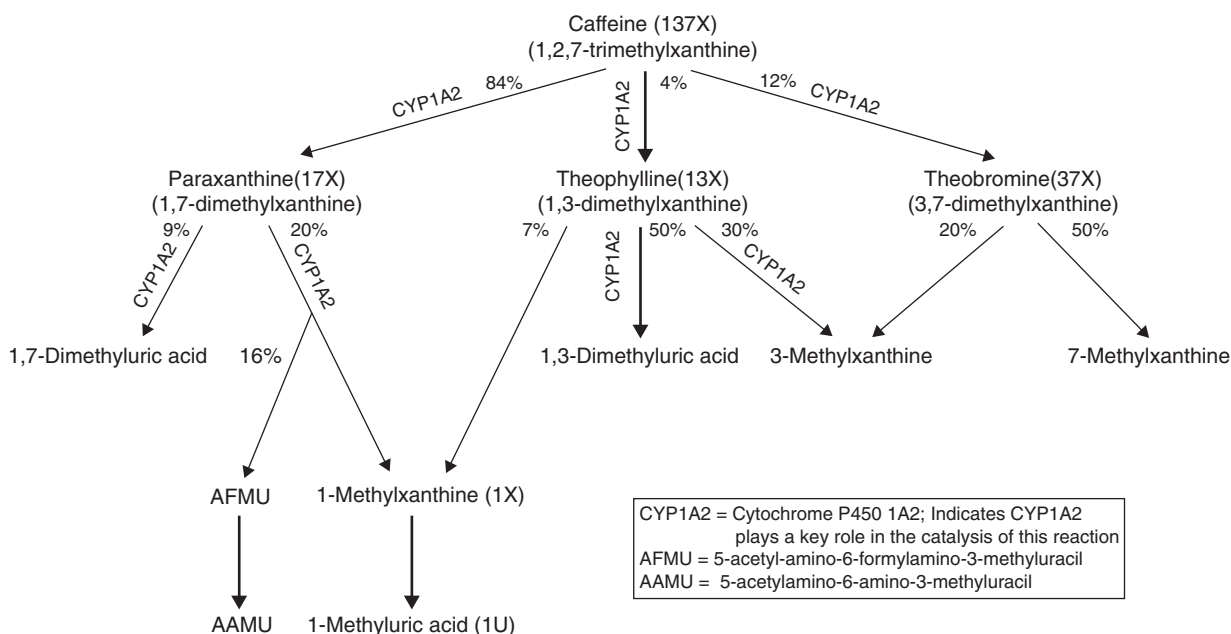


Figure 2 Metabolic pathways of caffeine and its derivatives. Only pathways that begin with N-demethylation are shown, which account for almost 95% of initial breakdown. Less than 3% of caffeine is excreted unchanged in the urine; the remainder is metabolized to 1,3,7-trimethyl uric acid (not shown). Source: From Ref. 17.

MECHANISM OF ACTION

Caffeine's behavioral, as well as ergogenic effects can be attributed to central adenosine receptors (20,21). Adenosine is an inhibitory neuromodulator in the central nervous system that has sedative-like properties. Under normal physiological conditions, caffeine is a nonselective competitive antagonist at these receptors. Four subtypes, A_1 , A_{2a} , A_{2b} , and A_3 , of G-protein-coupled adenosine receptors have been identified, each with a unique tissue distribution, signaling pathway, and pharmacological profile (22,23). Through the respective activation of G_i and G_s proteins, adenosine decreases adenylate cyclase activity, and hence, cAMP levels, when bound to A_1 or A_3 receptors, and increases activity when bound to A_{2a} or A_{2b} receptors (22,23).

Prior to discovery of caffeine's action on adenosine receptors, effects of caffeine were attributed to inhibition of phosphodiesterase (PDE) (24). However, the concentration of caffeine required to inhibit PDE substantially exceeds that achieved from consumption of caffeine in foods or dietary supplements. While caffeine blocks A_1 and A_{2a} receptors at concentrations in the low micromolar range (5–30 μ M), approximately 20 times as much caffeine is required to inhibit PDE, well above physiological levels as illustrated in Figure 3, which also presents the approximate caffeine concentration from consumption of a cup of coffee (25,26).

All four adenosine receptor subtypes are expressed to various extents in the brain and periphery (23). Adenosine A_1 receptors are widely distributed in the periphery, spinal cord, and brain, with high levels found in hippocampus, cortex, cerebellum, and hypothalamic nuclei; lower levels of the A_1 subtype are found in the basal

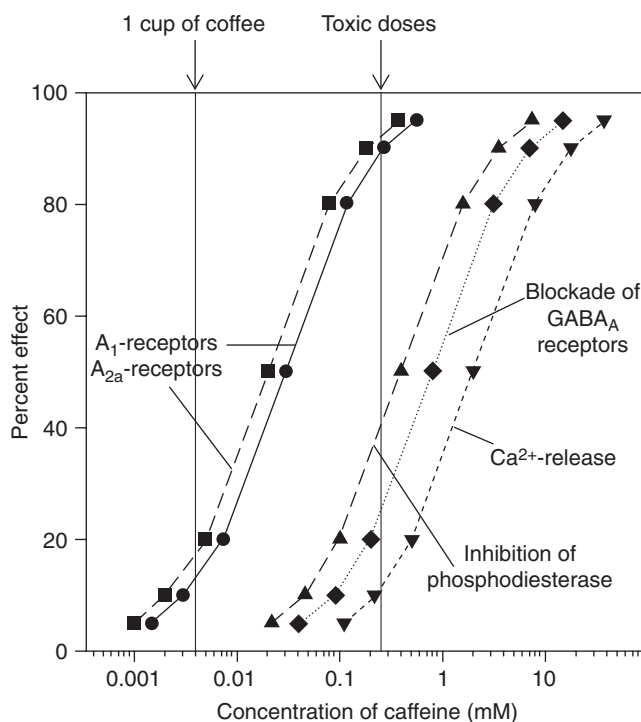


Figure 3 Concentration-effect curves for caffeine at various potential sites of action. Caffeine markedly affects A_1 and A_{2a} receptors at low micromolar concentrations. To inhibit phosphodiesterase (PDE), concentrations as large as 20-times are required. Approximate caffeine concentration resulting from a single cup of coffee and toxic doses of caffeine is indicated. Source: From Ref. 26.

ganglia (23). The A_{2a}, A_{2b}, and A₃ receptors are mainly expressed in the periphery; however, there is marked expression of the A_{2a} receptors in regions heavily innervated by dopamine-containing fibers, including the striatum, nucleus accumbens, and olfactory tubercle, where they are coexpressed with dopamine D₂ receptors (27).

Caffeine binds with highest affinity at A_{2a} receptor and has slightly lower affinity at the A₁ and the A_{2b} receptors; the A₃ subtype has little to no affinity (24). At standard physiological concentrations (i.e., low micromolar), effects of caffeine are due to blockade at A₁ and A_{2a} receptors, with binding at A_{2b} and A₃ receptors having a minor role, if any (22,27).

Both adenosine A₁ and A_{2a} receptors may be responsible for behavioral effects of caffeine, but the contribution of each is uncertain. The A₁ receptors are located predominantly on presynaptic nerve terminals and mediate release of several neurotransmitters, including glutamate, dopamine, and acetylcholine. Caffeine is thought to enhance arousal, vigilance, and attention by blocking inhibition by adenosine at these receptors, particularly those in the striatum (22). Caffeine may stimulate arousal via A₁ receptors by preventing inhibition of mesopontine cholinergic neurons that regulate cortical activity and arousal (22).

Unlike dopaminergic stimulants, such as cocaine and amphetamine, which facilitate dopamine D₂ receptor transmission, caffeine does not alter dopamine release in ventral striatum (22). This may explain why caffeine does not have the abuse potential of these stimulants.

Genetic Differences in Adenosine Receptors

Recently, it was shown in humans that a particular polymorphism (a T→C substitution at position 1976; also known as SNP rs5751876 or 1976T→C, and formerly known as 1083T→C) of the A_{2a} receptor gene (*ADORA2A*) is associated with effects of caffeine on sleep (28). It appears that 16% of individuals are homozygous for the T allele and roughly 35% are homozygous for the C allele (29). While inhibition of A₁ receptors may be partly responsible for wakefulness promoted by caffeine, most evidence suggests caffeine-induced arousal is due to blockade at A_{2a} receptors (23).

Individuals with the *ADORA2A* C/C genotype are more likely to report disturbed sleep following caffeine consumption compared with individuals with the T/T genotype (28). Consistent with these genetic differences, associations were observed between self-reported caffeine-sensitivity, assessed by questionnaire, and *ADORA2A* genotype. A higher proportion of sensitive subjects had the C/C genotype, whereas the T/T genotype was more frequent in insensitive subjects (28). In a sleep deprivation study conducted in a subset of the survey population, caffeine-sensitive men reported greater stimulant-like effects of caffeine compared with those who were caffeine-insensitive. In addition, ratings of caffeine sensitivity were positively correlated with psychomotor vigilance after sleep loss (28). Also, the C allele is associated with caffeine-induced insomnia, and the T allele appears to be related to caffeine-induced anxiety. Infrequent caffeine users consuming less than 300 mg/wk, who possess the *ADORA2A* 1976T/T genotype, experienced

greater anxiety following 150 mg caffeine compared with those who possessed at least one C allele (29). Individuals with the T/T genotype are significantly more likely to limit caffeine intake (i.e., consume <100 mg/day) than those who possess at least one C allele, with the probability of having the T/T genotype decreasing as caffeine intake increases (30).

CAFFEINE CONTENT OF VARIOUS PRODUCTS

In the United States, most of caffeine (approximately 80%) is consumed in coffee (31). Caffeine is also found in soft drinks, especially colas, energy drinks, tea, chocolate, over-the-counter (OTC) drugs, and dietary supplements (Table 1) (32). Some non-cola beverages also contain caffeine such as Mountain Dew[®], Dr Pepper[®], and Pibb Xtra[®]. There is tremendous variation in the caffeine in a cup of coffee. Instant coffee (5 oz) can have as little as 40 mg of caffeine, whereas drip-method brewed coffee can have as much as 150 mg (Table 1). There is considerable variation in coffee prepared using the same method, due to differences in caffeine content of different types of coffee beans, especially arabica versus robusta, and variations in brewing technique.

CAFFEINE INTAKE

Recent information on caffeine consumption in the United States is not available. The most current data are based on information collected between 1994–1998 (33) and 1999 (34). Using data from the nationally representative U.S. Department of Agriculture Continuing Survey of Food Intakes by Individuals ($n = 18,081$), Frary et al. reported, in 1994–1998, that 87% of the population was caffeine consumers, with an average caffeine intake of 193 mg/day in users. Adult males consumed more caffeine than females (268 mg/day vs. 192 mg/day). Coffee was the major source of caffeine for consumers of all ages (68 mg/day), followed by 15 mg/day from soft drinks and 12 mg/day from tea (33). However, Ahuja et al. (35) concluded that Frary et al. overestimated caffeine intake and revised their estimates downward by about 25%. According to Ahuja et al., average daily intake of caffeine in the U.S. population is 131 mg/day, with males and females (20+ years) consuming 193 and 149 mg/day, respectively (35). Knight et al. (34) estimated caffeine intake in caffeine consumers ($n = 10,712$) from beverages on the basis of data from the 1999 U.S. Share of Intake Panel as 141 mg/day for adults. There are other reports of higher caffeine consumption in the United States. Barone and Roberts (31) reported that U.S. caffeine intake for all consumers is about 210 mg/day.

Since these data were collected, changes in availability of caffeine-containing products have occurred and consumer preferences have changed (36). Energy drinks, introduced in the United States in 1997, contain caffeine (Table 1) and are a popular component of the diet, especially among young adults (36). Another new product containing high levels of caffeine (Table 1) termed “energy shots” is rapidly gaining in popularity.

High levels of caffeine are present in many dietary supplements (Table 1), particularly those intended to promote weight loss, for example, Zantrex 3[®] and new Hydroxycut Advanced[™] (37–39). Unfortunately, manufacturers of such products are not required to disclose their caffeine content, so this information can be difficult to obtain. These products have not been shown to increase weight loss, and recently some of the Hydroxycut[™] family of products was withdrawn from the market after the FDA issued a warning (40).

REGULATORY STATUS OF CAFFEINE

In the United States, complex regulations govern use of caffeine. Once caffeine is ingested and enters circulation, its source is of little physiological or health significance, but U.S. government agencies, in practice, regulate it on the basis of the medium in which it is consumed. Caffeine consumed in dietary supplements, occurring naturally in foods, caffeine added to foods, and caffeine in OTC and prescription drugs are all regulated differently. Multiple sources of caffeine ingestion and regulation can lead to peculiar consequences. For example, a large cup of coffee purchased at a coffee shop can contain more caffeine than the recommended dose of an OTC stimulant. The recent popularity of energy drinks has led to calls for additional regulation of such products, because it has been argued that these products are abused (36).

BEHAVIORAL EFFECTS OF CAFFEINE

It is likely that caffeine is the most widely studied behaviorally active compound not only in dietary supplements, but also in any exogenously administered compound. Caffeine's behavioral effects have been examined in a large number of laboratories and in well-controlled studies conducted with males, females, young, and older volunteers (41–43). Effects observed on specific aspects of cognitive function and mood state are usually consistent with the lay

perception of caffeine as a mild stimulant when consumed in moderate doses, just as Pietro della Valle recognized 400 years ago (44).

However, it can be difficult to detect effects of caffeine if insensitive behavioral tests that assess parameters not affected by caffeine are employed or doses that are too low or high administered. In addition, it is essential to control intake of caffeine before testing and monitor and control for habitual patterns of caffeine consumption, as these factors can have substantial effects on study findings. Controlling for tobacco use is also essential, because smoking significantly decreases caffeine's half-life. Furthermore, well-designed studies typically employ a range of doses, since caffeine's behavioral effects are dose dependent and nonmonotonic.

Effects on Cognitive Performance and Mood in Rested Volunteers

In rested volunteers, caffeine consistently improves both auditory and visual vigilance (3,41,45–48). When a dose of 200 mg of caffeine is given, effects on vigilance are seen for several hours and are so robust that they can be detected on a minute-by-minute basis (45) (Fig. 4). Such effects are present with doses equivalent to a single serving of a cola beverage, about 40 mg, up to multiple cups of coffee (3,43,46). However, when higher doses are administered (approximately 400–500 mg or above), cognitive performance begins to deteriorate, so optimal dose appears to be in the range found in foods (49,50). Caffeine also improves simple and choice reaction time in rested individuals (51). In general, it appears that sustained tests of vigilance or tasks with substantial embedded vigilance components are the most sensitive to behavioral effects of caffeine in rested individuals.

Mood state is also altered by doses of caffeine equivalent to those found in single and multiple servings of dietary supplements, foods, and drugs. Aspects of mood affected by caffeine are consistent with its effects on cognitive functions such as vigilance and reaction time. For example, the vigor and fatigue subscales of the Profile of Mood States (POMS) (52), a widely used self-report mood

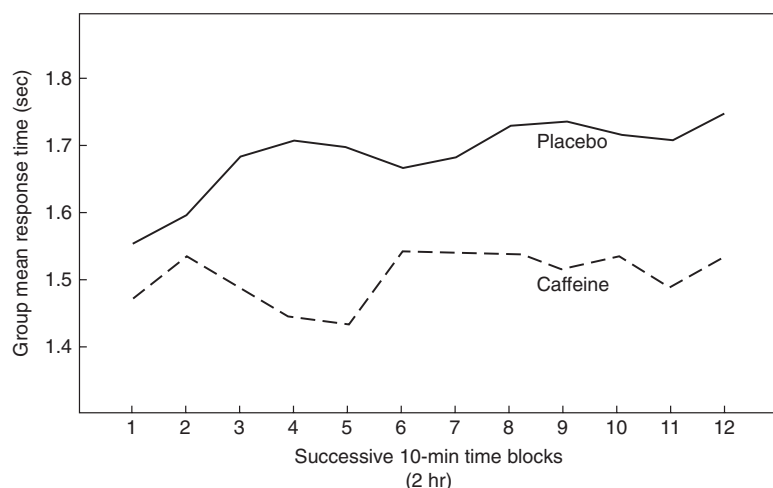


Figure 4 Effect of a 200-mg caffeine dose administered at time "0" on visual vigilance reaction time assessed continuously and plotted in 10 minute time blocks. Slower reaction time (higher number) indicates worse performance ($p < 0.002$; caffeine vs. placebo). Caffeine consistently improved cognitive performance for two hours. Source: From Ref. 45.

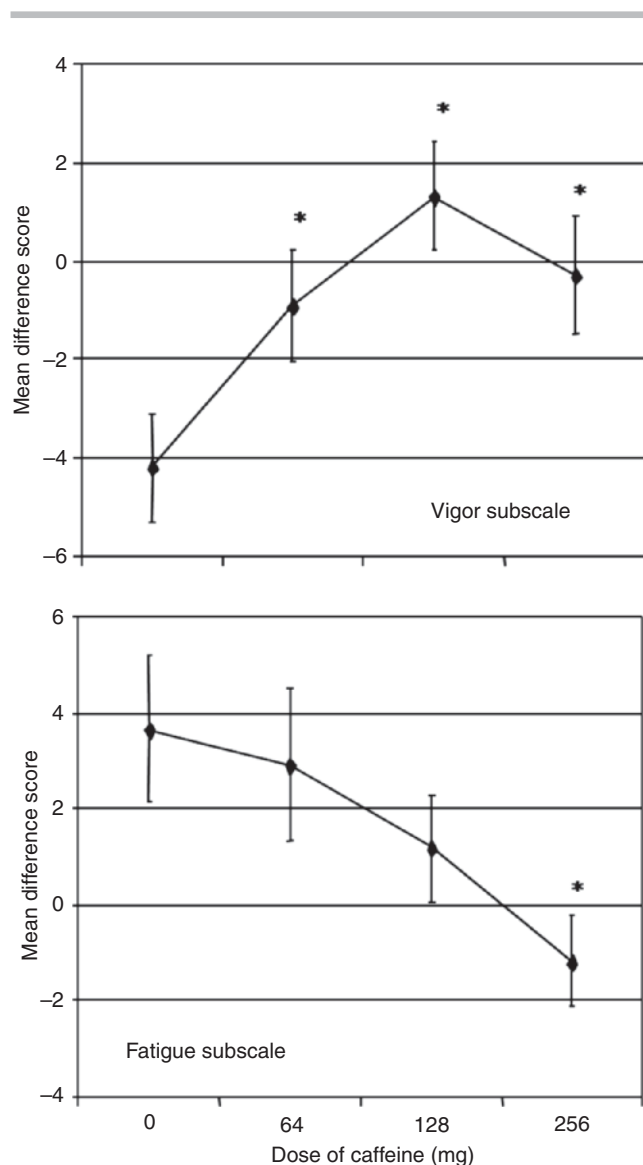


Figure 5 Effects of 64 to 256 mg of caffeine compared to placebo (mean \pm SEM) on the fatigue and vigor subscales of the Profile of Mood States (POMS). Difference scores were computed by subtracting the baseline from posttreatment values. Higher numbers on the vigor subscale indicate increased vigor; lower numbers on the fatigue subscale indicate lowered fatigue. * $p < 0.05$ caffeine vs. placebo Source: From Ref. 32.

questionnaire, are altered by caffeine in a dose-dependent manner (Fig. 5). Caffeine typically increases vigor and reduces fatigue. At higher doses, these beneficial effects may be reduced or disappear. An analog scale mood questionnaire designed to assess effects of caffeine consistently detects effects on moods such as tired/energetic, listless/full of go, and efficient/inefficient (41).

Fine Motor Performance

Caffeine consumption has been associated, at least anecdotally, with impaired fine motor performance. When administered in a dose of 160 mg, it disrupted hand steadiness in nonusers but not in users (53). In a study with low and moderate consumers, doses of 32 to 256 mg of caffeine

had no effect on tests of complex motor function (54). A recent study examined effect of caffeine on handwriting in caffeine consumers (54). In this study, subjects were administered caffeine in doses of 0, 1.5, 3.0, or 4.5 mg/kg and performed a writing exercise on a digitized tablet. Compared to placebo, high doses of caffeine improved aspects of handwriting, such as fluidity of movement (54). Caffeine has also been reported to improve marksmanship, a task that requires fine motor performance (55).

Caffeine and Anxiety

It appears that caffeine increases anxiety when administered in single bolus doses of 300 mg or higher, a dose not ordinarily found in single servings of beverages, although there are some exceptions. Generally, large servings of beverages, such as 16 oz of coffee, that contain high amounts of caffeine, are consumed slowly over time. Some products, for example certain brands of energy drinks or energy shots, do contain such high levels of caffeine and may be consumed quickly, especially energy shots (Table 1). The effects of caffeine on anxiety at lower doses are unclear, with both positive and adverse effects reported, perhaps due to differences in the testing environment (50,56).

Several papers suggest caffeine consumption can adversely affect individuals suffering from anxiety disorders. Also, consumption of more than 600 mg of caffeine per day may induce, in normal individuals, a syndrome known as "caffeinism," characterized by anxiety, disturbed sleep, and psychophysiological complaints (57).

Effects on Cognitive Performance and Mood During Sleep-Deprivation and Stress

Caffeine has substantial beneficial effects on cognitive performance and mood when individuals are sleep deprived or exposed to multiple stressors (50,58,59). Under such conditions, caffeine positively affects various behavioral parameters, including vigilance and mood state. For example, during a night of sleep deprivation, 200 mg of caffeine administered every two hours maintained vigilance performance (60). Other behavioral parameters, such as learning, memory, and reasoning, not altered when caffeine is administered to rested volunteers, are affected when individuals are sleep-deprived (50). When individuals were sleep-deprived for 30 hours but not exposed to additional stressors, 300 mg of caffeine per 70 kg of body mass improved working memory, logical reasoning, mathematical processing, pursuit tracking, and visual vigilance (61). In a study conducted with U.S. Navy SEAL trainees exposed to multiple stressors including cold, intense physical challenges, 72 hours of sleep deprivation, and severe psychological stress, caffeine in doses of 200 and 300 mg improved visual vigilance, choice reaction time, and self-reported alertness (50) (Fig. 6).

Simulator and Applied Behavioral Studies

Based, in part, on studies demonstrating caffeine has beneficial effects in laboratory studies of cognitive performance and mood, studies have been conducted to determine whether caffeine will have beneficial effects in simulated or real work environments. For example, Regina et al. (6) tested rested males in a realistic simulation



Figure 6 U.S. Navy SEAL trainees are exposed to multiple stressors during a segment of training known colloquially as “Hell Week.” This provided a unique opportunity to test the behavioral effects of caffeine during sustained exposure to severe stress (50). Cold stress serves as a key physiological stressor during most of Hell Week. As instructors look on, trainees are required to walk into the cold ocean water. Source: Photo courtesy of H.R. Lieberman.

of highway driving. Caffeine (200 mg) improved several aspects of driving performance including response time to accelerations and decelerations of a lead car. Philip et al. (63) examined effects of approximately 200 mg of caffeine administered in coffee on rested, young male volunteers driving a distance of 200 km late at night on a highway. Caffeine improved ability to maintain control of the vehicle as measured by deviation from the traffic lane. In a study simulating sentry duty, Johnson and Merullo (64) evaluated the effects of 200 mg of caffeine on marksmanship for three hours following caffeine administration. Soldiers in the study responded to infrequent appearance of a target by picking up a rifle, aiming, and firing as rapidly, and accurately, as possible. Caffeine decreased detection time but did not increase the number of targets hit.

Recently, a series of studies have been conducted by Kamimori and colleagues using a caffeine-containing gum to determine whether caffeine would enhance performance under conditions simulating combat, including intermittent or continuous sleep deprivation and extensive physical activity. These studies uniformly demonstrate that in a wide variety of circumstances, various aspects of cognitive, operational, and aerobic performance are enhanced by caffeine (58,59).

In aggregate, these behavioral studies have important practical implications. Use of caffeine in moderate doses can improve the performance of individuals who must drive automobiles or stand sentry duty for long periods of time during the day or night. These beneficial effects increase in situations where vigilance is reduced due to sleep loss, jet lag, or circadian variations in arousal. Recently, at the request of the U.S. Defense Department, an independent panel conducted a comprehensive review of the scientific literature and concluded that caffeine, in doses of 100 to 600 mg, could be used to maintain cognitive performance of military personnel. As a consequence, caffeine is currently available in certain field rations (2,65).

CAFFEINE AND MENTAL ENERGY

Considerable attention has focused on dietary supplements and foods that may increase “mental energy,” and most of these contain caffeine. As noted above, new product categories of “energy drinks” and “energy shots” have emerged as popular products among the population (36). Scientific literature on mental energy is quite limited (66–68), although related factors, such as fatigue and alertness, have been extensively examined. Scientists have typically used the term “energy” to describe the concept of physical energy measured in calories or joules. Mental energy, however, cannot be easily defined or measured, but a distinction between physical and mental energy clearly exists (32,68–70).

In the United States, surveys have observed a high prevalence of feelings of low energy. For the lay public, mental energy is perceived as critical for the conduct of daily activities and quality of life. On health-related Internet sites, “fatigue,” “tiredness,” or “absence of energy” are among the largest concerns for which remedies are sought. To address consumer demand for such products, dietary supplements and foods containing caffeine have been marketed asserting they enhance energy (36,69). Some have been evaluated by use of cognitive tests, and the results are usually consistent with the claims made (68).

Caffeine is one of the few constituents found in dietary supplements or foods that clearly increase mental energy (32,68). As noted above, low and moderate doses improve aspects of cognitive performance and mood associated with the perception of mental energy such as vigilance, reaction time, vigor, and fatigue (68,71). Beneficial effects of caffeine that appear related to mental energy are observed in simulations of real-world activities (58,59,62,64,72). Caffeine (100 mg) increases alertness and self-reported attention in college students attending a lecture (56). Epidemiological studies of large populations indicate caffeine consumption has positive effects on factors related to mental energy in large populations (73). In a sample of over 7000 British adults, a significant dose-response relationship between increased overall caffeine intake and improved cognitive performance was observed (73). Inclusion of caffeine in products intended to increase perception of mental energy therefore appears warranted. Caffeine may be the only active ingredient in such products, if mental as opposed to physical energy is the implied benefit (32,68).

EFFECTS OF CAFFEINE ON SLEEP

It is not surprising that caffeine may interfere with sleep, because it improves ability to sustain vigilance and increases alertness. Many individuals abstain from caffeine consumption in the afternoon and evening because they believe caffeine will disrupt nighttime sleep. Others report they consume caffeine-containing beverages before bedtime with no adverse impact on sleep (74). Genetic differences in sensitivity to caffeine, as discussed above, and acquired tolerance by individuals who consume caffeine, probably contribute to these differences. Consumers of 3 to 6 cups of coffee per day are less likely to report sleep

disturbances than individuals who consume 0 to 1 cups per day (74).

Anecdotal reports that caffeine interferes with sleep are supported by the scientific literature. In both high and low consumers, a high dose of caffeine (4.0 mg/kg) at bedtime reduced sleep tendency, as measured by the Multiple Sleep Latency test (75). A recent study examined effects of moderate doses of caffeine before bedtime on various sleep parameters assessed with polysomnography. Subjects received 100 mg of caffeine (or placebo) three hours and then one hour before sleeping in the laboratory. Caffeine lengthened sleep latency, increased stage 1 sleep (light sleep) and reduced slow-wave and stage 2 sleep (deeper sleep) (76).

BEHAVIORAL EFFECTS OF CAFFEINE: NONUSERS AND WITHDRAWAL

One of the most controversial issues regarding the behavioral effects of caffeine is whether it affects performance and mood independent of withdrawal symptoms. It has been suggested that behavioral effects of caffeine can only be observed in habituated individuals experiencing effects of caffeine withdrawal on performance and mood when treated with placebo (77). If this hypothesis is correct then caffeine should have no effects on individuals who are not regular users. Several studies that fail to find behavioral effects of caffeine in individuals who are not habitual users support this hypothesis (77,78). However, there are several problems with this hypothesis and the experimental evidence supporting it. No plausible mechanism has been advanced to explain how a substance could have no acute effects on cognitive function, yet have effects when it is withdrawn. Many substances produce tolerance when administered for sustained periods. However, these substances have acute behavioral effects consistent with behavioral consequences of their withdrawal. Examples include drugs of abuse, such as heroin, and therapeutic compounds such as the benzodiazepines. To test the hypothesis advanced by Rogers and colleagues that caffeine only has behavioral effects on habitual users, a number of laboratories have conducted studies. These demonstrate caffeine has behavioral effects on nonusers and affects users who continue with their typical patterns of caffeine consumption (47,48,51).

CAFFEINE AND PHYSICAL PERFORMANCE

Just as caffeine improves specific aspects of cognitive performance and mood, it also has positive effects on some aspects of physical performance; however, it is not clear that these effects occur in doses found in most dietary supplements or foods. Evidence that caffeine enhances aerobic performance is convincing. A review of the literature concluded that "caffeine effectively increases athletic performances in endurance events" (79). For example, when 4 mg/kg of caffeine was administered to eight male volunteers, time to run to exhaustion increased (80). In another study, 3 and 6 mg/kg of caffeine enhanced endurance but a higher dose, 9 mg/kg, did not (81). Beneficial effects of caffeine assessed with a bicycle ergometer were observed

at doses 5 and 9 mg/kg, a higher dose (13 mg/kg) was no more effective than lower doses (82).

These dose-dependent findings are similar to behavioral studies with caffeine, in which higher doses can have adverse effects on mood and do not enhance performance to the same extent as moderate doses (49,50). It appears that ergogenic effects of caffeine, like caffeine's behavioral effects, are attributable to its effects on central adenosine receptors (21).

CAFFEINE WITHDRAWAL

Sudden withdrawal of caffeine from the diet, if regularly consumed in substantial doses, can have adverse effects in approximately 50% of users. Most notable is headache, which is relieved by consumption of caffeine. Onset of symptoms typically occurs in 12 to 24 hours and may last for several days. Other symptoms can include fatigue, lower energy, and difficulty concentrating. Caffeine-withdrawal headaches are relieved by OTC analgesics (83,84). Individuals who wish to reduce or eliminate caffeine from their diet should probably do so gradually.

ABUSE POTENTIAL OF CAFFEINE

Although controversial, it has been suggested that caffeine should be considered an addictive compound and is similar to drugs that have substantial abuse potential, such as nicotine and cocaine (85). Evidence for this association includes adverse physical effects of caffeine withdrawal in animals and human studies of caffeine self-administration (84). Hirsh (83) has noted that addiction can best be defined as compulsion to use a drug, and specifically, involvement with the abused substances to the exclusion of other interests. The use of methylxanthines in foods and beverages would not appear to qualify as such behavior (83). Most individuals who are regular users of caffeine can readily halt its use and not feel compelled to continue consuming caffeine-containing products. It is much more difficult to stop using drugs of abuse despite the fact that these substances are known to be extremely harmful. Caffeine clearly has low abuse potential compared to more widely recognized drugs of abuse (86).

CONCLUSION

When caffeine is consumed in doses found in foods and dietary supplements, it improves ability to perform tasks requiring sustained vigilance, including real and simulated automobile driving, and activities that require maintenance of vigilance (50,58,59). In addition, caffeine increases self-reported alertness and decreases sleepiness. Caffeine positively affects a wide range of cognitive functions in sleep-deprived individuals, including learning, memory, and reasoning. Caffeine can be found in many dietary supplements, which are marketed to increase weight loss, but evidence to support this implied claim is lacking.

Adverse behavioral effects of caffeine occur when it is consumed in excessive doses or by individuals who are more sensitive to the substance. Genetic factors and an

individual history of caffeine consumption may be the key factors explaining individual differences. In high doses, caffeine can increase anxiety but its effects on fine motor performance vary with improvement and impairment reported. It also interferes with sleep when consumed by certain individuals at bedtime. Like many other drugs, regular caffeine consumption appears to produce tolerance to its behavioral effects. Sudden withdrawal of caffeine from the diet will lead to adverse symptoms, such as headache and undesirable changes in mood state, in approximately 50% of individuals. Some scientists believe that caffeine has properties that are similar to those exhibited by drugs of abuse; others strongly disagree with this hypothesis.

An evidence-based determination of the risk-to-benefit ratio of caffeine consumption is not possible. Positive behavioral consequences of caffeine are well documented. These beneficial effects generalize to highway driving, various military duties, and presumably other transportation and industrial operations. Use of caffeine in such circumstances could potentially prevent accidents attributable to lapses of vigilance such as "falling asleep at the wheel." Such accidents are a significant cause of motor vehicle accidents. However, adverse effects of caffeine on sleep quality have been observed, and some scientists believe that caffeine has characteristics of an addictive drug.

It must also be noted that a large and complex literature on possible beneficial and adverse effects of caffeine on the incidence of various diseases exists. There are many methodological concerns with both positive and negative studies. The difficulty in accurately assessing caffeine intake is a critical issue in such studies as is the lack of double-blind, placebo-controlled clinical trials. Therefore, both positive and negative findings regarding possible health risks and benefits of caffeine should be regarded with skepticism.

DISCLAIMER

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Calcium

Robert P. Heaney

INTRODUCTION

Calcium is an alkaline earth, divalent, cationic element, abundant in the biosphere, and widely distributed in nature. It exhibits intermediate solubility. As a solid, calcium forms crystalline minerals with various anions. These salts make up the bulk of limestone, marble, gypsum, coral, pearls, seashells, bones, and antlers. In solution, the calcium ionic radius (0.99 Å units) allows the ion to fit snugly into the folds of protein molecules.

ACTIONS AND PHARMACOLOGY/PHYSIOLOGY

Calcium is unusual—perhaps unique—among the nutrients in that its intake (whether from foods or supplements) is not related to its primary intracellular, metabolic function. Rather, calcium nutrition is centered almost exclusively on the secondary functions of the nutrient. Accordingly, the primary functions are described here for completeness, but only briefly. More information can be found in standard textbooks of cell physiology or in reviews of calcium signaling (1).

Primary Metabolic Functions

Calcium acts as a second messenger within cells, linking external stimuli acting on cells to the specific, internal responses a cell is able to make (e.g., nerve signals and muscle contraction). By forming up to 8 to 12 coordination bonds with oxygen atoms in amino acid side chains, calcium stabilizes the tertiary structure of numerous catalytic and structural proteins. Cytosolic calcium ion levels are normally maintained at very low concentrations [3–4 orders of magnitude below extracellular fluid (ECF) levels]. The second messenger response occurs when calcium ions flood into critical cytosolic compartments in response to first message stimuli.

Additionally, dissolved calcium in the circulating blood and ECF of all vertebrates supports such diverse functions as blood clotting and neuromuscular signal transmission. Calcium is not consumed in the exercise of these metabolic functions.

ECF $[Ca^{2+}]$ is tightly maintained at approximately 4.4 to 5.2 mg/dL (1.1–1.3 mmol/L). The regulatory apparatus behind this constancy consists of parathyroid hormone (PTH), calcitonin, and 1,25-dihydroxyvitamin D $[1,25(OH)_2D]$, acting jointly through control of intestinal

calcium absorption efficiency, bone resorption, and the renal excretory threshold for calcium.

Secondary Functions

Effects on the Size and Strength of the Nutrient Reserve (Bone Mass)

Calcium is lost continuously from the body through shed skin, hair, nails, sweat, and excreta. For this reason, land-living vertebrates, needing a continuous supply of calcium, have evolved an internal reserve, in the form of bone. Because bone also serves structural/mechanical functions, the reserve has become far larger than would be needed solely to protect calcium's primary functions. It is for this reason that the primary functions themselves are not threatened by deficient calcium intake, or enhanced by calcium repletion.

The bony reserves are accessed by a process termed “bone remodeling.” Bony tissue is continuously renewed by first resorbing preexisting volumes of bone and then subsequently replacing them with new bone. Mineralization of the new bone occurs at a rate that is the integral of the prior several days of osteoblast activity, and for that reason tends to be relatively constant over the short term. By contrast, osteoclastic bone resorption is controllable minute by minute. Thus, by modulating bone resorption, the body can, in effect, withdraw calcium from, or cause it to be taken up by, bone whenever ECF $[Ca^{2+}]$ departs from optimal levels.

When daily absorbed calcium intake is less than that needed to offset daily calcium losses, bone resorption exceeds bone formation and the bony reserves are depleted. This occurs by net destruction of microscopic volumes of bony tissue and scavenging of the calcium released in the process. Such decrease in skeletal mass results in a corresponding reduction in strength. Additionally, bone remodeling itself directly contributes to bony structural weakness (2), insofar as the remodeling locus is, for the several months of its life cycle, depleted of its normal complement of bony material, thereby greatly weakening the involved microscopic bony elements.

The principal purpose of calcium intake during growth is to support the accumulation of the skeletal mass called for in the genetic program, that is, the building of a large calcium reserve. During the adult years, intake serves to (i) offset daily losses, thus preventing unbalanced withdrawals from the skeletal reserves, with their inevitable, associated reduction in bony strength; and (ii) reduce the level of bone remodeling to the minimum

needed for optimum structural maintenance (2). These two effects are the basis for the protective effect of calcium with respect to osteoporosis.

Intraluminal Effects of Unabsorbed Dietary Calcium

Net absorption efficiency for ingested calcium is of the order of 10% to 15% (see later). Accordingly, up to 90% of dietary and supplemental calcium remains in the intestinal lumen and is excreted as a component of the feces. At high calcium intakes, unabsorbed calcium amounts to 1000 mg (25 mmol) per day or more. This unabsorbed calcium complexes with other constituents of the digestive residue, blocking their absorption or neutralizing their luminal actions (3). This occurs, for example, with oxalic acid, which may be either present in ingested plant foods or produced by bacterial degradation of unabsorbed food fatty acids. The formation of calcium oxalate in the gut lumen reduces oxalate absorption and hence the renal oxalate load. It thereby reduces the risk of kidney stones. Similarly, the calcium ion complexes directly with free fatty acids and bile acids in the digestate substances, which, in their free form, act as mucosal irritants. In colon cancer-prone individuals, these irritants would otherwise serve as cancer promoters. These intraluminal actions are the basis for the protective effects of high calcium intakes on risk of renal stone disease and colon cancer.

Additionally, calcium complexes with dietary phosphorus, blocking its absorption to some extent. This is the basis for the use of calcium salts as a part of the control of hyperphosphatemia in patients with end-stage renal disease (ESRD). Every 500 mg of ingested calcium (whether from foods or supplements) binds ≈ 166 mg of coingested phosphorus, preventing its absorption (4).

"Off-Loop" Effects of Alterations in Calcium Homeostasis

When calcium intake is low, PTH is secreted to improve renal calcium conservation and intestinal absorption efficiency, the latter through 1- α -hydroxylation of 25(OH)D to 1,25(OH) $_2$ D in the kidney. The calcium-conserving effects of these hormones are part of a classical negative feedback loop, in the sense that 1,25(OH) $_2$ D, by increasing calcium absorptive extraction from food, counteracts to some extent the original stimulus to PTH secretion and 1,25(OH) $_2$ D synthesis.

In addition to these functions within the feedback control loop, 1,25(OH) $_2$ D binds to membrane receptors in many tissues not directly involved in calcium regulation (3). These include vascular smooth muscle cells and adipocytes. These effects are termed "off-loop," because they occur as a result of reduced ECF $[Ca^{2+}]$ but do not act to change that level. Hence, they do not influence the signals that caused them in the first place, that is, they are not a part of the regulatory feedback loop. The cell membrane receptors are linked to calcium channels that open and let calcium ions into the cytosol, where they may trigger their usual second messenger function (but without the normal first messenger). The presence of high cytosolic calcium levels, when dietary calcium is low, has given rise to the term "calcium paradox disease." In individuals with limited control of cytosolic $[Ca^{2+}]$, this rise in cytosolic calcium triggers inappropriate, tissue-specific

cell activity, for example, smooth muscle contraction in arterioles and adipogenesis in fat cells. These relationships are the basis for the protective effects of high calcium intake against hypertension and obesity, and probably for premenstrual syndrome and polycystic ovary syndrome as well (3).

The Internal Calcium Economy

The adult human body contains approximately 1000 to 1300 g (25,000–32,500 mmol) of calcium, with more than 99% being locked up in bones and teeth. Low hydration of bone, together with the insolubility of hydroxyapatite (the principal form of calcium phosphate in mineralized tissues), means that most body calcium is effectively exterior to the ECF and accessible only by cellular action (e.g., osteoclastic bone resorption).

The ECF, which is the locus of all body calcium traffic, contains about 1 g (25 mmol) calcium (i.e., $\approx 0.1\%$ of total body calcium). Soft tissues contain another 7 to 8 g (175–200 mmol) of calcium, mostly locked up in intracellular vesicles, which store calcium for its critical, second messenger function. The calcium homeostatic regulatory apparatus functions solely to maintain the constancy of the concentration of the ≈ 1 g of calcium in the ECF. In healthy midlife adults, ECF calcium turns over at a rate of approximately 650 mg/day (≈ 10 mg/kg/day), with bone mineralization and resorption accounting for half to two-thirds of that traffic.

Figure 1 displays the principal organs involved in the transfers that comprise the traffic of the calcium economy, together with the sizes of those transfers in and out of the system. The values shown are typical of the

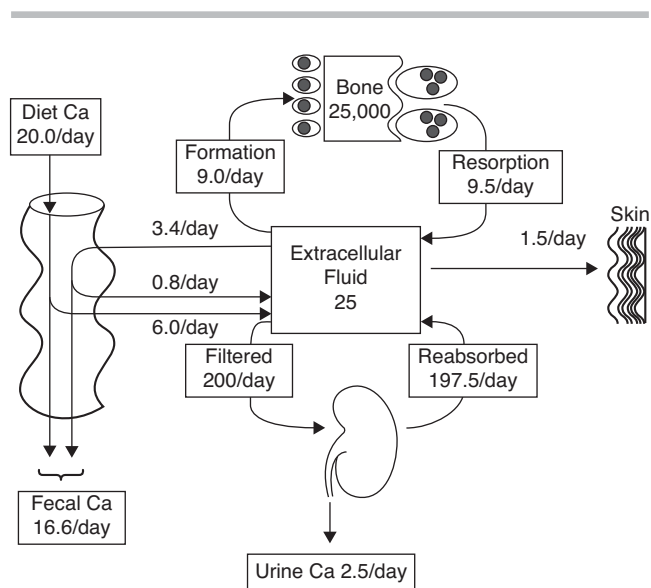


Figure 1 Principal routes of calcium entry into and exit from the extracellular fluid of an adult human. The values for bone and ECF are total masses (mmol); transfer rates are given in mmol/day and represent typical values. (To convert to milligrams, multiply values shown by 40.) Total body balance in this illustration is -0.5 mmol/day. Source: Copyright Robert P. Heaney, 1996, 2004; used with permission.

calcium economy of a middle-aged woman. In considering the magnitudes of these transfers, it is important to recognize that they do not vary independent of one another. An increase in absorption, for example, produces an immediate decrease in bone resorption. This linkage is mediated by the PTH-calcitonin-vitamin D regulatory apparatus.

Absorption

Calcium is absorbed mainly from the small intestine by a combination of active, transcellular transport and passive, paracellular diffusion. The active transport component is mediated by a vitamin D-dependent calcium-binding protein ("calbindin") that shuttles calcium ions from the luminal brush border to basolateral portions of the cell membrane, where calcium is released into the ECF. Calbindin activity is highest in the duodenum and drops along the length of the remaining bowel (including the colon). Accordingly active transport capacity is greatest in the duodenum. However, the residence time of the digestate in the duodenum is short, and most of the actual mass transport occurs in the jejunum and ileum, where residence time is longer.

The partition of absorption between the active and the passive mechanisms is not well studied, but data from various sources suggest that, at nutritionally relevant intake loads (i.e., 7.5+ mmol/meal), passive absorption amounts to approximately 15% of intake. Fractional absorption above that value thus reflects the vitamin D-mediated active transport component. The latter is highly variable, both because it is physiologically regulated in response to body need for calcium, and, in part, because it is often limited by vitamin D availability. The interactions between intake load of calcium and its active absorption are complex and are summarized in Figure 2. As measured fractional absorption is typically on the order of

0.30 to 0.32; it follows that the active transport component amounts to approximately 0.16 (16%). As is shown in Figure 2, the 16% isogram intersects the dashed line for 5 mmol (200 mg) net absorption at an intake of approximately 1200 mg (30 mmol), and thus designates the oral intake needed to maintain total body equilibrium. When vitamin D status is less than optimal, the body is generally unable to maintain active absorption at a 16% level and absorption occurs along lower and lower isograms until, at severe vitamin D deficiency, active absorption is zero. As is shown in Figure 2, intake at such absorption values would need to be in the range of 3000 mg/day to ensure absorption of sufficient calcium to offset obligatory losses.

Figure 3 shows the calcemic rise above baseline in healthy adults for a 500-mg calcium supplement source ingested as part of a low-calcium breakfast. It illustrates a number of features of calcium absorption: (i) a delay of approximately 30 minutes before serum calcium begins to rise, reflecting gastric residence time; (ii) peak calcemia at 3 to 5 hours after ingestion, indicating continuing absorptive input throughout that period of time; (iii) a degree of calcemia approximating a 1% rise for every 100 mg calcium ingested, that is, a perturbation that is within the usual normal range for serum calcium and hence effectively undetectable outside of a research context; and (iv) gradual return to baseline by 9 to 10 hours. Tracer studies show that calcium absorption is effectively completed by five hours after ingestion (5), and the slow fall to baseline after the peak reflects offsetting declines in other inputs into the ECF.

It is commonly considered that calcium salts must be dissociated to be absorbed, and hence that solubility predicts absorbability. However, this is probably incorrect. The pH of the digestate in the small intestine is close to neutral, and it is likely that most of the digestate calcium is complexed with prevailing anions in the digestate.

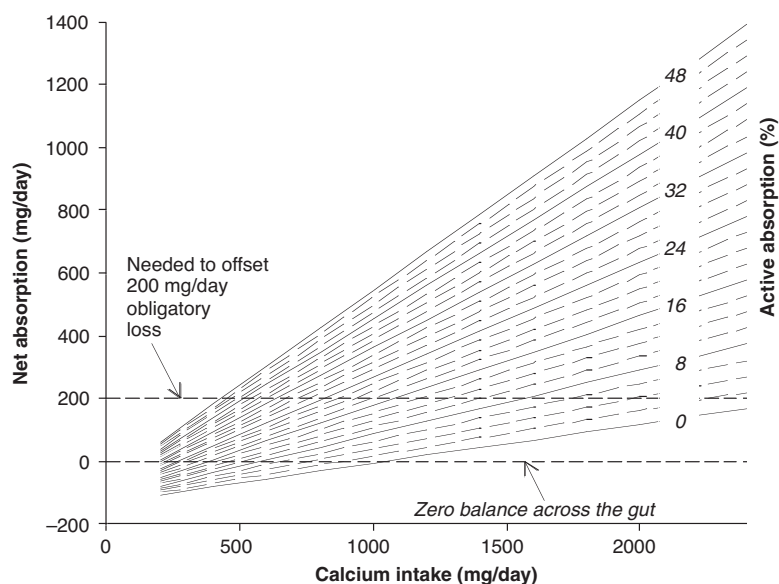


Figure 2 Relationship of vitamin D-mediated, active calcium absorption, calcium intake, and net calcium gain across the gut. Each of the contours represents a different level of active absorption above a baseline passive absorption of 12.5%. (The values along each contour represent the sum total of passive and variable active absorption.) The horizontal dashed lines indicate 0 and 5 mmol/day net absorption, respectively. The former is the value at which the gut switches from a net excretory to a net absorptive mode, and the latter is the value needed to offset typical urinary and cutaneous losses in mature adults. Source: Copyright Robert P. Heaney, 1999. Reproduced with permission.

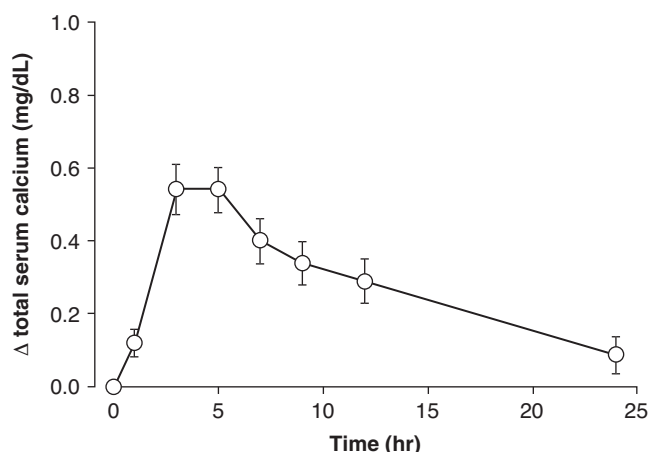


Figure 3 Time course of the rise in serum calcium following a single oral dose of a commercial calcium carbonate preparation (containing 500 mg calcium) taken as part of a light, low-calcium breakfast. Error bars are 1 SEM. Source: Copyright Robert P. Heaney, 2001, 2004; used with permission.

Aqueous solubility of calcium salts spanning 4 to 5 orders of magnitude has been shown to have little or no effect on absorbability if the calcium source is coingested with food (6). Double-tracer studies have demonstrated absorption of insoluble calcium complexes *without prior dissociation* (7). Thorough dispersion of calcium salts among food particulates is probably more important than actual solubilization. Additionally, continuous slow release of calcium from the stomach, exposing the duodenal mucosa to only small amounts of calcium at a time, substantially improves absorption (Figure 4) (8).

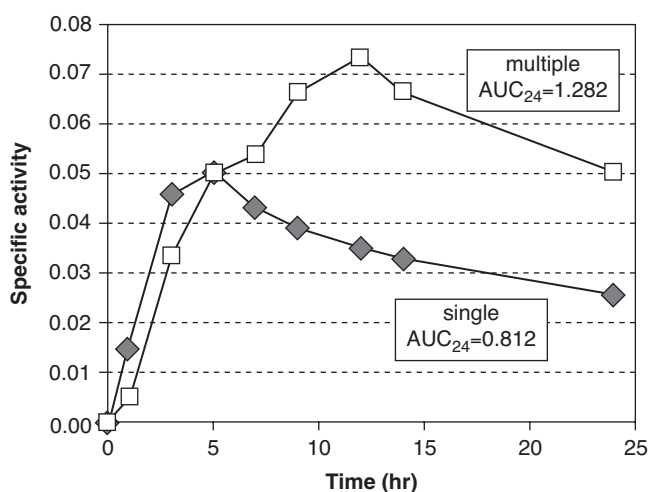


Figure 4 Time course of serum calcium tracer concentration following oral dosing of 1000 mg tracer-labeled calcium, either as a single bolus dose ("single") or as 17 divided doses of 59 mg each ("multiple") given over an 8-hour period. "Specific activity" is a measure of tracer concentration in the calcium contained in a serum sample. Source: Copyright Robert P. Heaney, 2002; used with permission.

Excretion

Calcium leaves the body through unabsorbed digestive secretions, through sweat and shed skin, hair, and nails, and through urine (Fig. 1). In nonexercising adult humans with typical calcium intakes, digestive calcium losses amount to approximately 120 mg/day, cutaneous losses amount to approximately 60 mg/day, and urinary losses amount to approximately 120 mg/day, with great individual variability around these figures. Only the urinary loss is physiologically regulated by the system controlling calcium homeostasis, and much of even the urinary calcium represents obligatory loss, that is, excretion determined by forces outside of the calcium regulatory system (9), such as salt intake and net endogenous acid production (as, for example, from metabolism of S-containing amino acids). On average, urine calcium rises by approximately 45 mg (1.1 mmol) for every 1000 mg (25 mmol) increase in calcium intake. This increase is a reflection of the small absorptive calcemia (Fig. 3), which produces a corresponding rise in the filtered load of calcium.

In adults, the primary purpose served by ingested calcium is the offsetting of obligatory excretory losses, thus protecting the skeletal reserves and thereby preserving their structural integrity. Ingested calcium, thus, does not so much "go" to bone as prevents net removal of calcium from bone.

INDICATIONS AND USAGE

Calcium is a nutrient and would normally be ingested as a component of food. However, except for dairy foods, modern diets, especially seed-based plant foods (which are the basis of most contemporary diets), are calcium-poor diets. Hence, for many individuals, achieving an adequate calcium intake may be difficult without recourse to supplements or calcium-fortified foods. (The latter are effectively equivalent to taking a supplement along with the otherwise unfortified food.)

Supplementation to Achieve Recommended Intake Levels

Diets free of dairy foods typically contain no more than 200 to 300 mg calcium, far below currently recommended intakes (Table 1). Supplementation (or fortification) will often be required to meet optimal intake objectives.

Table 1 Estimated Average Requirements (EARs) for Calcium and the Corresponding RDAs (mg/day)

Age range	EAR	RDA ^a
Infants, 7–12 mo	270	350
Children		
1–3 yr	500	600
4–8 yr	800	1000
Boys and girls, 9–18 yr	1300	1550
Men and women		
19–50 yr	1000	1200
>50 yr	1200	1450

^aUsing an estimated 10% coefficient of variation of individual requirements around the population mean.

Source: From Ref. 10.

Since absorption efficiency is inversely proportional to the logarithm of the ingested load (11), absorption is maximized by a divided dose regimen (e.g., 3× per day; Fig. 4). Also, because delivery of calcium to the absorptive sites in the upper small intestine is optimized under meal conditions, it is best to take calcium supplements with meals. (*N.B.*: Fortified foods tend, automatically, to meet both objectives.)

Preparations

The nutritional preparations of calcium include mainly salts with such anions as carbonate, citrate, phosphate, lactate, acetate, fumarate, and citrate-malate (CCM). In addition, salts with gluconic acid may occasionally be found, and calcium chelates with amino acids are also marketed. The calcium content (i.e., “elemental” calcium) varies from 40% for the carbonate salt to ≈13% for CCM. For phosphate binding in ESRD, the acetate salt is more commonly used. In the United States, most preparations come in the form of swallowable or chewable tablets, with calcium contents ranging from 200 to 600 mg per tablet.

Bioavailability is approximately the same for all the leading salts, although CCM and the chelates tend toward the high end of the range and the gluconic acid salts toward the low end. Absorbability of the salt is only very weakly related to solubility, and gastric acid is not necessary for calcium absorption if the supplement is taken (as recommended) with meals. The most extensive, side-by-side comparisons have involved the carbonate and citrate salts, and the bulk of the evidence for such comparisons indicates approximately equal absorbability for the two sources, with perhaps a slight edge for the carbonate salt (12). Poor pharmaceutical formulation will impede disintegration and hence impair absorption, a problem encountered with many generic calcium supplement products sold in the 1980s and early 1990s (13). For this reason, preference should be given to supplements that meet United States Pharmacopeia (USP) disintegration standards, and, even better, to those that have demonstrated bioavailability.

A growing variety of fortified foods has been available since 1999. As noted, fortification tends to improve the nutritional value of low-calcium foods and, to some extent, it can be thought of as equivalent to taking supplements with meals. However, interactions between added calcium and various food constituents during food processing and storage may alter the absorbability characteristics of the former. For example, it was noted during the early days of juice supplementation that CCM was well absorbed from orange and grapefruit juices, and even better from apple juice, but poorly from lemon juice. These differences could not have been predicted from what was known of food chemistry. Hence, with fortified foods as with supplements, actual bioavailability of the product reaching the consumer should be demonstrated.

When calcium is added to beverages (such as orange juice or soy beverage), an additional problem arises. Solubility of the principal calcium salts is relatively low, and serving size portions of such beverages would not sustain in solution more than a small fraction of the calcium content of, say, a comparable serving of milk. Hence, such fortification almost always requires physical suspension of a particulate. In some beverages, this suspension is so

poor that the calcium settles as a dense sludge at the bottom of the beverage container and may, accordingly, not be ingested at all (14).

Supportive Therapy as a Part of Antiosteoporosis Pharmacotherapy

Current antiosteoporosis pharmacotherapy includes bisphosphonates, selective estrogen receptor modulators (SERMs), estrogen, and anabolic agents such as the fluoride salts, PTH derivatives such as teriparatide, and RANK-ligand antibodies and cathepsin-K inhibitors. All have at least stabilization of bone mass as their goals. Some of them, such as the bisphosphonates, can lead to slow, steady-state bone gain (0.5–1.0% per year), and the anabolic agents can produce as much as 8% to 10% bone gain per year. To support this increase, especially for the anabolic agents, calcium intake from diet must usually be augmented by supplements. Optimal doses for calcium during pharmacotherapy have not been established. However, all the bisphosphonates and SERMs have been tested only with 500 to 1000 mg supplemental calcium, whereas fluoride has been shown to produce bone hunger calling for as much as 2500 mg of calcium per day. Only estrogen has been studied with and without supplemental calcium, and here the evidence is very clear: bony effects of estrogen are augmented two- to threefold, and estrogen dose can be reduced by half if calcium intake is above 1000 mg/day (15,16). With the more potent anabolic agents, a calcium phosphate preparation may be preferable, so as to ensure an adequate intake of both of the components of bone mineral and to compensate for the intestinal binding of diet phosphorus by high-dose calcium supplementation. The high phosphorus loads of the phosphate salts produce no adverse metabolic consequences, and calcium phosphate supports bone anabolism fully as well as the carbonate salts.

Ancillary Therapy for Prevention or Treatment of Miscellaneous Disorders

Hypertension, pre-eclampsia, colon cancer, renolithiasis, premenstrual syndrome, polycystic ovary syndrome, and obesity—all multifactorial disorders—have each been shown to have a calcium-related component (3), and for several of them, calcium supplementation has been shown in randomized-controlled trials to reduce incidence and/or severity. Optimal calcium intake for this protection has not been established for any of the disorders concerned, but several threads of evidence indicate that total intakes of 1200 to 1800 mg of calcium per day may be sufficient. The role of calcium in these disorders has been described earlier (see sections “intraluminal effects of unabsorbed dietary calcium” and “off-loop effects of alterations in calcium homeostasis”). Specific pharmacotherapy of any of the disorders concerned should always be accompanied by an adequate calcium intake, using supplements if necessary.

CONTRAINDICATIONS

There are few, if any, true contraindications to calcium supplementation. In general, supplementation moves

contemporary intakes into the range that would have been the Paleolithic standard, and hence helps to normalize modern diets. However, patients receiving calcitriol therapy or suffering from disorders such as sarcoidosis, in which calcium absorption may be high, should not take supplements except under medical supervision.

PRECAUTIONS AND ADVERSE REACTIONS

Calcium supplements may bind with tetracycline antibiotics and hence reduce their absorbability. The element has also been reported to interfere slightly with thyroxine absorption. Hence, a person requiring both calcium and thyroid replacements should take them at different times of the day or have plasma thyroxine and thyroid-stimulating hormone (TSH) levels checked to ensure that the thyroid dose produces the desired therapeutic effect. Both calcium salts and high-calcium foods reduce absorption of non-heme iron ingested at the same meal in unprepared subjects. However, chronic supplementation studies show no long-term deterioration in iron status in adults and no interference with augmenting iron status during growth (17). The single-meal tests that are used to demonstrate this interference could not have detected physiological upregulation of iron absorption.

Adverse reactions tend to be extremely rare and mostly idiosyncratic. Although constipation is often said to be a consequence of taking calcium carbonate, the evidence is scant (18), and in several randomized-controlled trials, the difference in degree of constipation between the calcium- and placebo-treated groups has generally been small and usually not statistically significant.

OVERDOSAGE

The Food and Nutrition Board of the Institute of Medicine, in its 1997 recommendations, set a tolerable upper intake level for calcium to be 2500 mg/day (9). However, it is important to note that there has never been a reported case of overdosage of calcium from food sources, even at continuing intakes over 6000 mg/day. Supplement intakes above 2500 mg/day are occasionally associated with a syndrome similar to the milk alkali syndrome. The pathogenesis of the hypercalcemia seen in this condition is complex, but there is usually hypoperfusion of both the kidneys and the skeleton, the two most important internal regulatory organs for calcium. The condition can usually be managed by giving attention to adequate hydration and maintenance of blood flow to these critical organs. Except as support for the most potent osteoporosis pharmacotherapy, or in management of the hyperphosphatemia of ESRD, there is no known reason to use supplements at a dose above 2500 mg of calcium per day.

REGULATORY STATUS

Calcium supplements are regulated as foods in the United States. Bioavailability is not a regulated characteristic of

marketed supplement products. Nevertheless, because of pharmaceutical formulation and food matrix effects on absorbability, bioavailability of different preparations of the same salt (e.g., calcium carbonate) may vary over a twofold range.

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L-Carnitine, Acetyl-L-Carnitine, and Propionyl-L-Carnitine

Charles J. Rebouche

INTRODUCTION

L-Carnitine [known chemically as *R*(-)- β -hydroxy- γ -(*N,N,N*-trimethylammonio)butyrate; molecular weight: 161.2 g/mol] is a water-soluble quaternary amine that facilitates lipid metabolism. Only the L isomer is biologically active. Humans acquire varying amounts of L-carnitine from dietary sources, but a dietary requirement has not been established. The goal of this chapter is to survey the literature on the clinical findings on L-carnitine and its esters acetyl-L-carnitine and propionyl-L-carnitine. Due to space constraints, this is not an exhaustive review. Readers are directed to the references for more information.

BIOCHEMISTRY AND FUNCTIONS

The human body synthesizes L-carnitine from the essential amino acids lysine and methionine in amounts that are limited but adequate for the maintenance of normal health (1). L-Carnitine participates in reversible transesterification reactions, in which an acyl group is transferred from coenzyme A to the hydroxyl group of L-carnitine (Fig. 1). Acetyl-L-carnitine, propionyl-L-carnitine, and other esters are biosynthesized in this manner. Carnitine and acetyl-L-carnitine (and lesser amounts of other esters of carnitine) are also obtained from the diet (1).

Transfer of Long-Chain Fatty Acids from Cytoplasm into Mitochondria

Long-chain fatty acids, as free acids or coenzyme A esters, cannot cross the mitochondrial inner membrane. In contrast, long-chain acylcarnitine esters rapidly cross this membrane, facilitated by a carrier protein, carnitine-acylcarnitine translocase (CACT) (2). In the cytoplasm, transesterification of long-chain fatty acids from coenzyme A to L-carnitine is catalyzed by carnitine palmitoyltransferase I (CPT I), an integral protein of the mitochondrial outer membrane. This enzyme serves as the primary regulator in partitioning fatty acids toward oxidation in mitochondria or triglyceride synthesis, and its activity is regulated principally through inhibition by malonyl-CoA (3). On the matrix side of the mitochondrial inner membrane, the acyl group of the carnitine ester is transferred to intramitochondrial coenzyme A and carnitine is released (2). This reaction is catalyzed by carnitine palmitoyltransferase II (CPT II), an enzyme bound to the surface of the membrane (2). L-Carnitine, either nonesterified or as a short-chain acyl ester, may then exit the mitochondrion via CACT (Fig. 2).

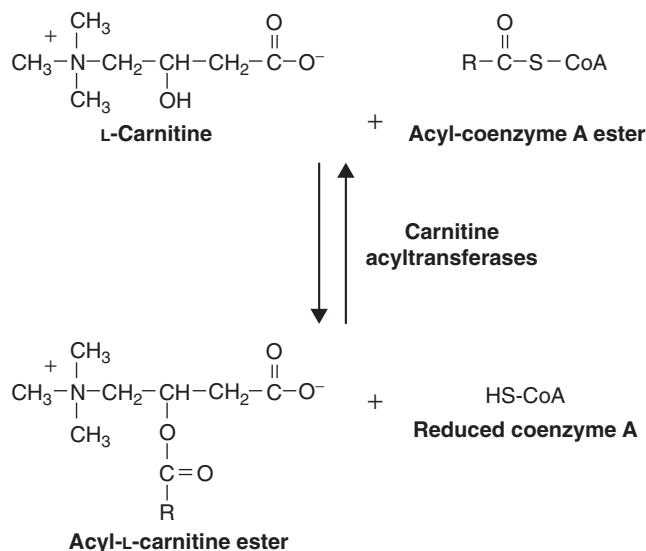


Figure 1 Transesterification reaction between L-carnitine and coenzyme A.

Transfer of Chain-Shortened Fatty Acids from Peroxisomes to Mitochondria

Very long-chain fatty acids are not metabolized in the mitochondria. Instead, they enter peroxisomes and undergo one or more β -oxidation cycles, leading to the generation of medium-chain acyl-CoA. These acyl groups are then transesterified by carnitine octanoyltransferase for export from the mitochondria (2). Medium-chain acylcarnitine esters exported from peroxisomes are transported into mitochondria by CACT, and the acyl moieties are transesterified to coenzyme A and oxidized (Fig. 2) (2).

Modulation of the Acyl-CoA/CoA Ratio in Cellular Compartments

Coenzyme A participates in many metabolic processes in the cellular cytoplasm and organelles. However, neither coenzyme A nor its thioesters can cross the membranes separating these compartments. Thus, in each compartment, sufficient nonesterified coenzyme A must be made available to maintain metabolic activities in that part of the cell. Because of its ability to be transported across organelle membranes and undergo rapid transesterification with coenzyme A, L-carnitine facilitates availability of adequate amounts of the deacylated coenzyme. In mitochondria, the amount of acetyl-CoA generated from rapid β -oxidation of fatty acids or carbohydrate utilization

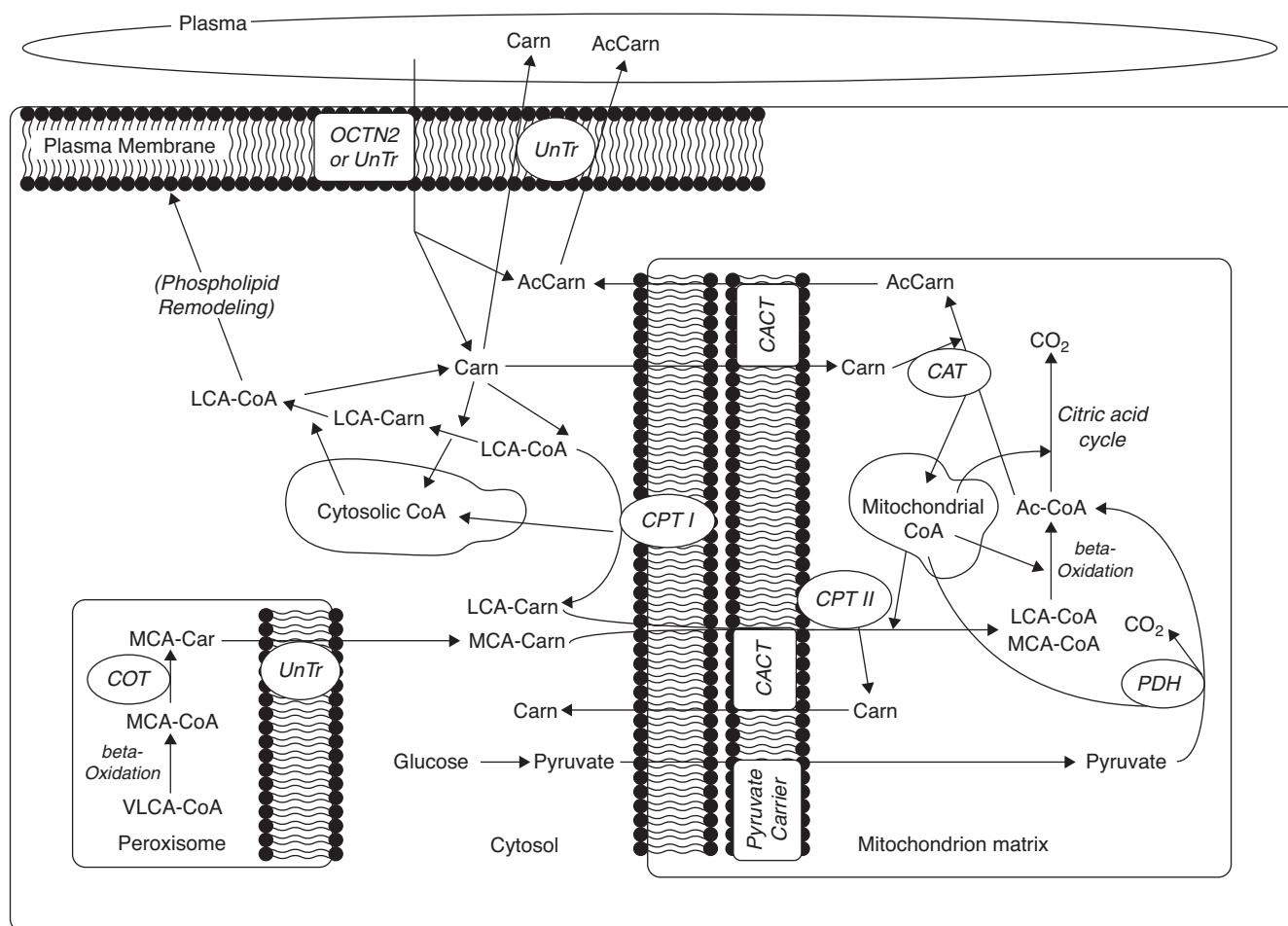


Figure 2 L-Carnitine and acetyl-L-carnitine transport and function. *Abbreviations:* Carn, L-carnitine; AcCarn, acetyl-L-carnitine; OCTN2, organic cation transporter 2; UnTr, unidentified transporter(s); LCA-CoA, long-chain acyl-coenzyme A; MCA-CoA, medium-chain acyl-coenzyme A; VLCA-CoA, very long-chain acyl-coenzyme A; LCA-Carn, long-chain acylcarnitine esters; MCA-Carn, medium-chain acylcarnitine esters; Ac-CoA, acetyl-coenzyme A; CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; CACT, carnitine-acylcarnitine translocase; PDH, pyruvate dehydrogenase complex.

may exceed the capacity of the citric acid cycle to release the coenzyme. Transesterification of acetyl units to L-carnitine, catalyzed by carnitine acetyltransferase (CAT), frees intramitochondrial coenzyme A for participation in subsequent cycles of substrate utilization (3,4). Lowering of the acetyl-CoA/CoA ratio stimulates pyruvate oxidation, secondary to an increase in pyruvate dehydrogenase complex activity (4). Acetyl-L-carnitine can be removed from the mitochondrion via CACT for use in the cytoplasm, for export and use in other cells or tissues, or for excretion (3,4). This function has long been viewed primarily as a means to dispose of acetyl units from mitochondria. However, export of acetyl-L-carnitine from some cells and tissues (e.g., liver and kidney) may be important for supply of this metabolite to other tissues (e.g., brain), where it may have specific functions in addition to its use as a substrate for energy production. Moreover, by modulating the intramitochondrial acetyl-CoA/CoA ratio, L-carnitine plays a significant role in regulating glucose metabolism in skeletal muscle and heart (4).

Membrane Phospholipid Remodeling

L-Carnitine and extramitochondrial CPT are important modulators of long-chain fatty acid utilization for membrane phospholipid biosynthesis and remodeling. L-Carnitine acts as a reservoir of long-chain fatty acids for incorporation into erythrocyte membrane phospholipids during repair after oxidative insult (2) and for use in the synthesis of dipalmitoylphosphatidylcholine, the major component of surfactant, in lung alveolar cells (2).

Other Reported Actions of L-Carnitine and/or Its Acetyl and Propionyl Esters

L-Carnitine may mimic some of the actions of glucocorticoids *in vivo*. In HeLa cells, L-carnitine reduces glucocorticoid receptor- α affinity for its steroid ligand and triggers nuclear translocation of the receptor (5). It suppresses glucocorticoid receptor-mediated tumor necrosis factor- α and interleukin-12 release by human primary

monocytes stimulated with lipopolysaccharide *ex vivo* (5). All of these effects of L-carnitine are concentration dependent. In rats and mice, L-carnitine is found to markedly suppress liposaccharide-induced cytokine production, improving their survival during cachexia and septic shock (5). In humans, L-carnitine supplementation of surgical and AIDS patients decreased serum tumor necrosis factor- α concentration (5). Glucocorticoids also increase the expression and activity of urea cycle enzymes. Hyperammonemia associated with chronic valproic acid therapy and with several inborn errors of metabolism, including CACT deficiency and medium-chain acyl-CoA dehydrogenase deficiency, is attenuated by L-carnitine administration. It has been experimentally found that L-carnitine supplementation protects against lethal ammonia intoxication in mice (6). One suggested mechanism for these effects of L-carnitine is increased synthesis and activity of urea cycle enzymes, a process also responsive to glucocorticoids.

L-Carnitine is a peripheral antagonist of thyroid hormone action in some tissues (7). It inhibits thyroid hormone entry into cell nuclei. In a controlled clinical trial, L-carnitine was shown to reverse or prevent some symptoms of hyperthyroidism (7).

Acetyl-L-carnitine may directly or indirectly reverse age-associated mitochondrial decay (8). It acts as a chaperone to protect macromolecules, including CAT, from structural alteration and/or loss of function. Acetyl-L-carnitine partially reverses age-associated loss of mitochondrial membrane potential and decline in membrane cardiolipin concentration, and protects against oxidative damage to mitochondrial DNA (8).

L-Carnitine inhibits arachidonic acid incorporation into platelet phospholipids, agonist-induced arachidonic acid release, and arachidonic acid-induced NADPH (nicotinamide adenine dinucleotide phosphate)-oxidase activation (9). It has been proposed and some evidence has been obtained to support this notion that acetyl-L-carnitine stimulates or upregulates expression of heat shock proteins, redox-sensitive transcription factors, and sirtuins that protect against oxidative cellular damage (10). These actions may be particularly important in modulating the aging process and in slowing the progression of neurodegenerative diseases.

PHYSIOLOGY

Dietary Intake and Biosynthesis

Meat, fish, chicken, and dairy products are rich sources of dietary L-carnitine (1). Plant-derived foods contain very small amounts of the substance. Most commercially available infant formulas contain L-carnitine, either provided from the milk component or supplemented, as in the case of soy-protein-based formulas. There is no recommended dietary allowance or dietary reference intake for L-carnitine.

In mammals, L-carnitine is synthesized from ϵ -N-trimethyllysine, which is derived from post-translationally methylated lysine residues in proteins, and protein turnover (1). In normal humans, the rate of synthesis is estimated to be approximately 1.2 $\mu\text{mol/kg}$ body weight/day (1). The rate of L-carnitine biosynthesis is regulated by the availability of ϵ -N-trimethyllysine. Thus,

conditions that increase protein methylation and/or protein turnover may increase the rate of L-carnitine biosynthesis (1).

Bioavailability

Dietary L-carnitine may be absorbed through active or passive mechanisms. Evidence from several *in vivo* and *in vitro* studies indicates that L-carnitine is actively transported from the small intestinal lumen into enterocytes (11). However, the preponderance of data suggests that intracellular L-carnitine in the intestinal mucosa does not cross serosal membranes by an active transport mechanism. Absorption of dietary L-carnitine and L-carnitine supplements appears to occur primarily by passive diffusion (11). In humans, approximately 54% to 87% of dietary L-carnitine is absorbed, depending on the amount in the diet. The bioavailability of dietary supplements (0.6–4 g/day) is 15% to 20% (11,12). Unabsorbed L-carnitine is degraded by microorganisms in the large intestine. Major metabolites identified are trimethylamine oxide in urine and γ -butyrobetaine in feces (11,12). Bioavailability of oral acetyl-L-carnitine has not been studied in normal healthy humans.

Distribution in Tissues, Fluids, and Cells

L-Carnitine and acylcarnitine esters are present in all tissues. In most tissues and cells, they are present in higher concentration than in the circulation. For example, in human skeletal muscle and liver, respectively, nonesterified L-carnitine is concentrated 76-fold and 50-fold from that in serum (estimated from data in Ref. 13).

Tissue Accumulation

L-Carnitine and acetyl-L-carnitine are concentrated in most tissues via high-affinity, Na^+ -dependent organic cation transporter OCTN2 (14). K_t for L-carnitine binding is 3 to 5 μM ; OCTN2 binds acetyl-L-carnitine and propionyl-L-carnitine with comparable affinity. This protein is highly expressed in heart, placenta, skeletal muscle, kidney, pancreas, testis, and epididymis and weakly expressed in brain, lung, and liver. L-Carnitine entry into the liver occurs via a low-affinity ($K_t = 5 \text{ mM}$) transporter, probably distinct from OCTN2. Several other L-carnitine transporters have been identified, including OCTN1, OCTN3, and ATB⁰⁺, and Oat9S (2,15). Specific roles for these transporters in carnitine metabolism in humans have not been determined.

Homeostasis, Renal Reabsorption, and Excretion

Circulating L-carnitine concentrations are maintained at a fairly constant level of around 50 μM , predominantly through efficient reabsorption by the kidney (11). At a filtered load of 50 $\mu\text{mol/L}$, the efficiency of L-carnitine and acylcarnitine ester reabsorption is 90% to 98%. However, as the filtered load of L-carnitine increases, for example, after consumption of a dietary supplement or after intravenous infusion, the efficiency of reabsorption declines rapidly. Physiologically, the efficiency of L-carnitine reabsorption is sensitive to the amount in the diet as well as the differences in the macronutrient content of the diet. Clearance of acylcarnitine esters is often higher than that of nonesterified L-carnitine. Experimental

studies have shown that in rats and humans, kidneys are able to synthesize acetyl-L-carnitine from L-carnitine and either acetoacetate or β -hydroxybutyrate, and that L-carnitine, acetyl-L-carnitine, and γ -butyrobetaine (also synthesized in human kidneys) are secreted from mucosal cells into the tubular lumen (11). Because the kinetics of transport of these metabolites by the sodium-dependent L-carnitine transporter are not different, the relative proportions appearing in urine reflect not only those in the glomerular filtrate but also those in the renal tubular epithelium that are secreted into the lumen. Thus, under conditions of rapid intracellular synthesis of acylcarnitine esters or direct accumulation from the circulation, secretion of these species will lead to a higher proportion of acylcarnitine esters in urine compared to that in the circulation. By inference, kidneys may be substantially involved in the regulation of circulating acylcarnitine ester concentrations (11).

L-CARNITINE DEFICIENCIES

L-Carnitine deficiency is defined biochemically as abnormally low concentration ($<20 \mu\text{M}$) of nonesterified L-carnitine in plasma (2). A concentration ratio of acylcarnitine esters/nonesterified L-carnitine of 0.4 or greater in plasma is also considered abnormal. Nutritional L-carnitine deficiency has not been shown to occur in the absence of other mitigating factors (2).

Primary L-carnitine deficiency occurs as a result of defects in the gene coding for the plasma membrane L-carnitine transporter OCTN2 (16). Characteristic features of this disease are cardiomyopathy, hypoketotic hypoglycemia, and muscle weakness. Secondary carnitine deficiency occurs in association with genetic diseases of organic acid metabolism and in genetic diseases involving defects in fatty acid oxidation. Secondary carnitine deficiency has been described in patients with end-stage renal disease requiring maintenance hemodialysis. Secondary carnitine deficiency has been observed during chronic use of drugs, including valproic acid, cisplatin, ifosfamide, zidovudine, and pivalic acid-containing prodrugs (17–20).

INDICATIONS AND USAGE

L-Carnitine, acetyl-L-carnitine, and/or propionyl-L-carnitine may be used for replacement therapy to restore normal carnitine concentrations and/or abnormal nonesterified-to-esterified carnitine ratio. They may be used as supplements to increase the carnitine load of the body and/or increase the flux of carnitine among compartments. In some conditions, both replacement therapy and supplementation are appropriate. For primary and some secondary carnitine deficiencies (see earlier), L-carnitine is used for replacement therapy.

L-Carnitine Supplementation in Neonatal Nutrition

L-Carnitine has been described as a conditionally essential nutrient for infants. For the last 25 years, commercial enteral infant formula products have included L-carnitine, where necessary, to achieve an L-carnitine concentration similar to that in human milk ($\sim 60 \mu\text{mol/L}$). Bovine milk-

based formulas typically contain a higher concentration of L-carnitine than does human milk. On the other hand, premature infant formulas, both enteral and parenteral, typically do not include L-carnitine at the time of manufacture, but are sometimes supplemented at the time of use. The rationale for supplementation is twofold: Infants utilize lipids as a primary source for energy and growth after birth, requiring a high rate of mitochondrial oxidation, and the concentration of L-carnitine in infant circulation and tissues typically is lower without supplementation than in infants fed human milk or formulas containing carnitine. Studies in premature infants typically have focused on the effect of L-carnitine supplements on growth rate and morbidity, with mixed results. In one double-blind, placebo-controlled, randomized trial of L-carnitine supplementation in premature infants 28 to 34 gestational weeks of age at birth, no differences in two-week weight gain over 8 weeks were observed between supplemented and nonsupplemented infants (21). More recently, in another prospective, randomized, placebo-controlled, double-blinded study, 29 premature neonates received carnitine supplementation (20 mg/kg body weight/day) or placebo for up to eight weeks. Supplemented neonates regained their birth weight more rapidly than placebo group neonates, indicating that L-carnitine supplementation may promote more rapid catch-up growth (22). An extensive review of these and many more relevant studies has been published (23). For infants expected to be on parenteral nutrition for seven days or longer, supplementation with L-carnitine 10 to 20 mg/kg body weight/day, given intravenously or orally, is recommended (23).

L-Carnitine Replacement Therapy and Supplementation in End-Stage Renal Disease

Regular L-carnitine supplementation in hemodialysis patients can improve lipid metabolism, antioxidant status, and anemia requiring erythropoietin and may reduce incidence of intradialytic muscle cramps, hypotension, asthenia, muscle weakness, and cardiomyopathy (24,25). The recommended dosage is 50 mg/kg body weight/day, to a maximum of 3 g/day.

L-Carnitine and Propionyl-L-Carnitine Supplementation for Cardiac Ischemia, Congestive Heart Failure, Cardiomyopathy, and Peripheral Arterial Disease

Experimental studies have shown L-carnitine to be an effective antianginal agent that reduces ST-segment depression and left ventricular end-diastolic pressure during stress in patients with coronary artery disease (25). Cardioprotective effects of L-carnitine have been observed following aortocoronary bypass grafting and following acute myocardial infarction. Carnitine administration initiated early after acute myocardial infarction attenuated left ventricular dilation and resulted in smaller left ventricular volumes (25).

L-Carnitine deficiency syndromes sometimes present with dilated cardiomyopathy and are often effectively treated with L-carnitine (26). Thus, it was suggested that cardiomyopathy progressing to congestive heart failure but not associated with inherited L-carnitine deficiency might respond to L-carnitine supplementation. A large-scale clinical trial of L-carnitine supplementation

versus placebo in 574 patients with heart failure produced promising results with regard to improvement in maximum duration of exercise, but other endpoints, including death and hospital admissions during the follow-up period, were not different between treatment groups (26). Utility of L-carnitine supplements for congestive heart failure not associated with inherited L-carnitine deficiency remains debatable (26,27).

Peripheral arterial disease is a common manifestation of atherosclerosis and is associated with reduced arterial circulation in the lower extremities. In five large, randomized, double-blind, placebo-controlled studies, with long duration of therapy, and three short-duration studies, propionyl-L-carnitine supplementation (1–3 g/day, orally) for up to one year improved initial claudication distance (distance walked before muscular symptoms appeared) and absolute claudication distance (distance at which patient stopped walking due to muscular cramps) (28). Quality of life outcomes were also improved with propionyl-L-carnitine treatment relative to placebo. Experimental and clinical studies suggest that the improvements observed in clinical trials are due to protection by propionyl-L-carnitine from the effects of oxidative stress and inflammation in ischemic tissue endothelium (28), as well as due to increased blood flow resulting from endothelium-dependent vasodilation elicited by propionyl-L-carnitine (29).

L-Carnitine Supplementation for Exercise Performance and Weight Reduction

L-Carnitine supplementation has been suggested to improve exercise performance in healthy humans. Proposed mechanisms include enhanced muscle fatty acid oxidation, altered glucose homeostasis, enhanced acylcarnitine production, modification of training responses, and altered muscle fatigue resistance (30). A review of published studies has led to the conclusion that L-carnitine supplements do not improve exercise performance in healthy humans (30–33). On the other hand, in conditions where the nonesterified L-carnitine concentration of skeletal muscle may be significantly reduced, such as in peripheral arterial disease and end-stage renal disease, L-carnitine supplementation has afforded some benefit to muscle function and exercise performance (33).

In a double-blind, placebo-controlled, crossover design clinical trial, oral administration of 4.5 g of propionyl-L-carnitine (as glycine propionyl-L-carnitine) to trained athletes significantly enhanced peak power production in resistance-trained males with significantly lower lactate accumulation (34). The incremental differences in power production were modest and were observed only after repeated short-duration Wingate cycle sprints. However, it is noted that, particularly for elite athletes, modest enhancement in performance can be highly significant in outcomes in competitive sports. Propionyl-L-carnitine may be more effective in enhancement of exercise performance than L-carnitine, because of its ability to increase nitric oxide production and vasodilation. Moreover, propionyl-L-carnitine may enhance citric acid cycle activity by providing propionyl units that can be converted to succinate.

Because of its role in facilitating fatty acid oxidation, L-carnitine has been suggested to aid in weight loss regimes. Two facts argue against this. First, there is no evidence that it facilitates, directly or indirectly, mobilization of fatty acids from adipose tissue. Second, in normal humans, the intracellular concentration of L-carnitine is not rate-limiting for transesterification of fatty acids by CPT I. Adding an increment of L-carnitine will not increase the rate at which this reaction occurs. There is no scientific evidence that L-carnitine supplements facilitate weight loss in humans.

L-Carnitine and Acetyl-L-Carnitine Replacement Therapy and Supplementation for Chronic Fatigue

L-Carnitine may improve symptoms of fatigue in humans. Use of the cancer chemotherapeutic agents cisplatin and ifosfamide is associated with fatigue. In a prospective, open-label study, improvement of symptoms of fatigue was observed in 50 nonanemic patients following L-carnitine supplementation to the chemotherapeutic regimen of cisplatin or ifosfamide (35). However, in a subsequent randomized, double-blind, placebo-controlled trial, no improvement in measures of fatigue was observed as a result of L-carnitine supplementation (36). The study included an open-label phase, in which fatigue symptoms did show some improvement with L-carnitine supplementation.

Chronic fatigue syndrome (CFS) in humans was found to be associated with low circulating acetyl-L-carnitine concentration and decreased accumulation in several brain regions (37). It has been suggested that acetyl-L-carnitine helps maintain neuronal metabolic activity by promoting glucose and lactate uptake and utilization through its role as a precursor of glutamate in neurons (38). In a randomized, open-label study of 30 patients with CFS, acetyl-L-carnitine and propionyl-L-carnitine showed beneficial effects on fatigue and attention concentration (39).

Acetyl-L-Carnitine Supplementation for Depression and Cognitive Function in the Elderly

Acetyl-L-carnitine appears to have specific and perhaps unique roles in brain metabolism. Animal studies and in vitro experiments suggest that this agent has promise in slowing or reversing memory and cognition decline as well as the decline in physical performance that normally occurs in the process of aging. In studies of the elderly, patients with depressive syndrome scored significantly lower on the Hamilton Rating Scale for Depression (modified for the elderly) following supplementation with acetyl-L-carnitine (40). Older subjects with mild mental impairment had improved scores on cognitive performance tests following such supplementation (41). A meta-analysis of the efficacy of acetyl-L-carnitine in mild cognitive impairment and mild Alzheimer disease included all identified double-blind, placebo-controlled, prospective, parallel-group studies using treatment doses of 1.5 to 3.0 g/day of acetyl-L-carnitine that were conducted between 1983 and 2000 (42). This analysis showed a significant advantage for acetyl-L-carnitine compared to placebo, with beneficial effects observed on both clinical scales and psychometric tests. The benefit of supplement use was

observed for three months, and it was found to increase over time. The typical usage recommended by vendors is 1 to 3 g/day.

L-Carnitine Supplementation in Liver Dysfunction with Hyperammonemia

Hyperammonemia occurs in some inborn errors of metabolism and as a result of drug- or toxicant-induced hepatotoxicity. Mortality and metabolic consequences of acute ammonium intoxication in mice are reduced by pharmacologic administration of L-carnitine (6). The mechanism for this effect may have two components. L-Carnitine administration normalizes the redox state of the brain (perhaps by increasing the availability of β -hydroxybutyrate and/or acetyl-L-carnitine to the brain), and it increases the rate of urea synthesis in the liver, perhaps, in part, by activation of the glucocorticoid receptor. At least part of the protective effect is associated with flux through the carnitine acyltransferases, as analogs of L-carnitine that are competitive inhibitors of carnitine acyltransferases enhance the toxicity of acute ammonium administration (6). Thus, it has been proposed that L-carnitine increases urea synthesis in the liver by facilitating fatty acid entry into mitochondria, leading to increased flux through the β -oxidation pathway, an increase of intramitochondrial reducing equivalents, and enhancement of ATP production (6). Carnitine supplementation may benefit individuals with hepatic dysfunction due to inborn errors of metabolism or chemical intoxication.

L-Carnitine and Acetyl-L-Carnitine Replacement Therapy and Supplementation in Diabetes

L-Carnitine infusion improves insulin sensitivity in insulin-resistant diabetic patients (43). Glucose oxidation is increased during L-carnitine administration, concurrent with lower plasma concentration of lactate. These observations suggest that L-carnitine activates normally depressed pyruvate dehydrogenase activity in insulin-resistant patients (43). Intravenous administration of acetyl-L-carnitine increases glucose disposal in Type 2 diabetic patients (44). Such administration appears to promote storage of glucose as glycogen, rather than increase in glucose oxidation (44).

L-Carnitine and Acetyl-L-Carnitine Replacement Therapy and Supplementation in HIV Infection

L-Carnitine and acetyl-L-carnitine ester concentrations are below normal in some human immunodeficiency virus (HIV)-infected patients undergoing antiretroviral therapy (45). L-Carnitine administration as part of antiretroviral therapy with either zidovudine or didanosine reduced lymphocyte apoptosis and oxidant stress compared to the antiretroviral regimens without L-carnitine (46).

L-Carnitine and Acetyl-L-Carnitine Supplementation in Male Reproductive Dysfunction

L-Carnitine and/or acetyl-L-carnitine supplementation may be beneficial in men with oligoasthenospermia, a condition in which low sperm count is associated with low sperm motility. Epididymal fluid contains the highest concentration of L-carnitine in the human body. L-Carnitine is secreted from the epithelium into epididymal plasma via

a testis-specific carnitine transporter (47). The very high concentration of L-carnitine in epididymal fluid provides for passive diffusion of L-carnitine into spermatozoa during transit and maturation through the epididymis. Mature spermatozoa acetylate L-carnitine to generate a pool of intracellular acetyl-L-carnitine (48). In semen obtained from 101 men with normal or abnormal spermiograms, concentrations of L-carnitine and acetyl-L-carnitine correlated positively with the number of spermatozoa, the percentage of motile spermatozoa, and the percentage of normal cells (49). A meta-analysis comparing L-carnitine and/or acetyl-L-carnitine to placebo treatment and including nine randomized, controlled clinical trials revealed significant improvements in pregnancy rate, total sperm motility, forward sperm motility, and presence of atypical sperm cells (50). The benefits of L-carnitine and acetyl-L-carnitine may be due to increased mitochondrial fatty acid oxidation, resulting in improvement in motility epididymal sperm, as well as due to the putative antiapoptotic effect(s) of carnitine in the testes (51).

ADVERSE EFFECTS

Transient diarrhea, nausea, vomiting, abdominal cramps, and/or "fish-odor syndrome" have been noted in rare cases after consumption of 2 to 6 g of L-carnitine (52).

COMPENDIAL/REGULATORY STATUS

L-Carnitine is approved as a pharmaceutical by the U.S. Food and Drug Administration for treatment of primary systemic carnitine deficiency, as well as for acute and chronic treatment of patients with inborn errors of metabolism that result in secondary carnitine deficiency (e.g., medium-chain acyl-CoA dehydrogenase deficiency, glutaric aciduria, Type 2 diabetes, methylmalonic aciduria, and propionic acidemia) (52). L-Carnitine is also approved, by the U.S. Food and Drug Administration, as a pharmaceutical for the prevention and treatment of carnitine deficiency in patients with end-stage renal disease who are undergoing dialysis (52).

CONCLUSION

L-Carnitine and esters of L-carnitine have a proven, essential role in cellular fatty acid metabolism. Beyond that, numerous other functions have been suggested by physiological and pharmacological studies in experimental animals and in humans. These include, but are not limited to, antioxidant and antiapoptotic properties, a role in membrane lipid remodeling, and modulation of gene expression. Identification of and evidence for these putative functions have led to hypotheses concerning a role for L-carnitine and its esters in promotion of physiological function (e.g., exercise performance), in prevention, slowing, or attenuation of progressive alteration or loss of physiological function (e.g., cognitive function in aging, cardiac or liver dysfunction, and male reproductive dysfunction). In some cases, establishment of a positive role for L-carnitine supplementation through blinded,

randomized clinical trials has been hampered by lack of accessibility of definitive, quantitative endpoint(s), and/or by the requirement for large sample sizes to detect small but meaningful physiological or pathological differences in a heterogeneous population. Nevertheless, it seems likely that new data will emerge from well-designed clinical trials to provide definitive answers regarding the efficacy of L-carnitine and/or its esters supplementation to promote better health and function in the human population.

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β -Carotene

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INTRODUCTION

β -Carotene (molecular formula: $C_{40}H_{56}$) is a fat-soluble plant pigment found in red, orange, and yellow vegetables and fruits. β -Carotene is converted to vitamin A (retinal, retinol, and retinoic acid), when it is in short supply in the body. It is an antioxidant—a compound that blocks the action of activated oxygen molecules that can damage cells. Dietary intake of foods containing β -carotene has been associated with cancer prevention. However, there is not enough evidence to support this. In fact, high-dose β -carotene supplementation may increase the risk of lung cancer among people already at high risk, such as smokers.

BIOCHEMISTRY AND FUNCTIONS

β -Carotene belongs to a large class of plant pigments referred to as carotenoids. It is made up of eight isoprene units that are cyclized at each end of the molecule. β -Carotene functions in plants and in photosynthetic bacteria as an accessory pigment in photosynthesis and protects against photosensitization in animals, plants, and bacteria. In humans, the only known function of β -carotene is its vitamin A activity. Other possible actions in humans include antioxidant activity, immunoenhancement, inhibition of mutagenesis and transformation, inhibition of premalignant lesions, and decreased risk of some cancers and some cardiovascular events. In the skin, β -carotene has been found to have protective effects against solar radiation damage. However, two human intervention studies that used high-dose β -carotene supplements reported an increased risk for lung cancer among smokers (1,2). In vitro and in vivo studies suggest the potential chemopreventive activity of β -carotene; that is, β -carotene itself may act as an anticarcinogen, but its oxidized products, which appear when β -carotene is present in tissue at high concentration, may facilitate carcinogenesis (3).

ABSORPTION AND METABOLISM

Because β -carotene is fat soluble, it follows the same intestinal absorption pathway as dietary fat. Release of β -carotene from the food matrix and its dissolution in the lipid phase are the important initial steps in the absorption process. β -Carotene is thought to be absorbed by the small intestinal mucosa via a passive, diffusion process. It is taken up by the mucosa of the small intestine and packaged into triacylglycerol-rich chylomicrons and is partly converted to vitamin A by a specific enzyme, β -carotene 15,15'-oxygenase (also known as carotene

mono-oxygenase or CMOI), in the intestinal mucosa. Both β -carotene and vitamin A (primarily as retinyl esters) are incorporated into chylomicrons and secreted into lymph for transport to the liver. A second cleavage enzyme (CMOII) cleaves β -carotene eccentrically at the 9'-10' position to yield β -apo-10'-carotenal and β -ionone. The significance of this reaction is uncertain, but it is clear that CMOII is a minor player with respect to the formation of vitamin A. Additional random oxidative cleavage at several double bonds in the polyene chain of β -carotene can also occur when there is not an adequate supply of antioxidants, for example, vitamin E. However, enzymatic central cleavage by CMOII plays the major role in β -carotene breakdown under normal conditions. In conditions of oxidative stress (e.g., smoking or diseases associated with oxidative stress) or when high concentrations of β -carotene are present, both central and random cleavage may occur (Fig. 1) (4).

The delivery of β -carotene to extrahepatic tissue is accomplished through the interaction of lipoprotein particles with receptors and the degradation of lipoproteins by extrahepatic enzymes such as lipoprotein lipase. β -Carotene is present in a number of human tissues, including adipose, liver, kidney, adrenal gland, and testes and is one of the major carotenoids in human diet, serum, and tissues.

In fasting serum, β -carotene is found primarily in low-density lipoproteins (LDL), but appreciable amounts are also found in high-density lipoproteins (HDL) (5-7). β -Carotene, being lipophilic, is located in the core of lipoproteins, which may explain why there is little transfer among them.

The concentration of β -carotene in human serum is highly variable and depends on a number of factors, including β -carotene intake, efficiency of absorption, and other components of the diet.

BIOAVAILABILITY

The bioavailability of a carotenoid is considered to be the fraction of ingested carotenoid utilized for normal physiological functions or storage. Information on carotenoid bioavailability is based largely on serum levels after ingestion. The bioavailability of β -carotene from food, concentrated extracts, or synthetic products is quite variable. Several human studies have reported on the serum response to β -carotene supplements. Factors that affect β -carotene bioavailability include vehicle type (supplement vs. food: processed vs. unprocessed food) and dietary factors (amount of β -carotene, fat, and fiber) (8).

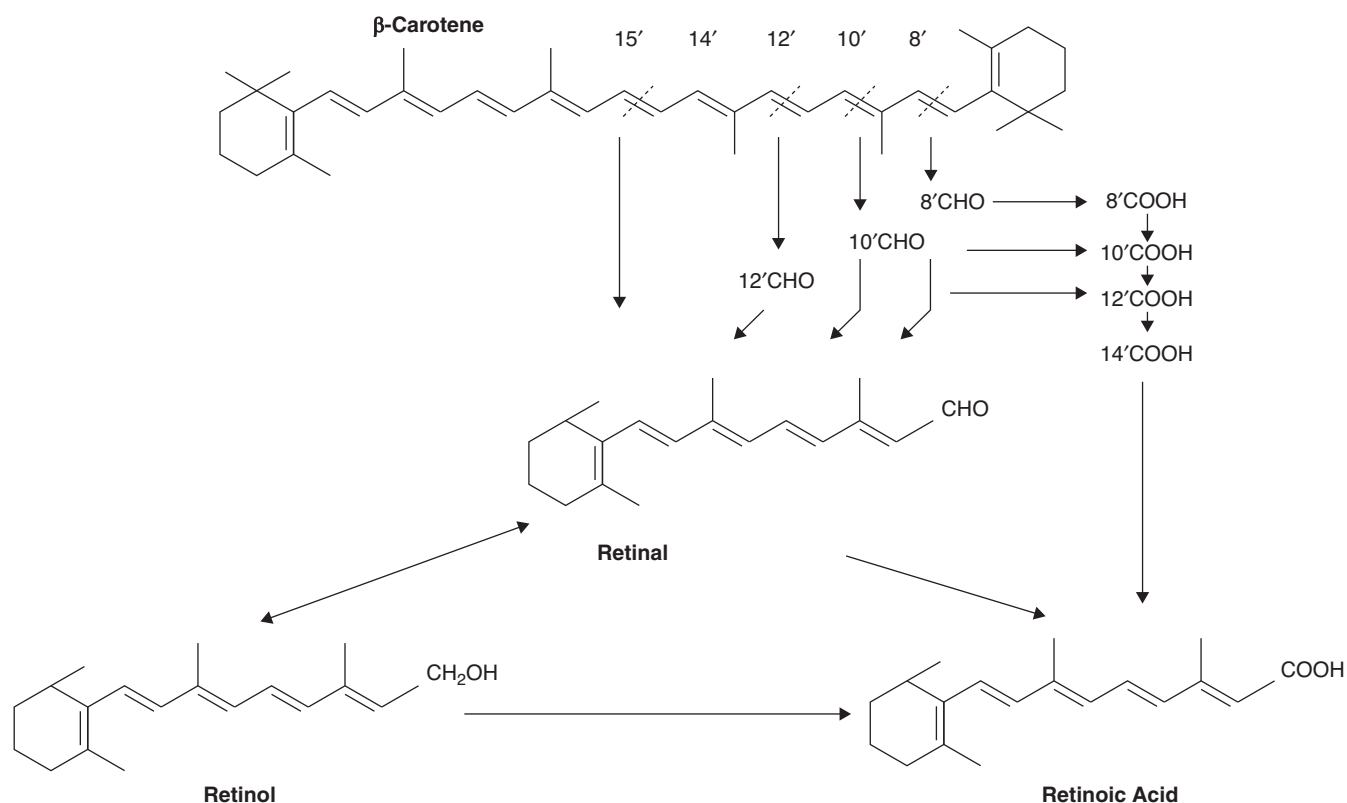


Figure 1 β -Carotene conversion to vitamin A (retinal, retinol, and retinoic acid). Central cleavage by carotene monooxygenase or CMOI between bonds 15 and 15' forms retinal directly. A second cleavage enzyme (CMOII) cleaves β -carotene excentrically at the 9'-10' position. Cleavage at other bonds forms apocarotenals (CHO). Apocarotenals may be oxidized to apocarotenoic acids (-COOH), which could form retinoic acid. Apocarotenals may be oxidized to apocarotenoic acids (-COOH), which could form retinoic acid.

Compared with carrots (a source of β -carotene), supplements suspended in oil or in water from gelatin-stabilized beadlets (the form used in the major clinical trials) raise the plasma concentration approximately six-fold. This may be because a pure form of β -carotene does not need to be released from a food matrix for intestinal absorption. β -Carotene may have twice the bioavailability from fruits compared with green leafy vegetables. The percentage absorption of a single dose of β -carotene (45 mg to 39 mg) has been reported to range from 9% to 22% (9-11), but the absorption efficiency decreases as the amount of carotenoids in the diet increases (12-15). Absorption of β -carotene at dosages greater than 20 to 30 mg is very limited because of the factors such as solubility (16).

Cooking and mechanical homogenization increase the bioavailability of carotenoids from foods. The mechanism by which this occurs is most likely the disruption of the food matrix to release the carotenoid from the matrix and from protein complexes. For example, the plasma response of β -carotene has been reported to be three times greater in spinach and carrots that were pureed and thermally processed than it was when these vegetables were consumed in raw, large pieces (17). Although dietary fat facilitates the absorption of β -carotene, the amount of dietary fat does not affect the postprandial increases in plasma β -carotene concentrations, as long as there is some fat in the diet (18). However, when

β -carotene is given in the absence of fat, no detectable change in serum level occurs (19). Studies involving daily supplementation with high-dose β -carotene on plasma concentrations of other carotenoids for several years find no overall adverse effect on plasma concentrations of other carotenoids (20). The β -carotene: vitamin A (retinol) equivalency ratio of a low dose (<2 mg) of purified β -carotene in oil is approximately 2:1. The water miscible form of β -carotene is presumed to be better absorbed than the carotenoid in oil and, therefore, may have a more efficient (i.e., lower) conversion ratio. However, the efficiency of absorption of β -carotene in food is lower than that of β -carotene in oil. The Institute of Medicine of the National Academy of Sciences proposes that 12 mg of β -carotene in food has the same vitamin A activity as 1 mg retinol (21).

INDICATIONS AND USAGE

Food Sources

β -Carotene is the most widely studied carotenoid and is one of the major carotenoids in our diet and in human blood and tissues (22,23). Major sources of dietary β -carotene include green leafy vegetables as well as orange and yellow fruits and vegetables (Table 1) (24). However, the bioavailability of β -carotene from green leafy

Table 1 β-Carotene Content of Foods (24)

Food	Content (mg/100 g wet weight) ^a
Carrots, raw	7.9
Carrots, cooked	9.8
Apricots, raw	3.5
Apricots, dried	17.6
Cantaloupe, raw	3.0
Kale	4.7
Pepper, red	2.2
Pumpkin	3.1
Spinach, raw	4.1
Spinach, cooked	5.5
Sweet potato, cooked	8.8
Winter squash, cooked	2.4

^aEdible portion.

vegetables such as spinach is thought to be low with a conversion factor of carotene to retinol of 20:1, whereas the conversion factor from fruit may be somewhat better (on the order of 12:1) (25). As discussed above, factors other than food vehicle are thought to be important in the bioavailability of β-carotene. These include cooking, chopping, and the presence of dietary fat, all of which improve the bioavailability (17,26). Of the 50 different carotenoids that can be metabolized into vitamin A, β-carotene has the highest provitamin A activity. Genetically engineered “Golden Rice” contains up to 35 μg of β-carotene per gram rice (27), with a the conversion factor of Golden Rice β-carotene to retinol in adults of 3.8:1, with a range of 1.9–6.4:1 by weight. Typical dietary intakes of β-carotene in the United States are 0.5 to 6.5 mg/day (28–30). However, intakes much higher than this are possible through over-the-counter supplements, which are commonly available in health food stores in doses of 3 to 20 mg/capsule.

Recommended Intakes

Although at present no dietary reference intakes (DRIs) are proposed for β-carotene, existing recommendations for increased consumption of carotenoid-rich fruits and vegetables are supported. Based on the evidence that β-carotene supplements have not been shown to provide any benefit for the prevention of major chronic diseases and may cause harm in certain subgroups (e.g., smokers and asbestos workers), it is concluded that β-carotene supplements are not advisable other than as a source of provitamin A. If there is adequate retinol in the diet, there are no known clinical effects of consuming diets low or moderate in β-carotene. β-Carotene is widely used in vitamin and mineral supplements at levels ranging from 0.4 to 20 mg/day. It is given medicinally in doses of up to 6 mg/day for dietary deficiency of vitamin A (although preformed vitamin A is usually used for this purpose) and up to 300 mg/day for the reduction of photosensitivity in individuals with erythropoietic protoporphyria.

Although no safe upper level of intake for β-carotene has been established in the United States, the European Expert Group on Vitamins and Minerals has established a safe upper level of β-carotene intake of 7 mg/day (31). The safe upper level applies only to the general population, that is, nonsmokers and those not exposed to asbestos and to β-carotene supplements only, given that there is no

evidence to suggest that β-carotene intake from foods are harmful.

Excessive dietary intake of preformed vitamin A has been associated with reduced bone mineral density and increased risk of hip fractures. β-Carotene may be a safe source of vitamin A in osteoporotic subjects, given that it is not associated with bone demineralization (32).

Cancer Prevention

Observational epidemiologic studies have been very consistent in showing that people who consume higher dietary levels of fruits and vegetables have a lower risk of certain types of cancer (33). The consistency of the results is particularly strong for lung cancer, in which carotenoid and/or fruit and vegetable intake has been associated with reduced risk in all of 8 prospective studies and in 18 of 20 retrospective studies (34). However, in three large randomized clinical trials using high-dose β-carotene supplements (20 mg/day, 30 mg/day, or 50 mg given every other day for 4–12 years), no protection was reported with respect to lung cancer or any other cancer (1,2,35). In fact, in two of these studies, there was an increased risk of lung cancer in heavy smokers and asbestos workers with β-carotene supplementation (1,2) (see “Contraindications”). More recently, it was reported that longer duration of use of individual β-carotene supplements (but not total 10-year average dose) was associated with statistically significantly elevated risk of total lung cancer (36). However, there was little evidence for effect modification by gender or smoking status.

Cardiovascular Disease

A body of evidence indicating that the oxidation of LDL plays an important role in the development of atherosclerosis has led investigators to consider a preventive role for β-carotene. Early in vitro studies of LDL oxidation showed that β-carotene carried in LDL is oxidized before the onset of oxidation of LDL polyunsaturated fatty acids, suggesting a possible role in delaying LDL oxidation. Epidemiologic studies, including descriptive, cohort, and case-control studies, suggest that β-carotene-rich diets are associated with a reduced risk of cardiovascular disease (37–39). Furthermore, inverse association between serum or adipose β-carotene levels and cardiovascular outcomes has also been observed. However, in a meta-analysis of eight β-carotene treatment trials involving 138,113 subjects, a dose range of 15 to 50 mg/day and follow-up range from 1.4 to 12.0 years, it was found that β-carotene supplementation led to a small but significant increase in all-cause mortality and a slight but significant increase in cardiovascular death (40).

Erythropoietic Protoporphyria

Erythropoietic protoporphyria is an inborn defect of ferrochelatase resulting in an increase in the protoporphyrin content of the erythrocytes, plasma, and feces. The disease is characterized clinically by photosensitivity, which generally appears within the first few years of life. These patients experience a burning sensation of the skin within a few minutes or hours of exposure to sunlight, followed by edema, erythema, and purpura. β-Carotene has been used therapeutically for the treatment of erythropoietic

Table 2 β -Carotene Supplementation Trials: Study Designs

Study (Ref.)	Population	Intervention	Duration (yr)
ATBC (2)	29,133 Finnish male smokers (50–69 yr of age)	β -Carotene, 20 mg/day; vitamin E, 50 mg/day	5–8
CARET (1)	18,314 men and women and asbestos workers (45–74 yr of age)	β -Carotene, 30 mg/day; vitamin A, 25,000 IU	<4
PHS (35)	22,071 male physicians (40–84 yr of age)	β -Carotene, 50 mg on alternate days	12
Linxian (42)	29,584 men and women, vitamin and mineral deficient (40–69 yr)	β -Carotene, 15 mg/day; selenium, 50 mg/day; α -tocopherol, 30 mg/day	5

protoporphyrin (41). This is based on the observation that carotenoids prevent photosensitivity in bacteria. On treatment of the condition with extremely high doses (up to 300 mg/day) of β -carotene, a marked improvement in skin photosensitivity has been reported in some, but not all, patients. No toxic effects have been in the limited number of patients reported.

CONTRAINDICATIONS

The epidemiologic observations of possible protective effects of high dietary (not supplemental) β -carotene intakes against cancer, along with what is known about carotenoid biochemical functions, has led to further study of the effect of β -carotene on cancer risk. Long-term large randomized intervention trials were designed to test the efficacy of high doses of β -carotene (20–30 mg/day) in the prevention of cancer (Table 2). As stated above, the results from two trials provided possible evidence of harm from β -carotene supplements in relation to cancer among high-risk individuals, such as smokers and asbestos workers (1,2), but no effect (either beneficial or detrimental) in a generally well-nourished population (34). Moreover, in the Linxian (Chinese) Cancer Prevention Study (42), it was found that supplementation with β -carotene doses, vitamin E, and selenium led to a significant reduction in total mortality (9%), especially from cancer (13%) and particularly stomach cancer (21%) (Table 3). The positive results of the Chinese study probably reflect the correction of a vitamin A deficiency in this study population. A number of mechanisms have been proposed to account for the association between β -carotene supplementation and lung cancer in smokers and asbestos workers, including an imbalance of other carotenoids or antioxidants, a pro-oxidant activity of β -carotene at the high oxygen tensions found in the lungs, induction of P450 enzymes, and the production of damaging β -carotene oxidation products by components of cigarette smoke (3).

The epidemiologic studies that led to these intervention studies reported an inverse relationship between diet

and/or blood β -carotene levels and cancer prevention. It is probable that β -carotene serves as a marker of increased fruit and vegetable intake and, therefore, of all components that have cancer prevention potential, for example, vitamin C, folic acid, other carotenoids, and polyphenols. Alternatively, low-dose dietary levels could have a protective effect against cancer, whereas high-dose supplement β -carotene could have a cancer stimulating effect.

TOXICITY/ADVERSE EFFECTS

β -Carotene obtained from eating fruits and vegetables is considered safe. β -Carotene first became available as a pharmaceutical product in the early 1970s. It can be purified from natural sources such as green plants or algae, or it can be manufactured synthetically. Purity of β -carotene may be a problem when derived from plant or algal sources. Preparations of crystalline β -carotene in oil are widely available. Although not harmful, high doses of β -carotene (from foods and supplements) can result in a skin condition known as carotenodermia, in which the skin turns to yellow–orange color due to an elevation of plasma and tissue carotene concentrations. Carotenodermia is reversible when β -carotene ingestion is discontinued. This condition has been reported in adults taking supplements containing 20 to 30 mg/day or more of β -carotene for long periods of time or consuming high levels of carotenoid-rich foods such as carrots (43) and is the primary effect of excess carotenoid intake noted in infants, toddlers, and young children (44). Carotenodermia is distinguished from jaundice in that the ocular sclerae are yellowed in jaundiced subjects but not in those with carotenodermia.

In the treatment of erythropoietic protoporphyria (180 mg/day), no toxic effects have been observed for very high doses of β -carotene (41). However, the number of patients studied has been small. There is no evidence that β -carotene is teratogenic, mutagenic, or carcinogenic in long-term bioassays in experimental animals (45). In humans, there have been no reports of reproductive toxicity or teratogenicity associated with high β -carotene intake, either before or during pregnancy. In addition, long-term supplementation with β -carotene to persons with adequate vitamin A status does not increase the concentration of serum retinol, as the metabolic conversion is regulated by vitamin A status, that is, the better the vitamin A status, the lower the conversion to vitamin A (20).

Doses of 20 to 30 mg/day of β -carotene for 4 to 12 years have been associated with an increased risk of lung cancer in high-risk groups (i.e., smokers and asbestos-exposed workers). Similar to the results in human

Table 3 β -Carotene Supplementation Trial: Cancer Outcomes

Study (Ref.)	Cancer outcome
ATBC (2)	18% increase in lung cancer; 8% increase in mortality
CARET (1)	28% increase in lung cancer; 17% increase in deaths
PHS (35)	No effect of supplementation on incidence of cancer
Linxian (42)	13% decrease in total cancers; 9% decrease in overall deaths

intervention studies, β -carotene supplementation for several months (at doses equivalent to a 30-mg dose in man) to ferrets exposed to cigarette smoke resulted in the development of squamous cell metaplasia in the lungs of ferrets (46). The development of squamous cell metaplasia was also observed in animals supplemented with β -carotene (at the same dose as above) without exposure to smoke, although the metaplasia was less prominent. Whether high, chronic doses of β -carotene in low-risk groups, for example, nonsmokers, would have toxic effects is not known at this time.

COMPENDIAL/REGULATORY STATUS

β -Carotene is in the generally recognized as safe (GRAS) list issued by the Food and Drug Administration.

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Carotenoids Overview

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INTRODUCTION

Carotenoids are a family of compounds of over 600 fat-soluble plant pigments, which provide much of the color seen in nature. For example, carotenoids are responsible for the red color of tomatoes and the orange color of carrots. Apart from their aesthetic role, carotenoids are considered to be beneficial in the prevention of various diseases, including certain cancers and eye disease. The beneficial effects of carotenoids are attributed to a small portion of the hundreds of carotenoids found in nature, given that only about two dozen are found in human blood and tissue, and only two in the eye. The carotenoids that have been most studied in this regard are α -carotene, β -carotene, cryptoxanthin, lycopene, lutein, and zeaxanthin. (See also separate chapters on " β -Carotene," "Lutein," and "Lycopene.") β -Carotene and lycopene belong to a class of carotenoids called carotenes and are highly fat soluble. Cryptoxanthin, lutein, and zeaxanthin belong to a class of carotenoids called xanthophylls. Because xanthophylls contain at least one hydroxyl group, they are more polar than that of carotenes. Thus, α -carotene, β -carotene, and lycopene tend to predominate in low-density lipoproteins (LDL) in the circulation, whereas high-density lipoproteins (HDL) are major transporters of cryptoxanthin, lutein, and zeaxanthin (1). In part, the protection against chronic disease by carotenoids is thought to be through their antioxidant activity. Lutein and zeaxanthin are thought to have an additional protective role of absorbing damaging blue light that enters the eye.

FOOD SOURCES

The most common carotenoids in the U.S. diet are α -carotene, β -carotene, β -cryptoxanthin, lycopene, lutein, and zeaxanthin (2). Rich sources of α - and β -carotene include carrots and winter squashes. Foods high in β -cryptoxanthin are orange and red fruits and vegetables such as pumpkin, papayas, and red peppers. Tomatoes account for approximately 80% of the dietary lycopene (3). Dark green leafy vegetables such as spinach and kale are rich sources of lutein and zeaxanthin. The relatively low bioavailability of carotenoids from foods is, in part, due to their association with proteins in the plant matrix (4). Disruption of the plant matrix with chopping, blending, or cooking can increase carotenoid bioavailability (5).

ABSORPTION AND METABOLISM

Carotenoids, being fat soluble, follow the same intestinal absorption path as dietary fat. Carotenoids are released

from food matrices and solubilized in the gut. This is done in the presence of fat and conjugated bile acids. As little as 3 to 5 g of fat in a meal is sufficient for carotenoid absorption (5,6). Absorption is affected by the same factors that influence fat absorption. Thus, the absence of bile or any generalized malfunction of the lipid absorption system (e.g., small intestinal disease, pancreatic disease) will interfere with the absorption of carotenoids. Chylomicrons are responsible for the transport of carotenoid from the intestinal mucosa to the bloodstream via the lymphatics for delivery to tissues. Carotenoids are transported in the plasma exclusively by lipoproteins, being carried predominantly by LDL (carotenes) and HDL (xanthophylls) (7). The delivery of carotenoids to extrahepatic tissues is accomplished through the interaction of lipoprotein particles with receptors and the degradation of lipoprotein lipase.

Plant sterols reduce the absorption of cholesterol in the gut, in part by competing with cholesterol when they are incorporated into the mixed micelles (8). Stanol and steryl esters reduce plasma concentrations of carotenoids (9,10). It is thought that, during the absorption process in the intestine, plant sterols could displace not only cholesterol but also these lipophilic molecules and replace them in incorporation into mixed micelles.

BIOLOGICAL ACTIVITIES

To date, the only known essential function of carotenoids is as a source of vitamin A. The carotenoids in this category are α -carotene, β -carotene, and β -cryptoxanthin (11). The vitamin A activity of β -carotene in food is 1/12 that of vitamin A (retinol), and vitamin A activity of both α -carotene and β -cryptoxanthin is 1/24 that of vitamin A (11).

Most carotenoids have antioxidant activity. β -Carotene and others carotenoids have antioxidant properties as shown in in vitro and animal models. Mixtures of carotenoids or associations with others antioxidants (e.g., vitamin E) can increase their activity against free radicals (12). The use of animal models for studying carotenoids is limited because most of the animals do not absorb or metabolize carotenoids similarly to humans. Epidemiologic studies have shown an inverse relationship between the presence of various cancers and dietary or blood carotenoid levels (13). However, three out of four intervention trials using high-dose β -carotene supplements did not show protective effects against cancer or cardiovascular disease. Rather, the high-risk populations (smokers and asbestos workers) in these high-dose intervention trials showed an increase in cancer and angina cases (14–17).

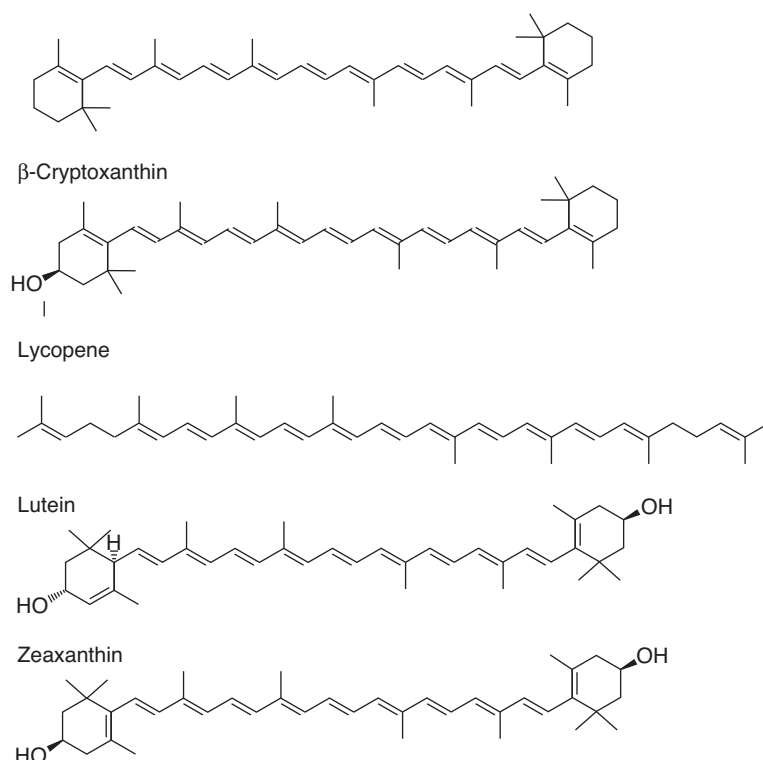


Figure 1 Structures of the major dietary carotenoids.

Therefore, carotenoids may promote health when taken at dietary levels but may have adverse effects when taken in high dose by subjects who smoke or who have been exposed to asbestos.

The long chain of alternating double and single bonds is a characteristic of all carotenoids (Fig. 1). This feature allows them to absorb light in the visible range of the light spectrum (18). This may be of particular importance in the macula of the retina, where lutein and zeaxanthin are highly concentrated to the exclusion of all other carotenoids (19). In the macula, lutein and zeaxanthin absorb blue light to reduce the amount of light that reaches critical visual structures, thereby providing some protection from light-induced oxidative damage (20).

CONCLUSIONS

Epidemiologic studies support a protective role of carotenoids in the prevention of certain major diseases, including certain cancer and eye disease. The hypothesis that these antioxidant nutrients may protect against certain diseases is plausible, given the putative role of oxidative damage in the etiology of these diseases. However, clinical trials have suggested that supplementation with high dose of β -carotene may have an adverse effect on the incidence of cancer in smokers and workers exposed to asbestos. Current recommendations include diets high in fruits and vegetables, which are rich sources of carotenoids.

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Cascara Sagrada

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INTRODUCTION

Rhamnus purshiana De Candolle is the largest species of buckthorn, occasionally growing up to 15 m in height; however, it is more commonly a large shrub or small tree (5–10 m) (1–5). *Rhamnus purshiana* is native to the Pacific Northwest United States and southwestern Canada (1–5). *Rhamnus* is the generic name for buckthorn, and the species name, *purshiana*, was given in honor of the German botanist Friedrich Pursh (4). The crude drug consists of the dried bark of the tree, which is officially known as Cascara or cascara sagrada, Spanish for “sacred bark” (1–4). The dried aged bark of the tree has been used by Native Americans for centuries as a laxative. It was accepted into medical practice in the United States in 1877 as a commonly used laxative and was the principal ingredient in many over-the-counter (OTC) laxative products. Cascara was first listed in the U.S. Pharmacopeia (USP) in 1890 as a laxative mild enough for use in treating the elderly and children. Products that were official in the USP included cascara sagrada extract, fluidextract, aromatic fluidextract, and tablets. In 2002, the U.S. Food and Drug Administration issued a final rule concerning the status of cascara sagrada (including casanthranol, cascara fluidextract aromatic, cascara sagrada bark, cascara sagrada extract, and cascara sagrada fluidextract) in OTC drug products (5). The final rule stated that cascara sagrada in OTC drug products is not generally recognized as safe and effective or is misbranded (6).

BACKGROUND

General Description

The shrub or small tree of *R. purshiana* De Candolle has elliptical leaves, greenish flowers, and black berries. It ranges in height from 4.5 to 15 m and has a reddish-brown bark (4). Most of the commercial production comes from Oregon, Washington, and southern British Columbia. The bark is collected in spring (April/May) and early summer by stripping from wild trees scattered throughout the native forests. It is removed by making longitudinal incisions and peeling off sections, which tend to roll into large quills. Trees are also felled and the bark is removed from the larger branches. The bark is then air dried, with the inner surface protected from the sun in order to preserve its yellow color. The dried bark is allowed to mature for 1 or 2 years before use in commercial preparations (4). The fresh bark contains chemical constituents that act as a gastrointestinal (GI) irritant and emetic; thus, the bark must be aged for at least 1 year prior to human use. Cascara bark and its preparations have been used for centuries by

the Pacific Northwest Native Americans, as well as the European settlers, and cascara preparations are now used worldwide as a laxative (5).

Commercial preparations of cascara (*Cortex Rhamni Purshianae*) consist of the dried, whole, or fragmented bark of *R. purshiana*. The bark and its preparations are official in the pharmacopeias of many countries (1,7–9). Cascara was first listed in the USP in 1890 as a laxative. The official listing of cascara in USP 25 (9) defined it as the dried bark (at least 1-year old) of *R. purshiana*, yielding not less than 7% of total hydroxyanthracene derivatives calculated as cascaroside A on a dried basis. Not less than 60% of the total hydroxyanthracene derivatives consist of cascarosides, calculated as cascaroside A (9).

CHEMISTRY AND PREPARATION OF PRODUCTS

The chemistry of cascara has been extensively investigated and numerous quinoid constituents are reported to be present in the bark (1). Much of the chemical and pharmacological research on cascara was performed over 50 years ago, and anthraquinone glycosides were established as the active constituents of the bark (5). Hydroxyanthracene glycosides make up 6% to 9% of the bark, of which 70% to 90% is C-10 glycosides, with aloins A and B and desoxyaloins A and B (= chrysaloins) accounting for 10% to 30% (1). The cascarosides A and B and cascarosides C and D are diastereoisomeric pairs derived from 8- β -O-glucosides of aloin A and B and 8-O-glucosyl-11-desoxyaloin, respectively, and constitute 60% to 70% of the total glycosides (1). Hydrolysis of the cascarosides cleaves the O-glycosidic bonds to yield aloins (barbaloin and chrysaloin). The cascarosides are not bitter, whereas most of their hydrolysis products (the aloins) are very bitter. Both the USP and the European Pharmacopoeia recognize the cascarosides and aloins as the active constituents of cascara and have chemical assay procedures for determining these glycosides (7–9).

Other major hydroxyanthracene glycosides include the hydroxyanthraquinones chrysophanol-8-O-glucoside and aloe-emodin-8-O-glucoside at a concentration of 10% to 20% (10). In the fresh bark, anthraquinones are present in the reduced form and are converted by oxidation to their corresponding parent anthraquinone glycosides during drying and storage (3).

Dosage Forms and Dose

Cascara sagrada is available as extracts, fluidextracts, and tablets (9). The average daily dose (taken at bedtime, or

one-half dose in the morning and at bedtime) of standardized preparations is 20 to 30 mg of hydroxyanthracene derivatives calculated as cascarioside A (dried aged bark, 0.25–1 g) (1). Do not exceed the recommended dose and do not use this dose for more than 1 to 2 weeks continuously.

PRECLINICAL STUDIES

Toxicity

While there are no specific data describing the carcinogenicity or mutagenicity for cascara sagrada, there are data available for emodin, one of the naturally occurring anthraquinones present in cascara (11–18). There are several studies reporting genotoxic and mutagenic effects both in vitro and in vivo for emodin and its derivatives, causing them to be classified as potential carcinogens (12–18). In vitro, the toxicity of 1,8-dihydroxyanthraquinones, such as emodin, may involve redox cycling between the quinone and the semiquinone radical generating reactive oxygen species (ROS), resulting in lipid peroxidation, protein damage, and DNA oxidation (16,19,20). For example, treatment of Reuber hepatoma and fibroblast Balb/3T3 cells with various anthraquinones resulted in the formation of 8-oxo-dG (16). In addition, concentrations of 50 μ M aloe-emodin increased DNA damage as measured by the single-cell gel electrophoresis assay (COMET assay) (21). Aloe-emodin and other anthraquinones also dose dependently induced tk-mutations and micronuclei in mouse lymphoma L5178Y cells and inhibited topoisomerase II-mediated decatenation in a DNA decatenation assay (21,22). The authors suggested that anthraquinones bind noncovalently to DNA and inhibit the catalytic function of topoisomerase II, which can lead to DNA breakage by competing with the DNA binding site of the enzyme (23). It is also possible that anthraquinones can covalently bind to DNA as observed with other quinones, such as *p*-benzoquinone (24,25). Binding of anthraquinones to DNA might also facilitate DNA oxidation due to their high potency of generating ROS. Besides the above-mentioned effects of redox cycling by anthraquinones, it is also reported that production of ROS by emodin can cause an immunosuppressive effect in human mononuclear cells and might result in apoptosis in A549 cells in vitro (19).

In vivo toxicology was assessed by the National Toxicology Program and published in 2001 (11). Reports that 1,8-dihydroxyanthraquinone caused tumors in the GI tract of rats led to the investigation of emodin in rodents, as this compound is structurally similar and was reported to be mutagenic in bacteria. The acute and chronic toxicities of emodin were investigated in rodents exposed to emodin in feed for 16 days, 14 weeks, or 2 years. In the 16-day study, rodents were fed diets containing average daily doses equivalent to 50, 170, 480, 1400, or 3700 mg/kg body weight for males and 50, 160, 460, 1250, or 2000 mg/kg body weight for females. The results showed that the mean body weights of males and females exposed to 480 mg/kg or greater were significantly lower than those of the controls. Macroscopic lesions were observed in the gallbladder and kidney of rats exposed to the highest doses of 1400 or 3700 mg/kg. In the 14-week study, rats were fed diets containing approximately 20, 40, 80, 170, or 300 mg/kg for males and females. Mean body

weights of males exposed to 170 mg/kg or greater and females exposed to 80 mg/kg or greater were significantly lower than those of the controls. In rats exposed to 170 or 300 mg/kg of emodin, increases in platelet counts and decreases in total serum protein and albumin concentrations were observed. Relative kidney weights of rats exposed to 80 mg/kg or greater and relative lung and liver weights of rats exposed to 40 mg/kg or greater were significantly increased compared to the control groups. The incidences and severities of nephropathy were increased in males and females exposed to 40 mg/kg or greater. In the chronic toxicity study (2 years), groups of 65 male and 65 female rats were fed diets containing emodin at an equivalent to average daily doses of approximately 110, 320, or 1000 mg/kg to males and 120, 370, or 1100 mg/kg to females for 105 weeks. Survival of exposed males and females was similar to that of the controls. There were negative trends in the incidences of mononuclear cell leukemia in both male and female rats and incidence of leukemia in the group fed 1000 mg/kg was significantly decreased. At the 12-month interim evaluation, nephropathy was slightly higher (11).

In terms of genetic toxicology, emodin was mutagenic in *Salmonella typhimurium* strain TA100 in the presence of S9 activation; however, no mutagenicity was detected in strain TA98, with or without S9 (11). Chromosomal aberrations were induced in cultured Chinese hamster ovary cells treated with emodin, with or without metabolic activation by S9. In the rat bone marrow micronucleus test, administration of emodin by three intraperitoneal injections gave negative results. Results of acute-exposure (intraperitoneal injection) micronucleus tests in bone marrow and peripheral blood erythrocytes of male and female mice were also negative. In a peripheral blood micronucleus test on mice from the 14-week study, negative results were seen in male mice, but a weak positive response was observed in similarly exposed females.

The results of these investigations show no evidence of carcinogenic activity of emodin in male F344/N rats in the two-year study. There was equivocal evidence of carcinogenic activity of emodin in female F344/N rats and male B6C3F1 mice. There was no such evidence in female B6C3F1 mice exposed to 312, 625, or 1250 ppm (11).

Other investigations of the carcinogenic potential of cascara have been carried out in rodents. In one study, the effects of the laxative bisacodyl (4.3 and 43 mg/kg) and cascara (140 and 420 mg/kg) on the induction of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and tumors in rats were investigated (26). Animals were treated with AOM and laxatives (alone or in combination) for 13 weeks. The results demonstrated that bisacodyl (4.3 and 43 mg/kg), given alone, did not induce the development of colonic ACF and tumors. However, bisacodyl (4.3 mg/kg) coupled with AOM increased the number of crypts per focus but not the number of tumors. Bisacodyl (43 mg/kg) significantly increased the number of crypts per focus and tumors. Cascara (140 and 420 mg/kg) did not induce the development of colonic ACF and tumors and did not modify the number of AOM-induced ACF and tumors (27). Results from another study were similar. Dietary exposure to high doses of these glycosides for 56 successive days did not induce the appearance of ACF or increase in incidence of ACF induced by

1,2-dimethylhydrazine (DMH). However, in rats treated with both DMH and the highest dose of glycosides, the average number of aberrant crypts per focus, considered a consistent predictor of tumor outcome, was higher than that in rats given DMH alone (26).

CLINICAL STUDIES

Laxative Effects

Cascara sagrada is an anthraquinone laxative and is used for short-term treatment of occasional constipation (1,28,29). The laxative effects of cascara are primarily due to the anthraquinone glycosides, the cascarosides A–D (1,5). Other anthranoid derivatives that may be active include emodin anthrone-6-O-rhamnoside (franguloside), and physcion and chrysophanol in glycosidic and aglycone forms (30,31). Anthraquinone laxatives are prodrugs in that after oral administration, the hydroxyanthracene glycosides are poorly absorbed in the small intestine, but are hydrolyzed in the colon by intestinal bacteria to form pharmacologically active metabolites, which are partly absorbed there (28,30); this acts as a stimulant and irritant to the GI tract (29).

The mechanism of action of cascara is similar to that of senna in that the action is twofold: (i) stimulation of colonic motility, resulting in augmented propulsion, and accelerated colonic transit (which reduces fluid absorption from the fecal mass); and (ii) an increase in the paracellular permeability across the colonic mucosa, probably due to an inhibition of Na^+ , K^+ -adenosine triphosphatase or an inhibition of chloride channels (30,32), which results in an increase in the water content in the large intestine (29,32). The laxative effect of cascara is generally not observed before 6 to 8 hours after oral administration. The hydroxyanthracene glycosides are excreted predominantly in the feces but are excreted to some extent in urine as well, producing an orange color; anthrones and anthranols also pass into breast milk (30).

Anthraquinone laxatives may produce an excessive laxative effect and abdominal pain. The major symptoms of overdose are gripes and severe diarrhea, with consequent losses of fluid and electrolytes (29). Treatment should be supported with generous amounts of fluid. Electrolytes should be monitored, particularly potassium. This is especially important in children and the elderly. Renal excretion of the compounds may cause abnormal coloration of urine (yellow–brown to reddish depending on the pH of the urine). Large doses may cause nephritis. Melanotic pigmentation of the colonic mucosa (pseudomelanosis coli) has been observed in individuals who abuse anthraquinone laxatives. Pigmentation is usually benign and reverses within 4 to 12 months of discontinuation of the products (29).

Contraindications and Precautions

Patients should be warned that certain constituents of cascara sagrada are excreted by the kidney and may color the urine (harmless). Rectal bleeding or failure to have a bowel movement after the use of a laxative may indicate a serious condition. Laxatives containing anthraquinone glycosides should not be used for periods longer than 1 to 2 weeks (29). Decreased intestinal transit time may result

in reduced absorption of orally administered drugs (1). Electrolyte imbalances such as increased loss of potassium may potentiate the effects of cardiotonic glycosides (e.g., digitalis). Existing hypokalemia resulting from long-term laxative abuse can also potentiate the effects of antiarrhythmic drugs that affect potassium channels to change sinus rhythm, such as quinidine. The induction of hypokalemia by drugs such as thiazide diuretics, adrenocorticosteroids, or liquorice root may be enhanced, and electrolyte imbalance may be aggravated (28).

Chronic use (>2 weeks) may cause dependence and need for increased doses, and an atonic colon with impaired function (29). It may also lead to pseudomelanosis coli (harmless) and to an aggravation of constipation with dependence and possible need for increased dosages. Chronic abuse with diarrhea and consequent fluid and electrolyte losses (mainly hypokalemia) may cause albuminuria and hematuria, and may result in cardiac and neuromuscular dysfunction (1).

Anthraquinone stimulant laxatives, such as cascara, should not be administered to patients with intestinal obstruction and stenosis, atony, severe dehydration states with water and electrolyte depletion, or chronic constipation (1,29). Cascara should not be administered to patients with inflammatory intestinal diseases, such as appendicitis, Crohn disease, ulcerative colitis, and irritable bowel syndrome, or in children younger than 12 years (1,29). As with other stimulant laxatives, cascara is contraindicated in patients with cramps, colic, hemorrhoids, nephritis, or any undiagnosed abdominal symptoms such as pain, nausea, or vomiting (29).

Because of the pronounced action on the large intestine and insufficient toxicological investigations, products containing cascara should not be administered to pregnant women (33,34). Furthermore, anthranoid metabolites are excreted into breast milk. Thus, cascara should not be used during lactation, due to insufficient data available to assess the potential for pharmacological effects in the breast-fed infant (33).

Adverse Reactions

In single doses, cramp-like discomfort of the GI tract may occur, which may require a reduction of dosage. Overdose can lead to colicky abdominal spasms and pain, as well as the formation of thin, watery stools. Long-term laxative abuse may lead to electrolyte disturbances (hypokalemia, hypocalcemia), metabolic acidosis, malabsorption, weight loss, albuminuria, and hematuria (35,36). Weakness and orthostatic hypotension may be exacerbated in elderly patients when stimulant laxatives are used repeatedly. Secondary aldosteronism may occur due to renal tubular damage after aggravated use. Steatorrhea and protein-losing gastroenteropathy with hypoalbuminemia have also been reported in laxative abuse (36). Melanotic pigmentation of the colonic mucosa (pseudomelanosis coli) has been observed in individuals taking anthraquinone laxatives for extended time periods (29,36–39). The pigmentation is clinically harmless and usually reversible within 4 to 12 months after the drug is discontinued (36–40). Conflicting data exist on other toxic effects such as intestinal–neuronal damage after long-term use (36). Use of the fresh drug may cause severe vomiting,

with possible spasms (30). Cases of allergic respiratory diseases after occupational exposure to cascara have been reported (41). Cascara sagrada is an etiologic agent of IgE-mediated occupational asthma and rhinitis. One case of cholestatic hepatitis, complicated by portal hypertension, has been attributed to the ingestion of cascara in one patient who was also known to abuse alcohol and take a number of other prescription medications (42).

CURRENT REGULATORY STATUS

Prior to June 1998, cascara sagrada was recognized by the Food and Drug Administration (FDA) as a category I (safe and effective) OTC preparation (monograph). In 2002, the U.S. FDA issued a final rule concerning stimulant laxatives including cascara sagrada (including casanthranol, cascara fluidextract aromatic, cascara sagrada bark, cascara sagrada extract, and cascara sagrada fluidextract) in OTC drug products, stating that they are not generally recognized as safe and effective or are misbranded (6). This final rule was based on a decision made by the agency after it had requested mutagenicity, genotoxicity, and carcinogenicity data on cascara in 1998. No comments or data were provided to the FDA for cascara; thus on the basis the lack of data and information and the failure of any persons to submit new data from carcinogenicity studies, the agency has determined that these laxative should be deemed not generally recognized as safe and effective for OTC use and has thus reclassified these ingredients to category II (nonmonograph) (6). According to the FDA, products containing aloe and cascara sagrada ingredients must be reformulated or discontinued; the stimulant laxatives must therefore be deleted or replaced. Reformulated products will also need to be relabeled. This final rule is part of FDA's ongoing OTC drug product review. However, these products may still be sold as dietary supplements under the Dietary Supplements Health and Education Act of 1994.

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Chaste Tree

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INTRODUCTION

Vitex agnus castus L. (Verbenaceae), commonly referred to as chaste tree or chasteberry, is a small shrubby tree, approximately 1 to 6 m in height and native to the Mediterranean region and Asia (1,2). The tree is also widely cultivated in warm temperate regions of the world. The name “chasteberry” may be derived from the traditional belief that the plant promoted chastity (3). The fruits of *V. agnus castus* (VAC) were used in ancient Greece and Rome, as well as by the monks of the Middle Ages, to suppress sexual desire (4,5). In the past, extracts of VAC have been used for the treatment of gynecological disorders, such as endometrial hyperplasia, hypermenorrhea, and secondary amenorrhea, as well as endocrine-dependent dermatoses (dermatitis dysmenorrhea symmetrica, acne vulgaris, eczema, and acne rosacea) (6–8).

Today, extracts of the dried ripe fruits of VAC are regulated in the United States as dietary supplements under the 1994 Dietary Supplement Health and Education Act. They are widely used as a botanical dietary supplement for the management of gynecological disorders including corpus luteum insufficiency (9,10), premenstrual syndrome (PMS) (11–13), menstrual problems (14,15), cyclic mastalgia (16–18), as well as to treat hormonally induced acne (19). In addition, VAC has been traditionally used to treat fibroid cysts and infertility, stop miscarriages caused by progesterone insufficiency (20), and treat indigestion (3).

CHEMISTRY AND PREPARATION OF PRODUCTS

Commercial products of VAC are prepared from the dried, ripe fruit, containing not less than 0.4% (v/w) of volatile oil and at least an 8% water-soluble extractive (1,21). To date, although the active constituents of VAC remain unknown, the European Pharmacopoeia recommends a minimum content of 0.08% casticin in the dried plant material (22). Two compounds are currently used as marker compounds for quality control: the iridoid glycoside *agnuside* and the flavonol *casticin* (23). Most VAC preparations used in European medicine are nonstandardized fluid extracts, tinctures, and/or native dry extracts. The “native” or “total” extract is an approximate 10:1 (w/w) drug-to-extract ratio containing 0.6% to 1.0% casticin (2).

The ripe, dried VAC fruit yields 0.4% to 0.7% (v/w) essential oil, depending on distillation time and comminution size. The oil is mainly composed of bornyl acetate, 1,8-cineole, limonene, α - and β -pinene, β -caryophyllene, and α -terpinyl acetate (24). Flavonoids,

iridoids, and diterpenes represent major groups of secondary constituents that are also found in the fruit (2). Casticin (up to 0.2%) is considered to be the major flavonoid, with chrysopenetin, chrysosplenol D, cynaroside, 5-hydroxy-3,4',6,7-tetramethoxyflavone, 6-hydroxykaempferol, isorhamnetin, luteolin, and luteolin 6-C-glycoside derivatives being other compounds of this class (24–26). Major iridoids found include agnuside (*p*-hydroxybenzoylaucubin, 0.0014%) and aucubin (0.0013%). Diterpene constituents include vitexilactone (0.001–0.004%), 6 β ,7 β -diacetoxy-13-hydroxylabda-8,14-diene, rotundifuran, vitexlabdines A–D, and vitexlactam A (24,25). The structures of the above-mentioned components are presented in Figure 1.

PRODUCTS AND DOSAGE

There is a wide range of VAC extracts and products available to consumers. The following examples are a general list of products used in clinical trials and listed in reference texts. This list is not complete and is not intended as a recommendation of one product over another. The dose as listed is intended for adults, and the products are not recommended for children.

- Dry native ethanolic extracts, 8.3–12.5:1 (w/w), approximately 1.0% casticin: one tablet, containing 2.6 to 4.2 mg native extract. The tablets should be swallowed whole with some liquid each morning.
- Dry native extract, 9.58–11.5:1 (w/w): one tablet containing 3.5 to 4.2 mg native extract each morning with some liquid (27).
- Dry native extract, 6.0–12.0:1 (w/w), approximately 0.6% casticin: PMS: one tablet containing 20 mg native extract daily with water upon awaking or just before bedtime, before meals.

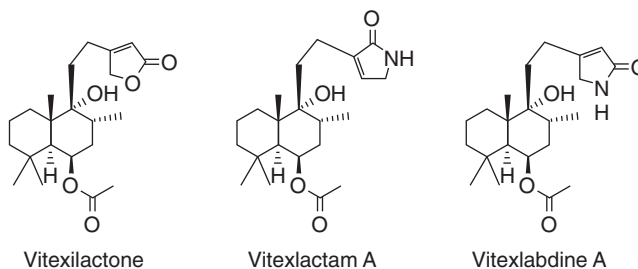


Figure 1 Compounds from *Vitex agnus castus* fruits.

- Fluid extract: 1:1 (g/mL), 70% alcohol (v/v): 0.5 to 1.0 mL.
- Fluid extract: 1:2 (g/mL): 1.2 to 4.0 mL.
- Tinctures, alcohol 58 vol% (100 g of aqueous-alcoholic solution contains 9 g of 1:5 tincture): 40 drops, one time daily with some liquid each morning.
- Tinctures, ethanol 19% (v/v) (100 g of aqueous-alcoholic solution contains 0.192–0.288 g extractive corresponding to 2.4 g dried fruit): 40 drops, once daily.
- Hydroalcoholic extracts (50–70%; v/v): corresponding to 30 to 40 mg dried fruit (2,28).

CLINICAL STUDIES

Extracts from VAC fruits are primarily used for the symptomatic management of corpus luteum insufficiency, hyperprolactinemia (9,10), PMS (11–13,24,27,29), and cyclic mastalgia (16–18). A few clinical studies have also indicated that VAC may also be a potential treatment for infertility due to hyperprolactinemia and luteal-phase defect (30), insufficient lactation, as well as to prevent miscarriages due to progesterone insufficiency (20).

Since the 1950s, over 35 human or clinical studies have assessed the safety and efficacy of various VAC extracts and tinctures (53–70% ethanol) for the treatment of acne, corpus luteum insufficiency, cyclic breast pain, hyperprolactinemia, menopausal symptoms, increasing lactation, PMS, uterine bleeding disorders, and miscellaneous menstrual irregularities. Most of these investigations are open, uncontrolled studies assessing the effects of VAC for the management of menstrual cycle irregularities or PMS. The results from randomized, controlled clinical trials are also published.

Premenstrual Syndrome

PMS refers to the regular occurrence of affective symptoms, such as depressive moods, irritability, anxiety, confusion, and social withdrawal, as well as somatic symptoms including breast tenderness or heaviness and breast pain (mastalgia), abdominal bloating, cravings, fatigue, and headache (31). The syndrome affects approximately 30% to 40% of menstruating women and is one of the most frequent complaints noted in gynecology practice (27). Approximately 13 clinical trials have assessed the safety and efficacy of VAC extracts for the symptomatic treatment of PMS (11,12,14,27,29,32–39). Of these investigations, only three were randomized, controlled trials and two were double blinded (27,36–39).

The most recent clinical trial assessing the safety and efficacy of VAC for the management of PMS was a prospective, randomized, multicenter placebo-controlled trial in Chinese women (39). After the screening and preparation phase lasting three cycles, eligible patients were randomly assigned into treatment or placebo groups and were treated with a VAC extract daily or placebo for up to three cycles. Efficacy was assessed using the Chinese version of the PMS-diary (PMSD) and Premenstrual Tension Syndrome (PMTS) scale. Two hundred and seventeen women were eligible to enter the treatment phase and were randomly assigned into the treatment group ($n = 108$) or the placebo group ($n = 109$), of these 208 provided the efficacy data (treatment: $n = 104$, placebo: $n =$

104) and 202 completed the treatment phase (treatment: $n = 101$, placebo: $n = 101$). The mean total PMSD scores decreased from 29.23 at baseline (0 cycle) to 6.41 at the termination (3rd cycle) for the treatment group and from 28.14 at baseline (0 cycle) to 12.64 at the termination (3rd cycle) for the placebo group. The total PMSD score of the 3rd cycle was significantly lower than the baseline in both groups ($P < 0.0001$). The difference in the mean scores from the baseline to the 3rd cycle in the treatment group (22.71 ± 10.33) was significantly lower than the difference in the placebo group (15.50 ± 12.94 ; $P < 0.0001$). Results of PMTS were similar in that the total scores for PMTS were significantly lower between the two groups ($P < 0.01$) and within each group ($P < 0.01$). The score was decreased from 26.17 ± 4.79 to 9.92 ± 9.01 for the treatment group and from 27.10 ± 4.76 to 14.59 ± 10.69 for the placebo group. A placebo effect of 50% was found in the present study. No serious adverse events were reported in either group. The study concluded that VAC was well tolerated and was efficacious for the treatment of severe PMS in Chinese women (39).

In a randomized, placebo-controlled study published by Schellenberg et al., women with PMS symptoms were randomized for treatment to either a VAC extract ($n = 86$; one tablet daily) or a placebo ($n = 84$) for three consecutive menstrual cycles (37). A PMS diagnosis was made according to the *Diagnostic and Statistical Manual for Mental Disorders* (DSM-III). The main efficacy variable measured was the change from baseline to the endpoint (end of cycle 3) in the patient's self-assessment (PSA) of six PMS symptoms (irritability, mood alteration, anger, headache, breast fullness, and other indications including bloating). The secondary efficacy variable measured was a change in the Clinical Global Impressions (CGI) score for the severity of condition, global improvement, and risk/benefit ratio. Mean improvement in PSA was significantly greater in the treatment group compared with placebo group ($P < 0.001$). CGI scores for each of the three factors also revealed significant superiority of the treatment relative to placebo ($P < 0.001$). The observed response rate ($> 50\%$ reduction in symptoms) was 52% and 24% for the treatment and placebo groups, respectively. Adverse events reported included treatment ($n = 4$): acne, multiple abscesses, intermenstrual bleeding, urticaria; placebo ($n = 3$): acne, early menstrual period, and gastric upset (37).

A randomized, double-blind, placebo-controlled trial involving 217 women with self-diagnosed PMS assessed the efficacy of the fruit in treating the syndrome. The self-diagnosis was made according to a modified version of the Menstrual Distress Questionnaire (MDQ), a rating scale covering most of the important PMS symptoms (38). Subjects were treated with either a powder of VAC (300 mg tablets; two tablets three times daily; $n = 105$) or a soy-based placebo ($n = 112$) for a period of three months, after which they all completed the modified MDQ again. Other than a statistically significant difference in effect between the VAC powder and the soy-based placebo for the symptom of "feeling jittery and restless" ($P = 0.05$), no other significant results were reported (38). Unfortunately, soy was a poor choice for a placebo in this study, as it is not considered to be biologically inert.

A multicenter, randomized, double-blind, controlled clinical trial compared the activity of a dried

ethanol extract of VAC fruit with that of pyridoxine (vitamin B₆) treatment of women with PMS (27). The intent-to-treat population included 127 women: 61 subjects were given one capsule of extract plus one placebo capsule daily for three cycles, whereas 66 were given one capsule of placebo twice daily on days 1–15 of their cycle, followed by one capsule (100 mg) of pyridoxine twice daily on days 16–35. Therapeutic response was assessed by using the PMTS scale, the CGI scale, and by recording six characteristic symptoms of PMS (breast tenderness, edema, inner tension, headache, constipation, and depression). Therapeutic efficacy was assessed by both patients and physicians, at the end of the trial. Initial mean PMTS scores were higher in the chaste tree group (15.2) compared with the pyridoxine group (11.9). By the end of therapy, the mean absolute change in PMTS score in each group was 5.1, representing a reduction of 10.1 and 6.8 for the chaste tree and pyridoxine groups, respectively ($P < 0.038$, both groups, 95% CI: -6.4261 to -0.1670). Therefore, no difference could be found between the two treatment groups. The CGI scale showed that 77.1% (chasteberry) and 60.6% (pyridoxine) of patients showed improvement. Adverse events were rare but included gastrointestinal complaints, skin reactions, and transient headache (27).

Six postmarketing studies assessed the safety and efficacy of various extracts of the fruit in 8391 female patients with menstrual abnormalities or PMS symptoms (11,14,29,33,34,36). Three open (uncontrolled) studies also assessed efficacy (12,32,35). The dose used ranged from 40 to 42 drops or one capsule daily, for 1 day to 9 years, and the outcomes measured included the physician's assessment and PSA. Elimination of symptoms was observed in 29% to 42% of patients, improvement in 51% to 59%, and no change in 1% to 10%. Adverse events were reported in 1% to 5% of patients but were generally not stated to be serious. The difficulty with these studies includes the lack of a control group, besides most of them not distinguishing between PMS and other menstrual disorders (12,32,35).

An open (uncontrolled) clinical trial involving 50 women (43 completed) with late-luteal phase dysphoric disorder (DSM-III) assessed the effect of an ethanol fruit extract on the management of PMS (32). Thirteen of the subjects were concurrently taking oral contraceptives. After two months of baseline observation, one tablet of the extract was administered daily for three cycles, followed by a posttreatment phase that lasted three cycles. Treatment effectiveness was evaluated using both the MDQ and the visual analogue scale (VAS). The MDQ was filled out by patients at the end of the first cycle and during cycles 3 and 6. The VAS was completed twice per cycle, once in the late-luteal phase when symptoms peaked and the other after menstruation during the follicular phase. By the end of the third cycle, the MDQ scores were reduced by 42.5% ($P < 0.001$), with a 50% reduction in the score in 20/43 patients. By the end of the posttreatment period, the scores remained approximately 20% below baseline ($P < 0.001$). The main symptoms that improved following treatment were breast tenderness, behavioral changes, negative feelings, and edema. The average late-luteal phase VAS score was reduced by 47.2% during the three-month treatment phase ($P < 0.01$) and remained at 21.7% below baseline ($P < 0.001$) during the posttreatment phase. By contrast, the follicular phase score did not significantly change. The

number of days with PMS symptoms was reduced from 7.5 to 6 days ($P < 0.001$), and the concomitant use of oral contraceptives had no significant effect on any of the parameters investigated. Twenty patients (47%) reported 37 adverse events during the treatment and posttreatment periods (32).

An open (uncontrolled) study involving 36 women with PMS assessed the effect of a 58% ethanol extract of the fruit for the management of PMS symptoms (12). The subjects were treated with 40 drops of the extract daily over three cycles and the outcomes measured were a reduction in physical symptoms such as headache, swollen breasts, breast tenderness, bloating, fatigue, and psychological changes such as increased appetite, sugar craving, nervousness and restlessness, anxiety, irritability, lack of concentration, depression, crying spells, mood changes, and aggressiveness. The duration of the luteal phase was also determined. After three months of treatment, 69% of women had a reduction in physical symptoms, where 80% showed a decrease in psychological symptoms ($P < 0.05$). The duration of the luteal phase lengthened from 5.4 to 11.4 days (12).

Mastalgia

Breast pain (mastalgia) is a common complaint and is usually classified as cyclical (associated with the menstrual cycle) or noncyclical (not related to the menstrual cycle). Mild premenstrual breast discomfort, lasting for one to four days prior to menstruation that resolves upon initiation, is considered cyclic mastalgia and is a symptom of PMS. In addition to the experiments reported above, a number of open studies (40–45) and four randomized-controlled clinical trials (16,17,39,46) have assessed the safety and efficacy of VAC extracts for the treatment of cyclic mastalgia.

A randomized, double-blind, placebo-controlled clinical trial involving 104 women with cyclic breast pain (for at least three cycles) assessed the efficacy of a VAC tincture (10 g tincture containing 2 g of crude drug in 53% ethanol VAC) for treatment of the pain (46). The patients were treated with placebo, VAC tincture (30 drops twice daily), or VAC tablets (one tablet twice daily) for three cycles. The subjects assessed the intensity of breast pain once per cycle using a VAS and recorded the presence of menstrual bleeding and the intensity of pain in a diary. Prolactin levels were measured during the premenstrual week of cycles 1 and 3. At the end of the third treatment cycle, a significant reduction in breast pain was observed in the treated patients as compared with placebo (VAC solution, $P = 0.006$; VAC tablets, $P = 0.007$). A significant decrease in prolactin levels ($P = 0.039$) was also noted in the treatment groups as compared with placebo (46).

A second randomized, placebo-controlled, double-blind study with a similar design compared VAC solution (30 drops twice daily for three cycles) with placebo in the treatment of 100 women (50 per group) who had breast pain at least five days prior to menses in the last cycle before the study (16). The treatment phase lasted three menstrual cycles (2×30 drops/day = 1.8 mL of VAC or placebo). Mastalgia for at least five days of the cycle before the treatment was the strict inclusion condition. For assessment of the efficacy, VAS was used. Altogether

97 patients were included in the statistical analysis (VAC: $n = 48$, placebo: $n = 49$). Intensity of breast pain diminished quicker in the VAC group. This study design and duration were similar to that of Wuttke et al. (46). The results of this experiment showed a decrease in the VAS scores in both the treatment and the placebo groups. However, as compared with the placebo, the treatment group had significantly lower VAS values at the end of each cycle ($P = 0.018$, 0.006 , and 0.064 for cycles 1, 2, and 3, respectively).

In a randomized, placebo-controlled trial, the effects of VAC solution and placebo (double-blind) were compared with that of gestagen (Lynestrenol®) in 160 women with mastalgia (18). A complete remission or improvement of symptoms was reported in 82.1%, 74.5%, and 36.8% of the patients in the Lynestrenol, VAC, and placebo groups, respectively. The difference in effect between treatment groups and placebo was significant ($P < 0.01$), but no significant discrepancy was found between the two treatment groups (18).

Open studies have been used to assess the effectiveness of VAC solution for the treatment of over 1700 women with mastalgia (40–45). All these investigations assessed the efficacy of one VAC solution, at a dose of 45 to 75 drops per day for 1 to 6 cycles. Two of these studies compared VAC treatment with Lynestrenol (5 mg daily on days 12–24 of each cycle). Elimination of symptoms was observed in 46% to 81.5% of the treated women, improvement in 12% to 39.6%, and no effect in 6.5% to 29%. Collective reported adverse events from these studies included circulatory disturbances, acne, and weight gain (40–45).

Menstrual Cycle Irregularity and Infertility

Since 1954, at least 17 investigations have assessed the efficacy of VAC extracts for the treatment of menstrual cycle disorders including amenorrhea, oligomenorrhea, polymenorrhea, corpus luteum insufficiency, and infertility (2). Two double-blind placebo-controlled clinical trials and several observational studies have investigated the effect of various fruit extracts on corpus luteal-phase dysfunction and infertility (10,30,47). The products tested were ethanol extracts (53–70% ethanol), and the dose administered was 20 drops twice daily, 15 drops three times daily, 30 drops twice daily, or one to two tablets or capsules daily.

In the first randomized, double-blind, placebo-controlled trial, the efficacy of a dried VAC fruit extract was assessed in infertile women (10). The objective of this study was to determine whether elevated pituitary prolactin levels could be reduced by treatment with VAC, and whether the deficits observed in the luteal-phase length and luteal-phase progesterone synthesis could be normalized. Blood was obtained for hormone analysis on days 5, 8, and 20 of the menstrual cycle, both before and after three months of VAC therapy. Latent hyperprolactinemia was analyzed by monitoring prolactin release 15 and 30 minutes after intravenous administration of 200 µg of thyroid hormone. Thirty-seven cases (placebo: $n = 20$, treatment: $n = 17$) were included in the statistical analysis. After three months of treatment, prolactin release was reduced, a significant increase in the length of the luteal phase (10.5 days; $P < 0.05$) was observed and deficits in luteal pro-

gesterone synthesis were decreased. These changes only occurred in the treatment group and were not observed in the placebo group. All other hormonal parameters did not change with the exception of 17β-estradiol, which was observed to increase during the luteal phase in the treatment group. The overall length of the menstrual cycles did not change, suggesting that there was a corresponding shortening of the follicular phase. Two women in the extract group became pregnant by the end of the study. No adverse events were reported (10).

In a second randomized, double-blind, placebo-controlled trial, the efficacy of a VAC fruit extract was assessed in 96 infertile women (30). The outcome criteria measured included pregnancy or menstrual bleeding in women with secondary amenorrhea or improved luteal hormone concentrations. The subjects were administered 30 drops of the extract twice daily for three months. Sixty-six patients completed the study, and overall positive outcomes were observed in 47% of women, with 61% in the treatment group and 38% in the placebo group, although the results did not reach statistical significance ($P = 0.069$). In women with amenorrhea or luteal-phase dysfunction, pregnancy resulted twice as often in the treatment group (15%) versus the placebo group (7%); however, no statistical analysis was reported (30).

In open (uncontrolled) trials involving 48 (45 completed) infertile women (due to luteal-phase dysfunction), the efficacy of a VAC fruit extract for the normalization of progesterone concentrations was determined (47). Inclusion criteria were normal prolactin levels (below 20 ng/mL), normal results in prolactin and thyroid stimulating stimulation (TSH) tests, and an abnormally low serum progesterone level below 12 ng/mL on the 20th day of the cycle. Treatment consisted of a fruit extract, 40 drops daily, without any other medication for three months. Forty-five patients completed the studies (three were excluded because of concurrent hormone use). The outcome of therapy was assessed by the normalization of the mid-luteal progesterone concentration and correction (lengthening) of any preexisting shortening of the phases of the cycle. Treatment was successful in 39 out of the 45 women. Seven subjects became pregnant. In 25 patients, serum progesterone was restored to normal (> 12 ng/mL), and in seven cases, there was a trend toward normalization of progesterone levels. However, no statistical analysis was performed on the resultant data (47).

Two larger postmarketing trials, involving 479 women, assessed the safety and efficacy of a VAC fruit extract for the treatment of oligomenorrhea or polymenorrhea (48). The subjects were treated with 30 drops of the extract twice daily and the outcome measured was the bleeding-free interval. A lengthening of the bleeding-free interval was observed for 35 days in 187/287 women receiving treatment for oligomenorrhea and 26 days in 139/192 patients being treated for polymenorrhea (48).

MENOPAUSAL SYMPTOMS

The efficacy of a combination product containing *Hypericum perforatum* (St. John's wort) and VAC (300 mg and 500 mg, respectively) for the management of menopausal

symptoms was investigated in a double-blind, randomized, placebo-controlled, parallel study (49). The trial was performed over 16-week period and involved 100 eligible late-perimenopausal or postmenopausal women experiencing hot flushes and other menopausal symptoms. The herbal combination therapy or placebo tablets were administered twice daily. The primary endpoint was a reduction in hot flush episodes. Secondary endpoints included Greene Climacteric Scale scores, Hamilton Depression Inventory scores, and Utian Quality of Life Scale scores. Of the 100 women that started the trial, 93 women completed the study. Data analysis on an intent-to-treat basis found no significant differences between the two groups for any of the endpoints. Analyses performed at interim data time points revealed no significant differences at week 4, 8, or 12 for daily weighted flushes or scores on the Greene Climacteric Scale scores or Hamilton Depression Inventory scores. No significant change was found for either group on quality of life. The herbal combination was well tolerated with no significant adverse events noted in the short term (49).

Endocrine-Dependent Dermatoses

Two uncontrolled clinical studies and one observational report have assessed the effects of a VAC fruit extract on acne caused by a hormone imbalance (6–8). In one open study, 118 cases of acne were treated with a VAC extract (20 drops twice daily for 4–6 weeks, and then 15 drops twice daily for 1–2 years) and compared with conventional acne treatments (8). Patients treated with the fruit extract reported a more rapid healing rate after six weeks and after three months of therapy, whereas 70% of subjects taking the VAC extract stated complete healing.

ADVERSE EFFECTS

In general, VAC products and extracts appear to be very well tolerated and there have been few accounts of adverse reactions (ARs). A review of 30 human studies, involving 11,506 subjects, reported a total of 246 adverse events, thus representing an AR rate of approximately 2% (2). The major ARs included acne, cycle changes, dizziness, gastrointestinal distress, increased menstrual flow, nausea, skin reactions, urticaria, and weight gain (2). Minor side effects include fatigue, hair loss, increased intraocular pressure, palpitations, polyurea, sweating, and vaginitis (2,46). One case of multiple follicular development was reported in a female patient after self-medication with a VAC-containing product for infertility (50).

Although the potential estrogenic effects of VAC extracts are weak (51,52), its use during pregnancy or in women with estrogen-dependent breast cancer should not be recommended. In addition, patients with a feeling of tension and swelling of the breasts or other menstrual disturbances should consult a healthcare provider for medical diagnosis (28). Although there are no drug interactions reported, the potential dopaminergic effects of VAC extracts may reduce the efficacy of dopamine-receptor antagonists (23,53). Furthermore, because of possible hormonal effects, VAC may interfere with the effectiveness of oral contraceptives and hormone therapy (2).

MECHANISM OF ACTION

Several potential mechanisms of action have been proposed to explain the activity of VAC extracts, including inhibition of prolactin secretion (50–51) and dopaminergic (53,54) and estrogenic effects (51,55–57). Extracts have been shown to act as a dopamine agonist in vitro and in vivo. The binding of an ethanol VAC extract and various fractions of the extract to the dopamine D₂ and other receptors were evaluated by both radioligand binding studies and by superfusion experiments (54). The extract bound to the dopamine D₂ and opioid (μ and κ subtype) receptors with a median inhibitory concentration ranges between 20 and 70 $\mu\text{g/mL}$. Binding was not observed for the histamine H₁, benzodiazepine and OFQ receptors, or the serotonin transporter. Two diterpenes, isolated from a hexane fraction of the extract, rotundifuran and 6 β ,7 β -diacetoxy-13-hydroxy-labda-8,14-diene (Fig. 2), exhibited inhibitory actions on dopamine D₂ receptor binding with a median inhibitory concentration of 45 and 79 $\mu\text{g/mL}$, respectively (26,54). While lipophilic fractions of the extract bound to the μ - and κ -opioid receptors, binding to delta opioid receptors was inhibited primarily by an aqueous fraction of the extract. In superfusion experiments, the aqueous fraction of a methanol extract inhibited the release of acetylcholine in a concentration-dependent manner. In addition, the D₂ receptor antagonist spiperone antagonized the effect of the extract suggesting a dopaminergic action mediated by D₂ receptor activation. A labdane diterpene, α -acetoxy-13-hydroxylabdadiene (Fig. 2), isolated from a fruit extract, was found to displace ¹²⁵I-sulpiride from recombinant human D₂ receptor binding sites in a dose-dependent manner (58). This group also demonstrated that rotundifuran, at a concentration of 100 μM , significantly ($P < 0.05$) inhibited the secretion of prolactin from cultured rat pituitary cells.

Several groups have demonstrated that extracts bind to the estrogen receptor and have weak estrogenic effects, suggesting that chasteberry may also affect the estrogen/progesterone balance (51,56–58). A methanol extract of the fruit bound to both ER α and ER β induced the expression of estrogen-dependent genes, progesterone receptor (PR), and presenilin-2 (pS2) in Ishikawa cells (51). Significant binding affinity for both ER α and ER β was observed, with a median inhibitory concentration of 46.3 and 64.0 $\mu\text{g/mL}$, respectively. However, the binding affinity of the extract for ER α and ER β was not significantly

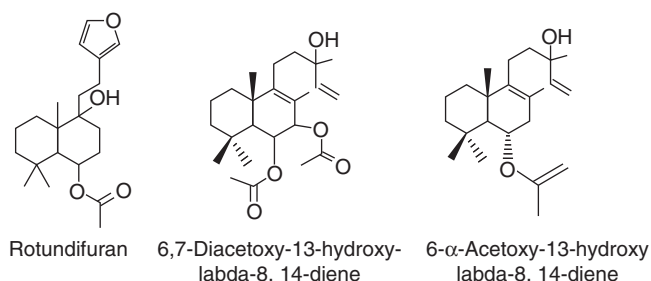


Figure 2 2- α -Acetoxy-13-hydroxylabdadiene, rotundifuran, and 6 β ,7 β -diacetoxy-13-hydroxy-labda-8,14-diene.

different (51). Based on bioassay-guided isolation, the "estrogenic" component from the fruit extract was identified as linoleic acid (LA), which also bound to ER α and ER β (52). Similar to the extract, LA also induced the expression of the PR mRNA in Ishikawa cells, at a concentration of 1 μ g/mL, indicating that binding produced a biological estrogenic effect in vitro. In addition, low concentrations of the extract or LA (10 μ g/mL) upregulate the expression of ER β mRNA in the ER plus hormone-dependent T47D:A18 cell line, a further indication of estrogenic activity (52). Recently, it has been suggested that methanol extracts of VAC may also activate the μ -opiate receptor, thereby exerting its effects on endogenous opiate peptides such as β -endorphin (50). This peptide assists in regulating the menstrual cycle through the inhibition of the hypothalamus-pituitary-adrenal axis through a complex feedback loop involving estrogen and progesterone (50). Levels of β -endorphin decrease along with estrogen in the late-luteal phase of the menstrual cycle, which correlated with the development of PMS symptoms (50). Thus, since VAC activates the μ -opiate receptor, it may increase the levels of β -endorphin, thereby having beneficial effects on PMS.

SAFETY

A recent review of the safety of VAC administration during pregnancy and lactation used database searches of published literature and case reports (59). The review concluded that in pregnancy, there is little evidence supporting the use of VAC during pregnancy based on theoretical and expert opinion and in vitro studies that chaste tree may have estrogenic and progestogenic activity, uterine stimulant activity, emmenagogue activity, and prevent miscarriages. In lactation, the data are conflicted as to whether chaste tree increases or decreases lactation. Thus, recommendations of VAC use during pregnancy and lactation are currently unsubstantiated and require further investigations for both safety and efficacy.

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Choline

Steven H. Zeisel

INTRODUCTION

Name and General Description

Choline, an essential nutrient for humans, is consumed in many foods. It is a constituent of all cell membranes and is necessary for growth and development. Also, as the major precursor of betaine, it is used by the kidney to maintain water balance and by the liver as a source of methyl groups for the removal of homocysteine in methionine formation. Finally, choline is used to produce the important neurotransmitter (nerve messenger chemical) acetylcholine, which is involved in memory and other nervous system functions (Fig. 1). Maternal diets deficient in choline during the second half of pregnancy in rodents caused decreased neurogenesis and increased neuronal apoptosis in fetal hippocampus (the memory center), resulting in permanent behavioral (memory) modifications in the offspring. Dietary deficiency of choline in rodents causes development of liver cancer in the absence of any known carcinogen. In humans, dietary deficiency of choline is associated with fatty liver and liver damage. The dietary requirement for choline is influenced

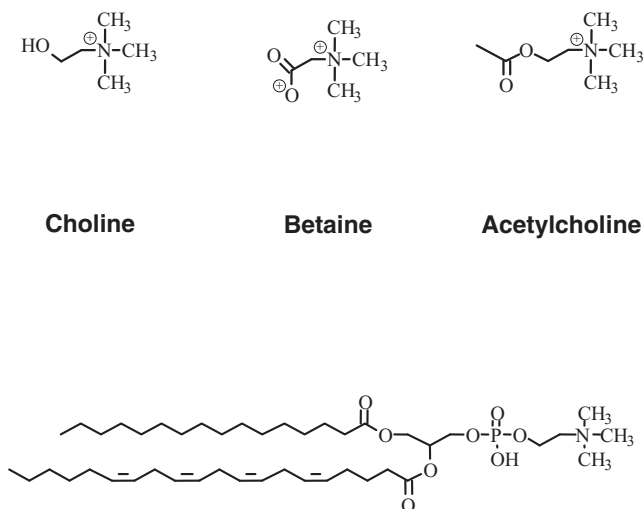
by gender as well as by genetic polymorphisms. Using a comprehensive database of the choline content of foods, a number of epidemiological studies identified associations between dietary choline intake and plasma homocysteine levels (risk factor for cardiovascular disease), cancer, and birth defects.

BIOCHEMISTRY AND RELATIONSHIPS WITH OTHER NUTRIENTS

Choline is needed for synthesis of several major phospholipids (phosphatidylcholine and sphingomyelin) in cell membranes and is also involved in methyl metabolism, cholinergic neurotransmission, transmembrane signaling, and lipid-cholesterol transport and metabolism (1) (Fig. 2). Choline can be acetylated, phosphorylated, oxidized, or hydrolyzed. There are several comprehensive reviews of the metabolism and functions of choline (1).

Cells absolutely require choline and die by apoptosis when deprived of this nutrient (2,3). Humans derive choline from foods, as well as from the *de novo* biosynthesis of the choline moiety via the methylation of phosphatidylethanolamine using (*S*)-adenosylmethionine as the methyl donor (most active in the liver). This ability to form choline means that some of the demand for choline can, in part, be met by using methyl groups derived from one carbon metabolism (via methyl-folate and methionine). Several vitamins (folate, vitamin B₁₂, vitamin B₆, and riboflavin) and the amino acid methionine interact with choline in 1-carbon metabolism. There has been renewed interest in these pathways during the past several years, engendered by recent insights that indicate that modest dietary inadequacies of the above-mentioned nutrients, of a degree insufficient to cause classical deficiency syndromes, can still contribute to important diseases such as neural tube defects, cardiovascular disease, and cancer (4).

Perturbing the metabolism of one of these pathways results in compensatory changes in the others (1). For example, methionine can be formed from homocysteine using methyl groups from methyl-tetrahydrofolate (THF), or using methyl groups from betaine that are derived from choline. Similarly, methyl-THF can be formed from one-carbon units derived from serine or from the methyl groups of choline via dimethylglycine, and choline can be synthesized *de novo* using methyl groups derived from methionine [via (*S*)-adenosylmethionine]. When animals and humans are deprived of choline, they



Phosphatidylcholine

Figure 1 Chemical structures of choline and related compounds.

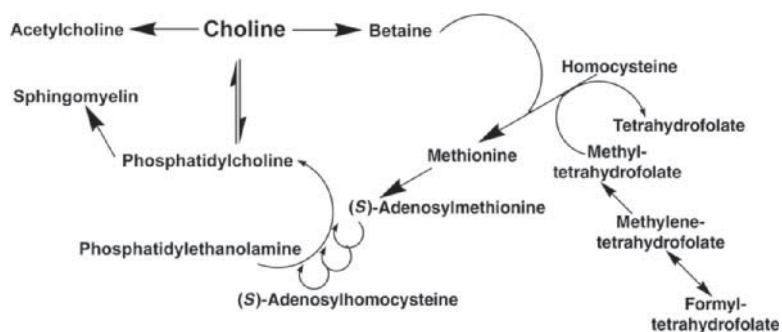


Figure 2 Pathways of choline metabolism

Choline can be a methyl-group donor and interacts with methionine and folate metabolism. It can be acetylated to form the neurotransmitter acetylcholine, and it can be phosphorylated to form membrane phospholipids such as phosphatidylcholine (lecithin) and sphingomyelin. Choline can be formed via the methylation of phosphatidylethanolamine (forming phosphatidylcholine, which can be hydrolyzed to make choline).

There is no estimate for percentage absorption of the various forms of choline in humans. The water-soluble choline-derived compounds (choline, phosphocholine, and glycerophosphocholine) are absorbed via the portal circulation, whereas the lipid-soluble compounds (phosphatidylcholine and sphingomyelin) are absorbed as chylomicrons. Lecithin is the most abundant choline-containing compound in the diet. About half of the lecithin ingested enters the thoracic duct, and the remaining is metabolized to glycerophosphocholine in the intestinal mucosa and then to choline in the liver. The liver takes up the majority of choline and stores it in the form of phosphatidylcholine and sphingomyelin. The kidney and the brain also accumulate choline. Although some free choline is excreted with urine, most is oxidized in the kidney to form betaine, which is responsible for maintaining the osmolarity in the kidney. A specific carrier is needed for the transport of free choline across the blood-brain barrier; the capacity is especially high in neonates.

Choline and other methyl donors are important dietary modulators of epigenetic marks on genes. The term “epi-

Examples of epigenetic effects of choline and other methyl donors include experiments in rodents in which pregnant dams were fed diets that were choline deficient versus normal, and DNA methylation in fetal brain was modified, resulting in over expression of genes that inhibit cell cycling in neural progenitor cells of developing brain (12,13). Gestational choline availability also affects histone methylation in the developing embryo, resulting in changes in expression of genes that regulate methylation and neuronal cell differentiation (14). Feeding pregnant Pseudoagouti *Avy/a* mouse dams a choline and methyl-supplemented diet altered epigenetic regulation of agouti expression in their offspring, as indicated by increased agouti/black mottling of their coats and by lean body phenotype (15,16). In another example, choline and methyl donor supplementation to dams increased DNA methylation of the fetal gene *Axin fused* [*Axin(Fu)*] and reduced incidence of tail kinking in *Axin(Fu)*/+ offspring by 50% (17). Thus, dietary manipulation of choline and methyl donors (either deficiency or supplementation) can have a profound impact upon gene expression.

HUMAN REQUIREMENT FOR CHOLINE

In one of the first clinical nutrigenomics studies, humans were phenotyped with respect to their susceptibility to developing organ dysfunction when fed a low-choline diet (18–21). Adult men and women (pre- and postmenopausal) aged 18 to 70 years were fed a standard diet containing a known amount of choline (550 mg/70 kg/day; baseline) for 10 days. On day 11 subjects were placed on a diet containing less than 50 mg choline/day for up to 42 days. Blood and urine were collected to measure various experimental parameters of dietary choline status, and markers of organ dysfunction and liver fat were assessed. If at some point during the depletion period, functional markers indicated organ dysfunction associated with choline deficiency, subjects were switched to a diet containing choline until replete.

Most men and postmenopausal women fed the low-choline diets developed reversible fatty liver (measure by mass resonance spectroscopy) as well as liver and muscle damage, whereas 56% of premenopausal women were resistant to developing choline deficiency (21). The fatty liver occurred due to lack of phosphatidylcholine synthesis in liver, which is required for very low density lipoprotein (VLDL) synthesis needed for export of excess triacylglycerol from liver (22). Choline deficiency liver damage was characterized by elevated serum aminotransferase (23) and muscle damage was characterized by elevated plasma serum creatine phosphokinase (19): both were due to increased rates of apoptosis in these tissues (also occurred in peripheral lymphocytes (24)). Choline-deficient subjects also had impaired ability to handle a methionine load, resulting in elevated plasma homocysteine concentrations (20,25).

Only 44% of premenopausal women develop signs of choline deficiency when deprived of dietary choline as compared with most adult men and postmenopausal women, suggesting their higher resistance to choline deficiency (19,20). Premenopausal women required less dietary choline because estrogen induces the phosphatidylethanolamine-*N*-methyltransferase (PEMT) gene to enhance the *de novo* biosynthesis of choline moiety (26). Estrogen binds to its receptors, and this complex interacts with estrogen response elements (EREs) in the promoter of the PEMT gene, resulting in an upregulation in PEMT mRNA expression and in hepatic upenzyme activity (26). Estrogen as the mediator of increasing PEMT activity in women is important, especially during pregnancy when fetal development uses a great deal of choline. Estradiol concentration rises from 1 to 60 nM during pregnancy (27,28), suggesting that the capacity for endogenous synthesis of choline should be highest when choline is needed most. Pregnancy and lactation are stages of life that demand high dietary choline intake and leave mothers extremely vulnerable to choline deficiency (29). In utero, the fetus is exposed to very high choline concentrations, with a progressive decline in blood choline concentration until adult levels of choline concentration are achieved after the first weeks of life (30). Plasma or serum choline concentrations are 6–7× higher in the fetus and newborn than those in adults (31,32). High circulating choline in the fetus and neonate ensures the availability of choline to tissues.

Less than 15% of pregnant women consume the recommended adequate intake for choline (33), and in case-control studies in California, women eating diets in the lowest quartile for choline were at fourfold increased risk for having a baby with a neural tube defect and at almost twofold increased risk for having a baby with a cleft palate; these risks were calculated after controlling for folate intake (34,35).

Genetics of Choline Requirements

Although premenopausal women are more resistant to choline deficiency, a significant portion of them (44%) still develops organ dysfunction when deprived of choline, suggesting individual differences in susceptibility to choline deficiency. In fact, some men and women require more than 850 mg/70 kg/day choline in their diet, whereas others require less than 550 mg/kg/day (21). Genetic variation likely underlies the differences in these dietary requirements. A single-nucleotide polymorphism (SNP) is a genetic variation occurring when a single nucleotide (A, T, C, or G) in the genome sequence is altered. These variations may affect metabolism. Only a few reports investigate whether SNPs in the genes involved in one carbon metabolism have roles in choline requirements (36,37). Premenopausal women with a SNP in 5,10-methylenetetrahydrofolate dehydrogenase (MTHFD1 rs2236225) were 15× more susceptible to choline deficiency than did noncarriers. This variant increased the use of choline perhaps by limiting the availability of methyl-folate for Hcy remethylation and increasing the demand for choline as a methyl-group donor. In addition, individuals with a SNP in PEMT (rs12325817) were more susceptible to choline deficiency, and women harboring this SNP were more affected than did men. SNPs in the PEMT gene alter endogenous synthesis of choline, thereby increasing the dietary requirement for choline.

Dietary Recommendations

In 1998, the Institute of Medicine (IOM) made recommendations for choline intake in the diet (4). At the time, there were insufficient data with which to derive an estimated average requirement for choline, thus only an adequate intake (AI) could be estimated. The IOM report cautioned, “this amount will be influenced by the availability of methionine and methyl-folate in the diet. It may be influenced by gender, and it may be influenced by pregnancy, lactation, and stage of development. Although AIs are set for choline, it may be that the choline requirement can be met by endogenous synthesis at some of these stages.” The IOM recommendations are summarized in Table 1.

Food Sources

In foods, there are multiple choline compounds that contribute to total choline content (choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, and sphingomyelin) (7). The U.S. Department of Agriculture (USDA) maintains a database of choline content in foods (38). Excellent sources of dietary choline are foods that contain membranes, such as eggs and liver. Average dietary choline intake on *ad libitum* diets for males and females are 8.4 and 6.7 mg/kg choline per day, respectively (39). Foods also contain the choline metabolite

Table 1 Recommended Adequate Intakes (AIs) of Choline for Humans

AI for infants	0–6 mo	125 mg/day, 18 mg/kg
	6–12 mo	150 mg/day
AI for children	1–3 yr	200 mg/day
	4–8 yr	250 mg/day
	9–13 yr	375 mg/day
AI for males	14–18 yr	550 mg/day
	19 yr and older	550 mg/day
AI for females	14–18 yr	400 mg/day
	19 yr and older	425 mg/day
AI for pregnancy	All ages	450 mg/day
AI for lactation	All ages	550 mg/day

Source: From Ref. 4.

betaine, which spare choline requirements (40). Human milk is rich in choline. Choline is routinely added to commercially available infant formulas. Until recently some infant formulas had inadequate choline content (especially soy-derived infant formulas), but in 2007–2008, many infant formula companies increased the choline content of their formulas so that they matched mature breast milk. These formulas still have different mixtures of the esters of choline than are present in human milk, perhaps resulting in different bioavailability as compared to human milk (41).

Adverse Effects

High doses of choline (>6 g) have been associated with excessive cholinergic stimulation, such as vomiting, salivation, sweating, and gastrointestinal effects (4). In addition, fishy body odor results from the excretion of trimethylamine, a choline metabolite from bacterial action (24). The tolerable upper limit for choline has been set at 3 g/day (4).

Assessing Choline Status

Measurement of choline and choline metabolites is useful in estimating choline status, but the measure is not definitive. Plasma choline concentration varies in response to diet and can rise as much as twofold after a two-egg meal. Fasting plasma choline concentrations vary from 7 to 15 μ M, with most subjects having concentrations of 10 μ M. Individuals that have starved for up to seven days have diminished plasma choline, but levels never drop below 50% of normal, probably because tissue phospholipids are “cannibalized” to prevent concentrations of choline from falling further (42). Note that children during the first year of life have normal plasma choline concentrations that are higher than 10 to 15 μ M (43). Plasma phosphatidylcholine concentration also decreases in choline deficiency (44), but these values are also influenced by factors that change plasma lipoprotein levels. Fasting plasma phosphatidylcholine concentrations are approximately 1 to 1.5 mM. Thus, measurements of choline or phosphatidylcholine in blood identify subjects with low dietary choline intake, but provide little help in differentiating the degree of deficiency.

CHOLINE AND CARDIOVASCULAR DISEASE

Choline and betaine may benefit heart health by lowering blood pressure, altering blood lipid profiling, and reducing plasma Hcy, a risk factor for cardiovascular disease

(CVD) (45). Dietary choline intake was found to have a statistically significant inverse relationship to circulating Hcy concentrations in the Framingham Heart Study (46) and in the Nurse’s Health Study (25), suggesting a protective effect of choline intake. However, when looking at the association between dietary choline intake and CVD incidence, no association was found (14) in the European Prospective Investigation into Cancer and Nutrition (EPIC) study (47), and a marginal positive association was found in the Atherosclerosis Risk in Communities (ARIC) study (48). It is important to note that in the ARIC study, most individuals in the cohort had choline intake below AI (49). Hence, the effects of choline supplementation on CVD risk remain unknown. Some human studies suggested that high betaine supplementation increases plasma low-density lipoprotein (LDL) cholesterol and triacylglycerol concentrations (50–52), effects that might counterbalance its Hcy lowering effects. However, the changes in serum lipid concentrations were not associated with higher risk of CVD. Moreover, the rise in LDL concentration may be an artifact of increasing VLDL and triacylglycerol excretion from fatty liver to plasma, which is not an adverse outcome (for critical review see Ref. 53). The relationship between choline and heart health warrants more study.

The choline-containing phospholipid phosphatidylcholine has been used as a treatment to lower the cholesterol concentrations because lecithin-cholesterol acyltransferase has an important role in the removal of cholesterol from tissue. Betaine, the oxidized product of choline, has been used to normalize the plasma homocysteine and methionine levels in patients with homocystinuria, a genetic disease caused by 5,10-methylenetetrahydrofolate reductase deficiency. Therefore, dietary choline intake might be correlated with cardiovascular disease risk. Many epidemiologic studies have examined the relationship between dietary folic acid and cancer or heart disease. It may be helpful to also consider choline intake as a confounding factor because folate and choline methyl donation can be interchangeable (7).

CHOLINE DEFICIENCY AND CANCER

An interesting effect of dietary choline deficiency in rats and mice has never been studied in humans. Dietary deficiency of choline in rodents causes development of hepatocarcinomas in the absence of any known carcinogen (54). Choline is the only single nutrient for which this is true. It is interesting that choline-deficient rats not only have a higher incidence of spontaneous hepatocarcinoma but also are markedly sensitized to the effects of administered carcinogens. Several mechanisms are suggested for the cancer-promoting effect of a choline-free diet. These include increased cell proliferation related to regeneration after parenchymal cell death occurs in the choline-deficient liver; hypomethylation of DNA (alters expression of genes); reactive oxygen species leakage from mitochondria with increased lipid peroxidation in liver; activation of protein kinase C signaling due to accumulation of diacylglycerol in liver; mutation of the fragile histidine triad (FHIT) gene, which is a tumor suppressor gene; and defective cell-suicide (apoptosis) mechanisms

(54). Loss of PEMT function may also contribute to malignant transformation of hepatocytes (55).

Only a handful of epidemiologic studies explore how choline and betaine intakes alter cancer risk in populations. This was perhaps due to the absence of food composition data, which has not been developed until recently (7). The Long Island Breast Cancer Study found that high choline consumption was associated with reduced breast cancer risk (56), and high choline and betaine consumption was associated with reduced breast cancer mortality (57). Moreover, individuals with *PEMT* rs12325817 and *CHDH* rs12676 SNPs had lower risk of developing breast cancer, whereas *BHMT* rs3733890 had lower breast cancer mortality. These data suggest the importance of nutrients and genetic interactions in the etiology of cancer. Alternatively, the Nurse's Health Study II found no association between choline intake and breast cancer risk (58), but a positive association between choline intake and colorectal cancer risk (59), suggesting different etiologies between breast and colorectal cancer. More research is warranted.

CHOLINE AND BRAIN

Choline and Brain Development

In rodents, maternal dietary choline intake during late pregnancy modulated mitosis and apoptosis in progenitor (stem) cells of the fetal hippocampus and septum and altered the differentiation of neurons in fetal hippocampus (60). Variations in maternal dietary choline intake (choline supplementation or choline deficiency) during late pregnancy were also associated with significant and irreversible changes in hippocampal function in the adult animal, including altered long-term potentiation (LTP) and altered memory (61). More choline (about 4× dietary levels) during days 11–17 of gestation in the rodent increased hippocampal progenitor cell proliferation, decreased apoptosis in these cells, enhanced LTP in the offspring when they were adult animals, and enhanced visuospatial and auditory memory by as much as 30% in the adult animals throughout their lifetimes (61). The enhanced maze performance appears to be due to choline-induced improvements in memory capacity. Indeed, adult rodents decrement in memory as they age, and offspring exposed to extra choline in utero do not show this “senility” (62). In contrast, mothers fed choline-deficient diets during late pregnancy have offspring with diminished progenitor cell proliferation and increased apoptosis in fetal hippocampus, insensitivity to LTP when they were adult animals, and decremented visuospatial and auditory memory (61).

Early postnatal choline supplementation significantly attenuated the effects of prenatal alcohol on a learning task, suggesting that early dietary interventions may also influence brain development (63). The mechanisms for these developmental effects of choline are not yet clear. Fetal alcohol syndrome (FAS) is an important concern of pediatricians, with 1 in every 750 infants born with FAS each year in the United States. Rats exposed to alcohol during the perinatal period had poor performance on memory tasks, which were improved by either prenatal or postnatal choline supplementation (64,65). Rett syndrome (RTT), a neurodevelopmental disorder associated with mutations

in the methyl-CpG-binding protein 2 (MeCP2) gene, is the second leading cause of mental retardation in girls. RTT girls experience a variety of deficits in cognitive, motor, and social functions. In mouse models of RTT, enhancing maternal or postnatal choline supplementation attenuates motor coordination deficits and improves neuronal integrity, proliferation, and survival (66,67). Choline supplementation also ameliorates the symptoms in rodent models of traumatic brain injury (68), status epilepticus (69–71), and schizophrenia (72).

Are these findings in animals likely to be true in humans? We do not know. Human and rat brains share many elements of brain development but they mature at different rates. In terms of hippocampal development, the embryonic days 12–18 in the rat correspond to approximately the last trimester in humans. Rat brain is comparatively more mature at birth than is the human brain, but human hippocampal development may continue for months or years after birth.

Choline and Adult Brain

Acetylcholine is one of the most important neurotransmitters used by neurons in the memory centers of brain (hippocampus and septum). Choline accelerates the synthesis and release of acetylcholine in nerve cells. Choline used by brain neurons is largely derived from membrane lecithin, or from dietary intake of choline and lecithin. Free choline is transported across the blood–brain barrier at a rate that is proportional to serum choline level; lecithin may be carried into neurons as part of an ApoE lipoprotein. Choline derived from lecithin may be especially important when extracellular choline is in short supply, as might be expected to occur in advanced age because of decreased brain choline uptake (73).

Results from studies using choline or phosphatidylcholine to treat adults with brain disorders have been very variable. Single doses of choline or lecithin in adult humans may enhance memory performance in healthy individuals, perhaps with greatest effect in individuals with the poorest memory performance. Studies in students showed that lecithin or choline treatment improved memory transiently for hours after administration (74). In humans with Alzheimer-type dementia, some studies report enhanced memory performance after treatment with lecithin (75), whereas other studies did not observe this. Buchman et al. recently reported that humans on long-term total parenteral nutrition may have verbal and visual memory impairment, which may be improved with choline supplementation (76). If lecithin is effective, it is in a special subpopulation in the early stages of the disease. Choline and lecithin have also been effectively used to treat tardive dyskinesia, presumably working by increasing cholinergic neurotransmission (77).

CONCLUSION

Choline in the diet is important for many reasons. Humans deprived of it develop liver and muscle dysfunction, and parenterally nourished patients need a source of choline. As our understanding of the importance of folate and homocysteine nutrition increases, there should be increased interest in how choline interacts with these compounds.

Recent findings about choline in brain development in animals should stimulate comparable studies in humans. The availability of food composition data now makes it possible to examine interactions between choline, folate, and methionine when considering epidemiological data.

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Chondroitin Sulfate

Karla L. Miller and Daniel O. Clegg

INTRODUCTION

Osteoarthritis (OA) is the most common arthropathy worldwide and a significant cause of morbidity and disability, especially in the elderly (1). Both biomechanical forces and biochemical processes are important in its pathogenesis, which is characterized by progressive deterioration of articular cartilage causing debilitating pain and loss of normal joint motion. Standard therapies can alleviate the symptoms of OA to some extent but have no ability to prevent disease progression. A number of alternative substances, collectively referred to as nutraceuticals, have been touted in the lay press as being beneficial for OA, with particular interest focused on glucosamine and chondroitin sulfate (2,3).

Chondroitin sulfate is a key component of normal cartilage that is substantially reduced in the cartilage of individuals with OA. This observation stimulated interest in its potential role as a therapeutic agent, and continuing investigations have now identified a number of apparent biologic actions. No consensus exists, however, as to its clinical efficacy or utility. While it has gained a measure of acceptance in Europe, physicians in the United States appear to be less convinced by the available clinical data. Nonetheless, the interest of the general population has been piqued, and owing to its universal availability as an over-the-counter supplement, present use of chondroitin sulfate, either with or without standard OA therapy, is not uncommon (4).

STRUCTURE, BIOCHEMISTRY, AND PHYSIOLOGY

Chondroitin sulfate is classified as a glycosaminoglycan (GAG) and is present abundantly in articular cartilage as well as in many other tissues, including bone, tendon, intervertebral disk, aorta, cornea, and skin. It is composed of alternating *N*-acetylgalactosamine and *D*-glucuronic acid residues, which form a long, unbranched chain. While the length of the chain is variable, it seldom exceeds 200 to 250 disaccharide units. Sulfation occurs at the 4 or 6 position of the *N*-acetylgalactosamine residue to produce chondroitin 4-sulfate (chondroitin sulfate A) and chondroitin 6-sulfate (chondroitin sulfate C), respectively, whereas the substitution of *L*-iduronic acid for *D*-glucuronic acid produces dermatan sulfate, formerly known as chondroitin sulfate B (Fig. 1).

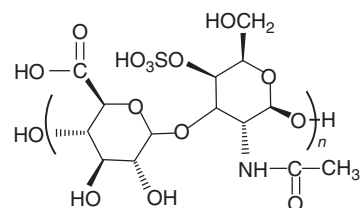
The significance of the sulfation position is not fully understood but appears to be associated with tissue age and location. Sulfation at the 4 position is seen more frequently in deeper, immature cartilage, while older, thinner cartilage is primarily sulfated at the 6 position (5). Addi-

tionally, abnormalities in sulfation appear to be present in OA cartilage (6), although their physiologic significance is uncertain.

The chondroitin sulfates comprise one of three primary divisions of GAGs, heparins and keratan sulfates being the other two. GAGs are synthesized intracellularly by chondrocytes, synoviocytes, fibroblasts, and osteoblasts. Following synthesis, multiple GAGs attach to a protein core within the Golgi apparatus to form a proteoglycan, which is subsequently secreted into the extracellular matrix (7). The factors that promote and regulate proteoglycan biosynthesis are complex, and it has been estimated that more than 10,000 enzymatic steps may be required (8).

The predominant proteoglycan in human articular cartilage is aggrecan, which contains both chondroitin sulfate and keratan sulfate side chains. Together, these side chains account for 80% to 90% of the mass of aggrecan. Chondroitin sulfate predominates over keratan sulfate, with more than 100 chondroitin sulfate side chains being present on a single aggrecan molecule. While there is some variability in the core protein, the physical and chemical properties of proteoglycans are largely attributable to the chondroitin sulfate side chains. One important feature of the proteoglycans is a marked negative electrical charge, which is created by the ionized sulfate groups within the GAG side chains.

Chondroitin sulfate A



Chondroitin sulfate C

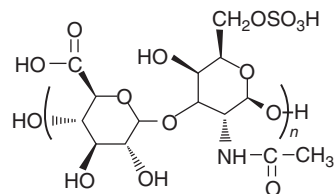


Figure 1 Structure of chondroitin 4-sulfate (chondroitin sulfate A) and chondroitin 6-sulfate (chondroitin sulfate C).

Articular cartilage consists of collagen fibers surrounded by a matrix containing aggregates of aggrecan and hyaluronate. Within the matrix, 100 to 200 aggrecan molecules bind to a single hyaluronate strand to form a supramolecular structure large enough to be seen by electron microscopy. The tensile strength of articular cartilage is the result of a network of collagen fibers, while the aggrecan-hyaluronate aggregates, which are rich in chondroitin sulfate chains, provide resiliency. Under normal circumstances, water is electrically attracted to cartilage by the negatively charged GAG residues and becomes entrapped within the aggregates. When a deforming force (such as occurs with weight bearing) is applied to the cartilage surface, minimal deformity occurs under normal conditions because the movement of water within cartilage is resisted by (i) its electrical affinity to the GAG residues, and (ii) the physical obstruction created by the bulky aggrecan-hyaluronate aggregates.

In OA, deterioration of articular cartilage is associated with a loss of proteoglycan, with a consequent change in water content and decrease in resilience. The pathogenetic events producing these changes remain uncertain but may result from changes in proteoglycan catabolism involving matrix metalloproteases, serine proteases, glycosidases, and chondroitinases secreted from chondrocytes and other connective tissue cells (9). Experimental models of OA suggest that synthesis of aggrecan increases early in the degenerative process in an apparent attempt at cartilage repair. The chondroitin sulfate side chains synthesized in this setting, however, are longer and more antigenic, suggesting that important GAG constitutional and/or conformational changes may be involved in the pathogenesis of OA (9). One such change appears to involve the terminal sulfation of chondroitin (10). Further study of the mechanisms that produce changes in the GAG synthesis may yet yield a site for therapeutic intervention that might have disease-modifying potential.

PHARMACOLOGY

The pharmacologic properties of exogenously administered chondroitin sulfate have been examined in a number of animal models and in humans with doses ranging from 60 mg/kg to 2 g/kg. Various routes of administration have been utilized in these studies, including oral, intraperitoneal, subcutaneous, and intravenous (11). In general, chondroitin sulfate appears to be well tolerated, and no significant adverse events have been reported with any route of administration. Determinations of oral bioavailability have yielded estimates of 5% to 15%, with blood levels reported to peak between 2 and 28 hours (12,13) following administration. No significant difference was observed between divided and single day dosing, while sustained dosing yielded serum levels only slightly higher than those seen following a single dose (12). The elimination half-life has been estimated at six hours. With a radiolabeled preparation of chondroitin sulfate administered orally to rats, more than 70% of the radioactivity was absorbed and subsequently identified in either the tissues or the urine. Radioactivity was found in every tissue examined at 24 hours, with levels variably diminished at 48 hours except in joint cartilage, the eye, the brain, and

adipose tissue, where levels were increased (12). There are very limited data for chondroitin sulfate pharmacokinetics when it is administered in conjunction with glucosamine.

The variability in pharmacokinetic derivations reported to date is considerable and appears to be principally due to methodological differences and limitations. Early studies that utilized radioactive forms of chondroitin sulfate (tritiated) in animals were complicated by the production of tritiated water, which introduced error into concentration determinations, while assays utilizing high-performance liquid chromatography (HPLC) methodology were unable to detect low concentrations of chondroitin sulfate. More recent work in humans is similarly problematic due to assay insensitivity, failure to account for endogenous chondroitin sulfate levels, and/or the use of diluents for anticoagulation. Newer technologies now permit the reliable quantitation of GAG at lower levels (14), and a pharmacokinetic study incorporating these techniques is being contemplated in conjunction with the Glucosamine/Chondroitin Arthritis Intervention Trial (GAIT).

CHONDROITIN SULFATE PREPARATIONS

Chondroitin sulfate is produced by several manufacturers and is readily available worldwide. It is derived by extraction from bovine, porcine, or shark cartilage. Various methods of extraction exist, but the specifics of each process are the proprietary information of the manufacturer. Most processes start with some form of enzymatic digestion followed by a variable number of washings, incubations, and elutions. In contrast to the procedure with prescription medications, the production process is not strictly regulated, and variations in quality and potency can occur from batch to batch and from manufacturer to manufacturer.

In a study conducted to identify a high-quality chondroitin sulfate dosage form for use in a clinical trial, three different sources of purified chondroitin sulfate were evaluated in a blinded fashion. While each sample exhibited similar disaccharide and GAG content overall, chondroitin sulfate potency varied by 15% to 20% (15).

In the United States, chondroitin sulfate is classified as a nutritional supplement and is widely available without a prescription in pharmacies and health and natural food stores. Not infrequently, it is manufactured in combination with glucosamine.

PUTATIVE MECHANISMS OF ACTION

A number of possible mechanisms of action for chondroitin sulfate in the treatment of OA have been suggested from pilot studies in animals and humans. Additional investigations are needed to confirm and extend these preliminary observations.

- a. *Inhibition of matrix proteases and elastases.* Articular cartilage is catabolized by proteinases and elastases that are elaborated from chondrocytes and leukocytes,

respectively. In both in vitro and in vivo studies with rodents, a modest decrease in elastase activity was seen following chondroitin sulfate administration. A similar chondroitin sulfate effect on neutral proteases has also been observed. The mechanism of this apparent inhibitory effect of chondroitin sulfate may be ionic disruption at the catalytic site of the enzyme. Chondroitin 6-sulfate may be more potent than chondroitin 4-sulfate (16).

- b. *Stimulation of proteoglycan production.* Several studies have shown that proteoglycan synthesis in vitro increases when chondroitin sulfate is added to cultures of chondrocytes and synoviocytes (17–19). The mechanism by which this occurs is unknown, but increased RNA synthesis has been observed, as well as TNF- α inhibition and IL-1 β antagonism.
- c. *Viscosupplementation.* An increase in synovial fluid viscosity has been reported following the administration of oral chondroitin sulfate to rabbits, rats, and horses (17,20,21). A more viscous synovial fluid may interfere physically with cartilage matrix catabolism, but the mechanism by which chondroitin sulfate might increase the viscosity of synovial fluid is uncertain.
- d. *Anti-inflammatory action.* Chondroitin sulfate has been reported to decrease leukocyte chemotaxis, phagocytosis, and lysosomal enzyme release in vitro. When administered orally to rodents, it appeared to decrease granuloma formation in response to sponge implants as well as attenuate the inflammatory response in adjuvant arthritis and carrageenan-induced pleurisy (22).

CLINICAL STUDIES

Interest in chondroitin sulfate as a therapeutic agent is longstanding and has primarily focused on the treatment of OA. Much of the available clinical data come from trials conducted in Europe, where it is now classified as a “symptomatic slow-acting drug in osteoarthritis” (SYSADOA) (23). Some have suggested that it may also have chondroprotective properties and thereby have properties of a “disease-modifying antiosteoarthritic drug” (DMOAD). Among physicians in the United States, however, there is considerable skepticism, and its role in the treatment of OA, if any, remains very controversial.

Most of the clinical experience with chondroitin sulfate has been in knee OA, which is an important patient subset due to its prevalence and resulting disability. Radiographic evidence of knee OA is present in approximately one-third of people older than 65 years, although not all have symptoms. Epidemiologic studies suggest that knee OA increases in frequency with each decade of life and affects women more often. Obesity, prior trauma, and repetitive occupational knee bending have also been identified as risk factors. The functional consequences of knee OA are considerable, as it produces disability as often as heart and chronic obstructive pulmonary disease (24).

The initial management of OA includes patient education, weight reduction, aerobic exercise, and physical therapy, and these should always be pursued before pharmacologic intervention is considered. Weight reduction and strengthening exercises may be of particular benefit in knee OA. Acetaminophen and nonsteroidal anti-

inflammatory drugs (NSAIDs) are the agents most often prescribed when nonpharmacologic measures prove insufficient. Local intervention with intra-articular corticosteroid injections and viscosupplementation may be of benefit in some patients.

Most rheumatologists would agree that present therapies for OA are suboptimal for the majority of patients. This was readily apparent in a representative two-year clinical trial comparing an NSAID and acetaminophen in knee OA, in which a majority of participants in both treatment groups withdrew prior to study completion because of toxicity or lack of efficacy. Given the shortcomings of standard therapy, it is not surprising that more than one-third of patients report that they have experimented with alternative and complementary treatments (25).

Nutraceuticals are produced and distributed in the United States under the authority of the Dietary Supplement Health and Education Act (DSHEA), which was enacted in 1994 as an amendment to the existing Federal Food, Drug, and Cosmetic Act. The provisions of DSHEA broaden the definition of dietary supplements and have removed the more stringent premarket safety evaluations that had been required previously. The act stipulates that the labels of dietary supplements list ingredients and nutritional information and permits manufacturers to describe the supplement’s effect on the “structure or function” of the body and the “well-being” that might be achieved through its use. However, representations regarding the use of the supplement to diagnose, prevent, treat, or cure a specific disease are expressly prohibited.

Legislation passed by the U.S. Congress in 1991 and 1993 (P.L. 102–170 and P.L. 103–43, respectively) established an office within the National Institutes of Health “to facilitate the study and evaluation of complementary and alternative medical practices and to disseminate the resulting information to the public.” This Office of Alternative Medicine became the forerunner of the present National Center for Complementary and Alternative Medicine (NCCAM), which was formally instituted in February 1999. With a present budget of more than \$125.5 million, the stated mission of NCCAM is to “explore complementary and alternative healing practices in the context of rigorous science.” One of the first clinical trials to be sponsored by NCCAM was GAIT, a Phase III evaluation of the efficacy and safety of glucosamine and chondroitin sulfate in knee OA.

Much of the clinical experience and study data with chondroitin sulfate suffers from poor study design, possible sponsor bias, inadequate concealment, and lack of intention-to-treat principles. More recent studies have sought to address these issues with larger trials that are more rigorously designed. Under the sponsorship of NCCAM, GAIT was a multicenter, randomized, double-blind, and placebo-controlled trial designed to evaluate the tolerability and efficacy of glucosamine and chondroitin sulfate in the treatment of knee OA (26). The study assigned 1583 patients to five treatment arms that consisted of glucosamine alone, chondroitin sulfate alone, a combination of glucosamine and chondroitin sulfate, celecoxib, and placebo. This trial was a two-part study designed to compare the efficacy of glucosamine and chondroitin sulfate alone and in combination with that of an active comparator (celecoxib) and placebo in alleviating

the pain of knee OA over 24 weeks. An ancillary study on a subset of GAIT patients was developed to determine whether radiographic benefit was evident after 24 months of agent exposure.

Overall, GAIT results revealed no difference in response to chondroitin sulfate alone or in combination with glucosamine. However, in the subgroup of patients with moderate-to-severe pain, there was a significantly higher rate of response with combined therapy. In addition, a statistically significant improvement in joint effusion was noted in the chondroitin sulfate group among the secondary outcome measures. Hochberg et al. (27) conducted a post hoc analysis of the GAIT data that specifically addressed the effects of chondroitin sulfate on joint swelling, and concluded that the patients with earlier disease based on symptoms and radiographic stage were most likely to benefit.

Two meta-analyses published in 2007 evaluated the recent data for the use of chondroitin sulfate for pain relief in OA (28,29). The first meta-analysis assessed randomized controlled trial (RCT) data on several medications used for short-term pain control in OA including NSAIDs, opioid analgesics, paracetamol, intra-articular steroids, glucosamine, and chondroitin sulfate (28). Data on chondroitin sulfate was limited to six RCTs (362 patients) and demonstrated a small effect on pain relief at four weeks that was statistically significant. Interestingly, a secondary outcome measure of pain relief at three months after the start of treatment showed a slight improvement in pain relief between weeks 4 to 12. This outcome differed from all other therapeutic interventions that showed no change or a decrease in pain relief from week 4 to 12. However, five of the six studies were sponsored by pharmaceutical companies, and the remaining trial did not show improvement in pain relief at 12 weeks. Overall, none of the available medications evaluated in this meta-analysis met criteria for a clinically relevant change in the primary outcome.

The second meta-analysis evaluated the use of chondroitin sulfate for pain in OA of the knee or hip as the primary objective (29). Joint space narrowing effects were analyzed as a secondary objective. Though a statistically significant effect size for pain relief was reported using the 20 trials (3846 patients) included in the analysis, this effect size approached zero when only the three larger trials with adequate concealment and intent-to-treat data were included in the analysis (1553 patients). Five studies reported data on joint space narrowing, and upon analysis showed a significantly lower rate of joint space loss with chondroitin sulfate over placebo. Though the effect size was statistically significant, it was small and of unclear clinical significance.

The first meta-analysis concluded that chondroitin sulfate was likely beneficial in alleviating the symptoms of knee OA to some degree but felt that the magnitude of the clinical effect was most likely less than that reported (28). The second meta-analysis determined that data from the larger, more rigorous trials suggested that symptomatic benefit from chondroitin sulfate was modest to nonexistent (29). Few trials addressing the effect of chondroitin sulfate on joint space narrowing were available for the latter analysis, and though a small effect was detected, the authors concluded that it was of uncertain clinical relevance and more study was necessary (29).

The ancillary radiographic report from GAIT published by Sawitzke et al. (30) assessed 572 patients with knee OA followed for two years for radiographic progression. These patients had been randomized to receive glucosamine 500 mg three times daily, chondroitin sulfate 400 mg three times daily, the combination of both supplements, celecoxib 200 mg daily, or placebo as part of the original GAIT study and were followed over 24 months with the primary outcome measure of mean change in joint space width (JSW) using metatarophalangeal semi-flexed radiography (31). No statistically significant difference in the loss of JSW in any of the treatment groups was found compared to placebo, but the study was limited by the smaller sample size and smaller than expected loss in JSW. Interestingly, loss of JSW was greater in the combination group compared to those taking either glucosamine or chondroitin sulfate alone, leading the authors to raise the possibility of interference with combined use.

The recently published results of the Study on Osteoarthritis Progression Prevention assessed the effects of chondroitins 4 and 6 sulfate on radiographic progression as well as symptomatic relief in knee OA over a two-year period (32). This study randomized 622 patients to receive 800 mg of chondroitin sulfate or placebo daily for two years. Loss in minimum JSW was the primary outcome, and symptomatic relief was a secondary outcome. A significant reduction in JSW loss was observed in the chondroitin sulfate group. This group also showed faster improvement in pain over the first nine months, but no significant difference was observed between the two groups thereafter or at the end of the two years.

It is important to recognize that OA trials designed to evaluate radiographic progression, may not be appropriate for detecting the symptomatic benefits of an intervention. Additionally, interventions that result in slowing of radiographic progression may not relieve symptoms, or symptom relief may not correlate with improvements in radiographic progression. Because OA is generally a slowly progressive disease, modification of the disease by an intervention such as chondroitin sulfate may not be evident for many years. Though some trials may report statistically significant changes in radiographic progression, the clinical importance of these changes remains uncertain and may become apparent with longer observational periods.

SAFETY

Information regarding the safety of chondroitin sulfate as a single agent, or in combination with other agents, suggests that adverse effects associated with chondroitin sulfate use are both minor and infrequent. In the randomized, controlled trials summarized above, the frequency of adverse effects reported in the chondroitin sulfate treatment arms was no greater than that with placebo arms. The side effects reported most often with chondroitin sulfate were epigastric distress, diarrhea, and constipation. Additionally, rashes, edema, alopecia, and extrasystoles have been reported, though infrequently.

An additional safety concern is the potential for transmission of bovine spongiform encephalopathy (BSE, or mad cow disease) from infected beef products. Despite

stringent safeguards put in place by the U.S. Department of Agriculture that banned the import of beef products from any at-risk country, a case was reported in an American herd. Those who elect to take chondroitin sulfate should be familiar with the animal source from which it has been extracted and, if bovine, assure themselves that it has come from a disease-free herd.

RECOMMENDATIONS

Considerable published medical literature is available suggesting that chondroitin sulfate is well tolerated and safe. Though it may be of benefit in alleviating the symptoms of OA in select patients, data demonstrating clinically relevant improvements in OA symptoms with chondroitin sulfate are sparse. This should be considered in the overall context that none of the currently available drugs for treatment of OA have shown dramatic improvements in pain relief. There are recent data suggesting that chondroitin sulfate may have effects on radiographic progression, but only studies of several year duration and sufficient scientific rigor will be able to determine the clinical significance of these findings. In light of the large number of studies documenting the favorable safety profile of chondroitin sulfate, patients who report benefit and would like to continue taking it can be assured that adverse effects are unlikely.

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Chromium

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INTRODUCTION

Chromium (Cr) is the 13th most common element in the earth's crust and 26th most common element in seawater. It is present in the environment in oxidation states ranging from -2 to $+6$ but principally as metallic (Cr^0), trivalent ($+3$), and hexavalent ($+6$) Cr. The valence state in which the Cr is present in the environment is of critical importance. Trivalent Cr, the most common and naturally occurring form of Cr, is an essential nutrient with very low toxicity and is the subject of this entry. This review will also focus on the role of Cr in humans. Hexavalent, the form of Cr used for industrial purposes, is largely a man-made product formed by the oxidation of the naturally occurring trivalent Cr. It is highly toxic and does not occur normally in biological tissues. Low amounts of hexavalent Cr can be reduced in biological systems to trivalent Cr.

BIOCHEMISTRY AND PHYSIOLOGIC FUNCTIONS

Essentiality

Chromium was shown to be an essential element in humans during the 1970s when a patient on total parenteral nutrition (TPN) developed severe signs of diabetes including weight loss, glucose intolerance, and peripheral neuropathy that were refractory to insulin (1). Since conventional treatments for diabetes, including 45 units of insulin per day, were unsuccessful, the patient was given supplemental Cr based on previous animal studies and preliminary human studies. Following two weeks of supplemental Cr, signs and symptoms of diabetes were reversed and exogenous insulin requirements dropped from 45 units per day to 0. Beneficial effects of Cr on patients on TPN have been confirmed on numerous occasions and documented in the scientific literature from three separate laboratories (2–4). Chromium presently added to TPN solutions may not be adequate for some patients. Peripheral neuropathy and glucose intolerance of a patient receiving recommended levels of Cr in these TPN solutions (total parenteral intake approximately $15\text{ }\mu\text{g}$ daily) were alleviated by an additional $250\text{ }\mu\text{g}$ daily dose of Cr as Cr chloride. Peripheral neuropathy was improved significantly within four days of additional Cr and normalization of nerve conduction within three weeks. Glucose intolerance was also normalized within three weeks of supplemental Cr (5). Recently, a patient developed severe insulin resistance following surgical repair of a thoracic aorta aneurysm. Postoperatively, the patient required 2110 units of insulin for more than 40 hours while receiv-

ing pressors and glucocorticoids. After the administration of intravenous Cr at $3\text{ }\mu\text{g/hr}$, the blood sugar normalized and insulin therapy was discontinued (6). This case represents a unique approach using intravenous Cr to achieve glycemic control in a patient with extreme insulin resistance and acute critical illness. Chromium is routinely added to TPN solutions as a daily administration for adults of 10 to $15\text{ }\mu\text{g}$ and 0.14 to $0.20\text{ }\mu\text{g/kg}$ for pediatric TPN patients (4).

Signs of Deficiency

Signs and symptoms of marginal Cr deficiency are not limited to patients on TPN and may be widespread in the general population. Insufficient dietary intake of Cr leads to increases in risk factors associated with diabetes and cardiovascular diseases including elevated circulating insulin, glucose, triglycerides, total cholesterol, reduced high-density lipoprotein cholesterol (HDL-C), and impaired immune function (7,8).

Chromium was shown to be an essential nutrient for animals almost five decades ago when it was shown that rats that fed on a *Torula* yeast-based diet developed impaired glucose tolerance that was reversed by an insulin potentiating factor whose active component was shown to be trivalent Cr (9). Chromium has subsequently been shown to be an essential element for fish, mice, squirrel monkeys, guinea pigs, pigs, cattle, and humans (Table 1).

Absorption and Excretion

Absorbed Cr is excreted primarily in the urine and only small amounts are lost in the hair, perspiration, or bile. Therefore, urinary Cr excretion can be used as an indicator of Cr absorption. Chromium absorption is inversely related to dietary intake. At daily dietary intakes of $10\text{ }\mu\text{g}$, Cr absorption is approximately 2% and at intakes of $40\text{ }\mu\text{g}$ is 0.5% (11). This leads to absorption of approximately $0.2\text{ }\mu\text{g/day}$, which appears to be a minimal basal level. At dietary intakes above $50\text{ }\mu\text{g Cr/day}$, Cr absorption is approximately 0.4%. The form of Cr also influences the absorption, that is, absorption of Cr from Cr chloride is usually in the region of 0.4% and Cr from Cr picolinate (the most common Cr supplement) is approximately 1.2%. Chromium incorporation into rat tissues was shown to vary widely depending upon its form. The highest concentrations of Cr were found in the kidney followed by liver, spleen, heart, lungs, and gastrocnemius muscle (12). The absorption of nutritional forms of Cr by human subjects was shown to be the greatest for Cr as Cr histidinate followed by Cr picolinate, Cr methionate, and Cr pidolate (13). In addition to its form, oxidation state and route of

Table 1 Signs and Symptoms of Chromium Deficiency

Function	Species
Impaired glucose tolerance	Human, rat, mouse, squirrel monkey, guinea pig, cattle
Elevated circulating insulin	Human, rat, pig, cattle
Glycosuria	Human, rat
Fasting hyperglycemia	Human, rat, mouse
Impaired growth	Human, rat, mouse, turkey
Hypoglycemia	Human
Elevated serum cholesterol and triglycerides	Human, rat, mouse, cattle, pig
Increased incidence of aortic plaques	Rabbit, rat, mouse
Increased aortic intimal plaque area	Rabbit
Nerve disorders	Human ^a
Brain disorders	Human ^a
Corneal lesions	Rat, squirrel monkey
Increased ocular eye pressure	Human
Decreased fertility and sperm count	Rat
Decreased longevity	Rat, mouse
Decreased insulin binding	Human
Decreased insulin receptor number	Human
Decreased lean body mass	Human, pig, rat
Elevated percentage of body fat	Human, pig
Impaired humoral immune response	Cattle
Increased morbidity	Cattle

^aThese effects have been observed only in patients on TPN.

Source: From Ref. 10.

administration, ascorbic acid, carbohydrates, phytate, oxalate, aspirin, antacids, and indomethacin also alter Cr absorption. Ascorbic acid was shown to significantly increase Cr absorption in humans with similar results in rats (14). Using radioactively labeled Cr chloride, animals that were fed starch were shown to have higher Cr absorption than those fed on sucrose, fructose, or glucose (15). Phytate has been reported to have either no effect on Cr absorption or an inhibitory effect. Oxalate also inhibits Cr absorption. Prostaglandin inhibitors such as aspirin and indomethacin enhance Cr absorption, and antacids such as Maalox[®] (trade name of Vovartis, Fremont, MI) and Tums[®] (Glaxo Smith Kline, Research Triangle Park, NC) inhibit Cr absorption.

Glucose Intolerance and Diabetes

Chromium supplementation to the general public, and in participants with diabetes in particular, is widespread but the efficacy of supplemental Cr is controversial. The controversy surrounding Cr supplementation stems from many factors, but the lack of definitive randomized trials is a major contributor. Specifically, many of the earlier studies evaluating Cr were open label and therefore generated substantial bias. Additional concerns from these earlier studies were the lack of "gold standard" techniques to assess glucose metabolism (e.g., euglycemic-hyperinsulinemic clamps), the use of differing doses and formulations, and the study of heterogeneous study populations. Based on these concerns, conflicting data have been reported that have contributed greatly to the confusion among health care providers regarding the routine use of Cr as a dietary supplement. More recent evidence, however, supports the concept that Cr supplementation yields more consistent clinical effects on carbohydrate

metabolism particularly when consumed at higher doses, for example, 200 µg or greater daily consumption (7,8,16–18). Subjects with varying levels of blood lipids have also been shown to improve following Cr supplementation, with the greatest improvements in total cholesterol, HDL cholesterol, and triglycerides in subjects with the highest initial levels. In the past decade, Cr has been shown to improve the signs and/or symptoms of diabetes in people with glucose intolerance and type 1, type 2, gestational and steroid-induced diabetes. The amounts of supplemental Cr shown to have beneficial effects in these studies ranged from 200 to 1000 µg/day. In a double-blind placebo-controlled study involving 180 subjects with type 2 diabetes mellitus, Cr effects were greater at 1000 µg/day than at 200 µg/day (19). The most dramatic improvements were shown in hemoglobin A_{1C} (HbA_{1C}), which is a reliable indicator of long-term glucose control. HbA_{1C} in the placebo group was 8.5 ± 0.2%, 7.5 ± 0.2% in the 200 µg group, and 6.6 ± 0.1% in the group of subjects receiving 1000 µg of Cr as Cr picolinate per day for four months (the upper level of reference range is approximately 6.5%). Similar results were observed in a double-blind placebo-controlled crossover study involving 50 subjects with type 2 diabetes supplemented with 200 µg of Cr twice daily as Cr picolinate (20). Improvements in fasting insulin levels and one-hour glucose and insulin levels following a 100 g glucose tolerance test in women with gestational diabetes were greater in the group receiving 8 µg/kg body weight per day compared with those receiving 4 µg/kg body weight (21). Steroid-induced diabetes that could not be controlled by oral hypoglycemic medications and/or insulin was also improved to acceptable levels in 47 of 50 people given 600 µg of Cr as Cr picolinate per day for two weeks followed by a daily Cr maintenance dose of 200 µg (22,23). Insulin sensitivity of obese subjects with a family history of diabetes also improved following 1000 µg of supplemental Cr daily as Cr picolinate (17).

A combination of Cr and biotin (600 µg as Cr picolinate and 2 mg of biotin) led to significant decreases in HbA_{1C} and glucose compared with the placebo group in a double-blind study involving 348 subjects with type 2 diabetes mellitus (DM). In subjects with high cholesterol and type 2 DM, there were also significant improvements in total cholesterol and low-density lipoprotein cholesterol (LDL-C) levels and atherogenic index in the group consuming the Cr biotin combination. Significant decreases in LDL-C, total cholesterol, HbA_{1C}, and very low-density cholesterol levels were also observed in the Cr biotin group taking statins (24). In a related study involving poorly controlled subjects with type 2 DM, there was a significantly greater reduction in the total area under the curve for glucose during a two-hour glucose tolerance test in the group receiving the Cr biotin combination. There were also reductions in fructosamine, triglycerides, and triglycerides/HDL-C ratio (25).

Chromium was found to be the most often studied nutritional supplement in type 2 diabetes (26). MEDLINE and EMBASE databases were searched using a systematic approach. Only double-masked randomized controlled trials were selected. A majority of the trials found a positive effect of Cr on fasting plasma glucose. Studies have involved individual case studies to studies with more than

800 subjects for more than one year (27). However, a meta-analysis of published studies on the effects of Cr on glucose and insulin (18) concluded, based upon the studies analyzed, that there were no significant effects of Cr. However, several positive studies were not included in this analysis owing to lack of specific data and inability to have an access to the original data. Several studies not reporting beneficial effects of supplemental Cr involved healthy normal subjects, with good glucose tolerance, who would not be expected to respond to additional Cr (8). Response to Cr is also dependent upon the amount and form of Cr consumed, and studies involving 200 μg of Cr or less or a form of Cr that is poorly absorbed would also not be expected to demonstrate effects of supplemental Cr (8).

In an attempt to determine who responds to supplemental Cr, 73 subjects with type 2 diabetes mellitus were assessed in a double-blinded, randomized, placebo-controlled study (28). Subjects were assessed at baseline for glycemic control with glycated hemoglobin measures, oral glucose tolerance tests, and body weight and body fat measures (dual-energy X-ray absorptiometry). The only subject variable significantly associated with the clinical response to Cr was the baseline insulin sensitivity, as assessed with the hyperinsulinemic-euglycemic clamp [partial $R(2) = 0.4038$] ($P = 0.0004$). Subject phenotype appears to be very important when assessing the clinical response to Cr because baseline insulin sensitivity was found to account for nearly 40% of the variance in the clinical response to Cr (28).

A follow-up study with use of "state-of-the-art" metabolic techniques and in a well-characterized cohort of individuals with type 2 diabetes representing a wide range of both phenotype and parameters assessing whole body insulin action, suggested that a consistent effect of Cr to improve insulin action and glycemia was not observed. However, this study is the first to show that Cr levels after supplementation do not differ between "responders" and "nonresponders," and provides the first comprehensive assessment of physiological and biochemical characteristics of individuals who responded to Cr. Specifically, "response" to Cr is more likely in insulin-resistant individuals who have more elevated fasting glucose and A_{1C} levels. Another novel finding was that tissue lipids are decreased in subjects randomized to Cr. Thus, it may be postulated that Cr alters insulin sensitivity through modulation of lipid metabolism in peripheral tissues and may represent a unique mechanism of action for trace minerals. The mechanism for this effect is the focus of ongoing studies (29).

Antioxidant Effects

Increased oxidative stress in relation to abnormal glucose metabolism is well documented in people with diabetes (30,31), and the antioxidative effects of Cr have also been demonstrated in animal studies (32). Chromium has also been shown to function as an antioxidant in people with type 2 diabetes (33) and confirmed in subjects with type 2 diabetes with HbA_{1C} values greater than 8.5% (34). Following six months of supplementation with Cr and Cr in combination with the antioxidant vitamins, C and E, there were significant reductions in both groups in thiobarbituric reactive substances in a double-blind study involving adult subjects with type 2 diabetes and $HbA_{1C} >$

8.5%. Total antioxidative status and glutathione peroxidase levels were also higher in the Cr group and the Cr plus antioxidative vitamins group (35). In addition, fasting glucose, HbA_{1C} , and insulin resistance also improved in groups consuming Cr.

Weight and Lean Body Mass

While there are a number of animal and human studies that suggest an effect of supplemental Cr on increasing lean body mass and decreasing body weight, there are also a number of studies showing no detectable effects of supplemental Cr (36,37). It may take more than six months to detect changes in lean body mass in humans and there may not be an effect on total body weight, as increases in lean body mass and decreases in fat may lead to small or minimal changes in total weight. Future studies involving body weight and lean body mass should be for 24 weeks or longer and involve 400 μg of Cr or more daily. Additional studies shorter than 24 weeks will be of minimal benefit and will likely serve to cloud the issue of whether supplemental Cr has an effect on body composition and weight loss in humans (36). Beneficial effects of Cr on lean body mass in humans have been substantiated in studies with pigs (38). The lack of the effect of Cr on lean body mass has been presented (39). A meta-analysis reported a body weight reduction of 1.1 to 1.2 kg during an intervention period of 10 to 13 weeks of Cr supplementation, considered too small to be clinically significant (37). However, long-term administration studies should be performed to determine whether the reduction in body weight could be maintained.

The controversial area of Cr, weight, and lean body mass was clarified when it was shown that goats eating a diet high in refined carbohydrates ate more than those eating the diet supplemented with Cr (40,41). Increases in weight were attributed to the antilipolytic effects of insulin leading to accumulation of triglycerides in the adipose tissue. Elevated insulin levels in the low Cr animals would also lead to decreased glucagon. As glucagon stimulates lipolysis, decreased glucagon may lead to decreased lipolysis and subsequent accumulation of body fat and weight gain and may explain the effects on lean body mass in humans.

In the above-mentioned study involving goats, it also took more than 28 weeks to detect significant changes in body weight in rapidly growing goats. This is consistent with human studies, usually 12 weeks or less in duration, which are unable to detect significant changes in weight of people eating conventional diets with supplemental Cr.

Supplemental Cr, 1000 μg of Cr as Cr picolinate, was also shown to decrease food intake ($P < 0.0001$), hunger levels ($P < 0.05$), and fat cravings ($P < 0.0001$), and tended to decrease body weight ($P = 0.08$) in 42 overweight women who reported craving carbohydrates. Study design was double-blind placebo controlled (42). In a related study involving rats, intraperitoneal administration of Cr resulted in a subtle decrease in food intake but when administered centrally, Cr picolinate dose dependently decreased food intake. The authors concluded that Cr has a role in food intake regulation, which may be mediated by a direct effect on the brain (42).

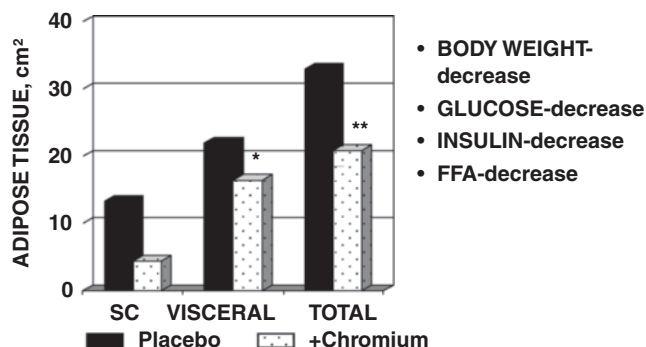


Figure 1 Chromium decreases visceral fat, subcutaneous fat, and total fat. Thirty-seven people with type 2 diabetes mellitus (DM) were placed on sulfonylurea medication for three months followed by six months of either 1000 μ g of Cr as Cr picolinate per day or placebo. *Significantly different at $P < 0.05$; **significantly different at $P < 0.01$. FFA, free fatty acids. Source: Adapted from Ref. 43.

A well-controlled study by the same group demonstrated that weight gain in people with type 2 diabetes was clearly regulated by supplemental Cr (43). Thirty-seven subjects with type 2 diabetes were placed on sulfonylurea drugs to control blood sugar for three months and then randomized to receive either Cr or placebo. Subjects receiving the supplemental Cr had smaller increases in body weight, percent body fat, and total abdominal fat compared with those in the placebo group. Subjects receiving Cr also had increased insulin sensitivity, corrected for fat-free-mass, and decreased free fatty acids (Fig. 1) (43).

Depression

A small pilot study suggested that Cr might be effective in the treatment of atypical depression. The study involved 15 patients with major depression, was double-blind, randomized, and placebo controlled (44). Seventy percent of the subjects responded to Cr with no negative side effects. Traditionally, depression has been treated with monoamine oxidase inhibitors; the toxicity and side effects of this class of drugs represent major limitations and the response is usually 50% or less. Depression has been associated with insulin resistance (45) and it is conceivable that increased insulin sensitivity leads to an enhanced central noradrenergic and serotonergic activity. Postsynaptic brain serotonin receptor downregulation by Cr in humans has also been reported, which could relate to insulin sensitivity and depression (46). However, larger and more comprehensive studies are required to address this problem.

In a double-blind, multicenter, eight-week replication study, 113 adult outpatients with atypical depression were randomized 2:1 to receive 600 μ g/day of elemental Cr, as provided by Cr picolinate, or placebo (47). Primary efficacy measures were the 29-item Hamilton Depression Rating Scale (HAM-D-29) and the Clinical Global Impressions Improvement Scale (CGI-I). The main effect of Cr was on carbohydrate craving and appetite regulation in depressed patients demonstrating that 600 μ g of elemen-

tal Cr may be beneficial for patients with atypical depression who also have severe carbohydrate craving. Further studies are needed to evaluate Cr in depressed patients specifically selected for symptoms of increased appetite and carbohydrate craving as well as to determine whether a higher dose of Cr would have an effect on mood.

The involvement of glutamatergic and serotonergic receptors in the antidepressant-like activity of Cr has been demonstrated in mice (48). The study confirmed the antidepressant-like activity of Cr in the mouse forced swim test and indicates the major role of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and participation of N-methyl-D-aspartate (NMDA) glutamatergic and 5-HT (1) and 5-HT (2 A/C) serotonin receptors in this activity.

Mechanism

How Cr serves as a cofactor for insulin action is not fully understood, and Cr remains as one of the only traceable minerals where a specific mechanism of action has not been identified. Several in vivo and in vitro studies have postulated a potential mechanism, but it must be stated that this mechanism has not been studied as to whether this is applicable to in vivo states. However, a low-molecular-weight chromium-binding substance (LMWCr) termed chromodulin (MW \approx 1500 Da) has been described, which appears to be involved in mediating the intracellular effects of Cr. It is composed of glycine, cysteine, glutamic acid, and aspartic acid (49). The principal carrier protein for Cr in the blood is suggested to be transferrin, which also is suggested to play a critical role in movement of Cr from blood to LMWCr. It has been postulated that an initial step in this process is the migration of transferrin receptors (Tf-R) to plasma membranes of insulin-insensitive cells after insulin stimulation. Transferrin containing the bound Cr binds to the Tf-R and is internalized by endocytosis. The pH of the internalized vesicle is reduced by adenosine triphosphate-driven proton pumps, Cr is released from transferrin, and the resulting free Cr is postulated to be sequestered by LMWCr (49,50). With this step, Cr is transferred from transferrin to LMWCr, which normally exists in insulin-dependent cells in the apo, or inactive, form. Binding with Cr ions converts inactive LMWCr to its holo, or active, form. It is proposed that LMWCr then participates as part of an insulin signal amplification system as it binds to insulin-activated insulin receptors and results in stimulating its tyrosine kinase activity. The end result of this process is postulated to be the activation of insulin receptor kinase and potentiation of the actions of insulin. Importantly, LMWCr without bound Cr or in the presence of other metal ions has been shown to be ineffective in activating insulin-dependent kinase activity and thus enhancing the actions of insulin (51). Thus, although this is an attractive hypothesis, definitive data for this mechanism as to whether it is operative in humans is lacking.

Chromium has also been reported to modulate phosphotyrosine phosphatase (52), the enzyme that cleaves phosphate from the insulin receptor leading to decreases in insulin sensitivity. The balance between kinase and phosphatase activity is suggested to facilitate insulin's role in rapidly moving glucose into cells. In addition, it

has been suggested that Cr enhances insulin binding, insulin receptor number, insulin internalization, and β -cell sensitivity (8). All of these effects contribute to improved insulin sensitivity.

Chromium, like insulin, also stimulates mRNA and protein levels for Ca^{2+} -ATPase, a protein involved in calcium transport. A combination of insulin and Cr as Cr picolinate caused a greater stimulation of the Ca^{2+} -ATPase mRNA than either insulin or Cr alone (53). Fluorometric analysis of the rate of ionized calcium recovery following stimulation with arginine also showed an effect of Cr; Cr picolinate alone increased recovery rate of 35% and Cr plus insulin 133%, compared to the increased recovery rate of 83% caused by insulin alone. In skeletal muscle cells, Cr was shown to stimulate tyrosine phosphorylation of the insulin receptor and insulin receptor substrate 1 (54). Wang et al. (55) also reported increased phosphorylation of the insulin receptor and concluded that cellular Cr potentiates insulin signaling by increasing insulin receptor kinase activity, separate from inhibition of phosphotyrosine phosphatase. Elmendorf and colleagues conducted a series of studies demonstrating that the cholesterol content of the plasma membrane regulates the response to Cr (56–59). They reported that a loss of plasma membrane phosphatidylinositol 4,5-bisphosphate-regulated filamentous actin structure contributes to insulin-induced insulin resistance. They also reported that Cr picolinate augments insulin-regulated glucose transport in insulin-sensitive 3T3-L1 adipocytes by lowering plasma membrane cholesterol. Insulin-induced insulin-resistant adipocytes display elevated plasma membrane cholesterol with a reciprocal decrease in plasma membrane phosphatidylinositol 4,5-bisphosphate. This lipid imbalance and insulin resistance was corrected by the cholesterol-lowering action of Cr picolinate (57). The plasma membrane lipid imbalance did not impair insulin signaling, nor did Cr picolinate amplify insulin signal transduction demonstrating that plasma membrane cholesterol is involved in the response to Cr and may be important in subjects who respond to supplemental Cr. In summary, although the precise mechanism of action of Cr is not known, the data that are available suggest effects on the insulin signaling processes that may be regulated by plasma membrane cholesterol content.

INDICATIONS AND USAGE

Food Sources

Not only is the total dietary intake of Cr important but also the total diet consumed. For example, increased intakes of simple sugars lead to increased losses of supplemental Cr (60). This becomes a double-edged sword, as high sugar foods are often also low in Cr. Diets high in simple sugars lead to elevated levels of circulating insulin and once insulin increases, Cr is mobilized. Chromium does not appear to be reabsorbed by the kidney and is lost in the urine. Other stresses such as acute exercise, pregnancy and lactation, infection, and physical trauma also increase Cr losses (61).

Foods high in Cr that are also low in simple sugars include broccoli, green beans, apples, and high fiber breakfast cereals (62). When nonprocessed fruits, vegetables, and high fiber foods are consumed, the requirement

for Cr is postulated to be lower because the Cr losses are lower. Chromium content of foods cannot be calculated from food composition tables, as there are large variations in individual foods owing likely to the contamination that occurs during growing and processing.

REFERENCE INTAKES

The estimated safe and adequate daily dietary intake (ESADDI) for Cr for children aged seven years to adult of 50 to 200 $\mu\text{g}/\text{day}$ was established by committees of the US National Academy of Sciences in 1980 and affirmed in 1989 (63). The Food and Drug Administration proposed a Reference Dietary Intake for Cr effective in 1997 of 120 $\mu\text{g}/\text{day}$. The new committee of the National Academy of Sciences has established that the normal intake of Cr should serve as the adequate intake of 20 μg for women and 30 μg for men older than 50 years and 25 μg for women and 35 μg for men aged 19 to 50 years (Table 2). It is unclear why the adequate intake for Cr is lower for people older than 50 years, other than total caloric intake. It is recognized that Cr is a cofactor for insulin action, and insulin action is known to be diminished with aging, but unsure whether this is a major reason. Indices of Cr status such as the Cr content of hair, sweat, and urine were shown to decrease with age in a study involving more than 40,000 people (64).

The proposed adequate intake appears to be the average intake as reported in 1985, which appears to be sub-optimal. Average daily intake (mean \pm SEM) of subjects consuming normal diets was 25 ± 1 μg for women and 33 ± 3 μg for men (11). There have been more than 30 studies reporting beneficial effects of supplemental Cr on people with blood glucose values ranging from marginally elevated to glucose intolerance and diabetes when consuming diets of similar Cr content.

Consumption of controlled normal diets in the lowest quartile of normal Cr intake, but near the new adequate intakes, led to detrimental effects on glucose (Fig. 2) in subjects with marginally impaired glucose

Table 2 Proposed Adequate Intakes for Chromium

Group	Proposed adequate daily intake (μg)
0–6 mo	0.2
7–12 mo	5.5
1–3 yr	11
4–8 yr	15
Boys, 9–13	25
Boys, 14–18	35
Girls, 9–13	21
Girls, 14–18	24
Men, 19–30	35
Men, 31–50	35
Women, 19–30	25
Women, 31–50	25
Men, 51–70	30
Men, >70	30
Women, 51–70	20
Women, >70	20
Pregnancy	30
Lactation	45

Source: From Ref. 65.

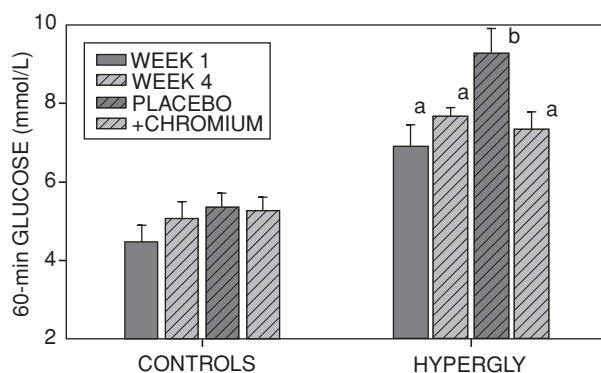


Figure 2 Effects of dietary Cr on people with good glucose tolerance and those with marginally impaired glucose tolerance consuming 20 μg or less of Cr daily. Subjects with good glucose tolerance, controls, are subjects with blood glucose levels less than 5.5 mmol/L (100 mg/dL), 90 minutes after consuming an oral glucose load of 1 g/kg body weight. Subjects defined as marginally hyperglycemic have 90-minute glucose levels between 5.5 and 11.1 mmol/L following an oral glucose load of 1 g/kg. Bars with different superscripts are significantly different at $P < 0.05$. Subjects with good glucose tolerance are able to maintain normal glucose levels at these low intakes, but not subjects with varying degrees of glucose intolerance. Source: From Ref. 66.

tolerance [90-minute glucose between 5.5 and 11.1 mmol/L (100–200 mg/dL) following an oral glucose load of 1 g/kg body weight]. The average person older than 25 years has blood glucose in this range. Consumption of these same diets by people with good glucose tolerance (90-minute glucose less than 5.5 mmol/L) did not lead to changes in glucose and insulin variables. This is consistent with previous studies demonstrating that the requirement for Cr is related to the degree of glucose intolerance and demonstrates that an intake of 20 μg /day of Cr is not adequate for people with marginally impaired glucose tolerance and certainly not for those with impaired glucose tolerance or diabetes.

ADVERSE EFFECTS

Safety of Chromium

Trivalent Cr, the form of Cr found in foods and nutrient supplements, is considered as one of the least toxic nutrients. The reference dose established by the US Environmental Protection Agency for Cr is 350 times the upper limit of the ESADDI as established in 1980 and affirmed in 1989, 3500 times the new adequate intake for women and 2333 times for men. The ratio of the reference dose to the required levels for most mineral and trace minerals is less than 10. The reference dose is defined as an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population, including sensitive subgroups, that is likely to be without an appreciable risk of deleterious effects during a lifetime. With these large safety factors, it is highly unlikely that there would be any reproducible signs of Cr toxicity at daily supplementation ranges of 1000 μg or less. There has been no evidence of toxicity in any of the nutritional studies involving Cr supplementation, but there have been

individual case studies reporting detrimental effects that have not been confirmed (67). Since the absorption of Cr is very low, it is likely that there would be indigestion and vomiting before enough Cr was absorbed to cause toxicity. However, Cr does bind to many biological substances including DNA and, at high enough levels, could (like almost all nutrients) lead to signs of toxicity in sensitive individuals. The toxic effects of Cr under nonphysiological conditions have been reviewed (39). The National Toxicology Program (NTP) (68) has completed independent in vitro and in vivo genotoxicity assays and evaluation of Cr picolinate, the most popular form of Cr in nutrient supplements, demonstrating that Cr picolinate did not produce chromosome damage in in vivo mouse assays and had no effect in two bacterial mutation assays (69). The absence of negative effects of Cr picolinate as reviewed by the NTP has led to the addition of Cr picolinate to generally recognized as safe list (68,69). Owing to the low toxicity of trivalent Cr, there is no upper limit established for Cr in the new National Academy of Sciences Guidelines (65).

CONCLUSIONS

The effects of Cr on glucose and insulin metabolism are well documented. Normal dietary intake of Cr appears to be suboptimal because several studies have reported beneficial effects of Cr on people with elevated blood glucose or type 2 diabetes eating conventional diets. Stresses that alter blood glucose often lead to increased mobilization of Cr that is subsequently lost from the body via the urine. The mechanism of action of Cr is largely through improvements in insulin sensitivity. Chromium makes insulin more effective and in the presence of Cr in a useable form, lower levels of insulin are required. There is no established upper limit for the supplemental Cr as it has very low toxicity and there have been no documented negative side effects in any of the more than 35 clinical studies. Number of subjects per study ranged from less than 10 to more than 800.

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Coenzyme Q₁₀

Gustav Dallner and Roland Stocker

INTRODUCTION

Coenzyme Q is a lipid with broad distribution in nature, present in plants, bacteria, fungi, and all animal tissues. Coenzyme Q refers to a general structure composed of a nucleus, that is, 2,3-dimethoxy-5-methylbenzoquinone, and, substituted at position 6 of this quinone, a side chain consisting of isoprene units (5 carbons), all in trans configuration and with one double bond. In human tissues, the major part of coenzyme Q is coenzyme Q₁₀, which has 10 isoprene units; only 2% to 7% is present as coenzyme Q₉.

NAME AND GENERAL DESCRIPTION

Coenzyme Q₁₀ (C₅₉H₉₀O₄) has a molecular weight of 863.3, melting point of 49°C, and redox potential of around +100 mV. The lipid is soluble in most organic solvents but not in water. The term coenzyme Q refers to both oxidized and reduced forms.

The oxidized form of coenzyme Q, ubiquinone (CoQ), has an absorption maximum at 275 nm, whereas its reduced form, ubiquinol (CoQH₂), has a small maximum at 290 nm. The absorption of CoQ at 210 nm is six times higher than that at 275 nm, but absorption at 210 nm is not specific for CoQ; this reflects the double bonds of the polyisoprenoid moiety and is therefore unspecific. The two major features of the lipid are the quinone moiety and the side chain. The quinone moiety is the basis for the redox function of this coenzyme, allowing continuous oxidation reduction (Fig. 1) as a result of enzymatic actions. The long polyisoprenoid side chain gives the molecule its highly hydrophobic character and influences its physical properties and arrangement in membranes.

EXTRACTION AND ANALYSIS

For analysis of the blood and tissue level of coenzyme Q, extraction is usually performed with organic solvents without previous acid or alkaline hydrolysis (1). The simplest procedure is using petroleum ether, hexane, or isopropyl alcohol and methanol. In this system, phase separation occurs, and the methanol phase retains all the phospholipids, which make up more than 90% of the total lipid in most tissues. The separated neutral lipids, among them coenzyme Q, are generally isolated and quantified by reversed phase high-performance liquid chromatography (HPLC) and UV detection. Both the sensitivity and the specificity of the method can be improved greatly by using electrochemical detection. In addition, this lat-

ter procedure makes it possible to analyze—under certain conditions—the ratio of oxidized/reduced coenzyme Q amount, reflecting the in vivo situation.

BIOCHEMISTRY AND FUNCTIONS

Biosynthesis

The biosynthesis of coenzyme Q in animal and human tissues is unique though the initial section, designated the mevalonate pathway, is identical for the production of coenzyme Q, cholesterol, dolichol, and isoprenylated proteins (2). After the branch point, however, the terminal portions of the biosynthetic pathways for each of the products are specific (Fig. 2).

The mevalonate pathway consists of eight enzymatic reactions, which lead to the production of farnesyl pyrophosphate, the common initial substrate for all terminal products mentioned earlier. The pathway starts with two enzymatic steps using three molecules of acetyl-CoA, resulting in 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The next reaction is a reduction to mevalonate by HMG-CoA reductase. This reaction is considered to be the main regulatory step in the pathway and also in cholesterol synthesis. Statins, drugs very commonly used in the treatment of hypercholesterolemia, are competitive inhibitors of HMG-CoA reductase. Mevalonate is phosphorylated in two steps to mevalonate pyrophosphate, which is then decarboxylated to isopentenyl pyrophosphate. Isopentenyl pyrophosphate is not only an intermediate but also the main building block for the synthesis of dolichol and the side chain of coenzyme Q. It is isomerized to dimethylallyl pyrophosphate, the substrate for farnesyl synthase. This enzyme mediates a two-step reaction, giving rise initially to the enzyme-bound, two-isoprenoid intermediate geranyl pyrophosphate, followed by a new condensation with isopentenyl pyrophosphate to the three-isoprenoid farnesyl pyrophosphate.

All branch-point enzymes utilize farnesyl pyrophosphate as substrate and they initiate the terminal part of the synthesis. These enzymes are considered for overall rate limiting and are consequently of utmost importance in the regulation of the biosynthesis of the lipid in question. In cholesterol synthesis, squalene synthase mediates the head-to-head condensation of two molecules of farnesyl pyrophosphate. *cis*-Prenyltransferase catalyzes the 1'-4 condensation of *cis*-isopentenyl pyrophosphate to all-trans farnesyl pyrophosphate, which, after additional modifications, generates dolichols with chain length between 16 and 23 isoprene units. *trans*-Prenyltransferase mediates a series of addition reactions of isopentenyl

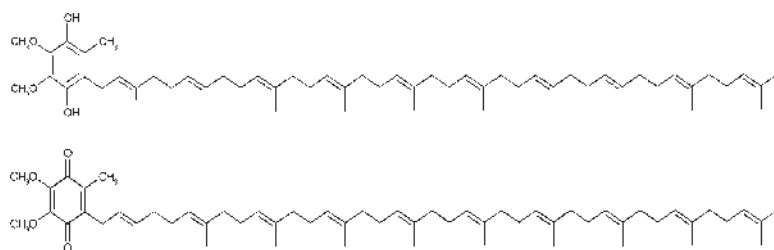


Figure 1 Coenzyme Q₁₀, shown in its reduced ubiquinol-10 (top) and oxidized ubiquinone-10 (bottom) forms, consists of a long hydrophobic side chain and a substituted benzoquinone ring.

pyrophosphate to farnesyl pyrophosphate, resulting in all-trans polyprenyl pyrophosphate, giving the side chain of coenzyme Q. The chain length varies between different species, and in humans, the chain is mostly decaprenyl pyrophosphate, with some solanesyl pyrophosphate.

The next step in the biosynthesis requires the precursor of the benzoquinone moiety, 4-hydroxybenzoate, which itself is produced from tyrosine and is present in excess amounts. After prenylation of 4-hydroxybenzoate, the ring is modified by C-hydroxylations, decarboxylation, O-methylations, and C-methylation. The final product of the biosynthetic process is reduced coenzyme Q, ready to serve as electron donor. The sequence of these reactions has been studied so far mainly in bacteria and yeast. In mammalian tissues, several genes have been identified through complementary recognition with yeast and the function for some of them was also established. Isolated enzymes are not available at present, although these will be required for the establishment of the details of coenzyme Q synthesis in animal tissues.

Enzymatic Reduction of CoQ

A major function of coenzyme Q is to serve as a lipid-soluble antioxidant. This requires CoQ to be present in its reduced form, CoQH₂, raising the question of how CoQ is maintained in its reduced form, CoQH₂. Ascorbate readily reduces benzoquinone in a catalytic process controlled by molecular oxygen, although this reduction is not likely of biological importance, as the benzoquinone moiety of the lipid-soluble CoQ₁₀, when localized in biological membranes, is not accessible to the water-soluble vitamin C. Similarly, cytosolic DT-diaphorase, an enzyme proposed earlier for CoQ₁₀ reduction, is not efficient in reducing benzoquinones containing long isoprene side chains. Based on studies with the inhibitors rotenone and dicoumarol, it is suggested that a cytosolic reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent CoQ reductase, different from the mitochondrial reductase and DT-diaphorase, is involved. More recently, the flavin adenine dinucleotide (FAD)-containing enzymes, lipoamide dehydrogenase and thioredoxin reductase, were found to reduce CoQ in vitro with high efficiency. These enzymes are homodimers, have a molecular weight of around 55 kDa, and belong to the family of pyridine nucleotide disulfide oxidoreductases.

Enzymatic Functions

The most thoroughly studied function of coenzyme Q is its participation in the mitochondrial electron transport chain. The lipid is essential in respiration as it shuttles electrons from nicotinamide nucleotide-linked (NADH) dehydrogenase and succinate dehydrogenase (complexes I and II) to the cytochrome system (complex III). During respiration, coenzyme Q is present in fully oxidized, fully reduced, and semiquinone forms. In the protonmotive Q cycle, there is a cyclic electron transfer pathway through complex III involving semiquinone that accounts for the energy conservation at coupling site 2 of the respiratory chain.

An electron transport system is also present in the plasma membranes of cells for transferring electrons across the membrane (3). The system is composed of a quinone reductase located on the cytosolic side and is thought to reduce CoQ in the presence of NADH. The resulting CoQH₂ then shuttles electrons to an NADH oxidase, located on the external surface of the plasma membrane, that reduces extracellular electron acceptors such as the ascorbyl radical, in this case to ascorbate. This oxidase is not related to the NADPH oxidase of phagocytes, which functions independent of coenzyme Q. The precise

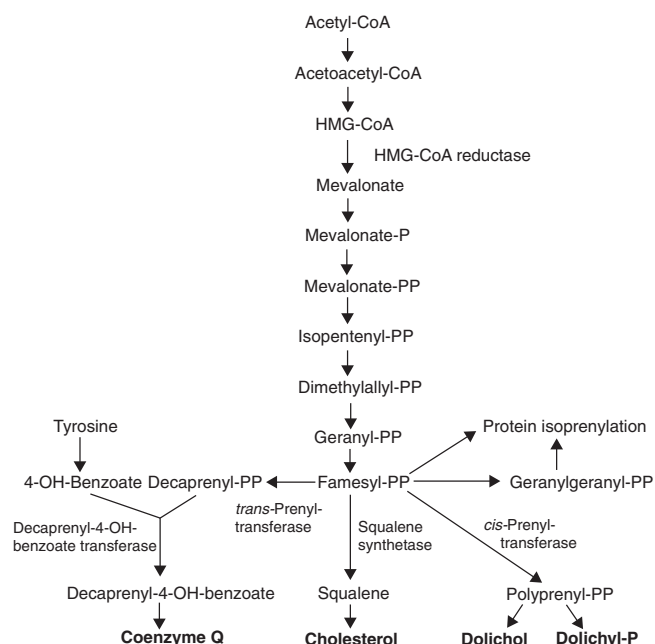


Figure 2 The mevalonate pathway leading to the biosynthesis of coenzyme Q, cholesterol, dolichol, and dolichyl phosphate.

function(s) of the NADH oxidase remain(s) to be elucidated, although it has been suggested to be involved in the control of cell growth and differentiation, the maintenance of extracellular ascorbic acid, the regulation of cytosolic NAD⁺/NADH ratio, the induction of tyrosine kinase, and early gene expression.

An electron transport system has also been proposed to be present in lysosomal membranes, transferring electrons from NADH to FAD, cytochrome *b*₅, CoQ, and molecular oxygen. This system could be involved in the translocation of protons into the lysosomal lumen.

Nonenzymatic Functions

Modulation of Mitochondrial Pore Opening

Ions and solutes may penetrate the inner mitochondrial membrane through specific transporters and ion channels. It has been observed *in vitro*, during the accumulation of Ca²⁺, that a permeability transition occurs and macromolecules up to the size of 1500 Da cross the membrane as a result of opening of an inner mitochondrial complex, the membrane transition pore. A large number of different compounds can open or close the membrane transition pore. An opening in the inner mitochondrial membrane is highly deleterious as it leads to loss of pyridine nucleotides, hydrolysis of adenosine triphosphate (ATP), disruption of ionic status, and elimination of the proton-motive force. Opening of the membrane transition pore is suggested to be an early event in apoptosis, causing activation of the caspase cascade through release of cytochrome *c*. On the other hand, the membrane transition pore may also have a physiological function by acting as a fast Ca²⁺ release channel in mitochondria.

Various coenzyme Q analogs that contain the benzoquinone moiety with or without a short saturated or unsaturated side chain are modulators of the membrane transition pore (4). They can inhibit, induce, or counteract the effects of inhibitors and inducers. Endogenous CoQ₁₀ may play an important role in preventing the membrane transition pore from opening, as it counteracts several apoptotic events, such as DNA fragmentation, cytochrome *c* release, and membrane potential depolarization.

Uncoupling Protein Function

It is well established that the inner mitochondrial membrane possesses uncoupling proteins that translocate protons from the outside to the inside of the mitochondria. As a result, the proton gradient established by the respiratory chain is uncoupled from oxidative phosphorylation and heat is produced instead of energy. In human tissues, five uncoupling proteins have been identified, but only uncoupling protein 1 has been studied in detail. It is present in brown adipose tissue and participates in thermogenesis. The content of uncoupling proteins in other tissues is low, since uncoupling is not a common event. Uncoupling protein 2 is found in most tissues, and uncoupling protein 3 is abundant in skeletal muscle.

By overexpressing uncoupling proteins 1, 2, and 3 from *Escherichia coli* in liposomes, it was demonstrated that coenzyme Q is an obligatory cofactor for the functioning of uncoupling proteins, with the highest activity obtained with CoQ₁₀ (5). Uncoupling proteins were able to transport protons only when CoQ₁₀ was added to the

membranes in the presence of fatty acids. Low concentration of ATP inhibited the activity. In this way, a proton is delivered from a fatty acid to the uncoupling protein with the assistance of CoQ₁₀ in the inner mitochondrial membrane. This is followed by the translocation of a proton to the mitochondrial matrix by the uncoupling protein.

Antioxidant Activity

Approximately 1% to 2% of the molecular oxygen consumed by mitochondria is converted to superoxide anion radical and hydrogen peroxide. In addition, reactive oxygen species are produced by other processes, including autoxidation reactions, and by the action of enzymes such as NADPH oxidases of phagocytes and other cells, mitochondrial monoamine oxidase, flavin oxidases in peroxisomes, and cytochromes P-450. Furthermore, nitric oxide, generated by nitric oxide synthases, can interact with superoxide and give rise to a number of reactive nitrogen species. These reactive species have the potential to damage lipids, proteins, and DNA, a process generally referred to as "oxidative damage." Antioxidants are enzymes, proteins, or nonproteinaceous agents that prevent the formation of reactive oxygen and nitrogen species, or remove these species or biomolecules that have been oxidatively damaged.

Coenzyme Q is the only lipid-soluble antioxidant synthesized endogenously (6). Its reduced form, CoQH₂, inhibits protein and DNA oxidation, but it is its effect on lipid peroxidation that has been studied in detail. Ubiquinol inhibits the peroxidation of cell membrane lipids and also that of lipoprotein lipids present in the circulation and in the walls of blood vessels. It has been suggested that CoQH₂ is a more efficient antioxidant than vitamin E, for two reasons. First, its tissue (but not blood) concentration exceeds several fold that of vitamin E. Second, and similar to vitamin C, CoQH₂ effectively reduces α -tocopheroxyl radical to α -tocopherol, and by doing so eliminates the potential pro-oxidant activities of vitamin E. In fact, CoQH₂ has been suggested to act as the first line of nonenzymatic antioxidant defense against lipid-derived radicals. In addition, CoQH₂ can inhibit the initiation of lipid peroxidation by scavenging aqueous radical oxidants.

As a result of its antioxidant action as a one-electron reductant, CoQH₂ is oxidized initially to its semiquinone radical (CoQH[•]), which itself may be oxidized further to CoQ, with the potential to generate the superoxide anion radical. Regeneration of CoQH₂ is therefore required for coenzyme Q to maintain its antioxidant activity. The effectiveness of cellular reducing systems is suggested by the fact that in most human tissues, the bulk of coenzyme Q is recovered as CoQH₂.

Effects on Atherosclerosis

Coenzyme Q₁₀ can theoretically attenuate atherosclerosis by protecting low-density lipoprotein from oxidation. Ubiquinol-10 is present in human low-density lipoprotein and, at physiological concentrations, prevents its oxidation *in vitro* more efficiently than vitamin E. The antiatherogenic effects are demonstrated in apolipoprotein E-deficient mice fed a high-fat diet (7). Supplementation with pharmacological doses of CoQ₁₀ not only increased aortic CoQ₁₀ levels but also decreased the absolute

concentration of lipoprotein-associated lipid hydroperoxides in atherosclerotic lesions. Most significantly, there was a clear decrease in the size of atherosclerotic lesions in the whole aorta. Whether these protective effects are solely due to the antioxidant actions of coenzyme Q remains to be established, as the tissue content of other markers of oxidative stress, such as hydroxylated cholesteryl esters and α -tocopherylquinone, did not decrease.

Oral administration of CoQ₁₀ to healthy humans results in increased concentrations of CoQ₁₀H₂ in circulating lipoproteins (8), with reduction most likely taking place in the intestine. Administration of CoQ₁₀ also results in uptake of the lipid into monocytes and lymphocytes but not into granulocytes, whereas this dietary treatment increases the vitamin E content in both mononuclear and polymorphonuclear cells (9). The phospholipid composition is modified selectively in mononuclear cells, which display elevated amounts of arachidonic acid. Basal and stimulated levels of β_2 -integrin CD11b and complement receptor CD35, distributed on the surface of monocytes, are also decreased by CoQ₁₀ supplementation. This may contribute to the antiatherogenic effect of dietary CoQ₁₀, since CD11b contributes to the recruitment of monocytes to the vessel wall during atherogenesis.

Effects on Blood Flow and Pressure

It is uncertain whether or not CoQ₁₀ reduces blood pressure in the long-term management of primary hypertension (10). It is possible that any blood pressure lowering effect is indirect—perhaps via improved diastolic and endothelial function. Endothelial dysfunction of the arteries has potentially serious consequences and is commonly seen in patients with established cardiovascular disease or elevated risk factors. Ubiquinone supplementation improves endothelial function measured as flow-mediated dilatation of the brachial artery in patients with uncomplicated type 2 diabetes and dyslipidemia but not in hypercholesterolemic subjects (11). In diabetic patients, CoQ₁₀ administration has also been found to decrease systolic blood pressure and HbA_{1C}, but not F₂-isoprostanes, suggesting that the protective effects may have been unrelated in the decrease of vascular oxidative stress.

Potential Anti-inflammatory Effects

There is some evidence that pharmacological doses of CoQ₁₀ may have anti-inflammatory effects *in vivo* under some conditions (12). This is an area worthy of further investigations, as inflammation is part of the etiology in many diseases, such as cardiovascular diseases, diabetes, and Alzheimer disease. An anti-inflammatory effect could help explain why positive health effects are reported in a number of investigations when uptake of the lipid into a specific organ was limited.

PHYSIOLOGY

Tissue Distribution

CoQ₁₀ is present in all human tissues in highly variable amounts (Table 1). The amounts are dependent on several factors, the most important under normal physiological conditions is the age (see sect. "Aging"). The highest amount is found in the heart (114 μ g/g wet weight) (13).

Table 1 Concentration of Coenzyme Q₁₀ in Different Adult Human Tissues

Tissue	CoQ ₁₀ (μ g/g tissue)
Brain	13
Thyroid	25
Lung	8
Heart	114
Stomach	12
Small intestine	12
Colon	11
Liver	55
Pancreas	33
Spleen	25
Kidney	67
Testis	11
Muscle	40

In the kidney, liver, muscle, pancreas, spleen, and thyroid, the CoQ₁₀ content is between 25 and 67 μ g/g, and in the brain, lung, testis, intestine, colon, and ventricle, it is between 8 and 13 μ g/g. This variation is explained by histological structure, and consequently there are great variations within the same organ. For example, in different regions of the bovine brain, the amount of CoQ₁₀ varies between 25 μ g/g (striatum) and 3 μ g/g (white matter). Rapid extraction and direct measurement by HPLC show that the major part of coenzyme Q₁₀ in tissues, with the exception of brain and lung, is the reduced form, CoQ₁₀H₂.

Intracellular Distribution

In rat liver, the highest amount of coenzyme Q₉ is found in the outer and inner mitochondrial membranes, lysosomes, and Golgi vesicles (1.9–2.6 g/mg protein); the concentration in plasma membranes is 0.7 μ g/g, and it is 0.2 to 0.3 μ g/g in the nuclear envelope, rough and smooth microsomes, and peroxisomes (Table 2) (13). The distribution pattern is quite different from that of other neutral lipids. For example, the major part of dolichol is localized in lysosomes, that of cholesterol in plasma membranes, and that of vitamin E in Golgi vesicles.

Within membranes, coenzyme Q₁₀ has a specific arrangement, with the decaprenoid side chain located in the central hydrophobic region, between the bilayer of phospholipid fatty acids. The functionally active group, the benzoquinone ring, is located on the outer or inner

Table 2 Concentration of Coenzyme Q₉ in Different Subcellular Organelles of Rat Liver

Organelle	CoQ ₉ (μ g/mg protein)
Nuclear envelope	0.2
Mitochondria	1.4
Outer membrane	2.2
Inner membrane	1.9
Microsomes	0.2
Rough microsomes	0.2
Smooth microsomes	0.3
Lysosomes	1.9
Lysosomal membrane	0.4
Golgi vesicles	2.6
Peroxisomes	0.3
Plasma membrane	0.7

surface of the membrane depending on the functional requirement. Because of this central localization, coenzyme Q₁₀ destabilizes membranes, decreases the order of phospholipid fatty acids, and increases permeability. These effects are in contrast to those of cholesterol, which is located adjacent to fatty acids on one side of the bilayer and that stabilizes the membrane, increases the order of its lipids, and decreases membrane permeability.

Transport

While the mevalonate pathway from acetyl-CoA to farnesyl pyrophosphate is mainly cytoplasmic, the terminal parts of coenzyme Q biosynthesis are localized in the mitochondria and endoplasmic reticulum (ER)-Golgi system. The mitochondrial inner membrane probably receives its lipid from the biosynthetic system associated with the matrix-inner membrane space. Newly synthesized very-low-density lipoproteins assembled in the ER-Golgi system also contain *de novo* synthesized coenzyme Q, which has to be synthesized at this location, like the other lipid and protein components of the lipoproteins. It is most probable that the various other cellular membranes also receive their constitutive coenzyme Q from the ER-Golgi system, as is the case with other lipids. Judging by studies in plants *in vivo* and with reconstituted cell-free systems, intracellular transport of coenzyme Q is a vesicle-mediated, ATP-dependent process, and cytosolic carrier proteins may also be involved.

Due to its hydrophobicity, the existence of a binding/transfer protein for coenzyme Q seems plausible, and recently saposin B has been suggested to serve this function (14). Aqueous saposin B was reported to extract and bind coenzyme Q dissolved in hexane to form a saposin B-coenzyme Q complex, with the lipid-binding affinity decreasing in the order: CoQ₁₀ > CoQ₉ > CoQ₇ >> α -tocopherol >> cholesterol (no binding).

Under normal conditions, all organs and tissues synthesize sufficient coenzyme Q, so that external supply is not required. Coenzyme Q present in small amounts in all circulating lipoproteins is derived from very-low-density lipoprotein newly synthesized and discharged by the liver. It likely functions as an antioxidant and protects lipoproteins, with restricted redistribution among them. In the case of dietary coenzyme Q, lipoproteins are the carriers in the circulation and interact with at least some types of tissues for cargo delivery. Thus, the situation differs from that of cholesterol, in which case several organs depend on external supply from the diet or the liver.

Bioavailability

Plasma

The uptake of coenzyme Q from the intestine occurs at a low rate, with only 2% to 4% of the dietary lipid appearing in the circulation. The uptake mechanism has not been studied so far but is probably similar to that of vitamin E and mediated by chylomicrons. In rats, dietary CoQ₁₀ appears as CoQ₁₀H₂ in mesenteric triacylglycerol-rich lipoproteins, which enter the circulation and are converted by lipoprotein lipase to chylomicron remnants, which are then cleared rapidly by the liver. Some of this diet-derived coenzyme Q reappears in the circulation, perhaps as a result of hepatic synthesis and release of very-

low-density lipoprotein. Depending on the diet, in healthy human controls the amounts of coenzyme Q in very-low-density, low-density, and high-density lipoproteins are 1.2, 1.0, and 0.1 nmol/mg protein, respectively. After dietary supplementation (3×100 mg CoQ₁₀/day for 11 days), the amounts are 3.2, 3.5, and 0.3 nmol/mg protein, respectively. These data are consistent with the notion that circulating coenzyme Q redistributes among lipoproteins to protect them against oxidation.

For most tissues, the low bioavailability of CoQ limits the ability of supplements to restore normal tissue levels of CoQH₂ where deficiency exist. There are several potential ways to approach this problem, including administration of the lipid in reduced form, and increasing bioavailability by either derivatization or administering CoQ in association with cyclodextrins. "Mitoquinone," a cationic modified form of CoQ attained by coupling to triphenylphosphonium and targeted to mitochondria to improve mitochondrial function, has received much interest recently (15). However, it is important to point out that mitoquinone is not a form of CoQ naturally occurring in human tissue, and the increase in superoxide production observed after uptake of mitoquinone into mitochondria is of potential concern (16). A potential alternative approach to increase CoQ in blood and tissues may be via drugs that stimulate the endogenous synthesis. This would not only elevate the amount of the lipid but possibly also direct it to the appropriate location. Polyisoprenoid epoxides in tissue culture and peroxisome proliferator-activated receptor- α agonists in rodents increase CoQ synthesis and amounts; however, no drug for this purpose is presently available for humans.

Blood Cells

Red blood cells contain very small amounts of coenzyme Q. In lymphocytes, the content of CoQ₁₀ is doubled after one week of dietary supplementation with this lipid, and this enhances both the activity of DNA repair enzymes and the resistance of DNA to hydrogen peroxide-induced oxidation (17). Two months of CoQ₁₀ supply to humans increases the ratio of T4/T8 lymphocytes (18), and an increase in the number of lymphocytes has been noted after three months of dietary supply of this lipid. Ten weeks of CoQ₁₀ administration to healthy subjects elevated the lipid content by 50% in monocytes, but no increase was observed in polymorphonuclear cells.

Tissues

There remains some controversy regarding the bioavailability of dietary coenzyme Q in different tissues. In rats, the liver, spleen, adrenals, ovaries, and arteries take up a sizeable amount of dietary coenzyme Q (19). Under normal physiological conditions, very limited uptake may also occur in the heart, pancreas, pituitary gland, testis, and thymus. No uptake is apparent in the kidney, muscle, brain, and thyroid gland. However, uptake into rat brain has been reported—possibly the outcome of the specific conditions employed. Similarly, in mice, some, but not all, investigators have reported uptake into tissues. Derivatization of coenzyme Q by succinylation and acetylation increases its uptake into blood but not into various organs.

What is clear is that under normal conditions, the bioavailability of dietary coenzyme Q in most tissues is

limited. This may be explained by its distribution and functional requirement. Under normal conditions, all cells synthesize sufficient lipid, so that external supply is not required. Exogenous coenzyme Q taken up by the liver does not appear in mitochondria, which house the bulk of this cellular lipid, but is found mainly in nonmembranous compartments, such as the lysosomal lumen.

The situation is, however, different in states of severe coenzyme Q deficiency. Genetic modifications causing low levels of coenzyme Q have serious consequences for neuronal and muscular function (20). In children with genetic coenzyme Q deficiency, dietary supplementation greatly alleviates pathological conditions and re-establishes mitochondrial and other functions. Limited studies with biopsy samples from patients with cardiomyopathy also indicate that the cardiac levels of coenzyme Q are decreased and may be increased by dietary supplementation with the lipid. Thus, it appears that uptake and appropriate cellular distribution of coenzyme Q occur if there is a requirement for the lipid.

Direct organ uptake of sizeable amounts is not necessarily the only way of action of coenzyme Q, as other redox-active substances can act by signaling, serving as primary ligands or secondary transducers. Thus, the presence of coenzyme Q in the blood may impact on the vascular system, the production of cytokines, the expression of adhesion molecules, and the production of prostaglandins and leukotrienes. The possibility that metabolites of coenzyme Q influence metabolic processes has not yet been investigated.

Catabolism

The short half-life of coenzyme Q, ranging between 49 and 125 hours in various tissues (Table 3), indicates that the lipid is subject to rapid catabolism in all tissues. The main urinary metabolites identified have an unchanged and fully substituted aromatic ring with a short side chain containing five to seven carbon atoms and a carboxyl group at the ω -end (21). Phosphorylated forms of these metabolites are also recovered from nonhepatic tissues. These water-soluble metabolites are transferred to the circulation and are excreted by the kidney through urine. In the liver, the coenzyme Q metabolites become conjugated to glucuronic acid for fecal removal via bile.

Table 3 Half-life of CoQ₉ in Rat Tissues

Tissue	Half-life (hr)
Brain	90
Thyroid	49
Thymus	104
Heart	59
Stomach	72
Small intestine	54
Colon	54
Liver	79
Pancreas	94
Spleen	64
Kidney	125
Testis	50
Muscle	50

Regulation of Tissue Coenzyme Q Content

In contrast to cholesterol, coenzyme Q does not appear to be subject to dietary or diurnal variations. However, a number of treatments decrease the content of the lipid in experimental systems. Administration of thiouracil, which inhibits thyroid gland function, decreases liver coenzyme Q. Oral administration of vitamin A also lowers hepatic coenzyme Q. In selenium-deficient rats, the coenzyme Q content of the liver is decreased by 50%, and the amount of the lipid is also lowered in the heart and kidney (but not muscle). A protein-free diet for three weeks lowers coenzyme Q content in the liver and heart but not in the kidney, spleen, and brain. As indicated earlier, HMG-CoA reductase controls cholesterol synthesis because the branch-point enzyme squalene synthase has a low affinity for farnesyl pyrophosphate, so that its pool size is the main regulatory factor (22). By contrast, the branch-point enzyme of coenzyme Q synthesis, *trans*-prenyltransferase, has a comparatively higher affinity for farnesyl pyrophosphate, so that a decrease in this substrate does not generally lower the rate of coenzyme Q synthesis. It appears, however, that the doses of statins employed for the treatment of hypercholesterolemia result in inhibition of synthesis, as the coenzyme Q concentration decreases in several tissues (23).

As mentioned earlier, the bioavailability of dietary coenzyme Q is limited. For this reason, it would be advantageous to find compounds that elevate tissue concentrations of coenzyme Q by increasing its biosynthesis. In rats and mice, treatment with peroxisomal inducers, such as clofibrate, phthalates, and acetylsalicylic acid, induces coenzyme Q synthesis in most organs and elevates its concentration in all subcellular organelles (24). The upregulation takes place by interaction with a nuclear receptor: peroxisomal proliferator receptor- α . This receptor interacts with a number of genes, resulting in the increased synthesis of several enzymes, many of them connected to lipid metabolism. However, peroxisomal proliferator receptor- α is poorly expressed in human tissue, and it is not known to what extent this transcription factor is involved in coenzyme Q metabolism. Agonists or antagonists to various nuclear receptors may be a future approach to the upregulation of coenzyme Q biosynthesis and its concentration in human tissues.

Hormones control coenzyme Q metabolism, but their method of action is not known in detail. Growth hormone, thyroxine, dehydroepiandrosterone, and cortisone elevate coenzyme Q levels in rat liver to various extents. A liver-specific increase of coenzyme Q occurs in rat and mice after two to three weeks stay in the cold room (+4°C). Vitamin A deficiency more than doubles the coenzyme Q level in liver mitochondria and more than trebles that in liver microsomes. Squalastatin 1, an inhibitor of squalene synthase, greatly increases coenzyme Q synthesis by increasing the farnesyl pyrophosphate pool and saturating *trans*-prenyltransferase.

COENZYME Q₁₀ DEFICIENCY

Genetic Disorders

Coenzyme Q deficiency is an autosomal recessive disorder that may present itself in the form of myopathy,

encephalopathy and renal disease, or ataxia (20). The myopathic form is characterized by substantial loss of muscle coenzyme Q, muscle weakness, myoglobinuria, ragged-red fibers, and lactic acidosis. Patients with encephalopathy and renal involvement possess a more general disease, with myopia, deafness, renal failure, ataxia, amyotrophy, and locomotor disability. In these cases, coenzyme Q is undetectable or present at very low levels in cultured fibroblasts. In the ataxic form of deficiency, weakness, cerebellar ataxia, cerebellar atrophy, seizures, and mental retardation dominate, and low levels of coenzyme Q are found in the skeletal muscle. Various types of mutations have been found to be responsible for decreased synthesis of CoQ (25). Most of the mutations are of the primary type, affecting proteins related to the biosynthesis of the lipid. COOQ1-PDSS1 and -PDSS2 (two subunits of decaprenyl diphosphate synthase), COOQ2 (decaprenyl-4-hydroxybenzoate transferase), COOQ8 (CABC1 or ADCK3, a putative protein kinase), and COOQ9 (nonidentified function) are genes established in this group. There are also secondary forms of deficiency caused by mutations in genes not involved in coenzyme Q biosynthesis. Mutations in APTX (encoding aprataxin) and ETFDH (multiple acyl-CoA dehydrogenase deficiency caused by defects in electron transfer flavoprotein or ETF-ubiquinone oxidoreductase) also result in CoQ deficiency.

The cases described in the literature probably represent extreme forms of coenzyme Q deficiency, seriously affecting mitochondrial functions. Moderate coenzyme Q deficiency is probably more common, though this requires verification by appropriate analysis of tissue biopsy samples. Unfortunately, the coenzyme Q content in blood often does not mirror the tissue concentration of the lipid, and it is highly desirable to develop methods to estimate moderate degrees of coenzyme Q deficiency. At present, diagnosis depends on measuring the coenzyme Q content in muscle biopsy samples, cultured fibroblasts, and lymphoblasts, or analyzing mitochondrial respiration and enzymes that require coenzyme Q as intermediate.

CoQ deficiency is of special interest since it is the only treatable mitochondrial disease and oral administration of CoQ re-establishes normal functions. Early diagnosis before development of clinical symptoms is of outmost importance since established kidney and brain damages may not be completely reversible. The treatment, however, stops the process and the improvement is dramatic as children leave the wheel-chair state and are able to perform various activities. The problem is that at present diagnosis requires a muscle biopsy and analysis of mitochondrial functions. This does not allow screening of larger populations. Therefore, development of simplified diagnostic procedures would be of great interest also for diagnosis of less severe cases, probably present in relatively high numbers.

Aging

In human organs, the coenzyme Q content increases three- to fivefold during the first 20 years after birth, followed by a continuous decrease, so that in some tissues the concentration may be lower at 80 years than at birth (Table 4) (26). The decrease is less pronounced in the brain, where

Table 4 Coenzyme Q₁₀ Content (μg/g) with Age in Human Organs and Human Brain

Human organs	Age				
	2 days	2 yr	20 yr	41 yr	80 yr
Lung	2.2	6.4	6.0	6.5	3.1
Heart	36.7	78.5	110.0	75.0	47.2
Spleen	20.7	30.2	32.8	28.6	13.1
Liver	13.9	45.1	61.2	58.3	50.8
Kidney	17.4	53.4	98.0	71.1	64.0
Pancreas	9.2	38.2	21.0	19.3	6.5
Adrenal	17.5	57.9	16.1	12.2	8.5
Human brain	34 yr	55 yr	70 yr	90 yr	
Nucleus caudatus	11.6	11.7	10.5	6.6	
Gray matter	16.4	16.2	16.0	13.5	
Hippocampus	14.5	13.8	12.6	8.0	
Pons	11.6	11.7	10.5	6.6	
Medulla oblongata	11.1	10.8	10.0	4.7	
White matter	5.0	5.0	4.9	2.0	
Cerebellum	13.2	13.0	12.9	11.0	

it mainly takes place between 70 and 90 years, and its extent, between 20% and 60%, depends on the localization. This pattern is different from that seen for other lipids. In most tissues, the content of cholesterol and phospholipids remains unchanged during the whole life period, whereas the amounts of dolichyl phosphate and especially dolichol increase greatly with age. It is unclear whether the decrease in coenzyme Q content is caused by its lowering in all or some selected cellular membranes or, alternatively, by other changes such as decreased number of mitochondria.

Cardiomyopathy

The uptake of dietary coenzyme Q into heart muscle is low in both rats and humans, but it may increase significantly in various forms of cardiomyopathy (27). A number of clinical trials performed during the last 30 years suggest that heart functional performance may be improved modestly by dietary coenzyme Q supplementation (28,29). In congestive heart failure, improvements have been reported for ejection fraction, stroke volume, and cardiac output. Patients with angina may respond with improved myocardial efficiency. Reperfusion injury, such as after heart valve replacement and coronary artery bypass graft surgery, includes oxidative damage, and treatment of patients with coenzyme Q prior to surgery may lead to decreased oxidative damage and functional improvement. However, the benefits reported have not been consistent, and despite the existence of a large body of literature, there remains a need for large, long-term, and well-designed trials to establish unambiguously whether CoQ₁₀ supplements are beneficial in the setting of cardiomyopathy and the failing heart.

Neurological Disorders

Judging by extensive animal studies, a number of neurological diseases involve mitochondrial dysfunction and oxidative stress. The positive effects obtained with coenzyme Q treatment in these models suggest that supplementation may also be beneficial in humans (30). Patients with early Parkinson disease were subjected to a trial

in which the placebo group was compared with groups supplemented for 16 months with coenzyme Q up to daily doses of 1200 mg. It was found that coenzyme Q slowed the progressive functional deterioration, with the best results obtained with the highest dose. Platelets from these patients had decreased coenzyme Q content and also showed reduced activity of mitochondrial complex I and complex II/III. The ratio of CoQ₁₀H₂ to CoQ₁₀ was also decreased in these platelets, indicative of the presence of oxidative stress. Upon supplementation, the CoQ₁₀ content in the platelets increased and complex I activity was also elevated. In Huntington disease, magnetic resonance spectroscopy detected increased lactate concentration in the cerebral cortex. Administration of CoQ₁₀ caused a significant decrease in lactate that reversed upon discontinuation of the therapy.

Deficiency of frataxin, a regulator of mitochondrial iron content, causes Friedreich ataxia. When patients with this disease were treated with coenzyme Q and vitamin E for six months, progression of their neurological deficits was slowed down, associated with an improvement in cardiac and skeletal muscle energy metabolism (31). Treatment of these patients with idebenone, an analog of coenzyme Q, reduced heart hypertrophy and improved heart muscle function. In several studies, patients with mitochondrial encephalopathy, lactic acidosis, and strokes (MELAS) displayed significant improvement after coenzyme Q or idebenone treatment (32). Several other trials were also performed during recent years with variable results. Since there are subtypes of individual neurodegenerative diseases, large numbers of patients are required to obtain reliable results, which is often difficult to accomplish.

Statin Therapy

Statins are the drugs most commonly used for the treatment of hypercholesterolemia, and, in addition to efficient cholesterol lowering, they also have anti-inflammatory activities. The basis for their use is that inhibition of HMG-CoA reductase decreases the farnesyl pyrophosphate pool to such an extent that squalene synthase, which catalyzes the terminal regulatory step in cholesterol synthesis, is no longer saturated, thereby inhibiting overall synthesis (22). It appears, however, that the extent to which the farnesyl pyrophosphate pool is decreased by therapeutic doses of the drug also affects the saturation of *trans*- and *cis*-prenyltransferases in spite of the fact that these latter enzymes have a higher affinity for farnesyl pyrophosphate. Consequently, synthesis of both coenzyme Q and dolichol is inhibited. Rats treated with statins exhibit decreased levels of coenzyme Q, dolichol, and dolichyl phosphate in heart and muscle, and the same is probably also true in humans. In humans, statin treatment significantly decreases blood coenzyme Q concentration (33), although the clinical significance of this phenomenon remains to be established. Various degrees of myopathy, myalgia, and rhabdomyolysis have been reported in statin-treated patients, and it is possible, but not proven, that these conditions are related to decreased muscle coenzyme Q content. Initial trials of CoQ₁₀ supplementation in patients with statin-induced myopathy have provided variable results (34). Given the widespread use of statins, it is important

that additional studies address a possible causal link between these side effects of statin treatment and altered tissue coenzyme Q content.

Exercise

During endurance exercise training, the coenzyme Q concentration increases in rat muscle on a weight basis due to an increase in mitochondrial mass. After four days of high-intensity training, the coenzyme Q content in the exposed muscles of healthy persons is unchanged (35). Supplementation (120 mg/day) doubles the coenzyme Q concentration in the plasma, but there is no change in the muscle content as judged by HPLC analysis of the tissue homogenate and isolated mitochondrial fraction in both control and trained subjects.

Dosage

So far, no toxic or unwanted side effects have been described for CoQ₁₀ supplements in humans, not even after ingestion in gram quantities. In most studies, 100 to 200 mg has been given per day in two doses. In genetic disorders, in the case of adults, the dose may increase to 300 mg/day and in neurological diseases, up to 400 mg/day or more. In the latter case, in the frame of large multicenter trials, doses up to 2400 mg have been supplied. A patent on the use of statins combined with coenzyme Q has expired recently, although this combined preparation has not been manufactured so far. Now it may be possible for the pharmaceutical industry to introduce capsules containing statins and coenzyme Q in order to decrease the potential for muscle damage. In this case, relatively low doses of CoQ₁₀ (e.g., 50 or 100 mg/day) appear to be appropriate.

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Conjugated Linoleic Acid

Kristina B. Martinez, Arion J. Kennedy, and Michael K. McIntosh

INTRODUCTION

Conjugated linoleic acid (CLA) consists of a group of positional and geometric fatty acid (FA) isomers of linoleic acid (C18:2; *cis*-9, *cis*-12 octadecadienoic acid). CLA isomers are found naturally in ruminant meats and dairy products due to biohydrogenation of linoleic or linolenic acids in the rumen of these animals. Larger quantities of CLA are chemically synthesized for use in dietary supplements or fortified foods. Initially identified as a potential anticarcinogen, CLA has been reported to prevent obesity, diabetes, or atherosclerosis in different animal and cell models, depending on the doses, isomers, and models used. Potential mechanisms for preventing these diseases include inducing cancer cell apoptosis, increasing energy expenditure and delipidating adipocytes, increasing insulin sensitivity, or reducing aortic lesions. However, unequivocal evidence in human participants is still lacking. Ironically, potential side effects of CLA supplementation include chronic inflammation, insulin resistance, and lipodystrophy. Long-term, well-controlled clinical trials and more mechanistic studies are needed to better understand the true potential health benefits versus risks of consuming CLA isomers and their mechanisms of action.

CHEMISTRY AND SYNTHESIS OF CLA

Natural Synthesis of CLA Isomers

CLA isomers are produced naturally in the rumen of ruminant animals by fermentative bacteria *Butyrovibrio fibrisolvens*, which isomerize linoleic acid into CLA isomers (Fig. 1). A second pathway of CLA synthesis in ruminants is in the mammary gland via δ -9-desaturase of *trans*-11, octadecanoic acid (1). Thus, natural food sources of CLA are dairy products including milk, cheese, butter, yogurt, and ice cream and ruminant meats such as beef, veal, lamb, and goat meat (2–4) (Table 1). The *cis*-9, *trans*-10 (9,11) isomer (i.e., rumenic acid) is the predominating CLA isomer in these products (~80%), whereas the *trans*-10, *cis*-12 (10,12) isomer represents approximately 10%. Although several other isoforms of CLA have been identified, the 9,11 and 10,12 isomers appear to be the most biologically active (5). Levels of CLA isomers in ruminant meats or milk can be augmented by dietary manipulation, including feeding cattle on fresh pasture (6) or by adding oils rich in linoleic acid (e.g., safflower oil) or ingredients that alter biohydrogenation of linoleic acid (e.g., ionophores) to their diet (7).

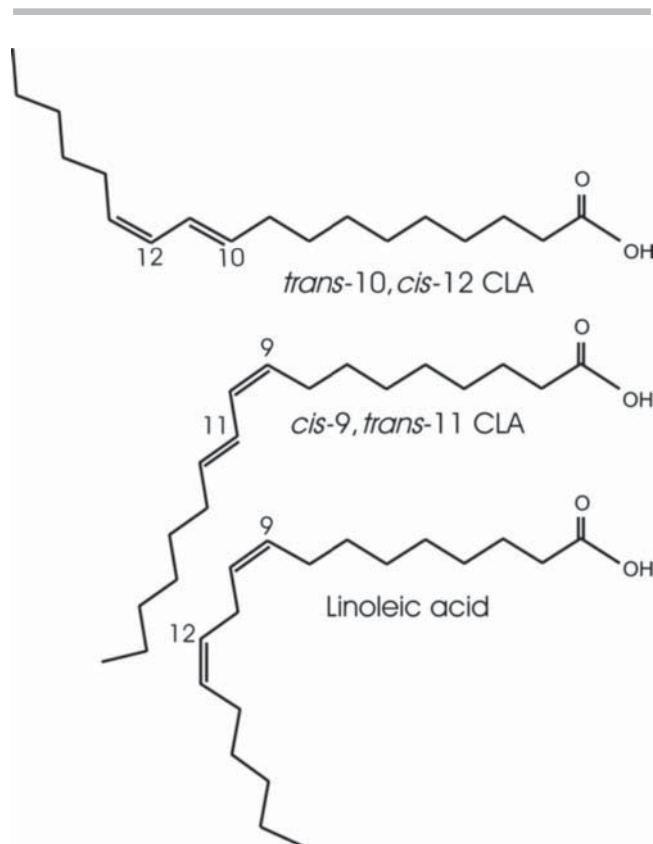


Figure 1 Structures of linoleic acid, *cis*-9, *trans*-11 CLA, and *trans*-10, *cis*-12 CLA.

Chemical Synthesis of CLA Isomers

Because of the relatively low levels of CLA isomers in naturally occurring foods that are high in fat content, the chemical synthesis of CLA has been developed for making supplements and for fortifying foods. CLA can be synthesized from linoleic acid found in safflower or sunflower oils under alkaline conditions, yielding a CLA mixture containing approximately 40% of the 9,11 isomer and 44% of the 10,12 isomer (reviewed in Ref. 8). Commercial preparations also contain approximately 4% to 10% *trans*-9, *trans*-11 CLA and *trans*-10, *trans*-12 CLA, as well as trace amounts of other isomers.

Table 1 CLA Content of Various Foods

Food	mg/g fat	Food	mg/g fat
Meats/fish		Dairy	
Corned beef	6.6	Condensed milk	7.0
Lamb	5.8	Colby cheese	6.1
Fresh ground beef	4.3	Butter fat	6.1
Salami	4.2	Ricotta	5.6
Beef smoked sausage	3.8	Homogenized milk	5.5
Knackwurst	3.7	Cultured buttermilk	5.4
Smoked ham	2.9	American processed cheese	5.0
Veal	2.7	Mozzarella	4.9
Smoked turkey	2.4	Plain yogurt	4.8
Fresh ground turkey	2.6	Butter	4.7
Chicken	0.9	Sour cream	4.6
Pork	0.6	Cottage cheese	4.5
Egg yolk	0.6	Low fat yogurt	4.4
Salmon	0.3	2% milk	4.1
Vegetable oils		Medium cheddar	4.1
Safflower oil	0.7	Ice cream	3.6
Sunflower oil	0.4	Parmesan	3.0
Peanut	0.2	Frozen yogurt	2.8

Sources: Based on values reported in Refs. 2–4; and the University of Wisconsin Food Research Institute (Dr. Pariza, Director).

PHARMACOKINETICS AND EFFICACY OF CLA

Human and Animal Studies

As with other long chain unsaturated fatty acids (FA)s, CLA is absorbed primarily in the small intestine, packaged into chylomicrons, and distributed to extrahepatic tissues having lipoprotein lipase (LPL) activity or returned to the liver via chylomicron remnants or other lipoproteins. The average daily intake of CLA is approximately 152 to 212 mg for nonvegetarian women and men, respectively (9), and human serum levels range from 10 to 70 $\mu\text{mol/L}$ after supplementation (10,11).

One major discrepancy between animal and human studies is the dose of CLA administered (i.e., equal levels of 9,11 and 10,12 isomers—referred to as a CLA mixture), when expressed per unit body weight. For example, most adult human studies provide 3 to 6 g/day of a CLA mixture, whereas rodent studies provide 0.5% to 1.5% of a CLA mixture (w/w) in the diet. When expressed per unit of body weight, humans receive approximately 0.05 g CLA/kg body weight, whereas mice received 1.07 g CLA/kg body weight, which is 20 times the human dose based on body weight. Thus, part of the discrepancy in results obtained from human and animal studies is likely due to this large difference in the dose of CLA administered. Supplementing humans with higher, or animals with lower, doses of CLA would address this issue. Other discrepancies in experimental designs include using CLA isomer mixtures versus single isomers, duration of CLA supplementation, and the age, weight, gender, and metabolic status of the subjects or animals.

Cell Studies

In vitro studies have been conducted in a variety of cells types, primarily using an equal mixture of 9,11 and 10,12 CLA, or each isomer individually. Doses used in cell studies generally range between 1 to 100 μM , reflecting the concentration found in human participants follow-

ing supplementation. Results from these studies suggest that these isomers are readily taken up by cells. For example, we found that 10,12 CLA is readily incorporated into neutral and phospholipid fractions of the primary human adipocyte cultures and reduced lipid and glucose metabolism (12). Similar to in vivo studies, 9,11 CLA acted more like the linoleic acid controls.

ANTICANCER PROPERTIES OF CLA

CLA Reduces Tumor Growth

Pariza's group initially discovered that CLA isomers in fried ground beef acted as anticarcinogens (13). Subsequently, numerous investigators have shown that CLA mixtures or individual isomers decrease tumor cell growth or increase cancer cell death in in vitro and in vivo models of mammary, gastric, or skin cancer (reviewed in Ref. 14). For example, feeding 0.8% to 1.0% individual CLA isomers or mixtures block the initiation or progression of chemically induced carcinogenesis in several rodent models (15–17). A 5 μM CLA mixture prevented cell growth and cytokine production in transformed human keratinocyte-like cells (18). Proposed anticarcinogenic mechanisms for CLA include decreasing nuclear factor (NF) κB and cyclooxygenase (COX) activity, thereby suppressing the levels of prostaglandin (PG)E₂, an inflammatory PG that promotes the progression of certain forms of cancer and induces human epidermal growth factor receptor 2 (HER2) oncogene expression (19).

CLA Induces Apoptosis of Cancer Cells

Several groups have reported that CLA isomers cause apoptosis or programmed cell death in cancer cells (reviewed in Ref. 11). For example, 32 to 128 μM CLA mixture prevented rat mammary cancer cell growth through apoptosis and decreased DNA synthesis in rat mammary cancer cells (20). Moreover, 40 to 80 μM 10,12 CLA induces apoptosis in breast cancer cells (19,21,22). Proposed proapoptotic mechanisms of CLA include inducing atypical endoplasmic reticulum (ER) stress, leading to caspase-12 activation (22).

In contrast to the cell and animal studies cited in the preceding text, a recent prospective cohort study conducted in Sweden found no evidence to support a protective effect of CLA consumption on the development of breast cancer in women (23). Furthermore, some studies show that 10,12 CLA enhances the risk of developing certain types of cancer (24). Thus, clinical studies examining the effects of purified CLA isomers on preventing or treating cancer, and safety issues, are needed.

ANTIOBESITY ACTIONS OF CLA

Due to the substantial rise in obesity over the past 30 years, there is a great deal of interest in CLA as a weight loss treatment, as it has been shown to decrease body weight and body fat mass (BFM). For example, supplementation with a CLA mixture (i.e., 10,12 + 9,11 isomers in equal concentrations) or the 10,12 isomer alone decreases BFM in many animal and some human studies (reviewed in Refs. 25 and 26). Of the two major isomers of CLA, the

10,12 isomer is responsible for the antiobesity properties (27–31).

CLA Decreases Body Weight and Body Fat Mass

Park et al. (32) were one of the first groups to demonstrate that CLA modulated body composition. Compared with controls, male and female mice supplemented with a 0.5% (w/w) CLA mixture had 57% and 60% less BFM, respectively. Since these findings, researchers have demonstrated that CLA supplementation consistently reduces BFM in mice, rats, and pigs (33–36). For example, dietary supplementation with 1% (w/w) CLA mixture for 28 days decreased body weight and periuterine white adipose tissue (WAT) mass in C57BL/6J mice (36).

In humans, some studies show that CLA decreases BFM and increases lean body mass (LBM), whereas others show no such effects. For example, supplementation of 3 to 4 g/day of a CLA mixture for 24 weeks decreased BFM and increased LBM in overweight and obese people (37). On the other hand, supplementation of 3.76 g/day of a CLA mixture in yogurt for 14 weeks in healthy adults had no effect on body composition (38). Supplementation with 3.2 g/day of a CLA mixture decreased total BFM and trunk fat compared with placebo in overweight participants, but not obese participants (39). These contradictory findings among human studies may be due to the following differences in experimental design: (i) mixed versus individual CLA isomers, (ii) CLA dose and duration of treatment, and (iii) gender, weight, age and metabolic status of the participants.

These antiobesity effects of CLA do not appear to be solely due to reductions in food intake in animals (40–42) or humans (43,44). Several mechanisms by which CLA decreases BFM will now be examined.

CLA Increases LBM

A recent meta-analysis of 18 human, placebo-controlled CLA studies found that consuming a CLA mixture increased fat-free mass (FFM) by 0.3 kg, regardless of the duration or dose (45). When these same 18 studies were examined for reductions in BFM, it was shown that CLA supplementation decreased BFM by 0.05 kg/week for up to one year (25). The average CLA mixture dose for these studies was 3.2 g/day. Collectively, these meta-analyses studies suggest that CLA supplementation of humans results in a rather small but rapid increase in FFM or LBM, and a much larger decrease in BFM over an extended period of time. The effects of CLA on FFM or LBM in humans may vary depending on baseline body mass index, gender, age, and exercise status of the participants.

Two proposed mechanisms by which CLA increases LBM are via increasing bone or muscle mass. 10,12 CLA supplementation for 10 weeks with a 0.5% (w/w) CLA mixture increased bone mineral density (BMD) and muscle mass in C57BL/6 female mice (46). CLA supplementation has been proposed to increase BMD via increasing osteogenic gene expression and decreasing osteoclast activity (46,47). Furthermore, CLA supplementation alone or with exercise increased BMD compared with control mice (48). An alternative mechanism could be that CLA decreases adipogenesis of pluripotent mesenchymal stem cells (MSC) in bone marrow, and instead promotes their

commitment to become bone cells. Indeed, 10,12 CLA has been shown to decrease the differentiation of MSC into adipocytes and increase calcium deposition and markers of osteoblasts (49). In contrast, 9,11 CLA increased adipocyte differentiation and decreased osteoblast differentiation. Consistent with these *in vitro* data, CLA mixture supplementation of rats treated with corticosteroids prevented reductions in LBM, BMD, and bone mineral content (50). Increasing LBM is directly linked to an increase in basal metabolic rate (BMR).

In addition to its effects on BMD, recent evidence supports a role of CLA in increasing endurance and muscle strength. For example, maximum swimming time until fatigue was higher in CLA fed versus control mice (51). Aging mice supplemented with a CLA mixture and 10,12 CLA had higher muscle weight compared with 9,11 CLA and corn oil controls (52). In addition, CLA isomers increased levels of antioxidant enzyme activity, ATP, and enhanced mitochondrial potential, indicating a protective effect against age-associated muscle loss (52). In humans, CLA increased bench-press strength in men supplemented with 5 g/day for seven weeks who underwent resistance training three days per week (53). Furthermore, supplementation with CLA combined with creatine monohydrate (C) and whey protein (P) led to greater increases in bench-press and leg-press strength than supplementation with C+P or P alone (54). Although preliminary, these data suggest that CLA may enhance exercise-induced muscle strength or prevent sarcopenia or age-related muscle loss.

CLA Increases Energy Expenditure

CLA has been proposed to reduce adiposity by elevating energy expenditure via increasing BMR, thermogenesis, or lipid oxidation in animals (27,42,55). In BALB/c male mice fed mixed isomers of CLA for six weeks, body fat was decreased by 50% and was accompanied by increased BMR compared with controls (42). Enhanced thermogenesis may be associated with increased uncoupling of mitochondria via uncoupling protein (UCP)s, which facilitate proton transport over the inner mitochondrial membrane thereby leading to dissipation of energy as heat instead of ATP synthesis. UCP1 is highly expressed in brown adipose tissue (BAT), and in WAT at lower levels. UCP3 is expressed in muscle and in a number of other tissues, whereas UCP2 is the form expressed at the highest level across most tissues. Supplementation with a CLA mixture or 10,12 CLA in rodents induced UCP2 mRNA expression in WAT (29,56). Recently, it was demonstrated that CLA increased mRNA and protein expression of UCP1 in WAT (57). Similarly, CLA supplementation induced UCP gene expression and elevated β -oxidation in muscle and liver (58–62).

CLA Increases Fat Oxidation

CLA has been shown to regulate the gene expression or activity of proteins associated with FA oxidation in adipose tissue, muscle, and liver. For example, CLA induced the expression of carnitine palmitoyl transferase 1 (CPT1) in WAT of obese Zucker *fa/fa* rats (63). Additionally, 10,12 CLA increased the expression of peroxisome proliferator-activated receptor (PPAR) γ coactivator-1 α

(PGC_{1α}) in WAT of mice (57). Consistent with these *in vivo* findings, 10,12 CLA increased β -oxidation in differentiating 3T3-L1 preadipocytes (64). Furthermore, 10,12 CLA treatment increased AMP kinase (AMPK) activity and increased phospho-acetyl-CoA carboxylase (ACC) levels in 3T3-L1 adipocytes, suggesting an increase in FA oxidation and a decrease in FA esterification to triglyceride (TG) (65).

In muscle, 10,12 CLA increased CPT1 expression in hamsters fed an atherogenic diet (60). Supplementation of a CLA mixture in high fat fed hamsters led to increased CPT1 activity in muscle (66). A CLA mixture increased CPT1b, UCP3, acetyl-CoA oxidase (ACO) 2, and PPAR α mRNA levels in skeletal muscle of Zucker rats (67). Consistent with these data, 10,12 CLA increased mRNA levels (63) and activity (68) of CPT1 in the liver. Additionally, 10,12 CLA increased hepatic peroxisomal fatty ACO activity (68), suggesting increased peroxisomal β -oxidation in addition to mitochondrial oxidation. These findings suggest CLA may reduce adiposity through increased energy expenditure via increased mitochondrial uncoupling and FA oxidation in WAT, muscle, and liver.

At least one report demonstrates that CLA increases FA oxidation in human participants (69). In this study, overweight adults supplemented with 4 g/day of a CLA mixture for six months had a lower respiratory quotient (RQ), indicating an increase in FA oxidation compared with placebo controls. However, others have shown no effect of CLA on energy expenditure or fat oxidation in humans (70,71). These discrepancies may be due to the length of treatment, time period of measurement, and time at which measurements are taken. For instance, CLA treatment for four to eight weeks had no effect on energy expenditure or FA oxidation, based on a 20-minute measurement during resting and walking (70). In contrast, the study by Close et al. (69) administered CLA for six months and measured FA oxidation over a 24-hour period and found that CLA increased FA oxidation and energy expenditure. Thus, discrepancies in this area may be due to insufficient duration of CLA treatment or measurements of energy expenditure or FA oxidation.

CLA Decreases Adipocyte Size

Lipolysis is the process by which stored TG is mobilized, releasing free fatty acids (FFAs) and glycerol for use by metabolically active tissues. C57BL/6J mice fed 10,12 CLA for three days had increased mRNA levels of hormone-sensitive lipase (HSL), a key enzyme for TG hydrolysis (56). Consistent with these data, acute treatment with CLA mixture or 10,12 CLA alone increased lipolysis in 3T3-L1 (32,72) and newly differentiated human adipocytes (73). *In vitro*, a CLA mixture and to a greater extent 10,12 CLA decreased TG content, adipocyte size, and lipid locule size in adipocytes (74). Similarly, mice fed 1% CLA displayed increased numbers of small adipocytes with a reduction in the number of large adipocytes (75). Furthermore, a CLA mixture reduced adipocyte size rather than cell number in Sprague Dawley (40) and fa/fa Zucker rats (76). Thus, CLA may reduce adipocyte size by increasing lipolysis.

CLA Decreases Adipocyte Differentiation

The conversion of preadipocytes to adipocytes involves the activation of key transcription factors such as

PPAR γ and CAAT/enhancer-binding proteins (C/EBPs). There is much evidence showing that CLA suppresses preadipocyte differentiation in animal (77–79) and human (12,80) preadipocytes treated with a CLA mixture or 10,12 CLA alone. 10,12 CLA treatment has been reported to decrease the expression of PPAR γ , C/EBP β , sterol regulatory element-binding protein-1c (SREBP-1c), liver X receptor (LXR α), and adipocyte FA-binding protein (aP2), thereby reducing adipogenesis and lipogenesis (12,29,79).

In rodents, supplementation of 10,12 CLA decreased the expression of PPAR γ and its target genes (79,81–83). In contrast, humans supplemented with a CLA mixture had higher mRNA levels of PPAR γ in WAT, but no difference in body weight or BFM (38). In mature, *in vitro*-differentiated primary human adipocytes or in mature 3T3-L1 adipocytes, 10,12 CLA treatment leads to a substantial decrease in the expression and activity of PPAR γ (82,83), and a decrease in PPAR γ target genes and lipid content (80). This shows that 10,12 is not only able to inhibit, but also to reverse the adipogenic process and indicates that this may be mediated by suppression of PPAR γ activity. In addition to its effect on PPAR γ , 10,12 CLA may also directly impact the activity of other transcription factors involved in adipogenesis and lipogenesis (i.e., LXR α , C/EBPs, SREBP-1c), which could contribute to CLA's anti-obesity actions.

CLA Decreases Glucose and FA Uptake and TG Synthesis

Conversion of glucose and FAs to TG is a major function of adipocytes. Genes involved in lipogenesis, such as a LPL, ACC, fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD), were decreased following supplementation with mixed isomers of CLA or 10,12 CLA alone (12,56,72,80). PPAR γ is a major activator of many lipogenic genes including glycerol-3-phosphate dehydrogenase (GPDH), LPL, and lipin as well as many genes encoding lipid droplet-associated proteins, such as perilipin, adipocyte differentiation-related protein (ADRP), and cell death-inducing DNA fragmentation factor of apoptosis-like effector c (CIDEC) (84). Thus, the antilipogenic action of 10,12 CLA may be explained by inhibition of PPAR γ activity. In addition, CLA repression of expression of SREBP-1 and its target genes may play an important role in delipidation. Finally, CLA suppression of insulin signaling may also impair insulin's ability to activate or increase the abundance of a number of lipogenic proteins including LPL, ACC, FAS, SCD-1, and the insulin-dependent glucose transporter GLUT4.

CLA Decreases Adipocyte Number

Apoptosis is another mechanism by which CLA may reduce BFM. Apoptosis can occur through activation of the death receptor pathway, ER stress, or the mitochondrial pathway. A number of *in vivo* and *in vitro* studies have reported apoptosis in adipocytes supplemented with a CLA mixture or 10,12 CLA alone (56,64,85,86). For example, supplementation of C57BL/6J mice with 1% (w/w) CLA mixture reduced BFM and increased apoptosis in WAT (75). Mice fed a high-fat diet containing 1.5% (w/w) CLA mixture had an increased ratio of BAX, an inducer of apoptosis relative to Bcl2, a suppressor of apoptosis (87).

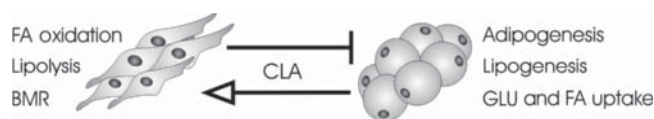


Figure 2 Reported mechanisms by which 10,12 CLA decreases adipose tissue mass and obesity.

Reported mechanisms by which CLA reduces adiposity are shown in Figure 2.

ANTI-DIABETIC PROPERTIES OF CLA

Feeding obese ob/ob C57BL/6 mice 0.6% 9,11 CLA for six weeks improved plasma levels of glucose, TG, and insulin and reduced the expression of markers of inflammation and insulin resistance in WAT (88). Furthermore, these authors demonstrated that 50 μ M 9,11 CLA prevented tumor necrosis factor (TNF) α -mediated insulin resistance in 3T3-L1 murine adipocytes. Their data suggest that 9,11 CLA improves insulin sensitivity by elevating GLUT4 levels or translocation to the plasma membrane, which are adversely affected by inflammation, thereby facilitating glucose disposal. Similarly, Wistar rats fed a high-fat diet supplemented with a 0.75% to 3.0% CLA mixture for 12 weeks had lower plasma levels of glucose, TG, and insulin compared with high-fat fed control rats (89). The CLA mixture enhanced the expression of PPAR γ target genes in WAT, which was proposed to be responsible for the improvement in insulin sensitivity. Consistent with these data, adiponectin, a WAT-specific, PPAR γ target gene that reduces blood glucose by enhancing its oxidation in liver and muscle, was increased in the plasma of Zucker diabetic fatty (ZDF) rats fed a 1% CLA mixture for eight weeks (55). Similarly, feeding 0.5% 9,11 CLA to insulin resistant C57BL/6J mice improved insulin sensitivity without affecting BFM (90). Conversely, these authors found that feeding 0.5% 10,12 CLA lowered BFM and increased LBM in these mice, but caused insulin resistance. Other studies have also reported that 10,12 CLA causes insulin resistance, especially in mice (81,99). Taken together, these data suggest that 9,11 and 10,12 CLA have opposite effects on insulin sensitivity, most likely due to their opposing effects on the activity of PPAR γ , vis-a-vis 9,11 CLA activates PPAR γ and 10,12 CLA inhibits PPAR γ .

ANTIATHEROSCLEROTIC ACTIONS OF CLA

CLA has been reported to decrease risk factors of atherosclerosis in several important animal models (reviewed in Ref. 91). For example, feeding 0.5% mixed or individual isomers of CLA to New Zealand White rabbits fed a high saturated fat and cholesterol-rich diet reduced blood lipids and atherosclerotic lesion area (92). Syrian Golden hamsters fed a high saturated fat and cholesterol-rich diet containing 1.0% mixed CLA isomers (93), 0.9% 9,11 CLA (94) or 1.0% 10,12 CLA (95), had decreased aortic

lipid accumulation or fewer fatty aortic streaks compared with controls. In apoE $^{-/-}$ deficient mice, feeding a 1.0% CLA mixture decreased aortic lesion area, and reduced macrophage infiltration and inflammatory gene expression in the lesions (96). In contrast to these animal studies, other animal and clinical trials with CLA mixtures have yet to show beneficial effects on reducing risk factors for atherosclerosis (reviewed in Ref. 97).

SAFETY

Adverse side effects have been reported for CLA supplementation such as elevated levels of inflammatory markers, lipodystrophy, steatosis, and insulin resistance. Most adverse side effects are due to the 10,12 CLA isomer.

CLA Increases Markers of Inflammation

Treatment with 10,12 CLA increases the expression or secretion of inflammatory makers such as TNF α , interleukin (IL)-1 β , IL-6, and IL-8 from adipocyte cultures (56,73,80,81,83). Moreover, CLA increases the expression of COX-2, an enzyme involved in the synthesis of PGs, and the secretion of PGF $_{2\alpha}$ (79,98). These inflammatory proteins are known to antagonize PPAR γ activity and insulin sensitivity (87,98–100).

Consistent with these in vitro data, 10,12 CLA supplementation increases the levels of inflammatory cytokines and PGs in humans (101,102). For example, women supplemented with 5.5 g/day of a CLA mixture for 16 weeks had higher levels of C-reactive protein in serum and 8-iso-PGF $_{2\alpha}$ in urine (44). 10,12 CLA supplementation in mice resulted in macrophage recruitment in WAT (81). In contrast, 9,11 CLA exhibits anti-inflammatory actions (6).

CLA Causes Insulin Resistance

Insulin resistance has been reported in vivo (56,102–104) and in vitro (12,73,79,98) following supplementation with a CLA mixture or 10,12 CLA alone. For example, 10,12 CLA supplementation of 3.4 g/day for 12 weeks in obese men with metabolic syndrome increased serum glucose and insulin levels and decreased insulin sensitivity (103). Supplementation with a CLA mixture in type-2 diabetics increased fasting plasma glucose levels and reduced insulin sensitivity (102). Mice fed 1% (w/w) 10,12 CLA displayed elevated fasted and feeding plasma insulin levels and had reduced insulin sensitivity (75). Consistent with these data, the mRNA levels of adiponectin, a key adipokine associated with insulin sensitivity, decrease following supplementation with 10,12 CLA in vivo (36,81,100) and in vitro (79,82,105,106).

CLA Causes Lipodystrophy

The combination of inflammation and insulin resistance results in reduced FA and glucose uptake in WAT, leading to ectopic lipid accumulation in the blood (hyperlipidemia), liver (steatosis), or muscle. CLA-mediated hyperlipidemia and steatosis has been reported in several animal studies (36,76,107). For example, 1% (w/w) CLA time-dependently increased insulin levels and led

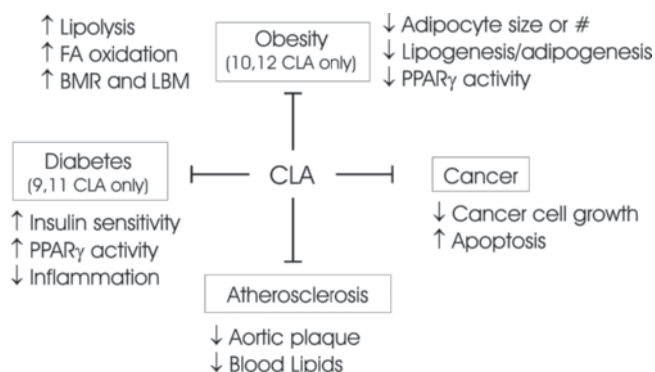


Figure 3 Reported mechanisms by which CLA reduces the risk of cancer, obesity, diabetes, and atherosclerosis.

to increased liver weight and liver lipid accumulation in C57BL/6J mice (36). Aging C57BL/6J mice fed 0.5% 10,12 CLA displayed increased insulin resistance and liver hypertrophy (107).

US Regulatory Status

Recently, the FDA approved CLA as GRAS (generally recognized as safe) for use in foods and beverages (not to exceed 1.5 g/serving) due its potential favorable effects. However, the use of CLA as a dietary supplement or ingredient should be cautioned based on the aforementioned safety issues.

CONCLUSIONS

There is an abundance of evidence in animals suggesting that CLA consumption may reduce the incidence or risk of developing cancer, obesity, diabetes, or atherosclerosis, depending on the type and abundance of CLA isomer consumed and the physiological status of the animal model (Fig. 3). Data on the antiobesity properties of 10,12 CLA in animals, especially mice, are the most reproducible. However, these potential benefits are not without risks, as the 10,12 isomer is associated with increased levels of inflammatory markers, lipodystrophy, and insulin resistance. More clinical studies are needed to determine the efficacy of CLA isomers in humans, and more mechanistic animal and cell studies are needed to determine the precise, isomer-specific mechanisms of action of CLA, and potential side effects.

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Copper

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INTRODUCTION

Since the discovery in 1928 that copper is an essential nutrient, hundreds of experiments to clarify its function have been performed with several species of animals and, under very controlled conditions, with adult human volunteers. People respond to copper depletion similar to animals (1).

The earliest experiments involved hematology, which preoccupied nutritional scientists for decades. Gradually, evidence for the adverse effects of copper deficiency on the cardiovascular and skeletal systems accumulated. Cardiovascular research related to copper deficiency, including associated lipid metabolism and cardiovascular physiology, now exceeds that on hematology. Early work on bone structure and function is being collected and extended.

Methods for assessing nutritional status for copper are poorly developed. However, there are a sufficient number of reports of low activities of enzymes dependent on copper and low copper values in important organs to suggest that a considerable number of people may be too low in this element. These data complement measurements of dietary copper suggesting that the Western diet, which is frequently low in copper, may be the source of this abnormal biochemistry. Some people with abnormal gastrointestinal physiology may absorb too little copper as well.

GENERAL DESCRIPTION

Copper is an essential and versatile nutrient that operates as the active site in 10 to 15 enzymes (1–3). These proteins moderate the chemistry of this metallic element to enhance various metabolic processes related to oxidation. There also are several other copper-binding proteins of physiological importance (3) in addition to some newly discovered proteins called metallochaperones (4). The latter proteins act in the intracellular transport of metallic elements and help to ensure that free copper ion is nonexistent in the body (5,6).

ACTIONS, BIOCHEMISTRY, AND PHYSIOLOGY

The essentiality of copper for mammals, including people, was discovered (7) when rats fed a milk diet with adequate iron became anemic and grew poorly. Copper

proved to be the active material in several foods that were curative and could prevent the condition. All the classic deficiency experiments with animals were performed with milk diets. Adequate copper permits normal utilization of dietary iron. In addition to preventing anemia, it assists in blood coagulation (8,9) and blood pressure control (10,11), crosslinking (2,3,12) of connective tissues of arteries, bones, and heart, defense against oxidative damage (1), energy transformations, myelination of brain and spinal cord, reproduction, and synthesis of hormones (13). Inadequate copper produces adverse effects (14–16) on the metabolism of cholesterol and glucose, on blood pressure control and heart function, on mineralization of bones, and on immunity. Isoprostanes are increased in deficiency (10).

Hypercholesterolemia in copper deficiency has been found in at least 30 independent laboratories (11,17), most recently in studies by Galhardi et al., Kaya et al., and Rosario et al. (18–20) since the original observation (21).

Glutathione is an effective regulator of 3-hydroxy-3-methylglutaryl coenzyme A activity (22,23). Copper deficiency disrupts glutathione metabolism (24), leading to increased activity of this enzyme (25–27) and contributing to the hypercholesterolemia that occurs. In contrast, decreased activities of lecithin: cholesterol acyltransferase (28) and lipoprotein lipase (29) also contribute to the hypercholesterolemia of deficiency.

Electrocardiograms of animals deficient in copper reveal human cardiovascular risk factors such as branch block and abnormalities of the ST segment (15); other heart blocks and wave pathologies are numerous (15). The heart blocks are probably caused by decreased activity of an ATPase isoform localized to the conduction system of the heart (30).

Copper deficiency depresses vasodilation via alterations in nitric oxide physiology (31,32). The mechanism has been reviewed (24,33) and may involve, *inter alia*, guanylate cyclase, which contains copper (34).

Paraoxonase, sometimes called PON1, is a homocysteine thiolactone hydrolase [activity (35) of which is decreased by copper deficiency] (36). The lactone accumulates when homocysteine is elevated and irreversibly inhibits lysyl oxidase (35), which depends on copper for crosslinking of connective tissue in arteries and bone.

There seems to be little doubt that copper deficiency can affect desaturase (and elongase) enzymes, but agreement is lacking on the details and directions of all the changes. Some of the data have been reviewed (14,24,37,38). These enzymes can alter the number of

double bonds in a fatty acid and can also increase its length. Prostaglandin metabolism is also affected (24).

Food Sources and Supplementation

As far as is known, food source does not affect copper absorption, in marked contrast to iron and zinc, which are more easily absorbed from animal, than from plant, products. Higher concentrations of copper in many plant foods can compensate if fractional absorption is slightly lower. Vegetarian diets are high in copper (39,40).

Phytates either have no inhibitory effect on copper, or have a markedly smaller effect than that on zinc (21,41). At intestinal pH, copper complexes with phytates are soluble whereas zinc complexes are not. Phytates can thus enhance the utilization of copper (42).

Copper absorption at 55% to 75% is considerably higher than that of other trace elements; absorption occurs mainly in the upper small intestine, but stomach and colon may absorb the element as well (1). Thus, the concentration of copper in foods is an important characteristic that determines nutritional usefulness. In order of increasing concentration on a weight basis, fats and oils, dairy products, sugar, tuna, and lettuce are low in copper; legumes, mushrooms, chocolate, nuts and seeds, and liver are high in copper (43,44). Bread, potatoes, and tomatoes are consumed in sufficiently large amounts by U.S. adults for these foods to contribute substantially to copper intake, although they are not considered to be high-copper foods (45). Copper and magnesium are highly correlated in U.S. diets (46). Food groups high in folate tend to be high in copper (35).

The Western diet typical of the United States, parts of Europe, and wealthy enclaves in the developing world is often low in copper. Approximately one-third of these diets are low in comparison with those used in successful depletion experiments of men and women (47–51) under controlled conditions and in comparison to the estimated average requirement (EAR) (52) and recommended dietary allowance (RDA) of the National Academy of Sciences (U.S.) (below).

Estimations of dietary copper intakes based on calculations, for example, about Canadian octogenarians (53), from the amount of copper in individual foods are too high according to eight published comparisons (54) to chemical analysis of composite diets; the mean error from calculation is an excess of 77% (54).

The calculated 25th, median, and 75th percentiles for intakes of 51- to 70-year-old men in a statistical sample of the U.S. population (Table C-15 in Ref. 55) are 1.19, 1.47, and 1.81 mg copper daily. Corrections based on the mean excess in copper found by calculation (54) decrease these estimates to 0.67, 0.83, and 1.02 mg daily. Although younger men seem to eat more copper, women eat less!

Data from several publications on dietary intakes of copper based on chemical analyses were pooled (40,44) and a frequency distribution curve was derived for 849 analyzed diets; approximately one-third of the diets contained less than 1 mg of copper daily. Further analytical confirmation of diets low in copper is available from men and women randomly selected in Baltimore. Thirty-six percent and 62% of the diets were below the respective dietary reference intakes for copper (56).

Three approaches to supplementation are available. Diets below the EAR and the RDA can be improved by avoiding foods low in copper and by selecting foods high in copper (43). A copper-deficient salad (lettuce, mayonnaise, oil, tuna, etc.) can be improved by adding sunflower seeds, mushrooms, legumes, etc (44). Soy products are increasingly popular and are high in copper (57), as are nuts (58) and chocolate (59). Beer enhances the utilization of copper in rats fed a deficient diet, resulting in a sixfold increase in longevity, with less cardiac damage and lower plasma cholesterol (60).

In contrast to iron, fortification of foods with copper is uncommon. Some new snacks and drinks promoted as products with exceptional nutritional properties are fortified with copper. A variety of tablets and capsules containing copper are available commercially.

Copper gluconate is the only copper supplement listed by the United States Pharmacopeial Convention and probably is the best supplement for oral use (61). We have used copper sulfate effectively in experiments with animals (21,62) and human volunteers (47,49,51). Others have used copper salts of amino acids (63). Other compounds containing copper, such as the orotate, for which there are no data on bioavailability should not be used. It is not easy to identify the chemical form of copper in some of the available supplements.

Cupric oxide is contained in some vitamin–mineral supplements; this form is no longer used in animal nutrition because the copper is utilized poorly (64). Cupric oxide is used in the preparations with many ingredients because of its high concentration of copper, not because of demonstrated efficacy.

Deficient people should be supplemented with several times the EAR or RDA. Daily supplements of 3 to 7 mg of copper have been tolerated for long periods (65,66).

INDICATIONS AND USAGE

The Western diet is associated with rapid growth in infancy, increasingly early sexual maturation, tall adults, and low rates of infection. This diet is also associated with common diseases of affluence such as cancer, heart disease, obesity, and osteoporosis etc. (67). Numerous anatomical, chemical, and physiological characteristics of people with some of these latter diseases have been found in several species of animals deficient in copper (15,16).

No single indicator provides an adequate assessment of copper nutriture (nutritional status) (52). Indices useful in experiments with animals have sometimes been helpful in depletion studies of people, but most do not seem to be altered by marginal deficiency. Circulating copper may not reflect the actions of enzymes inside cells in various organs where the metabolic processes affected by copper take place. Liver copper, generally impossible to assess in people, is the best indicator in animal experiments (62). Experiments with animals reveal that plasma copper can be normal or increased even though copper in liver or other organs may be low (68–76). Thus, normal or high plasma copper values in people may not be an accurate reflection of copper nutriture.

According to the Oxford Textbook of Medicine (77), low nutrient intakes can reduce nutrient concentrations in tissues and compromise metabolic pathways. Diagnosis then is relatively straightforward upon measurement of the nutrient in suitable tissues or testing of metabolic pathways. Numerous medical publications (some of which are summarized here) reveal low copper concentrations and impaired enzymatic pathways dependent on copper in people. As "nutritional state often alters the expression and course" (77) of illness, extra copper should be provided if low measurements related to copper values are found whether or not they are the cause or the result of the pathology under consideration.

Interpretation of copper or ceruloplasmin in serum or plasma in the assessment of nutriture may be difficult. Low values indicate impairment. Pepys (78) describes the acute phase response to acute and chronic inflammation: a number of plasma proteins, such as ceruloplasmin, are synthesized in liver under the influence of cytokines and are secreted into the circulation. Thus, any illness with a large inflammatory component may have falsely high values (78,79). Normal or high values cannot provide assurance that copper deficiency is not present. Clearly people with myelodysplasia and the new syndrome resembling the neurology of pernicious anemia (below) can be considered for supplementation.

Possibly deficient people should be evaluated with some of the newer, potentially more sensitive, indices of copper status such as erythrocyte and extracellular superoxide dismutases, leukocyte copper, platelet cytochrome c oxidase or serum lysyl oxidase (80–84).

Data on which to base dietary reference intakes for copper are elusive and, often, absent. Consequently, some of the values in Table 1 are rounded and values for males and females are combined. The adequate intake (AI) values are based on intakes of apparently healthy, full-term infants whose sole source of copper was human milk. Values for pregnancy are based on the amount of copper in the fetus and other products of conception. Those for lactation are the amounts needed to replace the average amount secreted in human milk. EARs are values estimated to meet the requirement of half of the healthy individuals of the group. Copper RDAs are based on the EAR plus an assumed coefficient of variation of 15%, which is larger than the 10% assumed for some other nutrients (57).

In the United States, dietary reference intakes are median values with an assumed symmetry of distribution (85). However, there is virtually no information about the

shape of the copper distribution; distributions for most nutrients are skewed to the high end (85). People who are deficient in copper without obvious cause (below) probably have a personal requirement for copper considerably higher than the median requirements reflected in the RDAs.

It seems clear that there is little or no copper deficiency in the industrialized world if one relies on traditional criteria of deficiency such as anemia with decreased plasma copper or ceruloplasmin. However, these markers are affected by the acute phase response and are easily increased by nondietary variables, such as inflammation, oral contraceptives, and pregnancy etc. Copper depletion experiments with men and women reveal unfavorable alterations in biochemistry and physiology with minimal or no changes in circulating copper and without anemia (above). Copper deficiency is the leading nutritional deficiency of agricultural animals worldwide; (86) can people be far behind?

A 2001 report on dietary reference intakes (55) and its predecessors, for example in Ref. (87), summarize the reasons why people may decide to take (or avoid) nutrient supplements. Growth and function are improved when nutrients are increased above levels just sufficient to prevent deficiency. There is little evidence that small surpluses of nutrients are detrimental, while small deficits will lead to deficiency over time. There is no evidence of unique health benefits from the consumption of a large excess of any one nutrient. Meeting recommended intakes for nutrients will not provide for malnourished individuals.

There seems to be little or no anemia responsive to copper in the United States, although this phenomenon does not seem to have been studied adequately in the last six decades. Copper deficiency can masquerade as the myelodysplastic syndrome, however (88–90). Supplementation of middle-aged Europeans with copper protected their red blood cells from oxidative hemolysis *in vitro* (63), indicating that extra copper improved the quality of the cells.

Alzheimer's disease is the leading cause of dementia in the elderly and is of unknown etiology. It is hypothesized that deficiency of dietary copper is the simplest and most general explanation for the etiology and pathophysiology of this disease because, *inter alia*, of numerous reports of low copper in the brain and low activity of enzymes dependent on copper in these patients (91). These findings are consonant with Golden's criteria for diagnosing deficiency (77).

Kumar (92) has reviewed and expanded upon a copper deficiency syndrome resembling the neuropathy of pernicious anemia (vitamin B₁₂ deficiency). Supplementation with cyanocobalamin is useless, but extra copper generally arrests the decline and sometimes reverses some of the signs.

Several of the classical risk factors for ischemic heart disease have been produced in animals deficient in copper. Similar changes have been found in more than 30 men and women in successful copper depletion experiments using conventional foods and have been reversed by copper supplementation (47–51). Copper intakes of 0.65 to 1.02 mg daily in these experiments were insufficient. Criteria of depletion included abnormal electrocardiograms

Table 1 Daily Adequate Intake (AI), Estimated Average Requirement (EAR), and Recommended Dietary Allowance (RDA) for Copper (mg)

Age	AI (mg)	EAR (mg)	RDA (mg)
0–6 mo	0.20 or 30 ($\mu\text{g}/\text{kg}$)		
7–12 mo	0.22 or 24 ($\mu\text{g}/\text{kg}$)		
1–3 yr		0.26	0.34
4–8 yr		0.34	0.44
9–13 yr		0.54	0.70
14–18 yr		0.685	0.89
19–70 yr		0.70	0.90
Pregnancy		0.80	1.00
Lactation		1.00	1.30

(47,48) and blood pressure regulation (51), dyslipidemia (50), glucose intolerance (49), and hypercholesterolemia (47). Two of these experiments were interrupted prematurely with early repletion with copper because of abnormal electrocardiography; all of the metabolic and physiological abnormalities disappeared with copper repletion. Low paraoxonase activity is found in conditions associated with increased risk of ischemic heart disease (36); isoprostanes are increased (93).

In contrast is a balance experiment using a formula diet that failed to confirm these results (94). Applesauce, cheese, chicken, cornflakes, crackers, lettuce, margarine, milk, orange juice, and rice provided less than 31% to 34% of dietary energy (calculated at 2400 kcal/day) (95). As actual energy intake ranged from 2415 to 3553 kcal (94), the food part of the formula was probably about 26%. Because formula diets are known to lower serum cholesterol (96), the potential increase in cholesterolemia from the low copper intake may have been obscured.

Activities of enzymes dependent on copper (97–103) and organ copper concentrations (104–113) have been found to be decreased in people with cardiovascular (mostly ischemic) diseases. There is a positive correlation between cardiac output and copper in heart tissue of patients with coronary heart disease (112). Decreased copper in organs and decreased enzyme activities are evidence of impaired copper nutriture (77,114,115).

No long-term copper supplementation has been performed in patients with cardiac arrhythmia, dyslipidemia, glucose intolerance, hypercholesterolemia, or hypertension. However, some dietary regimens found to alleviate some of these conditions may have included an increase in copper intake as a hidden variable: for example, the Lifestyle Trial (116), the protective effect of legumes on cholesterol, blood pressure, and diabetes (117) and the benefit of whole grain foods on coronary heart disease (118). Spencer (119) described two men and a woman whose premature ventricular beats, which had persisted for years, were thought to be due to coronary heart disease. These premature beats disappeared after they ingested 4 mg of copper (as copper gluconate) per day.

Witte et al. (120) explain how deficiencies of micronutrients, copper among them, can contribute to cardiovascular disease. Patients with heart failure in their supplementation trial had improved ventricular function and quality of life (121); copper in the supplement may have contributed (122). Supplementation trials with vitamins to lower homocysteine may show clinical benefit if extra copper is included (123); copper supplementation (with zinc) improved survival in a long-term, double-blind study of ocular disease (124).

Copper-deficient people have osteoporosis that can be cured with extra copper (reviewed in Ref. 16). This phenomenon has been found mainly in young children. Adults may have skeletal pathology from low copper status as well. Copper is decreased in bone in both osteoarthritis and ischemic necrosis of the femoral head (125). Low serum copper in patients with fractures of the femoral neck (126) or decreased lumbar bone density (14,127,128) may indicate covert copper deficiency. Plasma copper and bone mineral density are correlated (129). Healthy men fed a diet low in copper (0.7 mg/day)

experienced increased bone resorption that returned to normal when copper was replaced (130).

There can be no medical doubt that copper deficiency *can* cause osteoporosis in people. These references on osteoporosis from copper deficiency (131–141) have been found since the earlier review (16) of 17 articles. If copper deficiency turns out to be a major component of the osteoporosis of middle age, supplementation with copper alone is unlikely to be effective. If copper deficiency is corrected, another nutrient, particularly calcium and possibly zinc, may become limiting (77). Two double-blind, placebo-controlled trials have shown that trace element supplements including copper improved bone mineral density in postmenopausal women (65,142).

Premature infants and people with extensive burns may need extra copper. The former (143) are sometimes born before their mothers can load them with copper in the last trimester (12). Premature infants have lower superoxide dismutase activity in erythrocytes and plasma copper after 100 days of life than term infants (144); premature placentas are low in copper and copper-dependent enzymes (145).

In analogy to vitamin B₁₂ deficiency, any disruption of the gastrointestinal tract has the potential to impair copper nutriture. Copper deficiency is being reported with increasing frequency in patients who have had bariatric surgery (90,92,146–149). Some people with cystic fibrosis or pancreatic insufficiency may need extra copper (150–152). Copper-dependent enzyme activity and copper concentration have been found to be decreased in ulcerative colitis biopsies (153). Supplementation of people with these conditions should be performed under medical supervision.

If adults have unmet needs for copper to provide cardiovascular, hematopoietic, or skeletal benefit, neither the dose nor the duration of therapy is clear. A potential role for copper supplements in the treatment of rheumatoid arthritis and psoriasis has not been proved. There is probably no reason to exceed the tolerable upper intake level (UL) of 10 mg daily (Table 2).

Potential Toxicity and Precautions

All chemicals, including essential nutrients, are toxic if the dose is excessive. It seems that people have a 50- to 400-fold safety factor for copper considering usual dietary intakes and the tolerance level found with several species of experimental animals (154). The UL connotes an intake

Table 2 Daily Tolerable Upper Intake Level (UL) for Copper (mg)

Age group	UL (mg)
Children	
1–3 yr	1.00
4–8 yr	3.00
9–13 yr	5.00
Adolescents	
14–18 yr	8.00
Adults	
19–70+ yr	10
Pregnancy	8.00
Lactation	8.00–10.00

that can, with high probability, be tolerated biologically by almost all individuals.

Gastrointestinal signs and symptoms such as nausea are prominent in the setting of this limit. A small, double-blind study has revealed that adults are unaffected in 12 weeks by a daily supplement of 10 mg of copper (52). The UL values in Table 2 are based on this experiment; no value is available for infants less than 1-year-old. van Ravesteyn (155) administered 38 mg of copper daily to people for as long as 14 days; toxicity was not mentioned. Copper supplements should be taken with food (156,157) and should not be taken by people with biliary disease, liver disease, idiopathic copper toxicosis or Wilson's disease, or by people taking penicillamine or trientine.

Although copper can interfere with zinc utilization, this phenomenon does not seem to be of practical importance to people. In contrast, copper deficiency has been induced in people (and in numerous species of pets and animals in zoos) by the ingestion of recently minted pennies (United States), which are almost pure zinc (158). The dose of supplemental zinc that is excessive for adults is ill-defined, but the adult UL for zinc, 40 mg daily, is based on reduced copper nutrition from zinc in food, water, and supplements combined. A case of copper-responsive anemia has been reported in a patient with acrodermatitis enteropathica overtreated with zinc (159). This potential exists for patients with Wilson's disease treated with zinc, particularly children (160). Demyelination of the central nervous system has been reported from overzealous treatment of Wilson's disease with zinc (161). Denture creams high in zinc have led to copper deficiency (162,163).

Vitamin C is known to interfere with the utilization of copper (131,164–168), but its UL of 2 g daily is not based on copper effects. Adverse effects on blood pressure regulation and copper utilization were found in women fed 1.5 g vitamin C daily (51). Simple sugars such as fructose, glucose, and sucrose interfere with the utilization of copper (169,170): High-fructose corn syrup is found in many processed foods and beverages. Excessive ingestion of soft drinks (171,172) has contributed to copper deficiency. High iron intakes can disrupt copper utilization (85,131,173–175). People with iron overload (176,177) and lead poisoning (178–180) may benefit from copper supplementation. Copper supplements should not be used as emetics.

CONCLUSIONS

The Western diet often is low in copper. Statements to the contrary are based on dietary calculations, which are falsely high. The best way to ensure an AI of copper is to minimize the intake of foods low in copper and to increase that of foods high in it, such as cereals, grains, legumes, mushrooms, nuts, and seeds. Dietary copper can be increased by using the food pyramid as a guide. Only a few foods are fortified with copper. Copper gluconate is probably the best supplement.

There seems to be little copper deficiency in Western society if one considers anemia as its only sign. However,

adults with diseases of the cardiovascular, gastrointestinal, and skeletal systems have repeatedly been found to have low concentrations of copper in important organs and to have low activities of enzymes dependent on copper. These signs are consonant with deficiency. Premature infants may also be deficient in copper. Large intakes of vitamin C or zinc can impair the proper utilization of copper in people.

People deficient in copper are being reported with increasing frequency. Although many circumstances seem without explanation and because the clinical signs differ from those traditionally associated with copper deficiency, the reports are often scattered in medical journals that do not have the word "nutrition" in their titles.

Recognition of copper deficiency in the general population still seems rare enough to be published, but deficiency also is common enough that 10 cases are reported from one neurological clinic. The index of suspicion should be increased among those providing primary care. When obvious explanations such as bariatric surgery, dental adhesives high in zinc, hemochromatosis, lead poisoning, and soft drink excess are excluded one should consider the possibility that the patient has a dietary requirement higher than those mentioned among the dietary reference intakes. Some cases of myelodysplastic syndrome and heart failure respond to copper.

Evaluations should not rely only on plasma copper or ceruloplasmin. Supplementation should be done with substantial doses of easily absorbed, copper salts under medical supervision.

Some successful experiments in human copper depletion, particularly in relation to the cardiovascular system, are summarized here, as are some unsuccessful experiments. Others have been reviewed (181). Depletion experiments with positive results illustrate what is possible in the wider world, particularly when the potentially adverse effects were eliminated on repletion. Seemingly contrary or incongruous experiments should not promote denial or negativism; rather they should stimulate searches for explanations of differences.

Individual animals and individual people fed the same depleted diets do not respond uniformly. Signs of deficiency are variable in experimental pellagra and in animal experiments on biotin, thiamin, or copper deficiencies. Our success rate in inducing copper depletion in people resembles that of Goldberger in inducing pellagra. One should not expect all people to respond uniformly to a diet low in copper (15).

There is no information available on how copper requirements vary from person to person; dietary recommendations are based on assumptions of narrow variability. The amount of copper in the body and its variability are known, only inaccurately. Copper absorption is reasonably well defined, but data on copper losses are scant. Aside from some obvious causes of deficiency mentioned here, causes of human deficiency in some recent, medical reports are unknown. These reports have increased since the first edition and illustrate opportunities for research; it seems likely that people with unidentified high requirements for copper are eating too little. The field of copper nutrition is far from stagnant.

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Cordyceps

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ABBREVIATIONS

ATP, adenosine triphosphate; GM-CSF, granulocyte macrophage colony-stimulating factor; GRAS, generally recognized as safe; HDL, high-density lipoprotein; IFN, interferon; Ig, immunoglobulin; IL, interleukin; iNOS, calcium-insensitive nitric oxide synthases; LD₅₀, median lethal dose; LDL, low-density lipoprotein; NK cell, natural killer cell; PAH, polyaromatic hydrocarbons; PAMP, pathogen-associated molecular patterns; TCM, traditional Chinese medicine; TLR, Toll-like receptor; TNF, tumor necrosis factor.

INTRODUCTION

Species of the genus *Cordyceps* (Fr.) Link (also known as Chinese caterpillar fungi, or Tochucaso in Japanese; Clavicipitaceae, Ascomycetes) are the fungi found growing on insect larvae (Fig. 1), mature insects, or fruiting bodies of truffles of genus *Elaphomyces*. *Cordyceps* has a long history as a rare and exotic medicinal fungus. It has been a highly regarded cornerstone of Chinese medicine for centuries; one that reportedly has a number of far reaching medicinal effects (1). Most people in the West have only come to know of *Cordyceps* within the last twenty years, during which time, modern scientific methods have been increasingly applied to the investigation of its seemingly copious range of medicinal applications, in an attempt to validate what Chinese practitioners have noted for centuries (2).

BACKGROUND

Diversity and Artificial Cultivation

There are currently more than 680 documented species of *Cordyceps*, found on all six inhabited continents and in many climatic zones and habitats, and occurring parasitically or commensally with a range of hosts (2–3). Due to the rarity and high prices of the wild collected variety, attempts have long been made to cultivate *C. sinensis*. By the mid 1980s, the majority of *C. sinensis* available in the world's marketplace was artificially cultivated (4).

Many companies now produce artificially cultivated *C. sinensis* products, both from the mycelium as well as from the fruit bodies. The increase in supply has given rise to variations in purity and quality, creating a situation in which there are a large number of counterfeit and adulterated products being sold (3). Recently, there have been introduced, new methods for assaying the quality of *Cordyceps* spp. products (5). The large variations in quality

found in cultivated *C. sinensis* has lead many consumers to believe the wild collected variety is medicinally better than the cultivated type. But with new advances in biotechnology, this is rapidly changing (6).

Contamination and Adulteration of *Cordyceps*

As found in its natural state, *C. sinensis* is attached to the mummified body of the caterpillar, from which it arose. It is harvested whole in this way, dried, and supplied into the market. Because *C. sinensis* is sold by weight and intact fruiting bodies fetch higher prices in traditional markets, collectors have historically inserted a small bit of twig into many of the caterpillars, resulting in an increase in weight and the appearance of intact fruiting bodies (5). This is probably a harmless practice, as long as the object inserted is from a nontoxic source. However, modern collectors have inserted lead or other metal in order to boost the weight, so anyone who chooses to use the wild collected *C. sinensis*, rather than the cultivated variety, would be well advised to break each one of the caterpillars in half before use, so that any bits of foreign matter can be readily discerned and removed.

History and Traditional Uses

The first written record of the *Cordyceps* mushroom comes from China, in the year AD 620, at the time of the Tang Dynasty, bringing substance to the once intangible allegorical narrative, which spoke of a creature, whose annual existence alluded to a transformation from animal to plant, in summer, and then again from plant to animal, in winter (1). Tibetan scholars wrote of the healing animal/plant through the 15th to 18th centuries, and in 1757, the earliest objective and scientifically reliable depiction of the *Cordyceps* mushroom was written by the author Wu-Yiluo in the Ben Cao Congxin ("*New Compilation of Materia Medica*"), during the Qing Dynasty (2–3).

C. sinensis is found at high altitudes on the Himalayan Plateau, and thus, is difficult to harvest. Due to such difficulties, *Cordyceps* has always been one of the most expensive medicinal fungi known. Its high price had relegated it almost exclusively to members of the Emperor's court and others among the Chinese nobility, historically beyond the reach of the average Chinese subject. Despite its cost and rarity, the unprecedented litany of medicinal possibilities for *Cordyceps* spp. has made it a highly valued staple of the TCM.

The name *Cordyceps* comes from the Latin words, *cord* and *ceps*, respectively meaning, "club" and "head." The Latin word conjunction accurately describes the appearance of these club fungi, whose stroma and fruit body



Figure 1 *Cordyceps sinensis* in natural habitat (4550 m in Tibet, China).

extend from the mummified carcasses of insect larvae, usually that of the Himalayan ghost Moth, *Thitarodes armoricanus* (*Hepialis armoricanus*). In historical and general usage, the term "*Cordyceps*" normally refers specifically to the species *C. sinensis*. However, the name "*Cordyceps*" has come to be used for a number of closely related species over the last few years, which have been found to be much easier to cultivate. While *C. sinensis* may be the most well-known species, there are many other species in the genus *Cordyceps*, in which modern science may have uncovered potentially valuable medicinal properties.

The medicinal values of *Cordyceps* spp. have been recognized since ancient times in China and the surrounding Orient; but knowledge of this only reached Western scientific audiences in 1726, when it was introduced at a scientific meeting in Paris. The first specimens were carried back to France by a Jesuit priest, who chronicled his experiences with the *Cordyceps* mushroom during his stay at the Chinese Emperor's court (1,4).

The range of therapeutic uses claimed for *Cordyceps* spp. is far reaching; although most of them have yet to be sufficiently investigated. In TCM, *C. sinensis* has been used to treat conditions including respiration and pulmonary diseases, renal, liver, and cardiovascular diseases, hyposexuality, and hyperlipidemia. It is also used in the treatment of immune disorders and as an adjunct to modern cancer therapies (chemotherapy, radiation treatment, and surgery) (1). *C. sinensis* is believed by many, particularly in and around Tibet, its place of origin, to be a remedy for weakness and fatigue; and it is often used as an overall rejuvenator for increased energy while recov-

ering from a serious illness. Many also believe it to be a treatment for impotence, acting as an aphrodisiac in both men and women. *C. sinensis* is often prescribed for the elderly to ease general aches and pains. TCM practitioners also recommend the regular use of *C. sinensis* in order to strengthen the body's resistance to infections, such as colds and flu, and to generally improve the homeostasis of the patient.

CHEMISTRY AND PREPARATION OF PRODUCTS

Nutritional Components

Cordyceps spp. contains a broad range of compounds, which are considered nutritional. *C. sinensis* contains all of the essential 18 amino acids. The content of amino acids after hydrolysis is mostly reported in the range of 20% to 25%. The highest contents are glutamate, arginine, and aspartic acid, and the major pharmacological components are arginine, glutamate, tryptophan, and tyrosine (7). Also found are vitamins E, K, and the water-soluble vitamins B1, B2, and B12. In addition, *Cordyceps* spp. contain many sugars, including mono-, di-, and oligosaccharides, and many complex polysaccharides, proteins, sterols, nucleosides, macro- and microelements (K, Na, Ca, Mg, Fe, Cu, Mn, Zn, Pi, Se, Al, Si, Ni, Sr, Ti, Cr, Ga, V, and Zr) (2,5).

Polysaccharides

C. sinensis contains a large amount of polysaccharides, which can be in the range of 3% to 8% of the total weight, and usually comes from the fruiting bodies, the mycelium of solid fermentation submerged cultures and the broth (7). Four β -D-glucan exopolysaccharides from *C. militaris* with different molecular masses ranging from 50 to 2260 kDa were reported by Kim et al. (8). In the case of *C. sinensis*, most of the heteropolysaccharides contained mannose, galactose, glucose, and mannose in higher levels with smaller amounts of arabinose, rhamnose, fructose, and xylose, respectively. The average molecular mass varies between 7 and 200 kDa. *C. militaris* polysaccharides consisted mostly of glucose, galactose, and mannose with traces of rhamnose and xylose and average molecular weight approximately 60 kDa (9).

Proteins and Nitrogenous Compounds

Cordyceps spp. contain proteins, peptides, polyamines, all essential amino acids, some uncommon cyclic dipeptides, including cyclo-[Gly-Pro], cyclo-[Leu-Pro], cyclo-[Val-Pro], cyclo-[Ala-Leu], cyclo-[Ala-Val], and cyclo-[Thr-Leu]. Small amounts of polyamines, such as 1,3-diamino propane, cadaverine, spermidine, spermine, and putrescine, have also been identified (4). Many nucleosides have been found in *Cordyceps* spp., including uridine, several unique deoxyuridines, adenosine, dideoxyadenosine, hydroxyethyladenosine, cordycepin [3'-deoxyadenosine], cordycepin triphosphate, guanidine, deoxyguanidine, and other altered and deoxygenated nucleosides, many of which are found nowhere else in nature (Fig. 2). Chen and Chu (10) found cordycepin and 2'-deoxyadenosine in an extract of *C. sinensis*.

Sugar-binding proteins named lectins were isolated from *C. militaris*. N-terminal amino acid sequence differed

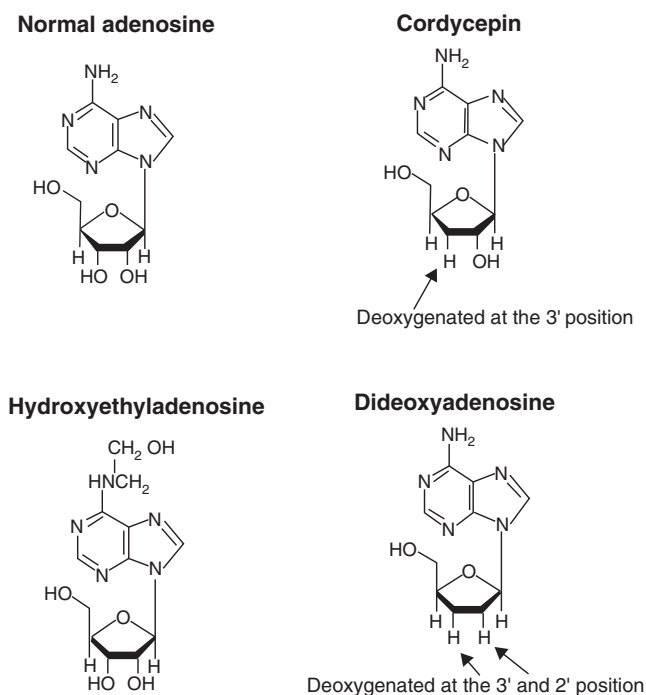


Figure 2 Some of the unique nucleosides found in *C. sinensis*.

greatly from other lectins (11). Production of the nonribosomal peptides cicapeptins I and II from *C. heteropoda* were reported by Krasnoff et al. (12).

Sterols

A number of sterol-type compounds have been found in *Cordyceps* spp.: ergosterol, δ -3 ergosterol, ergosterol peroxide, 3-sitosterol, daucosterol, and campesterol, to name a few (1). Another compound, sterol H1-A was found by Chen et al. (13) and it was claimed to be effective in the treatment of autoimmune disorders.

Other Constituents

Twenty-eight saturated and unsaturated fatty acids with the function of decreasing blood lipids and protecting against cardiovascular disease, and their derivatives, have been isolated from *C. sinensis*. The unsaturated fatty acid content includes C16:1, C17:1, C18:1, and C18:2 (7).

Polar compounds of *C. sinensis* extracts include many alcohols and aldehydes (1). Particularly interesting is the range of polycyclic aromatic hydrocarbons produced by many *C. sinensis* strains, named PAH compounds, for which it was proposed to react with the polypropylene used in common mushroom culture bags, resulting in the production of byproducts toxic to *C. sinensis* and stunting growth as time progresses (5). Of particular note are various immunosuppressive compounds found in *Cordyceps* spp., including cyclosporin from *C. subsessilis* (anamorph: *Tolypocladium inflatum*) (14), and also compounds found in *Isaria sinclairii*, a species closely related to the genus of *Cordyceps* (3).

Preparation of Products

Various pure compounds, extracts, whole fungus, and other preparations have been used in preclinical and clinical studies, and several products are now available in the market, mostly used as food supplements. In TCM, hot-water extraction of whole fruiting bodies is traditionally used. Nowadays, extracts of polysaccharides are mainly obtained by hotwater extraction followed by ethanol precipitation (15). For pure compounds, different types of chromatography are used, mainly affinity, ion-exchange or size-exclusion chromatography. It should be noted that different types of extracts give different results in the studies mentioned, but all of them show positive medicinal value.

PRECLINICAL STUDIES

The widespread use of *Cordyceps* spp. in TCM has been discussed above in the section on History and Traditional Uses. One of the most significant proposed activities of medicinal mushrooms is their role as immunomodulators. Other activities ascribed to *Cordyceps* spp. are anti-tumor, antimetastatic, immunomodulatory, anti-oxidant, anti-inflammatory, insecticidal, antimicrobial, hypolipidaemic, hypoglycemic, anti-aging, neuroprotective and renoprotective activities (2).

Cancer

A possibly valuable therapeutic application of *Cordyceps* spp. is its potential as a treatment for cancer, and as an adjunct to chemotherapy, radiation, and other conventional and traditional cancer treatments (2,4). The mechanism by which *Cordyceps* inhibits the growth of various cancer cells might occur by one of several means: by enhancing immunological function and nonspecific immunity; by selectively inhibiting RNA synthesis, thereby affecting the protein synthesis; by restricting the sprouting of blood vessels (angiogenesis); by inducing tumor cell apoptosis; by regulation of signal pathways; anti-oxidation and anti-free radical activity; anti-mutation effect; interfering with the replication of tumor-inducing viruses; and by inducing nucleic methylation (7).

Growth inhibition of various cancer cells by enhancing immunological function and nonspecific immunity is usually linked to polysaccharides, especially β -D-glucans, which present major cell wall structural components in fungi and are also found in plants and some bacteria but not in animals. Consequently, they are considered to be classic pathogen-associated molecular patterns, called as PAMPs (16). PAMPs potently trigger inflammatory responses in a host, as if it was infected by a fungus. Studies have shown that β -D-glucans initiate biological response with binding to complement receptor 3 (CR3) located on the surface of the immune system effector cells, like macrophages, thereby setting up different intercellular activities of the immune system and leading to production of cytokines, such as TNF- α , interleukins, interferons, and finally apoptosis of tumor cells (17). Toll-like receptors (especially TLR-2) and dectin-1 receptor play an important role in internalization and signaling responses to fungal β -D-glucans (18).

The anti-tumor effect also has been related to the inhibition of DNA and RNA synthesis (19). Studies (20) have demonstrated that cordycepin can selectively inhibit mRNA synthesis, which affects protein synthesis by competing with adenosine nucleoside phosphatase. The inhibition may be blocked by adenosine. Cordycepin can also kill leukemia cells and extend the period of mitotic cells in the S and G phases. Nakamura et al. (21) found that, with respect to cancer cells and normal cells, cordycepin caused an inhibition rate of cell division on cancer cells of 55% while only 1.5% on normal cells. These results show that cordycepin may have a very slight effect on the human body while treating cancer.

Another mechanism is inducing tumor cell apoptosis. Extracts of *C. militaris* inhibited cell growth of human leukemia cells in a dose-dependent manner (22), which was associated with morphological change and apoptotic cell death, such as formation of apoptotic bodies and DNA fragmentation. Results indicated that the antiproliferative effects were associated with the induction of apoptotic cell death through regulation of several major growth regulatory gene products (23).

In cancer research, there have been many studies made with *Cordyceps* spp. extracts using animal models. *C. militaris* inhibited the growth and metastasis of Lewis lung cancer cells and the growth of sarcoma S180 cells implanted in mice. In addition, the survival period of the mice was increased (24). A study using murine models verified that oral administration of a hot water extract of *C. sinensis* consequently resulted in the activation of macrophages, thereby increasing the production of GM-CSF and IL-6, which act on the systemic immune system (25). In another study (26), mice treated with cyclophosphamide, which suppresses immune function, and with *C. sinensis* hot water extract saw their immune function return to normal, as measured by the IgM and IgG response and macrophage activity.

Fatigue

Trials in the mouse swim test, conducted using *C. sinensis* added to a standard diet compared with use of the untreated standard diet, have invariably shown the use of *C. sinensis* to significantly increase the time to exhaustion in laboratory animals over their control groups (4). The use of *C. sinensis* by athletes stems from publicity surrounding the performance exhibited by the Chinese Women's Track and Field team at the Chinese National games in 1993. In this competition, nine world records were broken by substantial margins. The team's coach attributed their success to *C. sinensis* (27). An increase in cellular ATP level results in an increase in useful energy, in contrast to the perceived increase in energy, which occurs from the use of other stimulants, such as caffeine, ephedrine, and amphetamines, ultimately resulting in an energy deficiency (28).

Hypoglycemic Effects

In animal studies, isolated polysaccharides, have been shown to improve blood glucose metabolism and increase insulin sensitivity in normal animals, to lower blood sugar levels in genetically diabetic animals, and to positively effect blood sugar metabolism in animals with chemically

induced diabetes (29–31). The common thread throughout all these trials is the increase in insulin sensitivity and hepatic glucose-regulating enzymes, glucokinase and hexokinase.

Lung Ailments

Mice treated with *C. sinensis* were able to survive up to three times longer than those left untreated, demonstrating a more efficient utilization of the available oxygen. Such efficacy alludes to the use of *C. sinensis* as an effective treatment for bronchitis, asthma, and chronic obstructive pulmonary disease (32). A study was conducted using in vivo mouse model-induced acute pulmonary edema, which causes systemic lack of oxygen, acidic body, and death. Research results show that animals taking *C. sinensis* had a significantly greater survival rate of 20% mortality in comparison with 80% mortality of the control group (33).

Male/Female Sexual Dysfunction

C. sinensis has been used for centuries in TCM to treat male and female sexual dysfunction, such as hypolibidism and impotence. Preclinical data on the effects of *C. sinensis* on mice showed sex steroid-like effects (4), and human clinical trials have demonstrated similarly the effectiveness of *C. sinensis* in combating decreased sex drive and virility (34). Treatment of rats on a diet supplemented with *C. militaris* mycelium resulted in an increase of serum cordycepin concentration, serum testosterone, and serum estradiol-17 concentrations. They proposed that supplementation with *C. militaris* improves sperm quality and quantity in rats (35).

Antiviral Activity

The recognition of bacteria, viruses, fungi, and other microbes is controlled by host immune cells with many innate immunity receptors, such as Toll-like receptors, C-type lectin receptors, and immunoglobulin-like receptors. Studies indicate that the immune modulating properties of *C. sinensis* could be attributed to their polysaccharide components. These polysaccharides specifically interact with and activate surface receptors involved in innate immunity (36). It was shown that intranasal administration of an acidic polysaccharide, isolated from the extract of *C. militaris* grown on germinated soybeans, decreased virus titers in the bronchoalveolar lavage fluid and the lungs of mice infected with influenza A virus. Furthermore, it increased TNF- α , IFN- γ , IL-1, IL-6 and IL-10 levels, enhanced nitric oxide production, and induced iNOS mRNA expressions in murine macrophage cells (37).

CLINICAL STUDIES

Due to the historically high cost of the fungus and the only recently developed methods for artificial cultivation, clinical trials of *C. sinensis* and its extracts are still relatively new endeavors. Earlier trials, although few in number, have set the precedent from which modern trials are building, expanding, and cementing our understanding of *Cordyceps* spp. The majority of clinical trials mentioned in this section used standard double-blind placebo-controlled protocols. Approval was granted in the

countries where the trials were performed, but in most cases the trials were conducted in China.

Cancer

The belief in the efficacy of *C. sinensis* against cancer is widespread in the Orient, and many cancer patients in Japan, Korea, and China are taking *C. sinensis*, or some other mushroom-derived immunomodulators [such as PSK™, PSP™, Lentinan™, AHCC™, Immune Assist™ (a heteropolysaccharide complex formula), and arabinoxylnes (MGN3™)], while undergoing conventional treatment (1). Clinical studies involving cancer patients have been conducted mostly in China and Japan (38,39). In one study of 50 patients with lung cancer, who were administered *C. sinensis* at 6 g/day, in conjunction with chemotherapy, tumors were reduced in size in 46% of the patients studied. A trial involving cancer patients with several different types of tumors found that *C. sinensis*, taken over a two-month period at 6 g/day, improved subjective symptoms in the majority of patients. White blood cell counts were kept at 3000/μL, or higher; and even with radiation or chemotherapy, other immunological parameters showed no significant change, while tumor size was significantly reduced, indicating an improved tolerance for radiation and/or chemotherapy (1). In addition, natural *C. sinensis* has been shown to enhance the NK cell activity of normal patients by 74% and increased the NK activity of leukemia patients by 400% (39).

Fatigue

In a placebo-controlled clinical study of elderly patients with chronic fatigue, results indicated that most of the participants treated with *C. sinensis* reported a significant clinical improvement in the areas of fatigue, cold intolerance, dizziness, frequent nocturia, tinnitus, hyposexuality, and amnesia, while no improvement was reported in the placebo group (4,40–42). Another study involving healthy elderly volunteers, with an average age of 65, tested the output performance and oxygen capacity of participants while exercising on stationary bicycles. A portion of the volunteers consumed *C. sinensis* for six weeks, while others consumed a placebo. The results demonstrated that the *C. sinensis* group had a significant increase in energy output and oxygen capacity over the placebo group after six weeks of the study (43). The presence of adenosine, cordycepin, D-mannitol, polysaccharides, vitamins, and trace elements may be, at least partially, the cause for such effects.

Kidney Ailments

Traditional views of the *Cordyceps* spp. held that its consumption strengthened the kidneys. In a study of 51 patients suffering from chronic renal failure, it was found that *C. sinensis* significantly improved both the kidney function and overall immune function of treated patients, compared with the untreated control group (44). Patients with chronic renal failure or reduced kidney function often suffer from hypertension, proteinuria, and anemia. After a one-month treatment with *C. sinensis*, patients showed a 15% reduction in blood pressure, reduction in urinary protein, and increases in superoxide dismutase (44). Fifty-one percent improvement of chronic kidney diseases was

shown only one month after taking *C. sinensis* supplement (45). In another clinical study, treatment with *C. sinensis* of patients having gentamicin-induced kidney damage resulted in the recovery of 89% of their normal kidney function after six days, compared with only 45% recovery by patients treated with more conventional methods (1).

Hypoglycemic Effects

In a randomized trial, 95% of patients treated with *C. sinensis* showed improvement in their blood sugar profiles, while the control group showed only 54% improvement with treatment by other methods (46).

Lung Ailments

There have been many trials in humans, using *Cordyceps* spp. to treat many respiratory illnesses, including asthma and bronchitis, either alone or as an adjunct to standard antibiotic therapy, and it appears to be useful for all of these conditions (47–50). Extracts of *C. sinensis* have been shown to inhibit tracheal contractions, especially important in asthma patients, since it allows for increased airflow to the lungs. In addition, its anti-inflammatory properties may prove to bring further relief to asthma patients, whose airways become obstructed, due to an allergic reaction resulting in the swelling of the bronchial pathways (1). In a double-blind placebo-controlled study with 30 elderly volunteers, *C. sinensis* significantly improved the maximum amount of oxygen these people were able to assimilate (51).

Heart Ailments

It has been shown that *C. sinensis*, which often has a significant quantity of adenosine, along with adenosine-type nucleotides and nucleosides, has an effect on coronary and cerebral circulation (52,53). In studies of patients suffering from chronic heart failure, the long-term administration of *C. sinensis*, in conjunction with conventional treatments, promoted an increase in the overall quality of life (42). This included general physical condition, mental health, sexual drive, and cardiac function, compared with the control group. Studies have also shown the benefits of *C. sinensis* on heart rhythm disturbances, such as cardiac arrhythmias and chronic heart failure (54).

Liver Ailments

In the Orient today, *C. sinensis* is commonly used as an adjunct in the treatment of chronic hepatitis B and C. In one study, *C. sinensis* extract was used in combination with several other medicinal mushroom extracts as an adjunct to lamivudine, for the treatment of hepatitis B. The group receiving *C. sinensis* along with other medicinal mushroom extracts had much better results in a shorter period of time than the control group, who received only lamivudine (55). Treatment of 22 patients, diagnosed with posthepatic cirrhosis, with *C. sinensis* (56), showed improvement in liver function tests, and in another trial on patients with hepatitis B and patients with cirrhosis taking *C. sinensis* supplement showed around 80% improvement of liver functions (57).

Hypercholesterolemia

In both human and animal studies, administration of *C. sinensis* has been associated with cholesterol and triglyceride reduction and an increase in the ratio of HDL to LDL cholesterol (1). As such it may prevent, arrest, and even reverse coronary atherosclerosis (58). The studies have demonstrated that *C. sinensis* helps to lower total cholesterol up to 21% and triglycerides up to 26%. At the same time it helps to increase HDL cholesterol up to 30% (54).

Antiviral Activity

After three months of treatment of chronic hepatitis B patients using *C. sinensis*, CD4 and CD4/CD8 ratios increased significantly (59). The results suggest that beneficial effects might be obtained through adjustment of the T lymphocyte subsets level. Treatment of 65 cases (with 20 cases in the control group) of patients with posthepatic cirrhosis has shown similar results (60). Extracts of *Cordyceps* spp. are also effective against HIV infections. A *C. sinensis* containing formula named Immune Assist 24/7™ has recently been introduced throughout West Africa for use in treating HIV infections and other immune-deficient states (2), and is quite popular with both the doctors and the patients due to its low toxicity and cost when compared with other antiretroviral drug options.

Dosage

Because clinical data on *Cordyceps* spp. is relatively new, and even more so in Western Countries, recommended dosage requirements may vary, depending on the source. In general, clinical trials have been conducted using 3 to 4.5 g of *C. sinensis* per day, except in cases of severe liver disease, where the dosage has usually been higher, in the range of 6 to 9 g per day (4). There are some practitioners known to these authors, who keep their cancer patients on 30 to 50 g of *C. sinensis* per day. While this may seem excessive, the clinical results seen with this treatment regimen are promising, and *Cordyceps* spp. related toxicity has never been reported.

C. sinensis has been traditionally taken in tea or eaten whole, either by itself or cooked with a variety of meats. Today, in addition to the established traditional means of consumption, powdered mycelium and mycelial extracts are also available in capsulated and noncapsulated form. At present, there are no reliable standards by which to compare different brands, but in general, the quality of *Cordyceps* spp. is improving, as methods of more efficient cultivation are investigated; and as more clinical trials are conducted, a clearer picture of recommended dosages for a particular condition will become more standardized. Considering the quality of cultivated *Cordyceps* spp. available in the market today and the risk of lead exposure as well as the cost, such as with wild *C. sinensis*, the use of natural *Cordyceps* spp., over the artificially cultivated variety, is not recommended. Obtaining *Cordyceps* spp. from a reliable source, with complete analytical data provided, is the safest way to purchase species of *Cordyceps*.

Safety Profile

None known contraindications.

Drug Interactions

There is observational evidence that the alteration of the body's blood glucose metabolism in patients consuming *Cordyceps* spp. often results in the reduction of oral or injected anti-diabetic medications. It is also posited that the naturally occurring antiretroviral compounds found in *C. sinensis* (e.g., 2,3-dideoxyadenosine) are marketed as a major anti-HIV drug under the name Videx and Didanosine, as well as 3-deoxyadenosine (which has the same or at least similar activity); *C. sinensis* could result in increased effectiveness or decreased dosage requirements for patients undergoing concurrent therapy with other antiretroviral drugs. Caution should be exercised in these patients, especially considering the newer, more potent hybrid strains of *Cordyceps* spp. being developed, and the targeted medicinal compounds being selectively cultivated.

Adverse Side Effects

Very few toxic side effects have been demonstrated with *Cordyceps* spp. use, although a very small number of people may experience dry mouth, nausea, or diarrhea (1). One study reported that a patient had developed a systemic allergic reaction after taking a strain of cultivated *C. sinensis* called Cs-4 (61); however, this type of reaction is not common. There is little published data on the use of *Cordyceps* spp. in pregnant or lactating women, or in very young children, and appropriate precautions should be taken with these types of patients.

Toxicity

No human toxicity has been reported, and animal models failed to find an LD₅₀ (median lethal dose) injected IP in mice at up to 80 g/kg per day, with no fatalities after seven days (2). Given by mouth to rabbits for three months, at 10 g/kg per day ($n = 6$), no abnormalities were seen from blood tests or in kidney or liver function (62).

REGULATORY STATUS

Cordyceps spp. remains, in many nations throughout the world, an unrecognized substance. Other than import/export taxes and restrictions, which vary from country to country (many of which ban the import of any such substance), most governments do not require a prescription to purchase or use *Cordyceps* spp. Among the few countries that do require a doctor's prescription are Portugal, Romania, and Austria. Many governments require that vendors obtain a special license to distribute any product relating to human health.

In the United States, *Cordyceps* spp. are marketed privately and considered by the FDA as a dietary supplement. GRAS applications referring to *Cordyceps* spp. status as a food additive are unavailable; however, a premarket notification to the FDA regarding species of *Cordyceps*, containing in-depth information relating to preclinical trials and toxicology studies, has been available to the public, via the FDA website.

CONCLUSIONS

When a natural product, such as *C. sinensis*, has such a long history of use, it seems logical that there is quite likely some truth behind the myths. Our challenge in the modern age is to scientifically unravel the many claims and conflicts. With *C. sinensis* this challenge has been greater than with many other herbals due to the enormous cost and scarcity of the material. We are fortunate that we live in an age of such rapidly expanding biotechnological progress. For now, we have ways at our disposal to produce *Cordyceps* spp. in large enough volume, and at a low enough cost, that research becomes possible to nearly anyone interested in looking at this unique organism. As time passes, we may find that this once rare fungal species may hold the key to controlling some of our more difficult to manage diseases. More research is needed into this and other species of medicinal mushrooms.

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Cranberry

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INTRODUCTION

Cranberry (*Vaccinium macrocarpon* Aiton) is a native plant of North America. Today, it is one of the top selling herbal supplements in the U.S. market. Juice and dietary supplements derived from the berry reportedly exhibit various health benefits, including prevention and treatment of bacterial adhesion in urinary tract infections (UTIs) and stomach ulcers, prevention of dental caries, protection against lipoprotein oxidation, and anticancer activity. Some of these biologic effects have been linked to the presence of phenolic compounds. The composition of these compounds in cranberry is beginning to be assessed and quantified; however, their bioavailability and metabolism are for the most part not known. Interpretation of results from research on the efficacy/safety profile of cranberry is confounded by methodologic limitations. More research is needed to conclusively determine its health benefits.

BACKGROUND

V. macrocarpon Aiton, the cultivated species, is a member of the heath family (Ericaceae), which includes blueberry, huckleberry, and bilberry. The wild plants are distributed over eastern United States and Canada. Cranberry was of great economic value to the Native Americans, especially since it was the only edible fruit available late in the season (September–November). Various parts of the plant were used as dyes, food, and medicines. They used the berries in poultices for treating wounds and blood poisoning; the leaves for urinary disorders, diarrhea, and diabetes; and infusion of branches for pleurisy (1). In addition, the European settlers applied cranberries therapeutically for the relief of blood disorders, stomach ailments, liver problems, vomiting, appetite loss, and cancer. Sailors took barrels of the fruit to sea to prevent scurvy. Over 100 years ago, women in Cape Cod were known to use it for the treatment of dysuria. About four decades back, consumption of the berry for treatment of UTI received attention and support within the medical community (2,3).

Cranberry was first cultivated in the early 19th century. The principal areas of cultivation in North America are Wisconsin, Massachusetts, New Jersey, Oregon, and Washington, as well as parts of Canada. In the 1940s, cranberry juice cocktail became widely available and is the most common form of cranberry consumption today (1). This is a sweetened beverage of about 27% cranberry

juice by volume. As a dietary supplement, cranberry ranks among the top 10 selling herbal products in the U.S. market, with U.S. sales skyrocketing in 2007 by 15% (4). Also in 2007, cranberry supplements were among the top 20 supplements used by adults and children, who used non-vitamin, nonmineral, natural products for health reasons (5). Concurrent with increasing sales, publication of original scientific results, papers, and reviews almost doubled between 2004 and 2009 compared to the previous five-year period.

CHEMISTRY AND PREPARATION OF PRODUCT

The chemical composition for the nutrient constituents (Table 1) of cranberry has been well documented (6,7). Raw cranberries are relatively low in sugar content and minerals compared to other small fruits. They are a very good source of vitamin C, have a fair amount of vitamin A, but are relatively low in the B vitamins.

Most of the biologic effects of cranberry have been linked to its high level of phenolic compounds (8–11), higher than 20 other fruits tested (12,13). The major phenolics in the berry are flavonoids and phenolic acids. Chen et al. (11) found a total of 400 mg of total flavonoids and phenolic compounds per liter of sample in freshly squeezed cranberry juice. About 44% were phenolic acids and 56% flavonoids.

Phenolic acids include the cinnamic acids (C6–C3) and benzoic acids (C7). Cinnamic acids occur naturally in combination with other compounds, usually in the form of esters. The ester of caffeic with quinic acid is a classic example. On the contrary, benzoics usually occur as free acids. Benzoic acid is the major “phenolic” compound in cranberry (11). The fruits’ astringency is attributable to high levels of organic acids, primarily quinic, citric, malic, and benzoic.

Cranberries contain three major subclasses of flavonoids: flavanols, flavonols, and anthocyanidins (Table 2) (8,9).

Flavanols exist in the monomer form (catechin and epicatechin) and the oligomer or polymer form (proanthocyanidins). Proanthocyanidins, also known as condensed tannins, are polymeric compounds, the basic structural elements of which are polyhydroxyflavan-3-ol units linked together by carbon–carbon bonds (14). Unlike most fruits, cranberry contains a relatively high proportion of A-type proanthocyanidins (13,15). One subclass of proanthocyanidins is procyanidins. Cranberries contain a

Table 1 Nutrient Content of *Vaccinium macrocarpon*

Source (100 g)	Water (g)	Energy (kcal)	Total sugars (g)	Ca (mg)	Mg (mg)	K (mg)	Vitamin C (mg)	Thiamin (mg)	Riboflavin (mg)	Vitamin A (IU)	Vitamin E (mg)
Cranberries, raw	87.13	46	4.04	8	6	85	13.3	0.012	0.020	60	1.2
Cranberry juice cocktail	86.17	54	11.87	3	1	14	42.3	0.000	0.000	8	0.22

IU, International Units.

Source: From Refs. 6, 7.

variety of different procyanidins, mixtures of oligomers and polymers, with the last of these being the dominant procyanidins in cranberry (16). Procyanidins may contribute to organoleptic characteristics.

Flavonols include the glycosides of quercetin, kaempferol, and myricetin (11,17). Quercetin is the major flavonol in cranberry and is glycosylated mainly at the 3-position with arabinose, galactose, rhamnose, and rhamnose-glucose. Myricetin also exists and has been identified as conjugates of both arabinose and galactose (18). The wide-ranging flavonol content of cranberry is high, exceeding 150 mg/kg (17).

Anthocyanins, glycosylated anthocyanidins, are responsible for the fruit's bright red color. Early studies found a somewhat higher content of anthocyanins than flavonols (19). The pigments present are cyanidin-3-galactoside, -3-glucoside, and -3-arabinoside, as well as peonidin-3-galactoside, -3-glucoside, and -3-arabinoside (20). The major anthocyanins in cranberry are 3-galactosides and 3-arabinosides of cyanidin and peonidin (21).

Uniform requirements for the composition of cranberry products do not exist. In addition to the variable composition of the berries, processing and the product matrix contribute to product composition variability that may impact stability and bioactivity. Characterization and standardization of the bioactive constituents in cranberry products are needed to help in determining product stability and to allow comparison among studies. However, quantification is not always straightforward. A broad spectrum of methods is used to quantify the constituents, leading to differing results. Finally, no cranberry-standardized reference materials to which results of different analytic methods can be compared are available (22,23).

PRECLINICAL STUDIES

Bioavailability

The structural diversity of cranberry components has a major influence on their bioavailability, which in turn influences their biologic effects. Many studies have ignored

their achievable plasma concentration after ingestion as well as the possibility of conjugation and metabolism of bioactive components. In general, polyphenols reaching the colon are extensively metabolized by microflora into a wide array of low-molecular-weight phenolic acids. The concentration of intact polyphenols (parent compounds and their conjugated forms) in plasma rarely exceeds 1 $\mu\text{mol/L}$ (1 μM) after consumption of a single compound. However, measurement of plasma antioxidant capacity suggests that more phenolic compounds are present, largely in the form of unknown metabolites, produced either in the tissues or by gut microflora. Their urinary recovery has been found in the range of 1% to 25% of ingested amount (21).

The bioavailability of the major flavonoids from cranberry has not been studied. However, their bioavailability from other dietary sources (e.g., tea, cocoa or chocolate, red wine, onions, or fruits) has been analyzed (24–26). Less is known about absorption and metabolism of the proanthocyanidins than other flavanols, in part due to their complex structures and nonspecific analytic methods to detect them. Higher molecular weight polymers are considered to have poor absorption (27,28). Proanthocyanidins are degraded to low-molecular-weight metabolites by human colonic microflora (29). Although biologic activity is apparent after proanthocyanidin ingestion, only its metabolites have been measured in the urine and plasma (29).

Urinary Acidification

Cranberries contain quinic acid, which is excreted in the urine as hippuric acid. Early studies attributed the antibacterial nature of the fruit to the urinary acidifying activity due to the excretion of organic acids and increased concentration of hippuric acid (30–32). Other experiments showed no decreased pH, nor increased levels of hippuric acid or only a brief effect (33–35). Hippuric acid does have antibacterial effects if present in acidic urine (pH 5.0) and at concentrations of 0.02–0.04 M. However, cranberry juice rarely can achieve the bacteriostatic concentrations by itself without the addition of exogenous hippuric acid to the diet (36,37).

Table 2 Flavonoid Content of *Vaccinium macrocarpon* (mg/100 g)

Source	Anthocyanidins		Flavanols		Flavonols		Proanthocyanidins	
	Cyanidin	Peonidin	(–)-Epicatechin	(+)-Catechin	Myricetin	Quercetin	Monomers	Polymers
Cranberries, raw	41.81	42.10	4.37	0.39	6.78	15.09	7.26	233.48
Cranberry juice cocktail	0.38	NA	NA	0.19	0.51	1.27	0.56	8.33

NA, Not applicable.

Source: From Refs. 8, 9.

Antiadhesion in Urogenital Infections

In vitro and ex vivo studies indicate that cranberry products prevent adhesion of bacteria to the cell walls of the urinary tract, thus preventing UTIs. Emphasis has been on the role of components that act by interference with bacterial adherence of *Escherichia coli* to uroepithelial cells (38–40). Several ex vivo studies found antiadherence activity in mouse and human urine (15,38,41–43). Two compounds were identified that inhibited adherence. One was fructose and the other was a nondialyzed polymeric compound present only in cranberry. While fructose in vitro inhibits adherence (38,40), it is unlikely to contribute to in vivo antiadhesion activity in urine because it is metabolized before reaching the urinary tract. The nondialyzed polymeric compound proved to be A-type proanthocyanidins. This compound, but not B-type dimer or the (–)-epicatechin monomer, prevented uropathogenic *E. coli* from adhering to uroepithelial cells in vitro (14,39,44).

Subsequently, isolated proanthocyanidins and whole cranberry products have been shown to inhibit *E. coli* adherence to model systems of primary cultured bladder and vaginal epithelial cells in a dose-dependent fashion, including clinically achievable doses (240 mL cranberry juice cocktail). However, only a very small portion of a dose may reach the bladder (45) and possibly not even excreted intact in the urine (46). A new group of urinary marker compounds, discovered by a robust antiadhesion assay, include two new coumaroyl iridoid glycosides and a depside (47). Furthermore, it is not known if any cranberry constituents reach vaginal tissues (45).

In conclusion, to date no specific antiadherent cranberry constituents or metabolites, proanthocyanidin or otherwise, in the urine have been elucidated, and possible synergism among constituents needs to be considered.

Dental Plaque

Cranberry compounds, alone or combined, may have the potential to inhibit the development of dental plaque (biofilm) and to prevent or reduce the severity of periodontal disease. Nondialyzable, high-molecular-weight cranberry compounds (anthocyanins and proanthocyanidins in combination) may limit extracellular matrix degradation and other pathologic processes leading to periodontal disease. In vitro studies of this test material showed it having a high capacity to inhibit proteolytic enzyme activity of specific metalloproteinases and elastase, as well as to inhibit production of metalloproteinases (48). These enzymes play a major role in gingival tissue destruction, connective tissue remodeling, and alveolar bone resorption. Their secretion from host cells may, in part, be stimulated by components of the dental biofilm.

The pathogenesis of dental caries involves an interaction of diet constituents with microorganisms, which occurs within dental plaque. *Streptococcus mutans* is considered the primary microbial agent in this pathogenesis. It has two virulent traits: (i) synthesis of extracellular polysaccharides (glucans) through glucosyltransferases, and (ii) ability to produce and tolerate acids, both of which lead to cariogenic biofilms. Cranberry juice, crude extracts, and semipurified materials composed of low- and/or high-molecular-weight compounds have been shown in vitro to disrupt the virulent traits of *S. mutans* and

Porphyromonas gingivalis (49–52). Eleven isolated, highly purified, low-molecular-weight cranberry constituents (including flavonols, phenolic acids, and proanthocyanidins) were tested alone and in combination to investigate which compounds influenced the virulence properties of *S. mutans* associated with glucan synthesis and acidogenicity (53). Phenolic acids showed little effect. However, specific flavonoids and proanthocyanidins resulted in moderate, statistically significant effects. Furthermore, certain combinations of these low-molecular-weight compounds appeared to have an additive effect.

Helicobacter pylori Infection

Several mechanisms by which cranberry constituents may prevent or treat *Helicobacter pylori* infections have been examined and hypothesized, including (i) interference of bacterial adhesion, (ii) inhibition of cell growth and/or colonization, (iii) exerting bactericidal activities, (iv) induction of the bacteria to develop a coccoid (spheroid) form, and (v) neutralization of gastric pH.

Adhesins mediate adhesion of *H. pylori* to epithelial cells. Because cranberry or its constituents have been shown to inhibit adherence of *E. coli* to uroepithelial cells in vitro, it has been hypothesized that it would prevent adhesion of *H. pylori* to gastric mucus and cells. A high-molecular-weight, nondialyzable material from cranberry juice was demonstrated to restrain the adhesion of two-thirds of the tested strains of *H. pylori* to immobilized human gastric mucus and erythrocytes (54,55).

Preliminary results indicate that cranberry phenolics may disrupt energy production and cause cell death (56). In addition, cranberry phenolics may inhibit urease activity. *H. pylori* releases the enzyme urease, which converts urea into ammonia in the stomach. This neutralizes the pH and protects *H. pylori* from stomach acid. Finally, the *H. pylori* inhibiting factor may not be unique to cranberry but common to all polyphenol-rich fruits (56,57). These in vitro effects have been demonstrated in animal models, with the administration of cranberry juice resulting in the eradication of the pathogen; however, mechanisms and specific cranberry constituents remain to be elucidated.

Antioxidant

Antioxidant capacity is not restricted to a particular class of cranberry components but has been found in a wide range of fractions (58). Polyphenols are reducing agents, and together with others, such as vitamin C, they may protect the body's tissues against oxidative stress. The antioxidant activity of the berry in vivo cannot be accounted for on the basis of increased vitamin C alone (59). Crude cranberry fruit extracts have significant antioxidant activity in vitro (60). The total antioxidant activity of 100 g of cranberry was estimated to be equivalent to that of 3120 mg of vitamin C (12). Isolated polyphenolic compounds from whole cranberries are comparable or superior to that of vitamin E in their activity (18). Cranberry ranks higher than apple, peach, lemon, pear, banana, orange, grapefruit, pineapple, avocado, cantaloupe, melon, nectarine, plum, and watermelon (13,61,62). However, cranberry juices ranked lower in antioxidant potency using a variety of antioxidant tests than many other leading U.S. brands of ready-to-drink, polyphenol-rich beverages,

including pomegranate juice, red wine, Concord grape juice, blueberry juice, black cherry juice, and Acai juice (62). In comparing cranberry products to one another, it appears that processing decreases the quality of antioxidants. The quality is the result of changing the polyphenol composition and is independent of the quantity of antioxidants present (63).

The contribution of individual phenolics to total antioxidant capacity is generally dependent on their structure and content in the berry. The highest antioxidant activity has been noted in peonidin-3-galactoside (21% of antioxidant capacity). Quercetin-3-galactoside, cyanidin-3-galactoside, and peonidin-3-arabinoside each contribute about 10% to 11% (64). These four flavonoids have the most potent antioxidant activities compared to 16 other isolated compounds, including plant sterols, other flavonoids, derivatives of triterpenoids, and organic acids. The isolated compounds may have additive and synergistic effects (65). Animal model studies have shown whole-body antioxidant potential at clinically relevant doses and with dose-dependent responses (66,67).

Different methods of assessment of antioxidant capacity, varying substrate systems, divergent ways of extraction, length of storage, and differential concentrations of active antioxidants confound the antioxidant activity–chemical structure relationship. Given the diversity and abundance of phenolic antioxidants in cranberry, considerable potential exists for cranberry products to prevent oxidative processes related to cardiovascular disease and cancer at the cellular level and in vivo.

Atherosclerosis

Consumption of cranberry may decrease the risk of atherosclerosis (68). Possible mechanisms by which cranberry may reduce risk include: (i) inhibition of low-density-lipoprotein (LDL) oxidation (18,63), (ii) inhibition of platelet aggregation and adhesion, (iii) inhibition of the inflammatory response, (iv) induction of endothelium-dependent vasodilation, and (v) increase of reverse cholesterol transport and decrease of total and LDL cholesterol. Data supporting these mechanisms are preliminary and mostly from in vitro and animal model studies (69,70). In vitro studies suggest that molecules like quercetin, resveratrol, proanthocyanidin, anthocyanidin, hydroxycinnamic acid, and acetylsalicylic acid may contribute to an anti-inflammatory response. Human studies have shown that cranberry increases total antioxidant capacity, reduces plasma oxidized LDL (but not total LDL), and reduces cell adhesion molecules (71,72). It has been hypothesized that the potential effect of cranberry on atherosclerosis may result from additive or synergistic effects of multiple cranberry constituents due to various mechanisms and not just the antioxidant effect alone. The constituents contributing to the antioxidant effect were previously addressed.

Cancer

The antioxidant capacity alone of cranberry constituents may not account for the observed effects (61,73,74). A soluble-free cranberry extract had the highest antiproliferative activity and maximum calculated bioactivity index for dietary cancer prevention compared to 10 other fruits

(12). Many of the cranberry compounds are likely contributors, including the flavonols, anthocyanins, proanthocyanidins, catechins, various phenolic acids, triterpenoids (e.g., ursolic acid), and even stilbenes (e.g., resveratrol) although these are present in lesser quantities than the other constituents (65,70). Cranberry's effect on tumor initiation, growth, and metastases will depend largely on the bioavailability of its phytochemicals to the various target tissues.

Given the diversity of molecular structures and bioactivity among the classes of phytochemicals in cranberry, it is likely that they may fight cancer individually, additively, or synergistically by several different mechanisms. In vitro evidence in a variety of cell lines exists for possible mechanisms, including (i) induction of apoptosis in a variety of cancer cells, (ii) reduction of invasion and metastasis by inhibition of matrix metalloproteinases, (iii) inhibition of ornithine decarboxylase expression and activity, (iv) inhibition of angiogenesis, (v) inhibition of inflammatory processes, and (vi) inhibition of *H. pylori*, a risk factor for gastric cancer (58,61,70,72,74–76). In vivo carcinogenesis studies will need to be performed to further confirm antitumor promotion activity and identify individual components and mixtures responsible for activity.

Safety Studies

No animal toxicology studies of any cranberry products have been reported; however, two studies have reported on safety in animal models. A mouse model study of the effect of cranberry extract on cancer treatment reported weight loss indicative of toxicity (77). A safety study of a single oral dose of a proprietary multiberry supplement, including cranberry (66), did not cause any mortality and did not demonstrate any signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior in the treated rats. Similarly acute dermal toxicity, primary skin irritation, primary eye irritation via nonoral routes of administration caused no toxicity or harm in animal models.

CLINICAL STUDIES

Efficacy

Urinary Tract Infection

The use of cranberry, particularly as a juice or juice cocktail, to prevent or treat UTI is common. The accumulating evidence from small, noncontrolled, and controlled clinical trials suggests that the berry may relieve symptoms associated with UTI and may reduce the need for antibiotics. The Cochrane Library conducted separate reviews of the fruit for the prevention (78) and treatment (79) of UTI. For *treatment*, no trials meeting the inclusion criteria were found; only a few uncontrolled trials were found. The Cochrane Library concluded that there was no good quality or reliable evidence of the effectiveness of cranberry juice or other cranberry products for the treatment of UTI. For both prevention and treatment, the review authors concluded that more research was needed. For *prevention*, 10 studies were included in the review, of which only four were of sufficient methodological quality to include in the meta-analysis. Juice, juice cocktail, or concentrate was investigated in seven studies and capsules or tablets

studied in four trials (one study investigated both juice cocktail and tablets). Intervention duration ranged from four weeks to one year and dosage was quite variable. Several studies reported a high number of withdrawals, and poor adherence to the intervention was also reported. Side effects were common in all studies. The authors concluded that cranberry products may decrease the number of symptomatic UTIs over 12 months.

The National Institutes of Health (NIH) supported four, large Phase 2 clinical studies to investigate the effect of a research-grade, low-calorie cranberry juice cocktail on the prevention of UTI in men and nonpregnant women at high risk for UTI and in pregnant women. Subsequent to the Cochrane reviews, results of the cranberry juice cocktail study of asymptomatic bacteriuria in pregnancy have been reported (80). Similar to other studies, a high number of dropouts/withdrawals occurred and adherence to the intervention protocol was poor which led to a protocol change to reduce the dose of 240 mL (80 mg proanthocyanidin) from three to two times a day. Despite the limitation of the protocol change and problems with withdrawal, adherence, and intervention tolerability, the data suggest that cranberry juice cocktail may be protective of asymptomatic bacteriuria and symptomatic UTIs in pregnancy. Results from the other three NIH-supported trials will be reported.

Many of the clinical study reports, with the exception of the NIH-sponsored studies, suffer from major limitations. Many trials have not been controlled or randomized, and randomization procedures have not always been described. Crossover designs used in some research may not be appropriate for studies of UTI. Other limitations include no blinding or failed blinding, lack of controlled diets or dietary assessment, use of convenience samples, and small numbers of subjects. Trials have been faulted for the large number of withdrawals. Intention-to-treat analyses were not often applied. Most studies have been conducted in older or elderly patients. Very few have been conducted in younger patients, with or without comorbidities, or in men. Primary outcomes have differed from study to study and have often included urinary pH, as well as rate of bacteriuria, biofilm load, and urinary white and red blood cell counts, rather than UTI. It is also not clear what the optimum dosage or type of product is. There is limited evidence of efficacy or safety for forms of cranberry product other than juice or juice cocktail. Finally, the published articles do not describe the quality and composition of the products tested.

***H. pylori* Infection**

A few randomized controlled studies of *H. pylori* infected male and female adults and children have been undertaken in China, Israel, and Chile with treatment outcomes determined by the C urea breath test as the gold standard to noninvasively detect active *H. pylori* infection (81–83). Although study limitations exist and generalizability is limited, results are encouraging and suggest that regular consumption of cranberry juice as a complement or alternative to standard triple therapy (a combination of antibiotics and a proton pump inhibitor) may suppress *H. pylori* infection. The studies suggest that females may be more responsive and that the effect may not persist when cranberry treatment is discontinued.

Adverse Effects

The U.S. Food and Drug Administration granted generally recognized as safe (GRAS) status to cranberry foods and beverages. This means that their safety is well established when consumed in food amounts. The safety or harm of dosages higher than food amounts cannot be confirmed without further high quality clinical studies. The safety of cranberry capsules, tablets, and concentrates, for example, in which doses could reach pharmacologic levels, has not been established.

The Cochrane reviews of UTI prevention and treatment indicated that side effects were common in all cranberry juice cocktail studies included in the reviews (78,79). The reported side effects were primarily diarrhea or frequency of bowel movements and other gastrointestinal symptoms.

A review of the safety of cranberry consumption by pregnant and lactating women indicated that there were no clinical studies in the evidence-based medicine literature of cranberry being either safe or contraindicated during pregnancy or lactation (84). Subsequent to the review, the first randomized, controlled trial of cranberry juice cocktail for the prevention of bacteriuria in pregnancy reported about 20% withdrawal due to gastrointestinal upset, including nausea, vomiting, diarrhea, at a dose of 240 mL three times a day (80). When the dose was reduced to two times a day, the juice cocktail was somewhat better tolerated. There were no differences between the active and placebo groups with regard to obstetric or neonatal outcomes.

Observed Drug Interactions and Contraindications

There is insufficient reliable information available on cranberry dietary supplements or juice cocktail to assess their safety or their interaction with other dietary supplements, foods, medications, or laboratory tests.

Because of its oxalate levels, cranberry may be a causative factor in nephrolithiasis. The results of two small studies of juice cocktail and tablets are equivocal, showing differences in urine acidification, calcium and oxalate excretion, and other promoters and inhibitors of stone formation (85,86). A third study (87) was designed to specifically assess the influence of diluted cranberry juice on urinary biochemical and physicochemical risk factors for calcium oxalate kidney stone formation. Three key urinary risk factors were favorably altered: (i) oxalate (reported to not be readily bioavailable from cranberry juice) excretion decreased, (ii) phosphate excretion decreased, and (iii) citrate (an inhibitor of stone formation) excretion increased.

There is one report of an infant hospitalized for cranberry juice intoxication and acidosis (88).

Theoretically, the juice could interfere with the copper-reduction glucose test because ascorbic acid (a reducing agent) and hippuric acid have each been reported to cause a false-positive reaction with the copper-reduction glucose determination in vitro. However, the results of two small studies are equivocal and inconclusive indicating that interference may be variable and dependent on the type of reagent strip kit (89,90).

Limited studies have evaluated the drug interaction potential of cranberry juice; no studies of cranberry supplements are reported. The present hypothesis exerts that constituents of cranberry and/or their metabolites may

interact with liver CYP isoenzymes or with intestinal and renal drug transporters to alter the pharmacokinetics of drugs. Factors that alter the metabolism of drugs play an important role in dosing.

Only one study to date has examined the interaction of cranberry and antibiotics commonly prescribed for recurrent UTIs, amoxicillin and cefaclor (91). This study of healthy women showed a modest delay in amoxicillin absorption and a slight delay in cefaclor absorption, neither delay being clinically significant. Their total absorption and renal clearance were not affected.

Causal relationships cannot be proved by case reports; however, they often help in identifying adverse events and drug interactions. In 2003, the United Kingdom's Committee on Safety of Medicines alerted health care professionals about the possibility of an interaction between cranberry juice and warfarin, the most commonly prescribed oral anticoagulation therapy (92). Five unsubstantiated reported cases suggested an interaction (92). By 2004, the Committee had received 12 anecdotal case reports of suspected interaction and concluded that there was sufficient evidence of interaction, even though the evidence was not credible (93).

It now appears that reports of enhanced antithrombotic effects of warfarin associated with cranberry juice administration may be a coincidence; however, there is inconsistency among study findings. To address the effect of cranberry on CYP2C9, evaluations have been conducted in vitro and in vivo. In vitro studies have shown that cranberry juice potentially inhibits CYP3A and CYP2C9 (94,95). On the other hand, a number of in vivo human studies reported no alteration of warfarin pharmacokinetics (95–99). In addition, studies of coadministration of cranberry with other drugs primarily metabolized by CYP2C9 (95,99) or metabolized by CYP3A (100) or CYP1A2 (96) similarly show no pharmacokinetic change. There does remain, nevertheless, potential drug interaction liability with cranberry (101), because inconsistencies among studies may be due to participant characteristics, dosing, intervention duration, variability of cranberry test materials, physiochemical effects of cranberry on drug absorption, study design, and sample size. Worthy of further investigation is the new evidence of genotype-dependent interactions with warfarin (97).

Because information concerning the influence of cranberry juice on the pharmacokinetics of CYP2C9 substrates is limited, it may be premature to reach a definite conclusion about the effect of cranberry juice on warfarin pharmacokinetics. Nevertheless, patients who are coadministered warfarin and especially large doses of cranberry (102) should be monitored for the most appropriate therapeutic range.

REGULATORY STATUS

In the United States, cranberry is classified as a food when sold as juice, juice cocktail, and other conventional forms. Cranberry products, such as encapsulated powders, tablets, or tinctures, are regulated as "dietary supplements" in the United States. In Canada, conventional forms are sold as foods, whereas products promoting a health claim are sold as "natural health products."

CONCLUSIONS

There is a need for comprehensive chemical analyses of all classes of compounds present in cranberry. Individual structures and composition vary significantly among cranberry products and its isolated constituents. Composition varies by ripeness of the fruit, plant variety, growth conditions, extraction method, and processing. This suggests that bioactivities will also vary. However, quantitation of complex polyphenols has been and continues to be limited because of the lack of appropriate standardized analytical methods. Consequently, the precise estimation of cranberry constituent intake is hampered. Furthermore, the bioavailability, metabolism, stability, purity, and composition of cranberry products tested in clinical studies have not been established or published. Therefore, the ability to infer epidemiological relationships with health and disease can be confounded.

Evidence for health benefit of cranberry is preliminary and inconclusive. Current evidence from in vitro and clinical studies has been conflicting. This could reflect differences among sources of cranberry or its constituents, form of product consumed, and level of intakes. In addition, clinical studies performed to date have had many methodological limitations and few have assessed safety. Nevertheless, results of clinical studies are encouraging for the relief of symptoms associated with and the prevention of UTI.

The complex composition of cranberry creates problems in extrapolation of research results on dietary intake of individual constituents to intake of whole fruits or extracts of whole fruits. Synergistic effects of the whole may enhance the health benefits beyond what can be achieved by the individual constituents. The complex mixture of compounds could also protect against side effects. More research on potential synergistic and protective effects among the classes of compounds in cranberry and with other food constituents and pharmaceuticals is necessary.

For these reasons, it is important to understand the composition of cranberry, determine the bioavailability and metabolism of its constituents in isolation and as part of the whole mixture, and rigorously examine the biologic effects of cranberry on disease conditions in order to establish its potential for being safe and providing health benefit.

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Creatine

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INTRODUCTION

Creatine (Cr)—methylguanidino acetic acid—is a naturally occurring compound that was first described by Chevreul in 1832. Its name is derived from the Greek word *kreas* (flesh). Creatine is found in abundance in skeletal muscle (red meat) and fish. It is essential in energy transmission and storage via creatine kinase (CK). The daily Cr dosage is obtained by both endogenous synthesis and via nutritional intake, followed by absorption in the intestine (1). Creatine supplementation is widespread among sportspersons because of its documented and/or presumed ergogenic effects (2–4). In addition, supplementation with Cr has proven to be instrumental for the treatment of rare inborn errors of metabolism due to defects in Cr biosynthesis enzymes (5–8).

Creatine is stored in high concentrations in skeletal and heart muscles and to a lesser extent in the brain. It exists in both free and phosphorylated form [phosphocreatine (PCr)] and is important for maintaining high ratios between adenosine triphosphate (ATP) and adenosine diphosphate (ADP). Upon increases in workload, ATP hydrolysis is initially buffered by PCr via the CK reaction. During high-intensity exercise, PCr in muscle is depleted within several seconds. Whether *de novo* Cr biosynthesis occurs in the brain or whether Cr is taken up into the brain through the blood–brain barrier, is currently a matter of debate.

DEFICIENCY AND SUPPLEMENTATION

Patients with Cr deficiency syndromes (CDS), that is, patients with a Cr biosynthesis defect or a Cr transporter defect, have developmental delay and mental retardation (MR), indicating that Cr is crucial for proper brain function. Surprisingly, however, CDS patients do not suffer from muscular or heart problems. Those with a Cr biosynthesis defect, in contrast to Cr transporter-deficient subjects, can partly restore their Cr pool in brain upon Cr treatment (5–10).

Creatine supplementation, due to its ergogenic effects, has become a multimillion dollar business (3). In the Western world, Cr has received wide public interest. A simple search on “creatine” in the World Wide Web using common database search engines results in more than 500,000 entries. Besides the use by sportspersons, Cr supplementation is explored in several animal models of neuromuscular disease (i.e., Huntington and Parkinson disease, amyotrophic lateral sclerosis) and in human disease (3,6,11,12). A recent study suggests that Cr supplementa-

tion increases intelligence and memory performance tasks (13).

The goal of this entry is to provide an overview on Cr and its metabolism in health and disease. The functions of Cr and PCr, Cr biosynthesis, its degradation, tissue distribution, transport and molecular aspects, as well as the benefits and risks of Cr supplementation are discussed. (For in-depth reviews, see Refs. 2, 3, 6 and references therein.)

BIOCHEMISTRY AND FUNCTION

Creatine Structure

Creatine is a naturally occurring guanidino compound. Its chemical structure is depicted in Figure 1. Creatine is a hydrophilic, polar molecule. Phosphocreatine is zwitterionic, with negatively charged phosphate and carboxylate groups and a positively charged guanidino group.

Creatine Synthesis

Biosynthesis

The transfer of the amidino group of arginine to glycine yielding L-ornithine and guanidinoacetic acid (GAA) represents the first step in the biosynthesis of Cr and is performed by L-arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1). This reaction is reversible and occurs in mitochondria, into which arginine has to be taken up for guanidinoacetate biosynthesis. The human *AGAT* mRNA encodes a 423-amino acid polypeptide including a 37-amino acid mitochondrial targeting sequence. The *AGAT* gene is located on chromosome 15q15.3, is approximately 17 kb long, and consists of 9 exons.

The second step involves the methylation of GAA at the amidino group by (S)-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2), whereby Cr is formed. The methyl group is provided by (S)-adenosylmethionine. The human *GAMT* mRNA encodes a 236-amino acid polypeptide. The gene is located on chromosome 19p13.3, is approximately 12 kb long, and consists of 6 exons.

Chemical synthesis

Creatine is produced by chemical synthesis, mostly from sarcosine and cyanamide. This reaction is prone to generation of contaminants such as dicyandiamide, dihydrotriazines, or Crn (14). Some manufacturers may fail to separate these contaminants from Cr. The toxicological profiles of these contaminants are often not known. Dicyandiamide liberates hydrocyanic acid (HCN) when exposed to strongly acidic conditions (such as in the stomach). For human consumption, only pure preparations of Cr should thus be allowed. Unfortunately, no generally accepted and

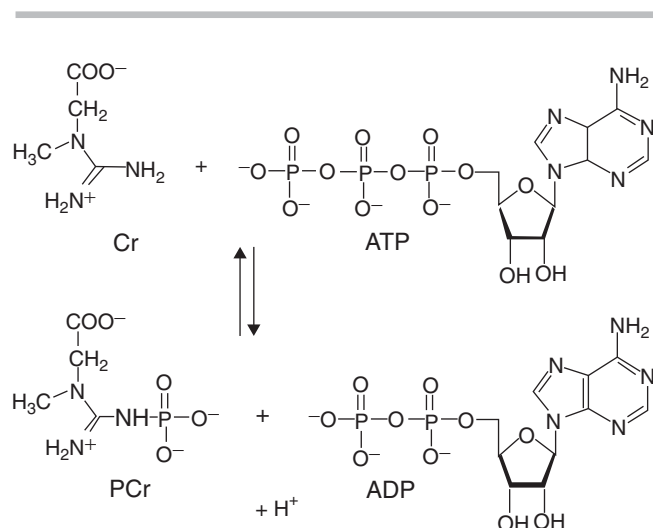


Figure 1 Schematic representation of the creatine kinase (CK) reaction, and chemical structures of creatine (Cr) and phosphocreatine (PCr).

meaningful quality labels are yet in place that would allow a consumer to judge the origin and quality of Cr in a given commercial product. Moreover, for most studies published so far, it is not possible to correlate the presence or lack of ergogenic, preventive, or adverse side effects with the quality of the many Cr preparations used.

Creatine Function (CK Reaction)

Creatine is involved in ATP regeneration via the CK reaction. The phosphate group of PCr is transferred to ADP to yield Cr and ATP, the “universal energy currency” in all living cells. The CK reaction serves as an energy and pH buffer and has a transport/shuttle function for high-energy phosphates.

Several CK subunits exist that are expressed in a tissue- and/or spatial-specific manner. In mammals, four CK isoforms exist: the cytosolic M-CK (M for muscle) and B-CK (B for brain) subunits form dimeric molecules, that is, the MM-, MB-, and BB-CK isoenzymes. The two mitochondrial CK isoforms, ubiquitous Mi-CK and sarcomeric Mi-CK, are located in the mitochondrial intermembrane space and form both homodimeric and homo-octameric interconvertible molecules.

In fast-twitch skeletal muscles, a sizeable pool of PCr is available for immediate regeneration of ATP, which is hydrolyzed during short periods of intense work. In these muscles, the cytosolic CK activity is high and “buffers” the cytosolic phosphorylation potential that seems to be crucial for the proper functioning of a variety of reactions driven by ATP. Slow-twitch skeletal muscles, the heart, and spermatozoa depend on a more continuous delivery of high-energy phosphates to the sites of ATP utilization. In these tissues, distinct CK isoenzymes are associated with sites of ATP production (e.g., Mi-CK in the mitochondrial intermembrane space) and ATP consumption [e.g., cytosolic CK bound to the myofibrillar M line, the sarcoplasmic reticulum, or the plasma membrane] and fulfill the function of a “transport device” for high-energy phosphates. The γ -phosphate group of ATP, synthesized within the mitochondrial matrix, is transferred by Mi-CK

in the mitochondrial intermembrane space to Cr to yield ADP and PCr. ADP may directly be transported back to the matrix where it is rephosphorylated to ATP. Phosphocreatine leaves the mitochondria and diffuses through the cytosol to the sites of ATP consumption. There, cytosolic CK isoenzymes locally regenerate ATP and thus warrant a high phosphorylation potential in the vicinity of the respective ATPases. Subsequently, Cr diffuses back to the mitochondria, thereby closing the cycle. According to this hypothesis, transport of high-energy phosphates between sites of ATP production and ATP consumption is achieved mainly by PCr and Cr. The CK system is required to allow most efficient high-energy phosphate transport, especially if diffusion of adenine nucleotides across the outer mitochondrial membrane is limited.

Physiology

Tissue Distribution of Creatine and of Its Biosynthesis Enzymes

In a 70-kg man, the total body creatine pool amounts to approximately 120 g (1). Creatine and PCr are found in tissues with high and fluctuating energy demands such as skeletal muscle, heart, brain, spermatozoa, and retina. In skeletal and cardiac muscle, approximately 95% of the total bodily Cr is stored, and the concentration of total creatine may reach up to 35 mM. Intermediate levels are present in brain, brown adipose tissue, intestine, seminal vesicles and fluid, endothelial cells, and macrophages. Low levels are found in lung, spleen, kidney, liver, white adipose tissue, blood cells, and serum (25–100 μ M) (2).

Until recently, GAA biosynthesis was presumed to occur mainly in the kidney (and pancreas), where AGAT is highly expressed, followed by its transport via the blood and uptake of GAA into the liver, the presumed major site of the second reaction, the methylation of GAA by GAMT. Current knowledge suggests that AGAT and GAMT expression is not limited to these organs. Synthesis outside of these organs may allow local supply of Cr (e.g., in brain; see creatine biosynthesis in mammalian brain) and may, to a minor extent, contribute to the total Cr content in the body.

Creatine Accumulation: Transporter-Mediated Creatine Uptake

Cellular transport is of fundamental importance for creatine homeostasis in tissues devoid of Cr biosynthesis. Creatine needs to be taken up against a steep concentration gradient [muscle (mM), serum (μ M)]. The Cr transporter gene (*SLC6A8*) (MIM300036) has been mapped to chromosome Xq28. Northern blots indicated that this gene is expressed in most tissues, with the highest levels in skeletal muscle and kidney, and somewhat lower levels in colon, brain, heart, testis, and prostate. The *SLC6A8* gene product is a member of a superfamily of proteins, which includes the Na⁺-dependent and Cl[−]-dependent transporters responsible for uptake of certain neurotransmitters. The Cr transporter gene spans approximately 8.4 kb, consists of 13 exons, and encodes a protein of 635-amino acids.

Creatine/Creatinine Clearance

Creatine can be cleared from the blood via either uptake into different organs by the Cr transporter or by excretion via the kidney. There is evidence that tissue uptake

of Cr may be influenced by carbohydrates, insulin, caffeine, and exercise and that transporter molecules located in kidney are able to reabsorb Cr. Nevertheless, Cr is found under normal conditions in urine in various amounts. The main route for clearance of Cr is via creatinine excretion. Creatine and PCr are nonenzymatically converted to creatinine. The rate of creatinine formation, which mainly occurs intracellularly, is almost constant (~1.7% per day of the Cr pool). Because muscle is the major site of creatinine production, the rate of creatinine formation is mostly a reflection of the total muscle mass. Creatinine enters the circulation most likely by passive transport or diffusion through the plasma membrane, followed by filtration in kidney glomeruli and excretion in urine.

Creatine Deficiency Syndromes

Both AGAT and GAMT deficiencies are autosomal recessive inborn errors of metabolism. This is in contrast to the third disorder of Cr metabolism, which is an X-linked inborn error due to a defect in the Cr transporter (Table 1).

GAMT Deficiency

The first inborn error of Cr biosynthesis, GAMT deficiency (MIM601240), was identified in 1994. The absence of a Cr signal in the proton magnetic resonance spectroscopy (¹H-MR) spectrum of brain, the low amounts of urinary creatinine, and the increased levels of GAA in plasma and urine led to the diagnosis of this disease. In addition to creatinine, Cr is also reduced in body fluids. Clinical symptoms are usually noted within the first eight months of life. Possibly Cr is provided in high amounts in utero via the umbilical cord and in newborns via the mother's milk, thereby delaying the clinical signs. All patients identified so far have developmental delay, MR to various degrees, expressive speech and language delay, epilepsy, autistic-form behavior, and very mild-to-severe involuntary extrapyramidal movements. The disorder has a highly het-

erogeneous presentation, varying from very mild signs to severe MR, accompanied by self-injurious behavior.

AGAT Deficiency

In 2001, the first family with AGAT deficiency (MIM602360) was identified. The two sisters, four and six years old presented with MR, developmental delay from the age of eight months, and speech delay. GAMT deficiency was ruled out because GAA was not increased in urine and plasma. Creatine supplementation (400 mg/kg body weight per day) increased the Cr content in the brain to 40% and 80% of controls within three and nine months, respectively. A homozygous nonsense mutation in the AGAT gene, predicting a truncated dysfunctional enzyme, was finally identified. Lymphoblasts and fibroblasts of the patients indicated impaired AGAT activity. A third related patient was identified with similar clinical presentation. The biochemical hints to detect this disorder are reduced levels of GAA (and creatinine) in plasma, cerebrospinal fluid (CSF) and possibly urine, together with reduced undetectable levels of Cr in the brain.

SLC6A8 Deficiency (Creatine Transporter Deficiency)

Like AGAT deficiency, the X-linked Cr transporter defect was unraveled in 2001. An X-linked Cr transporter (MIM300352) defect was presumed because of: (i) the absence of Cr in the brain as indicated by proton magnetic resonance spectroscopy (MRS); (ii) elevated Cr levels in urine and normal GAA levels in plasma, ruling out a Cr biosynthesis defect; (iii) the absence of an improvement on Cr supplementation; and (iv) the fact that the pedigree suggested an X-linked disease. The hypothesis was proven by the presence of a hemizygous nonsense mutation in the male index patient and by impaired Cr uptake by cultured fibroblasts. The hallmarks of this disorder are MR, expressive speech and language delay, epilepsy, developmental delay, and autistic-form behavior. The age at diagnosis of the affected males identified so far (>50) (9,15)

Table 1 Overview of CDS Based on the Listed Number of Patients

Deficiency	No. of patients	Trait	Clinical hallmarks	Metabolites	Treatment	References
AGAT (MIM602360) ^a	3, related	AR	MR Dysphasia Autistiform behavior Epilepsy	Brain: Cr ↓ ↓ in H-MRS Plasma, CSF (urine?): GAA ↓, Cr ↓	Cr supplementation	(8)
GAMT (MIM601240)	20	AR	MR Dysphasia Autistiform behavior Extrapyramidal signs Epilepsy	Brain: Cr ↓ ↓ in H-MRS Urine, plasma, CSF: GAA ↑ ↑, Cr ↓	Cr and ornithine supplementation + arginine restriction	(7,8)
SLC6A8 (MIM300036)	> 50 (15 families)	X-linked	Males MR Dysphasia Autistiform behavior Epilepsy Female carriers 50%: learning and behavioral disabilities 50%: no clinical signs	Males Brain: Cr ↓ ↓ in H-MRS Urine: Cr/Crn ratio ↑ CSF: Crn ↓ ? ~ 50% of female carriers Brain: Cr ↓ in H-MRS Urine: Cr/Crn ratio normal	Cr supplementation: not successful in affected males	(9,10)

^aMIM Victor A. McKusick: Online Mendelian Inheritance in Man: <http://www.ncbi.nlm.nih.gov>.

Abbreviations: AR, autosomal recessive; Cr, creatine; Crn, creatinine; H-MRS, proton magnetic resonance spectroscopy; MR, mental retardation.

varies from 2 to 66 years. In two cases, the disease-causing mutation had arisen *de novo*. In mothers and sisters who are carriers of the disease, learning and behavioral disabilities are noted in about 50% of the cases. Unfavorable skewed X-inactivation is likely the cause of the difference in severity of the clinical signs in females.

Intriguing Questions Linked to CDS

Does a Muscle-Specific Creatine Transporter Exist?

It is noteworthy that the *SLC6A8*-deficient patients do not seem to suffer from muscle and/or cardiac failure. This could indicate sufficient endogenous Cr biosynthesis in muscle. Alternatively, Cr uptake is taken over by other transporters, or a yet unknown Cr transporter exists that is specifically expressed in skeletal and cardiac muscle.

Creatine Biosynthesis in Mammalian Brain

It is a matter of debate whether Cr biosynthesis occurs in mammalian brain. The following findings suggest that it actually does: (i) In rat brain, *AGAT* and *GAMT* mRNA and protein were detected (16), (ii) The Cr content in brain of mice treated with guanidinopropionic acid, an inhibitor of the Cr transporter, was—in contrast to muscle tissues—hardly decreased. (iii) In contrast to skeletal muscle, Cr supplementation in *AGAT*- and *GAMT*-deficient patients requires months to result in an increment in Cr concentration in the brain. These findings make it unlikely that the brain is entirely dependent on Cr biosynthesis in the liver or on its nutritional intake, followed by transport through the blood–brain barrier into the brain.

However, why do Cr transporter deficient patients also reveal Cr deficiency in the brain? One explanation could be that Cr synthesis in the brain, although present, is too low to be relevant physiologically. Alternatively, the expression of *AGAT* and *GAMT* may be separated spatially (i.e., *AGAT* and *GAMT* molecules may be found in the same or different cell types, but may not be expressed in one and the same cell). This is in line with data of Braissant et al. (17) showing such spatial separation in rat brain at both the mRNA and protein level. These findings suggest that GAA needs to be taken up into the appropriate cells prior to GAA methylation, which in case of the transporter defect is not feasible. This would explain the incapability to synthesize Cr in the brain of *SLC6A8*-deficient patients. Clearly, more thorough investigations are needed to study these discrepancies toward a better understanding of Cr metabolism in the human brain.

Significance of CDS/relevance for Health Care

Mental retardation occurs at a frequency of 2% to 3% in the Western population. In 25% of MR cases, a genetic cause is suspected, of which Down syndrome and fragile X syndrome are the most common. Mutations in the *SLC6A8* gene may be, together with other X-linked MR genes, partly responsible for the skewed ratio in sex distribution in MR, autism, and individuals with learning disabilities. *SLC6A8* deficiency appears to be a relatively common cause of X-linked MR, though not as common as fragile X. Creatine biosynthesis defects may be less common. Because the damage incurred in these three diseases is irreversible to a large part and an effective treatment

is available at least for the Cr biosynthesis defects, early diagnosis of these patients is highly important.

To date, the clinical phenotype appears to be non-specific and suggests that all MR patients should be tested in diagnostic centers by ^1H -MRS, metabolite screening, and/or sequence analysis of the *SLC6A8* gene. In the case of X-linked MR or X-linked autism due to a genetic, but unknown, cause, the parents are confronted with a risk of recurrence (50% chance that the mother passes the mutant allele on to her child). The diagnosis of *SLC6A8* deficiency or a Cr biosynthesis defect allows prenatal diagnosis for subsequent pregnancies.

Creatine Supplementation/Therapeutic Use

Creatine Sources

Creatine is present in high amounts in meat (4.5 g/kg in beef, 5 g/kg in pork) and fish (10 g/kg in herring, 4.5 g/kg in salmon), which are the main exogenous Cr sources in the human diet. Low amounts of Cr can be found in milk (0.1 g/kg) and cranberries (0.02 g/kg) (17). As discussed earlier, Cr is also synthesized endogenously, which supplies around 50% of the daily requirement of approximately 2 g. This suggests that in vegetarians, who have a low intake of Cr, the bodily Cr content is reduced, unless its endogenous biosynthesis is largely increased. Indeed, in vegetarians, the Cr concentration in muscle biopsies was reported to be reduced (18).

Dosing as an Ergogenic Aid

Creatine can be obtained as nutritional supplement in the form of various over-the-counter creatine monohydrate products, which are supplied by many manufacturers. Commercial Cr is chemically produced. The majority of consumers are sportspersons, due to Cr's documented and/or presumed ergogenic and muscle mass increasing effects. Usually, a loading phase of five to seven days of 20 g/day (in four portions of 5 g) is recommended, followed by a maintenance phase with 3–5 g Cr per day.

Benefits

Benefits in Sportspersons

Creatine supplementation is common among cyclists, mountain bikers, rowers, ski jumpers and tennis, handball, football, rugby, and ice hockey players. While there is a large body of evidence supporting the ergogenic effects of Cr in high-intensity, intermittent exercise, the situation is more controversial in sports involving single bouts of high-intensity exercise, such as sprint running or swimming (2,19). In endurance exercise, there is currently no reason to believe that Cr supplementation has any benefit. There is a widespread contention that Cr supplementation, by accelerating recovery between exercise bouts, may allow more intensive training sessions. Similarly, supplementation seems to enhance recovery after injury.

In most studies, a significant weight gain has been noted upon Cr supplementation. The underlying basis for this weight gain is still not entirely clear, and may be due to stimulation of muscle protein synthesis or increased water retention. The proportion of fat tends to decrease. Most likely, the increase in body weight reflects a corresponding increase in actual muscle mass and/or volume. Therefore, it is not surprising that Cr use is popular among

bodybuilders and wrestlers. On the other hand, in mass-sensitive sports like swimming and running, weight gain due to Cr supplementation may impede the performance, or may at least counteract the ergogenic effects of Cr.

Creatine supplementation may improve muscle performance, especially during high-intensity, intermittent exercise, in four different ways by: (i) increasing PCr stores, which is the most important energy source for immediate regeneration of ATP in the first few seconds of intense exercise; (ii) accelerating PCr resynthesis during recovery periods; (iii) depressing the degradation of adenine nucleotides and possibly also the accumulation of lactate; and (iv) enhancing glycogen storage in skeletal muscle.

Benefits in Neuromuscular Disease

Besides its ergogenic effects, supplementary Cr has a neuroprotective function in several animal models of neurological disease, such as Huntington disease, Parkinson disease, and amyotrophic lateral sclerosis (ALS) (2,3,6,11). The rationale could be that these disorders, due to different causes, hamper cellular energy metabolism in the brain. In animal studies, Cr also protected against hypoxic and hypoxic-ischemic events. Therefore, Cr may be useful in the treatment of a number of diseases, for example, mitochondrial disorders, neuromuscular diseases, myopathies, and cardiopathies. Currently, the first clinical studies with Cr supplementation in neuromuscular disease are emerging. In two studies on patients with mitochondrial myopathies or other neuromuscular diseases, Tarnopolsky's group showed increased muscle strength upon Cr supplementation (11). A randomized, double-blind, placebo-controlled trial to determine the efficacy of creatine supplementation did not show a significant beneficial effect on survival and disease progression in a group of 175 ALS patients. These data are in contrast to what was suggested from animal models of ALS and tissue specimens of ALS patients (12). Studies on single subjects and small groups of neuromuscular disease patients have been reported to show both the presence and absence of beneficial effects of Cr supplementation. Recent publications on Cr supplementation in Huntington disease showed difficulty in proving the effect of Cr on the deterioration of cognitive function (20,21). In Duchenne muscular dystrophy, enhanced muscle strength upon treatment was shown; whereas, for example, in myotonic dystrophy type 2/proximal myotonic myopathy, no significant results were seen (22,23). Future studies with enough statistical power are warranted to unravel the relevance of Cr supplementation in these disorders. Clinical trials of patients with ALS, Parkinson, and other neurological diseases are currently ongoing (<http://clinicaltrials.gov/>).

Benefits in Creatine Biosynthesis Disorders

Oral supplementation with 350 mg to 2 g/kg body weight per day has been used in patients with GAMT and AGAT deficiencies. In these patients, the Cr concentration in their brains increased over a period of several months (5). In GAMT deficiency, the GAA concentration in plasma, urine, and CSF decreased with Cr supplementation, but still remained highly elevated. Guanidinoacetic acid was

found to be toxic in animals and may be partly responsible for some of the clinical signs (i.e., involuntary extrapyramidal movements). Combination therapy of Cr plus ornithine supplementation with protein (arginine) restriction reduced GAA in CSF, plasma, and urine, and almost completely suppressed epileptic seizures (7). In general, all patients with a Cr biosynthesis defect who were treated with Cr alone or in combination therapy showed improvements. Clearly, younger patients will experience the largest benefits, because less irreversible damage is to be expected. However, even older patients showed remarkable improvements (7).

Adverse Effects

Weight gain is the only consistent side effect reported. Gastrointestinal distress, muscle cramps, dehydration, and heat intolerance have been reported repeatedly. Most of these complaints may be due to water retention in muscle during the loading phase of Cr supplementation. Although a causal relationship with fluid intake has not been proven yet, subjects should take care to hydrate properly to prevent these side effects. The French Agency of Medical Security of Food (www.afssa.fr/ftp/basedoc/2000sa0086.pdf) released a statement in January 2001 that the health risk associated with oral Cr supplementation is not sufficiently evaluated, and that Cr may be a potential carcinogen. Because at present there is no scientific basis for the assertion (both Cr and Cr analogs were actually reported to display anticancer activity), this in turn has resulted in a wave of protest from suppliers and defenders of oral Cr supplementation. In fact, based on the current scientific knowledge in healthy individuals, Cr supplementation at the recommended dosages (see dosing as an ergogenic aid) should be considered safe. Unfortunately, almost nothing is known about the use of Cr in pregnancy, nor are appropriate studies in children available. Furthermore, a potential health hazard is the possible presence of contaminants in some commercial Cr preparations (see chemical synthesis).

CONCLUSIONS

Oral Cr supplementation is known or presumed to have a number of favorable effects. For example, it prevents or ameliorates clinical symptoms associated with inherited Cr biosynthesis defects, it may protect against neurological and atherosclerotic disease, (2,6) and it increases sports performance, particularly in high-intensity, intermittent exercise. Despite widespread use of Cr as an ergogenic aid and the significant public interest, the majority of studies on the properties, metabolism, and function of Cr have focused on physiological questions rather than on pharmacokinetics. As yet, the pharmacokinetics is difficult to interpret due to different (and incomplete) study designs. Currently, therefore, it is not adequately known whether Cr supplementation causes any long-term harmful effects. Some precaution is warranted based on the fact that the daily recommended dosage for ergogenic effects (i.e., 20 g during the loading phase, 3–5 g during the maintenance phase) cannot be met by normal food intake.

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Dong Quai

Roy Upton

INTRODUCTION

The root of dang gui (*Angelica sinensis*; also known as dong quai; Fig. 1) is one of the primary botanicals used in traditional Chinese medicine (TCM) for the treatment of gynecological and circulatory conditions. In TCM its primary use is to both build and promote the movement of blood, and on the basis of these actions it is utilized for a myriad of conditions. Despite its widespread use among practitioners of TCM, there have been few clinical studies regarding its efficacy, although preclinical data support many of these traditional uses as well as suggest benefit for numerous other uses.

BACKGROUND

Traditional and Modern Uses

Dang gui grows at high altitudes in comparatively cold, damp, mountainous regions in China and other parts of East Asia. The plant is a fragrant perennial that has smooth purplish stems and bears umbrella-shaped clusters (umbels) of white flowers that grow to approximately 3 ft in height. Dang gui produces winged fruits in July and September. In the earliest known herbal text of China, the *Divine Husbandman's Classic of the Materia Medica* (*Shen Nong Ben Cao Jing*), dang gui is described as a herb to "supplement nature" (1). In the monumental 52-volume *Compendium of Materia Medica* (*Ben Cao Gang Mu*), written by Li Shizhen in the 16th century, dozens of uses for dang gui were elaborated. These included the following: to tonify the five major viscera, especially the heart; to generate flesh; to stop headache, back pain, menstrual pain, toothache, and pain associated with the "belt channel" (*dai mai*); to treat a wide range of skin sores and rashes; and to correct menstrual problems such as irregular menstruation, amenorrhea, and dysmenorrhea (2).

Modern research has focused on the use of dang gui for its ability to enhance circulation and oxygenation in hypoxic conditions specifically in regard to brain and cardiovascular effects. Despite the widespread popularity and use of dang gui in gynecology, there is a lack of research in modern English language journals regarding this use, though some data suggest estrogenic and both uterine relaxant and uterine stimulatory activity, depending on the fraction studied. A number of studies report on the ability of dang gui to promote the healing of tissues, specifically in ulcerative colitis and gastric ulcers, and other studies have focused on its anticancer and hepatoprotective effects, among others.

Chemistry and Preparation of Products

The primary analytes of interest in dang gui are the Z-alkylphthalides, most notably ligustilide (Fig. 2), low- and high-molecular-weight polysaccharides, and ferulic acid (Fig. 2). The alkylphthalides are present in the essential oil and are strongly aromatic. Both the crude extract and individual compounds have been correlated with biological activity (see preclinical studies; clinical studies). The crude extract has been associated with positive human clinical effects for the treatment of chronic obstructive pulmonary disease (COPD) and COPD with hypertension, increasing blood volume in postischemic patients, and decreasing platelet aggregation (3). The alkylphthalides and ferulic acid inhibit platelet aggregation and the formation of platelet thromboxane A_2 (4–6) and elicit in vitro spasmolytic activity, increase coronary blood flow, slightly decrease myocardial contractility, and markedly prolong the effective refractory period (7). Total extracts have also been associated with hepatoprotective effects. Thus dang gui is utilized in portal hypertension and veno-occlusive disease. At least part of this activity is associated with the demonstrated antioxidant activity of ligustilide, ferulic acid, and polysaccharides (8–10), as well as the ability of dang gui to promote hepatic microcirculation. In human clinical trials both an aqueous extract and ligustilide have been found to be effective in treating dysmenorrhea (11). Dang gui polysaccharides stimulate hematopoiesis and, along with ferulic acid, elicit immunomodulatory activity (e.g., increased phagocytosis) (12,13). Ligustilide and ferulic acid elicit a strong uterine spasmolytic effect (14–17). All of these actions are consistent with the use of dang gui in traditional Chinese medicine.

Investigations of polysaccharides derived from dang gui, specifically in conjunction with their potential immunomodulatory effects have been conducted. The polysaccharides, named *A. sinensis* polysaccharide fractions (APF 1, APF 2, APF 3) and crude angelica polysaccharide consist of rhamnose, galacturonic acid, glucose, galactose, mannose, and arabinose in various ratios (18,19).

In TCM, the roots of dang gui are commonly prepared as a tea, extract, syrup, tablet, or capsule. In supplement form, dang gui occurs predominantly in tablets and capsules, and occasionally in tinctures. As with the majority of Chinese herbs, dang gui is most often used in combination with other botanicals and is predominantly featured in formulas for promoting healthy gynecological and cardiovascular systems and for a healthy liver. Different portions of the roots are used for different indications. The whole roots are said to "harmonize" the



Figure 1 Whole dang gui (*Angelica sinensis*) roots. Source: Courtesy of Roy Upton, Soquel, California. (View this figure in color at www.dekker.com)

blood; the dang gui root bodies (dang gui tou; 当归头) are used to build and nourish the blood and are commonly included in soups for convalescence and blood deficiency; the tails (dang gui wei; 当归尾) are predominantly used to “break the blood” and prevent and treat abnormal blood stagnation.

Pharmacokinetics

There are limited data on the pharmacokinetics of some of the compounds contained within dang gui. In a study of the bioavailability of ferulic acid in humans ($n = 5$), the peak time for maximal urinary excretion of ferulic acid following the consumption of 360 to 728 g tomatoes (providing approximately 21 to 44 mg ferulic acid) was 7 to 9 hours (20). A considerable proportion of ferulic acid was excreted as glucuronide in all subjects. The recovery of ferulic acid in the urine, on the basis of total free ferulic acid and feruloyl glucuronide excreted, was 11% to 25% of that ingested. The bioavailability of ferulic acid from beer is consistent with the uptake of ferulic acid from other dietary sources, such as tomatoes (21). Urinary and biliary metabolites of ferulic acid were primarily glucuronic acid and glycine conjugates of ferulic acid and vanillic acid (22).

The pharmacokinetic parameters of ferulic acid following IV injection to mice fit a one-compartment open

model and suggest that ferulic acid is rapidly and almost completely absorbed from the intestinal tract (23). It has also been reported that ferulic acid crosses the blood–brain barrier, although in very low concentrations (24). The major metabolites of ferulic acid are nontoxic and water soluble, being excreted through the urine and bile as free acids and acid conjugates.

PRECLINICAL STUDIES

Pharmacodynamics

Dang gui is one of the most widely used of all Chinese botanicals. Historically and in modern Chinese medicine, it has been primarily used as a general blood tonic for the TCM diagnosis of blood deficiency, a syndrome closely related, but not exactly analogous or limited, to anemia. Dang gui has also been used for a myriad of gynecological indications, although there has been very little research done in this regard in English language journals. More recently, pharmacological research has focused on the potential of constituents of dang gui to elicit cardiovascular, hematopoietic, hepatoprotective, antioxidant, antispasmodic, and immunomodulatory effects. Chinese botanicals are most often used in multi-ingredient formulas rather than as single agents. Therefore, there are very few clinical trials on dang gui alone, although numerous preclinical studies exist. Due to the lack of primary English language literature, it is difficult to adequately access or adequately review the available data by non-Chinese language readers. Another difficulty in reviewing the available studies is that many of the investigations are of disease patterns that are unique to TCM and do not have well-defined corresponding Western diagnoses, or vice-versa; studies are conducted for indications not synonymous with TCM indications. While the TCM findings are relevant to TCM practitioners, their importance may be ignored or even criticized by non-TCM practitioners. Study of non-TCM indications often is conducted for purposes of modern drug discovery and therefore can be criticized on different grounds. Lastly, it has been reported that up to 99% of studies presented in the Chinese medical literature show results favoring test intervention, suggesting the potential for a positive publication bias and hence the need for caution in interpreting the available data (25). Conversely, publication bias against dietary supplement research in the primary medical literature of the United States has been reported and may similarly limit a critical review of investigations of herbal products (26).

The bioactive compounds most studied in dang gui are phthalides, polysaccharides, and ferulic acid. Studies using these compounds have reported a number of therapeutic effects, some of which are consistent with the use of dang gui in TCM and some of which are not. The contribution of ferulic acid to the therapeutic effect of dang gui is unlikely given its low concentration in crude dang gui (0.05–0.09%). The compounds used in pharmacological studies are often administered at doses exceeding those available from typical dosages of dang gui root preparations. While these data are presented, it is not possible to extrapolate results from such studies to clinical efficacy of orally administered crude drug products; hence, the reported findings must be evaluated critically.

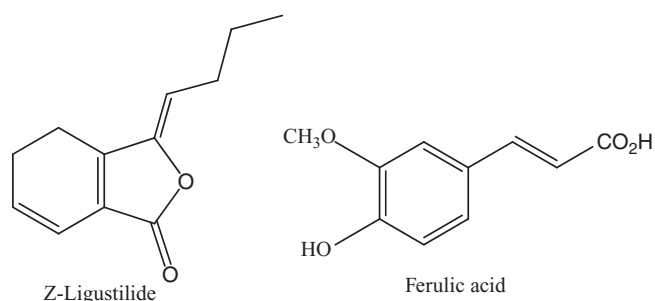


Figure 2 Major constituents of dang gui.

Cardiovascular and Hemorheological Effects

Clinically, dang gui is widely used for the treatment of cardiovascular disease, specifically conditions that can benefit from enhanced circulation and a decrease in platelet aggregation. Preclinical studies using dang gui and some of its constituents suggest actions and mechanisms by which it may exert a cardiovascular effect. These include stimulation of circulation, platelet aggregation inhibition, decrease in myocardial oxygen consumption, and vasorelaxation (measured as a decrease in vascular resistance; see also effects on smooth muscle).

A number of animal studies and in vitro assays support some of the putative cardiovascular effects of dang gui. These include an increase in myocardial perfusion, decrease in myocardial oxygen consumption, increase in blood flow, decrease in vascular resistance, and inhibition of platelet aggregation, ventricular fibrillation, and arrhythmias (3). However, a direct extrapolation of these findings to humans cannot be made without confirmatory human studies. A review of the activity of sodium ferulate reported that it both inhibits platelet aggregation and elicits a thrombolytic activity in vitro and in vivo. These effects were due to inhibition of cyclooxygenase and thromboxane A₂ synthase with improvements in blood viscosity, reduction in the concentration of plasma fibrinogen, and increase in coronary perfusion. Additional cardiovascular effects reported include reduction of cholesterol biosynthesis and lowering of triglycerides, improvements in myocardial oxygen consumption, and antiarrhythmic, antioxidant, and antiatherogenic activity (24).

Hepatoprotective Effects

A number of preclinical studies indicate that dang gui, dang gui polysaccharides, ferulic acid, and sodium ferulate have antioxidant effects that can protect the liver against damage due to chemically induced toxicity. Part of this action is due to the ability of dang gui polysaccharides to reduce the levels of nitric oxide (24.6%), serum alanine aminotransferase (40.8%), and serum glutathione S-transferase (18.4%) in animals with acetaminophen-induced or carbon tetrachloride-induced liver damage (8,9,27,28).

Gynecological Effects

Dang gui is one of the most important herbal medicines in TCM for the treatment of menstrual disorders, especially when used in combination with other botanicals. It has traditionally been used to treat conditions associated with the TCM diagnosis of "blood stasis" and "blood vacuity," which can be correlated with Western syndromes such as amenorrhea, dysmenorrhea, endometriosis, uterine fibroids, and certain forms of infertility. Its efficacy appears to have been demonstrated over the 750-year history of its use for these indications and its continued, and apparent, successful use by modern practitioners of TCM. However, there are few studies substantiating these effects (11,29), and those that are available lack methodological rigor.

Using ELISA-type immunoassays of two steroid-regulated proteins, presenilin-2 and prostate-specific antigen, in breast carcinoma cell line BT-474, researchers reported that dang gui extract showed "weak" estrogen and androgen antagonistic effects of 50% and 71% blocking activity, respectively, and no progestational activity (29). In

contrast to these findings, another group of researchers found no estrogen receptor binding, cell proliferation, or progestin activity of an aqueous-ethanol extract of dang gui (30).

A study of ovariectomized rats showed that an extract of dang gui (300 mg/kg SC; 1% ligustilide) resulted in a thickening of the luminal epithelium suggesting an estrogenic activity, but one much lower than comparison with estradiol. The extract also suppressed luteinizing hormone secretion. The researchers considered ligustilide to be the active compound on the basis of previous in vitro research they conducted (31). Several preclinical studies have investigated the estrogenicity of dang gui or ferulic acid, with mixed, but largely negative, results. Some in vitro assays have reported that dang gui extract exhibited a significant dose-dependent inhibition of estrogen receptor binding, indicating that it competed with estradiol for receptor sites (32). In the same study, dang gui extract dose-dependently induced reporter gene expression in estrogen-sensitive rat uterine leiomyoma cells, suggesting a potentially proliferative effect on these cells. However, when tested in conjunction with the maximum stimulatory dose of estradiol, the extract inhibited estradiol-induced reporter gene expression, suggesting the possibility that dang gui may act as an estrogen antagonist when in the presence of physiological levels of estradiol. Another group of researchers reported similar findings (33).

Effects on Smooth Muscle

Dang gui and its constituents have been shown to relax the smooth muscle tissue of the vascular system, trachea, intestines, and uterus. The spasmolytic effects of dang gui on trachea and uterine tissues are consistent with TCM indications. While the mechanism of the relaxant action has not been fully elucidated, preclinical studies suggest that it may be due, in part, to histamine receptor blocking activity, calcium ion channel effects, or modulation of cholinergic receptors. Both relaxing and stimulating effects on uterine tissue have been reported, with various constituents eliciting different actions. The therapeutic relevance of in vitro findings to humans is unknown given the lack of clinical evidence. Ex vivo studies demonstrate that ligustilide and butylidenephthalide isolated from the volatile oil of dang gui exhibit a strong spasmolytic effect on isolated uteri (34,35). Ligustilide was shown to relax early pregnant and nonpregnant uteri of experimental animals (34). Ligustilide and butylidenephthalide showed an inhibitory effect on prostaglandin F₂-, oxytocin-, or acetylcholine-induced contraction of nonpregnant rat uteri (14,36). This could explain the spasmolytic effect of the volatile oil. Other studies indicated that the observed spasmolytic effect may be due to an effect on calcium channels (14,37). Three of the available studies reviewed found that ferulic acid elicited a uterine spasmolytic effect. At oral doses of 300 to 1000 mg/kg and IV doses of 30 to 300 mg/kg, ferulic acid inhibited spontaneous uterine contraction in rats (16,17). The inhibitory effect of IV ferulic acid was not blocked by either propranolol or by cimetidine and it strongly inhibited the uterine contraction induced by oxytocin (0.3 unit/kg), but not that induced by acetylcholine (0.1 mg/kg) or serotonin (10 µg/kg). It was suggested that the uterine relaxant effect of ferulic acid is partially due to the oxytocin receptor

system rather than its inhibitory effect on prostaglandin biosynthesis (38). Another study, however, suggested that ferulic acid may not be responsible for the spasmolytic effect of dang gui, since its content in raw material is low (approximately 0.03% to 0.06%) (39).

Hematopoietic Effects

One of the traditional applications of dang gui in TCM is its use in the treatment of "blood vacuity," which closely, but not completely, corresponds to a Western medical diagnosis of anemia. Limited clinical and preclinical data support this use. One proposed mechanism of action is its reported effect in stimulating hematopoiesis. These actions appear to be primarily associated with the polysaccharide fraction (13,40). One study demonstrated the hematopoietic effects to at least partially be associated with proliferation of bone marrow mononuclear cells through signal transduction pathways (e.g., MAPK/ERK pathway) (41).

Antioxidant Effects

There have been numerous studies demonstrating an antioxidant effect of dang gui and its constituents. Much of these have focused on the antioxidant activity of ferulic acid, which is well known for its ability to prevent lipid peroxidation, inhibit superoxide anion radical formation, scavenge free radicals, and protect against radiation damage (42–44). Dang gui contains only trace amounts of ferulic acid, so these in vitro findings cannot be extrapolated to the use of crude dang gui preparations. There are, however, animal studies showing that dang gui polysaccharides have a protective effect against chemically induced ulcerative colitis and inflammation. In one study, dang gui polysaccharides elicited anti-inflammatory effects in the gastrointestinal mucosa through inhibition of neutrophil infiltration in the stomach (45). In another study, dang gui polysaccharides (5 mg and 10 mg/mL in drinking water) attenuated colonic lesions caused by oxidative damage induced by 2,4-dinitrobenzene sulfonic acid in rats in a dose-dependent manner. This action was associated with a preservation of endogenous glutathione levels. Other studies reported tissue-healing effects of dang gui to be associated with ornithine carboxylase activity, c-Myc protein expression, and epidermal growth factor-mediated pathway (27,46). A follow-up study by the same group of researchers showed that crude extract of dang gui (50 mg/kg PO) significantly accelerated the healing of gastric ulcers in animals and showed an anti-angiogenic activity and a quicker restoration of mucosal synthesis and mucosal cell proliferation (47).

Studies suggest that the antioxidant activity of dang gui may reduce ischemia-reperfusion induced injury (48,49), ameliorate cognitive dysfunction associated with postischemic brain damage (49), and inhibit the damage associated with aggregation of amyloid- β peptide, suggesting a possible use of dang gui in Alzheimer's. These effects were reported to be correlated with both ferulic acid and Z-ligustilide (50).

Other studies show that dang gui provides antioxidant protection against free radical induction of rat adrenal medulla (PC12) cell lines (51) and suppressed radiation-induced expression of tumor necrosis factor- α and tumor growth factor- β -1 (52), and prevented

doxorubicin-induced cardiotoxicity, without decreasing the antitumor activity of the drug (53).

Wound-Healing Effects

In addition to the beneficial effects of dang gui's antioxidant activity on tissues noted earlier, specific wound-healing properties have been reported. One group of researchers found that a crude extract of dang gui (characterization and dosage not available) significantly accelerated epithelial cell proliferation in wounds (27,46,54). The activity was reportedly associated with an increase in DNA synthesis and epidermal growth factor mRNA expression. The same researchers observed direct wound-healing effects of dang gui crude extract, with activity associated with increased ornithine carboxylase activity and increased c-Myc expression. Another study found that dang gui prevented bleomycin-induced acute injury to rat lungs. Alveolitis and the production of malondialdehyde were all reduced ($P < 0.01$ or $P < 0.001$), suggesting immunomodulatory and antioxidant effects (55).

Immunomodulatory Effects and Potential Anticancer Activity

Limited animal and in vitro studies have reported on specific immunomodulatory effects of dang gui, including stimulation of phagocytic activity and interleukin-2 production, and an anti-inflammatory effect. There is evidence to suggest that the polysaccharide fraction of dang gui may contribute to these effects. However, there is no clinical evidence supporting these effects, and there appears to be no direct correlation between TCM use of dang gui and immunomodulatory activity (56–58).

A new direction in investigation of the use of dang gui is for its potential anticancer activity. Ligustilide has been shown to have direct cytotoxic activity against several human and animal cell lines (59–61). In the absence of clinical and directly applicable toxicological investigation, little emphasis should be placed on these in vitro findings. There have, however, been a number of animal studies suggesting immunomodulatory and anticancer activity. In one study, *n*-butyridenephthalide suppressed growth of subcutaneous rat and human brain tumors, reduced tumor volume, and significantly prolonged survival in treated rats. This activity was reported to be due to an induction of cell cycle arrest and apoptosis (62). Polysaccharides have similarly been shown to inhibit growth of murine tumors (S180, EAC, L1210) in vivo, resulting in a prolonged survival of treated animals. In vitro, dang gui polysaccharides were shown to inhibit the metastasis of human hepatocellular cancer cell lines (63).

A variety of immunomodulatory activities have been reported for dang gui polysaccharides, including enhanced macrophage and T cell numbers, increased production of interleukin and interferon, improved CD4/CD8 ratios, and a general regulation of Th1- and Th2-related cytokines (18). Other actions reported for polysaccharides include release of nitric oxide from peritoneal macrophages and enhanced cellular lysosomal enzyme activity (64,65).

Effects on Bone Cells

Dang gui is traditionally used in formulas for bone and tendon injuries. A recent study investigated the

pharmacology behind this indication by testing the *in vitro* effects of a 1% aqueous extract of dang gui on human osteoprecursor cells. Cells were incubated for five days in medium with (12.5–1000 $\mu\text{g/mL}$) and without the extract. Compared to untreated control cell cultures, cell proliferation was enhanced at extract concentrations less than 125 $\mu\text{g/mL}$ ($P < 0.05$), whereas it was inhibited at concentrations greater than 250 $\mu\text{g/mL}$ ($P < 0.05$ at 1 mg/mL). Protein secretion in osteoprecursor cells and type-I collagen synthesis were significantly increased ($P < 0.05$) (66).

CLINICAL STUDIES

The clinical data regarding the use of dang gui alone are scarce and of poor methodological quality.

Cardiovascular and Hemorheological Effects

One study reported that 0.08 g/day/IV of sodium ferulate relieved symptoms of angina pectoris after three to seven days of treatment (24). Limited clinical studies have investigated the use of dang gui for the treatment of patients with acute ischemic stroke or COPD with pulmonary hypertension. Results provide fairly weak evidence that dang gui exerts hypotensive and cardioprotective effects. In general, the study design of the available reports was poor and the patient populations extremely limited.

One study looked at the effects of dang gui in 60 patients with COPD (67). In the dang gui group, levels of blood endothelin-1, angiotensin II, endogenous digitalis-like factor, mean pulmonary arterial pressure, and pulmonary vascular resistance were decreased significantly ($P < 0.05$ or $P < 0.01$) compared to those in the controls ($20 \pm 6\%$, $36 \pm 9\%$, $38 \pm 11\%$, $17 \pm 5\%$, and $27 \pm 8\%$, respectively). Another study showed that dang gui decreased the mean pulmonary arterial pressure in patients with COPD without changing blood pressure and heart rate, suggesting a vasodilatory effect on pulmonary vessels without effect on systemic circulation (3).

In another study, it was suggested that dang gui and dextran exhibited positive effects on neurological and hemorheological symptoms in patients recovering from stroke (68). However, no control group was included, and so any claimed effects are questionable. Other clinical studies with very small numbers of patients (11,69) have reported on an ability of dang gui to decrease blood viscosity, an effect consistent with its traditional use. While this effect may be real, the mechanisms by which this may occur and the constituents involved have not been well articulated.

Hepatoprotective Effects

There is some evidence to suggest that dang gui and its constituents can decrease portal hypertension in patients with liver cirrhosis without affecting systemic hemodynamics. This use is consistent with the traditional actions of dang gui in improving circulation, because portal hypertension is thought to be due to the obstruction of hepatic microcirculation (70,71).

Hormonal Effects and Effects on Menopausal Symptoms

Because of the putative effects of dang gui in gynecological imbalances, various studies have investigated its potential for eliciting hormonal effects. In one human study (72), one of the few double-blind, placebo-controlled trials with dang gui, no statistically significant differences in endometrial thickness, vaginal cell maturation, or menopausal symptoms were observed between subjects taking dang gui and those taking placebo. This contrasts with a study of ovariectomized rats which showed that an extract of dang gui (300 mg/kg SC ; 1% ligustilide) resulted in a thickening of the luminal epithelium suggesting estrogenic activity, but one much lower than comparison with estradiol (see gynecological effects).

Analgesic Effects

Two uncontrolled clinical trials were found that addressed the traditional Chinese use of dang gui as an analgesic for pain due to "blood stasis"; both used injectable preparations. In one, an ethanol extract was administered (intramuscularly) on alternate days for a total of 20 doses into the pterygoideus externus of 50 patients with temporomandibular joint syndrome. A 90% cure rate was claimed (73). Thirty cases of refractory interspinous ligament injury were treated by local injection of 2 mL of 5% or 10% dang gui twice weekly for two to three weeks. Twenty-four (80%) of these patients reported a disappearance of pain, no tenderness, and the ability to work as usual; four (13%) patients reported alleviation of pain; two (7%) reported no improvement (74). These uses are consistent with the traditional use of dang gui in TCM. However, the effects of injectable preparations cannot be extrapolated to oral use of dang gui.

Dosages

- Crude herb: 6 to 12 g daily to be prepared as a decoction.
- Fluid extract (1:1): 3 to 5 mL three times daily (75).

SAFETY PROFILE

Side Effects

On the basis of a review of the available traditional and scientific data, dang gui is a very safe herb with a low probability of side effects when used within its normal dosage range. One review article that claimed to cover 200 reports on dang gui pharmacology stated that dang gui had no major side effects (35). Individual case reports regarding the potential of dang gui to promote bleeding have been prepared.

Contraindications

On the basis of a review of the available literature and the experience of practitioners, dang gui is contraindicated prior to surgery and, generally speaking, in those with bleeding disorders.

Precautions

Precautions regarding the use of dang gui and other botanicals used in traditional systems of medicine must be differentiated between those recognized in the scientific literature and those recognized traditionally. There is evidence suggesting an anticoagulant effect for dang gui, and there

are two published reports on its ability to enhance the effects of chronic treatment with warfarin (see interactions). A few unpublished case reports suggest that high doses or chronic administration of dang gui alone during pregnancy may be associated with miscarriage. There are also anecdotal reports of administration of dang gui alone causing increased blood flow during menses (R.U., personal communication). Therefore, patients should consult with a qualified health care professional prior to using dang gui if they have bleeding disorders, are using anticoagulant medications, or wish to use it during menses or in the first trimester of pregnancy. It must, however, be noted that in TCM, dang gui is specifically indicated for certain bleeding disorders that are due to an underlying diagnosis of blood stasis and in certain cases of threatened miscarriage. For such uses, dang gui must be used according to TCM principles under the guidance of a qualified TCM practitioner.

Interactions

Two reports are available suggesting that dang gui can enhance the effects of the anticoagulant warfarin. According to one of these, a 46-year-old woman with atrial fibrillation who had been stabilized on warfarin for almost two years (5 mg daily) consumed a dang gui product concurrently for four weeks (565–1130 mg daily). She experienced a greater than twofold elevation in prothrombin time (from 16.2 to 27 sec) and international normalized ratio (from 2.3 to 4.9). No other cause for this increase could be determined. Within one month of discontinuing dang gui use, coagulation values returned to acceptable levels (76).

An animal study investigated the interaction of dang gui and a single dose or a steady-state dose of warfarin (77). Six rabbits were administered a single dose of warfarin (2 mg/kg SC). Seven days later, the same animals were given an aqueous extract of dang gui (2 g/kg PO, twice of a 2 g/mL extract daily) for three days, after which they were again given a single dose of warfarin. Plasma warfarin concentrations were measured at intervals up to 72 hour after each warfarin dose, and prothrombin time was measured daily during dang gui treatment and after the warfarin doses. Mean prothrombin time did not change significantly during the dang gui treatment period. However, when measured after coadministration of dang gui and warfarin, prothrombin time was significantly lowered at 24, 36, and 48 hours compared to that with warfarin treatment alone ($P < 0.05$ or $P < 0.01$). No significant variations in the single dose pharmacokinetic parameters of warfarin were observed after treatment with dang gui. Hence, the mechanism of decrease in prothrombin time could not be correlated to the pharmacokinetics of warfarin. Another group of six rabbits was given 0.6 mg/kg of warfarin SC daily for seven days; a steady-state plasma concentration was achieved after day 4. On days 4, 5, and 6, the rabbits were treated as above with dang gui. Mean prothrombin time was again significantly increased after coadministration with dang gui and two rabbits died at days 6 and 7 after the dang gui treatment had begun. Plasma warfarin levels did not change after dang gui treatment. The authors suggested that these results indicate that precautionary advice should be given to patients who medicate with dang gui or its prod-

ucts while on chronic treatment with warfarin. Another study reported that dang gui acted synergistically with aspirin (24).

General enhancement of cytochrome P450 isoforms has been reported for both water (CYP2D6 AND 3A) and ethanol extracts (CYP2D6) of dang gui in animal models (78).

One study reported that dang gui might enhance the antitumor effect of cyclophosphamide in mice with transplanted tumors (79).

Pregnancy, Mutagenicity, and Reproductive Toxicity

Because of its blood-moving properties, dang gui should be used in pregnancy only under the supervised care of a qualified health professional. According to TCM practice, dang gui is used in combination with other herbs in various stages of pregnancy (29). Formula traditionally used in pregnancy are prescribed within the context of specific diagnoses in which the use of dang gui in pregnancy is clearly indicated. In the West, dang gui is often used alone out of this traditional medical context. Because of this, several Western sources consider dang gui to be contraindicated in pregnancy. Data regarding the effect of dang gui preparations on the fetus are lacking.

Lactation

There are three unpublished case reports of a rash in infants of lactating mothers who were taking dang gui. The rashes reportedly resolved upon discontinuation of the preparation by the mother. Specific details regarding the preparations used were lacking (Romm, August 1, 2002, oral communication to AHP). Dang gui is a member of the botanical family Apiaceae, a group of plants that contain many types of photoreactive compounds known to cause rashes.

Carcinogenicity

Data regarding the effects of dang gui in relationship to carcinogenicity are mixed with both tumorigenic and antitumorigenic activity reported. Antitumor activity due to an induction of cell cycle arrest and apoptosis has been reported for ligustilide (62). Polysaccharides have been shown to inhibit growth of murine tumors (S180, EAC, L1210) in vivo. This was accompanied by a prolonged survival of animals and an inhibition of metastasis in vitro (63). Another animal study identified a possible antitumor effect of dang gui applied to mice with Ehrlich ascites tumors (80). Regarding the potential effects of dang gui on estrogen-positive tumors the data are mixed. One in vitro assay found that dang gui stimulated the growth of MCF-7 breast cancer cell lines 16-fold, with no measurable effect on estrogen receptors (81), while another found a possible antitumor effect in T-47D and MCF-7 cell lines (82). Data regarding the potential estrogenic effects of dang gui have been mixed.

Influence on Driving

On the basis of the experience of modern herbal practitioners, no negative effects are to be expected.

Overdose

On the basis of the available literature, its use as a "food" ingredient in soups, and the experience of modern herbal

practitioners, dang gui appears to be safe when used at recommended doses.

Treatment of Overdose

No data available.

Toxicology

The following lethal dose (LD₅₀) values have been reported for dang gui extract (8:1 or 16:1), 100 g/kg PO in rats (83,84); dang gui aqueous extract, 100 g/kg IV in mice (85); dang gui 50% ethanol extract, greater than 40 g/kg PO in mice (86); dang gui total acids, 1.05 ± 0.49 g/kg IP in mice (87). The LD₅₀ of ferulic acid IV in mice was reported to be 856.6 mg/kg, (16) and that of ligustilide, approximately 410 mg/kg IP (88). In a review of the toxicology literature on dang gui, it was reported that IV injection of the volatile fraction of dang gui could cause kidney degeneration (76).

REGULATORY STATUS

Regulated as a dietary supplement (USC 1994).

CONCLUSIONS

Dang gui is one of the most important herbal drugs in TCM, primarily being used for blood tonification and the treatment of gynecological disorders. More recently, interest has focused on dang gui's possible cardiovascular, hepatoprotective, hematopoietic, antioxidant, antispasmodic, and immunomodulatory effects. Despite its long tradition of use and current widespread clinical utility, there has been very little clinical work verifying the therapeutic efficacy of dang gui when used alone, primarily due to the fact that in TCM, botanicals are generally used in combinations rather than as single agents.

On the basis of the literature available and keeping many of its limitations for an English readership in mind, there is limited clinical support for the use of dang gui alone for the following indications: pulmonary artery and portal hypertension, acute ischemic stroke, dysmenorrhea, infertility, and pain due to injury or trauma. The use of dang gui for most of these indications is consistent with TCM. One trial on menopausal symptoms found no effect of dang gui on hormonal activity. Most of the trials available are of poor methodological quality.

Clinical and preclinical studies provide some support for a wide variety of actions of dang gui. These include the promotion of circulation, vasodilation/relaxation, and the inhibition of platelet aggregation, all of which are consistent with the "blood quickening" properties ascribed to dang gui in TCM. Similarly, the hematopoietic effect of dang gui is consistent with its use in TCM to "nourish blood." Its smooth muscle (uterus, vessels, trachea) relaxant effects are consistent with its use for dysmenorrhea, asthma, and coughing. Dang gui may relax or stimulate the uterus depending on a variety of factors. In general, the volatile oil fraction appears to be a uterine relaxant, while the nonvolatile constituents appear to stimulate contractions. There is some support for the traditional use of dang gui as an analgesic and vulnerary. The radiation protective effect of dang gui in animals

is most likely due to its antioxidant activity. Assays for an estrogenic effect of dang gui have had mixed, but largely negative, results. The relevance of many of these actions to the therapeutic use of dang gui in humans has not yet been demonstrated.

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Dehydroepiandrosterone

Salvatore Alesci, Irini Manoli, and Marc R. Blackman

INTRODUCTION

DHEA is the acronym used to designate the hormone “dehydroepiandrosterone,” also referred to as “prasterone.” The chemical name for DHEA is 5-androsten-3 β -ol-17-one (Fig. 1).

DHEA is available as an over-the-counter dietary supplement in the United States, as the Food and Drug Administration did not include it in the 2004 Anabolic Steroid Control Act, which reclassified other related agents as anabolic steroids and hence, controlled substances. As a dietary supplement, it is marketed under different trade names (e.g., Nature’s Blend DHEA, Nature’s Bounty DHEA, DHEA Max, DHEA Fuel, etc.). A pharmaceutical-grade preparation, currently available only for experimental use, has been assigned the trade name Prestara (previously known as Aslera or GL701).

HISTORICAL OVERVIEW AND GENERAL DESCRIPTION

Discovered in 1934, DHEA is the most abundant steroid hormone, and is produced by the adrenal glands in humans and other primates. It acts as a weak androgen and serves as a precursor of other steroids including more potent androgens and estrogens. To date, however, the exact functions of this hormone remain unknown. DHEA is broadly traded on the Internet, under the claim of being a “marvel hormone.” Despite the growing popularity of its use, there is insufficient scientific evidence supporting the purported potential health benefits, and little information regarding the potential short- and long-term adverse risks of consuming exogenous DHEA. Moreover, variations in quality control and manufacturing practices of dietary supplements result in differences in concentrations and purity of the marketed compounds, and insufficient surveillance for side effects.

BIOCHEMISTRY AND PHYSIOLOGY

Biosynthesis and Metabolism

DHEA is primarily produced in the zona reticularis of the adrenal cortex. In healthy women, the adrenal gland is the principal source of this steroid, whereas in men, 10% to 25% of the circulating DHEA is secreted by the testes (1). It can also be synthesized within the central nervous system (CNS), and can be considered a “neurosteroid” (2). Pregnenolone, the immediate precursor of

DHEA, is derived from cholesterol through the action of the cytochrome P450 side-chain cleavage enzyme (CYP-scc). It is converted into DHEA by cytochrome P450 17 α -hydroxylase (CYP17), while hydroxysteroid sulfotransferase (DHEAST) catalyzes the transformation of DHEA into its 3-sulfated metabolite DHEAS (Fig. 2). This can be converted back to DHEA by the action of sulfohydrolases (DHEASH), located in the adrenal gland and peripheral tissues.

Human plasma contains DHEA-fatty esters (DHEA-FA), which are formed from DHEA by the enzyme lecithin-cholesterol acyltransferase. Newly formed DHEA-FA are incorporated into very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) and may be used as substrates for the synthesis of active oxidized and hydroxylated metabolites in the periphery, such as 7 α / β -hydroxy-DHEA in the brain, and androstenedione, androstenediol, and androstenetriol in the skin and immune organs (3,4).

Regulation of DHEA Production

Release of DHEA by the adrenals is mostly synchronous with that of cortisol, under the stimulus of the hypothalamic corticotrophic-releasing hormone (CRH) and pituitary adrenocorticotrophic hormone (ACTH). However, the finding of dissociation between DHEA and cortisol secretion during several physiologic and pathophysiologic states suggests that other non-ACTH-mediated mechanisms may be involved in the modulation of DHEA secretion (Table 1).

Estrogens, growth hormone, insulin, and prolactin stimulate DHEA secretion by human adrenal cells. However, these findings have not always been replicated in animal or clinical studies. A complex intra-adrenal network involving vascular and nervous systems, local growth and immune factors, and a “cross talk” between cells of the cortex and medulla, the other component of the adrenal gland, also regulate DHEA secretion. The existence of a specific “adrenal androgen-stimulating hormone” has also been postulated, but remains controversial (5).

Adrenarche and Adrenopause

At birth, DHEAS is the predominant circulating steroid. However, a dramatic involution of the fetal adrenal zone, starting in the first postnatal month and continuing through the first year of life, is paralleled by a sudden decrease in DHEA/DHEAS, which remains unchanged for the next six years. By the age of six to eight years, the adrenal gland matures, culminating in the creation of

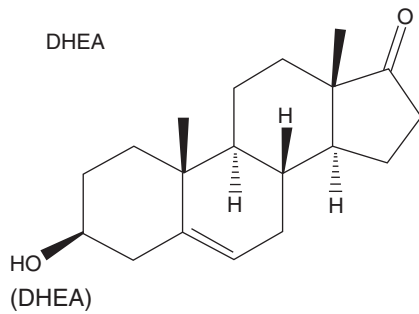


Figure 1 Chemical structure of dehydroepiandrosterone (DHEA).

the zona reticularis, followed by an abrupt elevation in DHEA and DHEAS concentrations, termed the “adrenarche” (6). Peak concentrations of DHEA (180–800 ng/dL) and DHEAS (45–450 μ g/dL) are reached during the third decade of life. Subsequently, there is a progressive 2% decline per year in DHEA and DHEAS secretion and excretion, with concentrations equal to 20% of the peak by the age of 80, and values lower in women than in men (5,7). This marked decline has been termed “adrenopause.” DHEA and DHEAS levels are higher in men than in women at all ages.

Mechanisms of Action

Despite the identification of high-affinity binding sites for DHEA in rat liver, T-lymphocytes, and endothelial cells, the search for a specific, cognate DHEA receptor has been unsuccessful. Multiple mechanisms of action have been proposed for DHEA. Most important among these are that DHEA can be metabolized into more potent androgens [testosterone and dihydrotestosterone (T and DHT)] and estrogens (estradiol and estrone) in the periphery, which

Table 1 Dissociation of Cortisol and DHEA/DHEAS Secretion During Physiological and Pathological Conditions

Condition	DHEA	DHEAS	Cortisol
Physiological (age-related)			
Fetal stage		↑	N
Birth		↑	N
Infancy and childhood		↓	N
Adrenarche (6–8 yr)	↑	↑	N
Puberty	↑	↑	N
Adrenopause (50–60 yr)	↓	↓	N or ↑
Pathological			
Anorexia nervosa	↓	↓	↑
Chronic/severe illness		↓	↑
Burn trauma		↓	↑
Cushing disease	N	N	↑
Congenital adrenal hyperplasia		↑	↓
Ectopic ACTH syndrome	N, ↑, or ↓	N, ↑, or ↓	↑
Idiopathic hirsutism		↑	N
Partial hypopituitarism without ACTH deficiency	↓	↓	N
End-stage renal diseases		↓	↑
Stress	↓	↓	↑

N = normal serum levels; ↑ = increased serum levels; ↓ = decreased serum levels.

Source: From Ref. 5.

can interact with specific androgen and estrogen receptors. DHEA itself can bind to the androgen and estrogen receptors, but its affinity is extremely low compared with those of the native ligands. It has been estimated that DHEA and DHEAS function as precursors of 50% of androgens in men, 75% of active estrogens in premenopausal women, and 100% of active estrogens in postmenopausal women (8). Lipophilic DHEA, but not hydrophobic DHEAS, can be converted into both androgens and estrogens intracellularly in target tissues by “intracrine” processes (4). This conversion depends on the levels of different steroidogenic and metabolizing enzymes, and on the hormonal milieu. For example, DHEAS and sulfatase are present in high concentrations in the prostate, and the resultant metabolism of DHEA to DHT accounts for up to one-sixth of the intraprostatic DHT (9).

DHEA can also function as a neurosteroid, by modulating neuronal growth, development, and excitability, the latter via interaction with γ -aminobutyric acid ($GABA_A$), *N*-methyl-D-aspartate (NMDA), and sigma receptors (10). It is known to be a potent inhibitor of glucose-6-phosphate dehydrogenase (G6DPH), thus interfering with the formation of mitochondrial nicotinamide adenine dinucleotide phosphate [NADP(H)] and ribose-6-phosphate and inhibiting DNA synthesis and cell proliferation (11). The steroid hormone has also been proposed to exert antigluccorticoid, cytokine modulatory, potassium channel and cyclic guanylyl monophosphate (cGMP) stimulatory, and thermogenic effects.

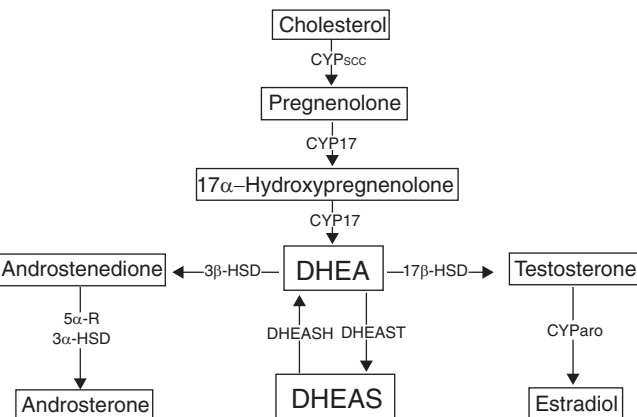


Figure 2 Schematic diagram of DHEA/DHEAS biosynthesis and metabolism. Abbreviations: CYPsc, cytochrome P450 side-chain cleavage enzyme; CYP17, cytochrome P450 17 α -hydroxylase; 3 β /17 β /3 α -HSD, 3- β /17- β /3 α hydroxysteroid dehydrogenase; 5 α -R, 5- α reductase; DHEASH, DHEA sulfohydrolase; DHEAST, DHEA sulfotransferase; CYParo, cytochrome P450 aromatase.

PHARMACOKINETICS

Absorption and Tissue Distribution

While DHEA is marketed as an oral product, it has also been shown to be absorbed when administered by the transdermal, intravenous, subcutaneous, and vaginal

routes. Crystalline and micronized formulations result in higher DHEAS serum concentrations, possibly due to an enhanced rate of absorption (12). After absorption in the small intestine, DHEA is mainly sulfated in the liver. The nonoral route averts first pass liver degradation, resulting in higher serum levels. DHEA concentrations are high in the brain, with a brain-to-plasma ratio of 4–6.5:1 (13) and plasma, spleen, kidney, and liver concentrations follow in descending order. Cerebrospinal, salivary, and joint fluid levels are directly related to those of serum.

Bioavailability, Metabolism, and Clearance

Pharmacokinetic studies on DHEA reveal a clearance rate compatible with a two-compartment model. The initial volume of distribution is 17.0 ± 3 L. DHEA disappears from the first compartment in 17.2 ± 6.2 minutes and from the second in 60.2 ± 12.3 minutes (14). DHEAS follows a one-compartment model of disappearance. Its volume of distribution is 4.6 ± 0.9 L, while the half-life from that compartment is 13.7 hours (15). In men, 77.8 \pm 17.3% of the DHEAS that enters the circulation will reappear as DHEA, while in women, it is 60.5 \pm 8.2%. The opposite conversion of DHEA to DHEAS is much smaller, 5.2% \pm 0.7% in men, and 6.25% \pm 0.54% in women.

Mean metabolic clearance rates (MCRs) were calculated using the constant infusion technique: The DHEA MCR is 2050 ± 160 L/day in men and 2040 ± 160 L/day in women, whereas the MCR for DHEAS is 13.8 ± 2.7 L/day in men and 12.5 ± 1.0 L/day in women. The differences in the clearance rates of DHEA and DHEAS are partly explained by different binding efficiencies with albumin. Circulating DHEA is primarily bound to albumin, with only minimal binding to sex hormone-binding globulin (SHBG); the remaining small amount is free. There is no known specific DHEA-binding protein. In comparison, DHEAS is strongly bound to albumin but not to SHBG, and an even smaller amount is protein free. Obesity results in increased MCR for DHEA from 2000 to 4000 L/day in women. A rise in MCR is also caused by insulin infusion in men (16).

Supplementation

Considerations related to the metabolism of DHEA become more complicated when it is administered as a dietary supplement (in the United States) or as a drug (in some other countries). The steroid is usually given orally in a single morning dose, as its constant interconversion to DHEAS and the long half-life of DHEAS make multiple dosing unnecessary. In addition, morning dosing mimics the natural rhythm of DHEA secretion. Doses ranging from 25 to 1600 mg/day have been used in different studies.

After an oral dose, the half-lives of DHEA/DHEAS are longer (24 hours) than those reported in intravenous tracer studies, which may reflect the conversion of DHEAS to DHEA (17). Oral administration of 20 to 50 mg of DHEA in patients with primary or secondary adrenal insufficiency restores serum DHEA and DHEAS concentrations to the range observed in normal young individuals, while a dose of 100 to 200 mg/day results in supraphysiological concentrations.

Different metabolic pathways for exogenous DHEA in relation to gender and age have been reported. DHEA levels after oral administration of 25 or 50 mg DHEA for eight days were persistently higher in women versus men (17). Similarly, oral administration of 200 mg of micronized DHEA in single or multiple doses for 15 days in healthy adult men and women resulted in higher serum concentrations and bioavailability (measured by DHEA C_{\max} and AUC) in women. The net increase in DHEAS levels was 21-fold in women and 5-fold in men (18). The metabolic fate of exogenous DHEA also differs by gender and age. While in pre- and postmenopausal women, DHEA is mostly transformed into androgens, in men it is preferentially metabolized into estrogens. Higher serum concentrations of DHEA, testosterone, and estradiol are achieved in elderly subjects (19).

THERAPEUTIC APPLICATIONS

DHEA Replacement in Adrenal Insufficiency

Patients with primary (Addison disease) and secondary adrenal insufficiency, typically exhibit very low, often undetectable, serum concentrations of DHEA(S) compared with values in age-matched control subjects. Despite optimal glucocorticoid and mineralocorticoid replacement, however, these patients often experience chronic fatigue, reduced sense of well-being, and lack of sexual interest.

Some studies have suggested that DHEA replacement exerts a positive effect on mood, vitality, sexuality, and overall well-being mostly in women and, to a lesser extent, in men, with chronic adrenal insufficiency (20–23). For example, oral administration of 50 mg/day of DHEA for four months to 24 women with adrenal insufficiency increased serum levels of DHEAS, androstenedione, testosterone, and androstenediol glucuronide, and improved overall well-being, mood, and sexual activity (20). In another study of 15 men and 24 women with Addison disease, administration of 50 mg/day of DHEA for three months corrected the hormonal deficiency and improved self-esteem, while it tended to enhance overall well-being, mood, and energy (21). However, other studies have failed to show similar effects (24–26). Moreover, a meta-analysis of 10 randomized placebo-controlled studies concluded that DHEA may improve health-related quality of life and depression in women with adrenal insufficiency, though the effect size (0.21; 95% confidence interval, 0.08–0.33; inconsistency (I^2) = 32%) was too small to support the routine use of DHEA in this patient group (27).

In the earlier studies, a single oral dose of DHEA (25–50 mg) was sufficient to keep serum concentrations of DHEA within the normal range, while signs of over-replacement included acne, hirsutism, or alopecia. Moreover, concerns have been raised about the effects of DHEA supplementation on the lipid profile and specifically on high-density lipoproteins (HDL) levels in hypoadrenal women (28), though it has been reported to improve their insulin resistance (29).

Because a pharmaceutically controlled DHEA preparation, as well as proof of effectiveness and safety in long-term phase III trials, are still lacking, replacement of DHEA in patients with adrenal insufficiency should be

restricted to those individuals whose well-being or libido is severely impaired despite adequate glucocorticoid and mineralocorticoid replacement.

DHEA Replacement in Adrenopause and Age-Related Disorders

As noted, aging is accompanied by profound decreases in circulating concentrations of DHEA and DHEAS in both sexes (5,7). Epidemiological studies suggest an association between the DHEA and DHEAS declines and the adverse effects of aging, albeit with gender differences.

One large, prospective observational study reported a small, but significant, inverse correlation between serum DHEAS concentrations and risk of cardiovascular mortality in men at 19-year follow-up, whereas in women, high DHEAS levels were associated with an increased risk of cardiovascular death at 12-year follow-up, and this trend lost significance at 19-year follow-up (30). Similar results were reported in another study of 963 men and 1171 women aged >65 years: all-cause and cardiovascular mortality were highest in men with DHEAS levels in the lowest quartile, whereas no significant association between circulating DHEAS and mortality was found in women (31). Other studies failed to demonstrate this inverse relationship in men (32). Positive correlations between low circulating DHEAS concentrations and depressed mood and bone loss have been reported in aged women (33). In comparison, DHEAS levels are reduced in men, with noninsulin-dependent diabetes mellitus (NIDDM) (34). Reports of an association between low DHEA levels and Alzheimer disease are conflicting.

It remains uncertain as to whether the DHEAS decline is simply a biomarker of aging, or is causally related to morbidity and mortality in the elderly. One study of the effects of oral administration of 50 mg/day of DHEA for six months to 13 men and 17 women aged 40 to 70 years showed restoration of DHEA and DHEAS levels to young-adult values, with improvement in physical and psychological sense of well-being, but not sexual interest, in both genders using a questionnaire for self-assessment. These effects were accompanied by increased serum levels of insulinlike growth factor 1 (IGF-I), reduced IGF-I binding protein-1 (IGFBP-1), and a significant decrease in apolipoprotein A1 and HDL-cholesterol in women, but no change in insulin sensitivity and body composition (35). Another study using 100 mg/day of DHEA for six months in 9 men and 10 women aged 50 to 65 years reported decreased fat mass and enhanced muscle strength in men, whereas increased levels of downstream androgens were detected in women (36). In a third study of 39 elderly men treated for three months with 100 mg/day of oral DHEA, no treatment effect on body composition or subjective well-being was found, whereas a significant reduction in HDL-cholesterol was reported (37).

In the largest study to date, 280 men and women aged 60 to 79 years were treated for 12 months with 50 mg/day of oral DHEA. No improvement in well-being was detected using a variety of validated tools. In addition, there were no significant changes in body composition, metabolic parameters, or muscle strength. However, a slight but significant increase in bone mineral density (BMD) at the femoral neck and the radius,

and an increase in serum testosterone, libido, and sexual function were observed in women >70 years old (38). Similar changes were reported in 14 women aged 60–70 years who were treated for 12 months with a 10% DHEA skin cream. In addition to a 10-fold increase in DHEA levels, the authors described increased BMD at the hip, and decreased osteoclastic and increased osteoblastic bone markers. Other changes included improved well-being, a reduced skinfold thickness, and lower blood glucose and insulin levels, with no adverse change in lipid profile (39). DHEA replacement did not affect BMD in other studies (36).

Little information is available regarding the effects of DHEA therapy on cardiovascular function and insulin sensitivity from interventional studies, other than that provided in the preceding text. The effects of DHEA treatment on lipid profile were evaluated in more than a dozen studies performed in men and women with various doses and routes of administration. The majority of these studies showed no effect of DHEA on plasma lipids (40).

In one study of 24 middle-aged men, administration of 25 mg/day of oral DHEA for 12 weeks decreased the plasma levels of plasminogen activator inhibitor type 1 (PAI-1), and increased dilatation of the brachial artery after transient occlusion (41).

In rodent models of NIDDM, dietary administration of DHEA consistently induced remission of hyperglycemia and increased insulin sensitivity. Some clinical studies in aged men and women have shown improved insulin sensitivity after DHEA replacement (39,42), whereas others have not confirmed those findings in women (35,43).

The effects of DHEA supplementation on cognitive function in healthy elderly people were recently reviewed in depth by Grimley et al. All randomized placebo-controlled trials enrolling people aged >50 without dementia to whom DHEA had been administered for more than one day were considered for inclusion. Five studies provided results from adequate parallel-group data. The authors concluded that findings from these controlled studies did not support any beneficial effect of DHEA supplementation on cognitive function in nondemented elderly people (44). Moreover, DHEA replacement did not affect memory in any of the controlled studies in healthy elderly, as previously discussed. Therefore, the use of DHEA supplementation to improve cognitive function should not be recommended in the elderly population at this time.

Potential Beneficial Effects of DHEA Supplementation

DHEA administration, often in large doses, has been proposed in the management of numerous disorders, including obesity, cancer, autoimmune diseases, AIDS, mood disorders, as well as in enhancing physical performance. The scientific evidence supporting the benefits of DHEA therapy in these conditions is, however, very limited.

Pharmacologic treatment with DHEA in mice genetically predisposed to become obese reduced weight gain and fat cell size. In obese rats, the steroid decreased food intake by 50%. In humans, some observational studies indicate a relationship between circulating DHEA and

DHEAS levels, body mass index (BMI) and weight loss, whereas others do not confirm this (45). Similar inconsistencies are encountered in interventional studies. Administration of a high oral dose of DHEA (1600 mg/day) decreased body fat and increased muscle mass, with no net change in body weight, in a small group of healthy young men. As mentioned earlier, a reduction in fat mass was also reported in healthy elderly men after six months administration of 100 mg/day of DHEA (36), whereas topical application of a 10% DHEA cream for one year decreased femoral fat and skinfold thickness in postmenopausal women (39). Other studies in healthy elderly individuals and in obese men failed to demonstrate changes in body fat after DHEA treatment (37,43).

DHEA has been reported to exhibit chemopreventive activity in mouse and rat models, although it has also been found to be hepatocarcinogenic in rats. Epidemiological research has revealed increased DHEA levels to be associated with a rise in risk of ovarian cancer and breast cancer in postmenopausal women, whereas decreased levels are linked with increased risk of bladder, gastric, and breast cancer in premenopausal women. To date, we are unaware of clinical studies documenting the effects of DHEA intervention on cancer initiation or propagation. Fluorinated DHEA analogs that cannot be converted into androgens or estrogens appear to have antiproliferative effects and have been tested in several pre-clinical cancers including bowel polyposis (46) and used without side effects in doses up to 200 mg/day orally for four weeks (47).

Increased antibody production in response to bacterial infections and decreased mortality from endotoxic shock were reported in DHEA-treated mice. In a study of 71 aged individuals, however, DHEA administration did not enhance antibody response to influenza vaccine (48). In contrast, 50 mg/day of oral DHEA increased the activity of natural killer cells by twofold, with a concomitant decrease in T-helper cells in postmenopausal women (49). In 28 women with mild-to-moderate systemic lupus erythematosus (SLE), oral treatment with 200 mg/day of DHEA for three to six months improved well-being and decreased disease activity and prednisone dosage requirements. The same DHEA dose administered orally for 56 days to patients with Crohn disease or ulcerative colitis decreased disease activity in a small, uncontrolled study (50). DHEA did not separate itself from placebo in a randomized controlled trial in patients with Sjögren syndrome (51). The effect of DHEA supplementation in SLE was recently reviewed more systematically. Analysis of the results from seven randomized placebo-controlled trials of at least three months duration revealed a modest but clinically significant beneficial effect of DHEA on measures of quality of life in SLE patients, whereas effects on measures of disease activity were inconsistent (52).

Serum DHEA and DHEAS levels in patients infected with HIV are directly related to CD4 cell counts and disease stage and progression. This observation has stimulated self-administration of DHEA as an adjunct to antiviral treatment by AIDS patients. In the only published placebo-controlled trial of DHEA in patients with advanced HIV disease, treatment with 50 mg/day orally

for four months resulted in increased DHEAS levels and improved mental function, with no change in CD4 cell count (53). These results were consistent with those from a previous open label study in 32 HIV-positive patients treated with DHEA doses of 200 to 500 mg/day for eight weeks (54).

Studies in adults and adolescents with major depressive disorders have revealed a blunted DHEA circadian variation, with low DHEA and high cortisol/DHEA ratio at 8:00 AM. In 22 patients with medication-free or stable major depression, supplementation with 30 to 90 mg/day of oral DHEA for six weeks decreased Hamilton depression scale scores as much as 50% (55). Similar results were reported in a well-controlled study in 15 patients aged 45 to 63 years with midlife-onset dysthymia who, after a three-week administration of 90 mg of DHEA, reported improvements in depressive symptoms (56). In a recent double-masked trial in schizophrenic patients with predominant negative symptoms, supplementation with 100 mg/day of DHEA also led to improvement in depression and anxiety (57). DHEA administration improves memory test results and decreases serum levels of β -amyloid in aging mice. In contrast, treatment with 100 mg of oral DHEA did not improve cognitive performance in patients with Alzheimer disease, similar to the findings reported in healthy elderly subjects (58).

DHEA is a popular dietary supplement among athletes. Nevertheless, at 150 mg/day orally, it did not improve body mass or strength in young male athletes and weight lifters (59,60). In contrast, increased quadriceps and lumbar strength were reported in healthy elderly men on DHEA replacement (36), and an increase in lean body mass of 4.5 kg was observed in healthy young men taking 1600 mg/day of DHEA for four weeks (61).

A summary of the various potential therapeutic applications of DHEA replacement/supplementation is presented in Table 2.

Table 2 Clinical Conditions for Which DHEA Use Has Been Proposed

Condition	Effect	References
Adrenal insufficiency	Improved general well-being, mood, sexual function	20-23
	No significant effects	24-27
Aging	Improved physical well-being, bone mineral density or sexual function	35,38,39
	Decrease in HDL or no effects	36,37
Autoimmune disease	Improved well-being, fatigue, disease activity in SLE patients, enhanced immune response	52
	No effect in HIV patients	53,54
Body composition	Decreased fat and increased muscle mass	36,61
	No changes	37,43
Insulin resistance	Improved insulin sensitivity	39
	No change	35
Depression	Improved mood	55-57
Alzheimer disease	No effect on cognitive function	58
Cardiovascular	Improved endothelial function	38
	No effect on lipids	40

CLINICAL PHARMACOLOGY AND TOXICOLOGY

Dietary/Nondietary Sources and Available Preparations

There are no known dietary sources of DHEA, although it was suggested that the supplement chromium picolinate could stimulate endogenous DHEA secretion. The Mexican plant "wild yam" contains some natural DHEA precursors, which cannot be converted into DHEA. However, sterol extracts of "wild yam" (such as diosgenin and dioscorea) are used to produce various forms of synthetic DHEA, including tablets, capsules, injectable esters (Gynodian Depot, Schering), sublingual and vaginal preparations, topical creams, lozenges, and herbal teas. DHEA is usually sold in tablets of 5 to 50 mg. A pharmaceutical-grade preparation (Prestara) has been developed for potential use as a prescription drug. During the manufacture of DHEA, other steroid-like compounds, like androstenedione, may be produced and could contaminate DHEA. Moreover, the real steroid content of the DHEA preparation sold over the counter may vary from 0% to 150% of the amount claimed (62). In addition, there is a lack of information about comparability or bioequivalence among the many products on the market or information about lot-to-lot variability of any particular product in terms of characterization (content) and standardization (contaminants).

Dosage and Administration

There is no consensus on a recommended dietary allowance (RDA) or optimal treatment dose for DHEA. Replacement with oral doses of 20 to 50 mg/day for men and 10 to 30 mg/day for women appears adequate to achieve DHEA/DHEAS levels similar to those in young adults, as suggested by most studies in subjects with adrenal or age-related DHEA/DHEAS deficiency, though oral doses as low as 5 mg/day have been reported to be effective. Higher DHEA doses may be necessary for patients with very low endogenous DHEA levels secondary to glucocorticoid administration or chronic disease. Doses of 200 to 500 mg and 200 mg/day have been used in patients with HIV and SLE, respectively. Serum DHEAS levels and its androgenic and estrogenic metabolites must be closely monitored during replacement to enable appropriate dose adjustments. Rigorous dose ranging studies are needed to determine the optimal doses to achieve a beneficial effect.

Adverse Reactions, Long-Term Effects, and Contraindications

DHEA appears to elicit few short-term side effects when used in the recommended doses. Women may experience mild hirsutism, increased facial sebum production, and acneiform dermatitis. Circulating concentrations of downstream androgens rise above young-adult values in healthy elderly women treated with 100 mg/day DHEA; however, the long-term consequences of this increase are unknown. No significant changes in complete blood count, urinalysis, hepatic, and thyroid indices were found in women after 28 days of treatment with 1600 mg DHEA/day (43). A dose escalation study of 750 to 2250 mg

oral DHEA conducted in 31 HIV-positive men revealed no serious dose-limiting toxicity (63).

Because of its potent androgenic and estrogenic effects, it would appear prudent to avoid DHEA replacement/supplementation in individuals with a personal or family history of breast, ovarian, or prostate cancer. This would seem especially important for postmenopausal women, considering the demonstrated direct correlation between circulating concentrations of DHEA and breast and ovarian cancers in this group (46), and the reported increase with DHEA supplementation in free IGF-1 (64). Caution may also be appropriate in HIV patients, since high DHEA levels have been implicated in the pathogenesis of Kaposi's sarcoma. Moreover, increased insulin resistance and decreased cholesterol and HDL after administration of a high dose 1600 mg/day of DHEA for four weeks have been reported in aged women (43).

DHEA administration is not recommended for people younger than 40 years of age, unless there is a documented deficiency state. It should be avoided during pregnancy, lactation, and in persons younger than 18 years, as dosage and safety of the treatment under these conditions have not been evaluated.

Known Drug Interactions

Drugs known to interfere with DHEA and/or DHEAS include various hormone preparations, drugs acting on the CNS, cardiovascular drugs, adrenergic and adrenergic blocking agents, and anti-infective agents. Synthetic glucocorticoids such as dexamethasone are the most potent suppressors of DHEA and DHEAS. DHEA and/or DHEAS levels are known to be decreased in patients taking aromatase inhibitors, oral contraceptives, dopaminergic receptor blockers, insulin, troglitazone, and multivitamins, or fish oil. They are also decreased due to the induction of the cytochrome P450 enzymes, by carbamazepin, phenytoin, or rifampicin. Metformin (a biguanide antihyperglycemic drug) and calcium channel blockers are shown to increase DHEA and DHEAS levels. The effect of alcohol is controversial (65).

Compendial/Regulatory Status

In the early 1980s, DHEA was widely advertised and sold in U.S. health food stores as an "anti-aging," "anti-obesity," and "anti-cancer" nonprescription drug. However, on the basis of unknown potential long-term risks, and following the ban by the International Olympic Committee, in 1985 the U.S. Food and Drug Administration reclassified DHEA as a prescription drug. In October 1994, the U.S. Dietary Supplement Health and Education Act allowed DHEA to be sold again as an over-the-counter dietary supplement, as long as no claims are made regarding therapeutic efficacy. DHEA remains available over the counter in the United States, as it was not reclassified as an anabolic steroid, and hence a controlled substance, in the 2004 Anabolic Steroid Control Act.

CONCLUSIONS

Despite a considerable amount of research related to DHEA, and its alleged utility to sustain "eternal youth," several key questions remain unanswered. For example, the physiologic function(s), regulation, and mechanisms of actions of DHEA remain unknown, and a causal link between age-related declines in DHEA and adverse effects of aging has not been proven. Results from clinical studies suggest that DHEA may be beneficial in some patients with adrenal insufficiency, whereas data from intervention studies in healthy older people have been inconclusive, save for some modest gender differences in selected outcome measures. Well-characterized and standardized products need to be evaluated for safety and efficacy, starting with dose ranging to determine an optimal dose. Because DHEA can be converted to estrogen and testosterone, its potential adverse effects in patients with breast or prostate cancer need to be determined. Long-term, well-designed clinical studies, with clear end points, will be necessary before the beneficial and/or detrimental effects of "replacement" or "pharmacological" DHEA therapies in human aging and disease can be firmly established.

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Echinacea Species

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INTRODUCTION

Echinacea is a herbal medicine that has been used for centuries, customarily as a treatment for common cold, coughs, bronchitis, upper respiratory infections (URIs), and inflammatory conditions. It belongs to Heliantheae tribe of the Asteraceae (Compositae) family, and the nine species of these perennial North American wildflowers have a widespread distribution over prairies, plains, and wooded areas.

BACKGROUND

Three species of *Echinacea* are used medicinally: *E. purpurea* (Fig. 1), *E. pallida* (Fig. 2), and *E. angustifolia* (Fig. 3). In the United States of America, *Echinacea* preparations have been the best selling herbal products in health food stores. However, an analysis reveals that completely different preparations are sold under the name *Echinacea* using different plant parts and different extraction solvents (1). The most investigated preparation, which is mainly available on the German market, contains the expressed juice of *E. purpurea* aerial parts. Besides this, hydroalcoholic tinctures of *E. purpurea* aerial parts and roots, as well as from *E. pallida* and *E. angustifolia* roots, can be found (2,3). In North America, especially, it is also common to sell encapsulated powders from aerial parts and roots of these three species.

Preclinical studies indicate that *Echinacea* constituents modulate immune mechanisms and there is increasing evidence that *Echinacea* preparations containing alkamides can suppress stress-related cellular immune responses. As reviewed by Chicca et al. (4), the interaction of alkamides with the cannabinoid receptor type-2 (CB₂), which is a modulator of inflammation, may provide a mechanistic basis for the anti-inflammatory and immunomodulatory effects exerted by purple coneflower. *Echinacea* alkamides are now considered as a new class of cannabinomimetics (5,6). Recent findings suggest that *Echinacea* has a modulatory role on the innate immune system, able to both stimulate and inhibit the immune response. Since *Echinacea*-derived alkamides significantly suppressed T-lymphocytes (7), it is apparent that the multiplicity and diversity of parts of various plants, methods of extraction, and solvents used, as well as the components on which the extracts have been standardized, have hampered clear recommendations regarding *Echinacea* usage.

Several reviews on the evidence regarding the effectiveness of orally ingested *Echinacea* extracts in reduc-

ing the incidence, severity, or duration of acute URIs have been published. The majority of trials investigated whether *Echinacea* preparations shorten the duration or decrease the severity of symptoms of the common cold (for reviews see 5,8–12). A recent Cochrane review (10) concluded that especially preparations based on the aerial parts of *E. purpurea* might be effective for the early treatment of colds in adults, but results are not fully consistent. Beneficial effects of other *Echinacea* preparations for preventative purposes might exist, but have not been shown in independently replicated rigorously randomized trials. Therefore, further clinical studies are needed to evaluate the therapeutic role of *Echinacea* preparations. Many of the in vivo studies performed so far used phytochemically insufficiently characterized *Echinacea* preparations.

The regulatory status of *Echinacea* products is variable. In the United States, they are considered as dietary supplements, and manufacturers can therefore produce, sell, and market herbs without demonstrating safety and efficacy first, as is required for pharmaceutical drugs. In Canada, they are regulated as Natural Health Products (NHPs), and in several European countries they have drug status. The EMEA (European Medicines Agency) Committee on Herbal Medicinal Products (HMPCs) has published the following guidance documents for *Echinacea* products (www.emea.europa.eu):

- Community Herbal Monograph on *Echinacea purpurea* (L.) Moench, herba recens (13)
- Community Herbal Monograph on *Echinacea purpurea* (L.) Moench, radix (14)
- Community Herbal Monograph on *Echinacea pallida* (Nutt.) Nutt., radix (15)
- Community List Entry for *Echinacea purpurea* (L.) Moench, herba recens (16)

For HMPCs published as a Community Herbal Monograph, the industries in general have still more possibilities in change. In contrast, entries in the Community List are legally binding to applicants and competent authorities.

ACTIVE PRINCIPLES, PHARMACOLOGICAL EFFECTS, AND STANDARDIZATION

The constituents of *Echinacea*, as in any other plant, cover a wide range of polarity, ranging from the polar polysaccharides and glycoproteins, via the moderately polar caffeic acid derivatives, to the rather lipophilic polyacetylenes



Figure 1 *Echinacea purpurea*.

and alkamides. This makes it necessary to study separately the activity of different polar extracts of *Echinacea*, such as aqueous preparations, alcoholic tinctures, and hexane or chloroform extracts.

Polysaccharides and Glycoproteins

Systematic fractionation and subsequent pharmacological testing of the aqueous extracts of the aerial parts of *E. purpurea* led to the isolation of two polysaccharides (PS I and PS II) with immunostimulatory properties. They were shown to stimulate phagocytosis in vitro and in vivo, and enhance the production of oxygen radicals by macrophages in a dose-dependent way. Structural analysis showed PS I to be a 4-*O*-methylglucuronarabinoxylan with an average MW of 35,000



Figure 2 *Echinacea pallida*.



Figure 3 *Echinacea angustifolia*.

Da, while PS II was demonstrated to be an acidic arabinorhamnogalactan of MW 45,000 Da. A xyloglucan, MW 79,500 Da, was isolated from the leaves and stems of *E. purpurea* (17), and highly water-soluble fructans have been recently isolated from *E. purpurea* (L.) Moench roots (18). Polysaccharides from *E. angustifolia* have also been found to possess anti-inflammatory activity (19). In a Phase-I clinical trial, a polysaccharide fraction (EPO VIIa), isolated from *E. purpurea* tissue culture and injected at doses of 1 and 5 mg, caused an increase in the number of leukocytes, segmented granulocytes, and tumor necrosis factor- α (TNF- α) (20). Three glycoproteins, MW 17,000, 21,000, and 30,000 Da, have been isolated from *E. angustifolia* and *E. purpurea* roots. The dominant sugars were found to be arabinose (64–84%), galactose (1.9–5.3%), and glucosamines (6%). The protein moiety contained high amounts of aspartate, glycine, glutamate, and alanine (21). An enzyme-linked immunosorbent assay (ELISA) method has been developed for the detection and determination of these glycoproteins in *Echinacea* preparations (22). In 2005, an arabinogalactan protein (AGP) and an arabinan were isolated from the roots of *E. pallida* (Nutt.) Nutt (23). Most AGPs contain mostly carbohydrate moieties (>90%) along with a small amount of protein (<10%). Also information concerning the protein-polysaccharide linkage type has recently been published,

and hydroxyproline (42.9% w/w) was detected as the dominant amino acid and was identified as the major amino acid responsible for the binding between the protein and the AG subunits via an *O*-glycosidic linkage (24). Purified extracts containing these glycoprotein-polysaccharide complexes exhibited B-cell stimulating activity and induced the release of interleukin-1 (IL-1), TNF- α , and interferon- α,β (IFN- α,β) in macrophages, which could also be reproduced in vivo in mice (21).

In a study performed in 2002, the influence of an oral administration of a herbal product, consisting of an aqueous-ethanolic extract of the mixed herbal drugs *Thujae summities*, *Baptisia tinctoriae* radix, *E. purpurea* radix, and *E. pallidae* radix, standardized on *Echinacea* glycoproteins, on cytokine induction, and antibody response against sheep red blood cells was investigated by Bodinet et al. (25) in mice. This administration caused a significant enhancement of the antibody response against sheep red blood cells, inducing an increase in the numbers of splenic plaque forming cells and the titers of specific antibodies in the sera of the treated animals (25).

The influence of the same extract on the course of Influenza A virus infection in Balb/c mice was also tested. The data show that the oral treatment with this aqueous-ethanolic extract induced a statistically significant increase in the survival rate, prolonged the mean survival time, and reduced lung consolidation and virus titer (26). An *Echinacea* polysaccharide enriched fraction isolated from *E. purpurea* aerial parts was able to exert an antiviral action on the development of recurrent herpes simplex virus type-1 (HSV-1) disease when supplied prior to infection (27). Hwang et al. (28) showed that macrophages respond to purified polysaccharide and also alkamide preparations. Adherent and nonadherent mouse splenocyte populations were incubated in vitro with *E. purpurea* liquid extract (fresh *Echinacea* root juice, mature seed, fresh leaf, and fresh fruit juice extracted in 40–50% alcohol), or with water or absolute alcohol (25 mg dry powder/mL of solvent) soluble *E. purpurea* dried root and leaf extract preparations. The immune stimulatory ability of components contained within these *Echinacea* extracts offers insight into possible therapeutic potential to regulate nonadherent lymphocytes in immune responses and activation events. The use of flow cytometry demonstrates a link between the polysaccharides in *Echinacea* and the immunostimulatory effect that has therapeutic relevance. It was suggested that the main immunostimulatory activity of *Echinacea* resides in the water-soluble materials rather than the lipoidal small molecules (29).

Caffeic Acid Derivatives

Alcoholic tinctures of *Echinacea* aerial parts and roots are likely to contain caffeic acid derivatives (Figs. 4–6). Extracts of different species and plant parts of *Echinacea* can be distinguished by thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC) analysis (2). Also, capillary electrophoresis [micellar electrokinetic chromatography (MEKC)] has been successfully applied for the analysis of caffeic acid derivatives in *Echinacea* extracts and enables the discrimination of the species (30). The roots of *E. angustifolia* and *E. pallida* have been shown to contain 0.3–1.7% echinacoside (1) (2). Both species can

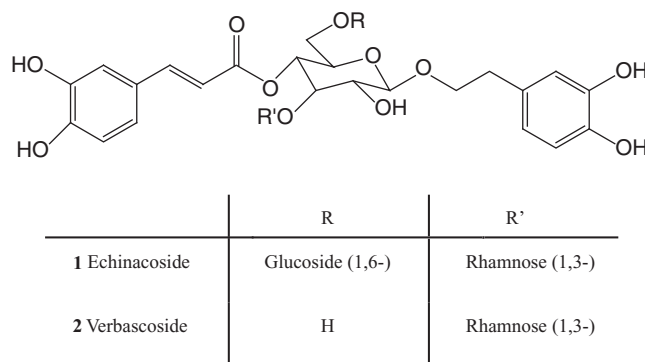


Figure 4 Main phenylpropanoid glycosides found in *Echinacea* species.

be discriminated by the occurrence of 1,3- and 1,5-O-dicaffeoyl-quinic acids (3, 4), which are only present in the roots of *E. angustifolia*. Recently, it has been reported that cynarin, the 1,3-dicaffeoyl-quinic acid, blocked the interaction between the CD28 of T-cell receptor and CD80 of antigen presenting cells under the condition of 1:1 ratio of T-cell and B-cell in vitro. This *Echinacea* component is the first small molecule that is able to specifically block the CD28-dependent pathway of T-cell activation and has therefore great potential as an immunosuppressive agent (31). Echinacoside has anti-oxidant (32), low antibacterial, and antiviral activity, but does not show immunostimulatory effects (17). It is also reported that echinacoside inhibits hyaluronidase (33) and protects collagen type III from free radical-induced degradation in vitro (34). The aerial parts of *E. angustifolia* and *E. pallida* have been shown to contain verbascoside (2), a structural analog of echinacoside (1). The roots of *E. purpurea* do not contain echinacoside, but cichoric acid (2*R*,3*R*-dicaffeoyl-tartaric acid; 6) and caftaric acid (monocaffeoyl tartaric acid; 5). Cichoric acid (6) is also the major polar constituent in the aerial parts of *Echinacea* species. Echinacoside (1) and cichoric acid (6) have also been produced in tissue cultures of *E. purpurea* and *E. angustifolia* (17). The latter acid (6) has been shown to possess phagocytosis stimulatory activity in vitro and in vivo, while echinacoside (1), verbascoside (2), and 2-caffeoyl-tartaric acid (5) did not exhibit this activity (17). Robinson described cichoric acid as an inhibitor

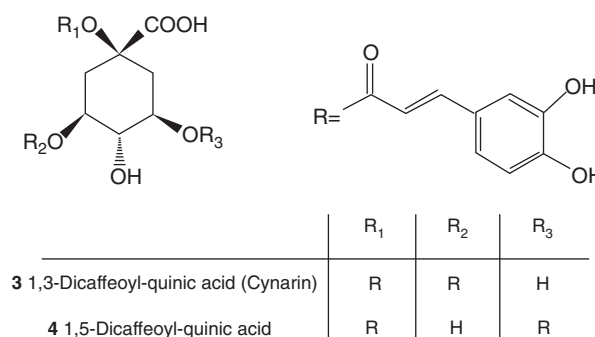


Figure 5 Quinic acid derivatives from *Echinacea* species.

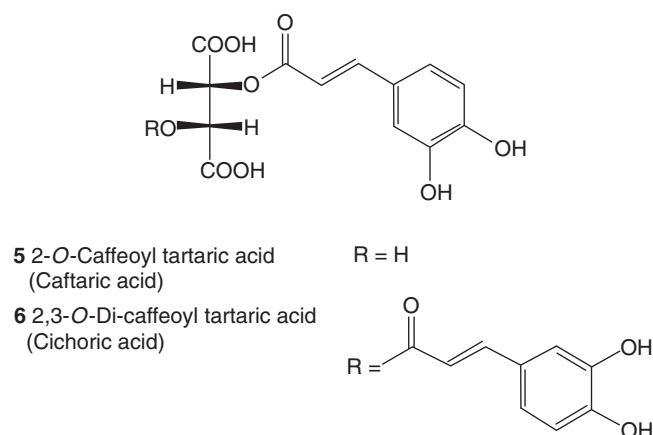


Figure 6 Tartaric acid derivatives from *Echinacea* species.

of human immunodeficiency virus type-1 (HIV-1) integrase (35). It is especially abundant in the flowers of all *Echinacea* species and the roots of *E. purpurea* (1.2–3.1% and 0.6–2.1%, respectively). Much less is present in the leaves and stems. *E. angustifolia* contains the lowest amount of cichoric acid (2). The content, however, strongly depends on the season and the stage of development of the plant and is highest at the beginning of the vegetation period and decreases during plant growth (36). Nüsslein et al. found that polyphenol oxidases (PPO) are responsible for the oxidative degradation of exogenous and endogenous caffeic acid derivatives (37,38). Apart from cichoric acid, echinacoside (1) and cynarin (3) from *E. angustifolia* roots are also highly susceptible to enzymatic degradation and oxidation in hydroalcoholic solutions during the extraction process. During the 16 days after extraction of *E. angustifolia* roots with 60% (v/v) ethanol/water and storage at 4°C, a decline to 0 was observed in the content of echinacoside (1) and cynarin (3) from 0.25 mg/mL extract and 0.09 mg/mL extract, respectively (39). In order to standardize *Echinacea* preparations and guarantee a consistent content of caffeic acid derivatives, it is vital to control this enzymatic activity.

Alkamides

Striking differences have been observed between the lipophilic constituents of *E. angustifolia* (alkamides; Fig. 7) and *E. pallida* roots (ketoalkenynes; Fig. 8). *E. purpurea* roots also contain alkamides, however, mainly with two double bonds in conjugation to the carbonyl group, while *E. angustifolia* primarily has compounds with a 2-monoene chromophore. The chief lipophilic constituents of *E. pallida* roots have been identified as ketoalkenes and ketoalkynes with a carbonyl group in the 2-position (2). The main components are tetradeca-8Z-ene-11,13-diyn-2-one (17), pentadeca-8Z-ene-11,13-diyn-2-one (18), pentadeca-8Z,13Z-diene-11-yn-2-one (19), pentadeca-8Z,11Z,13E-triene-2-one (20), pentadeca-8Z,11E,13Z-triene-2-one (21), and pentadeca-8Z,11Z-diene-2-one (22). They occur only in trace amounts in *E. angustifolia* and *E. purpurea* roots. Therefore, they are suitable as markers for the identification of *E. pallida* roots. However, it has been observed

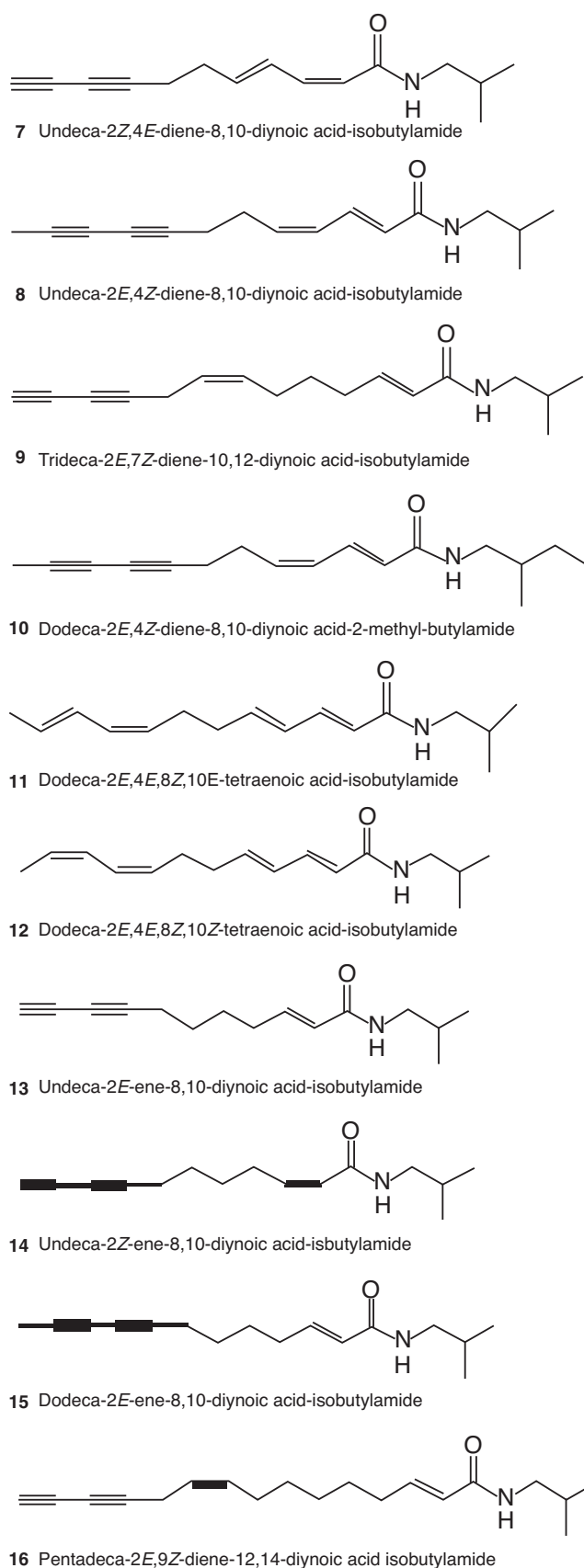


Figure 7 Main alkamides found in *Echinacea* aerial parts and *E. purpurea* and *E. angustifolia* roots.

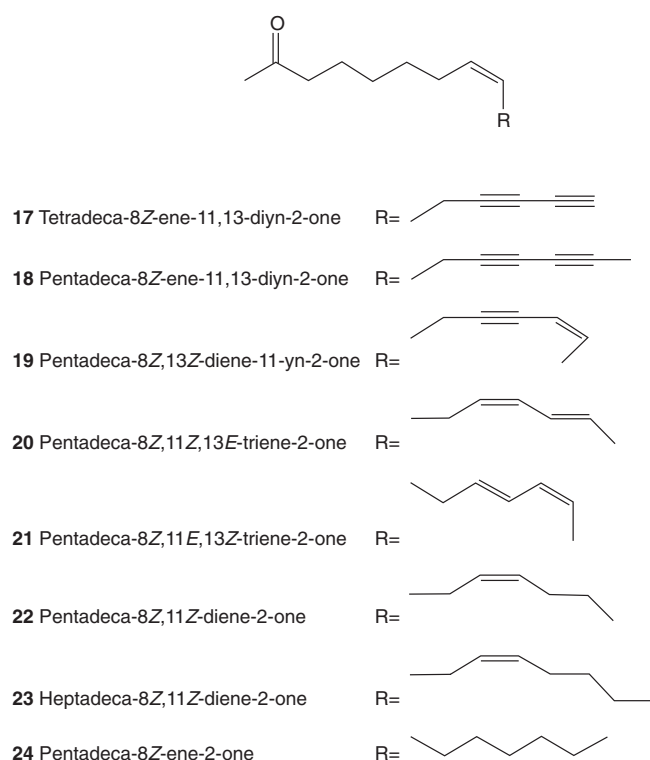


Figure 8 Ketoalkenes and ketoalkynes found in *E. pallida* roots.

that these compounds undergo auto-oxidation when the roots are stored in powdered form. Then, the hydroxylated artifacts, 8-hydroxy-9E-ene-11,13-diyn-2-one (25), 8-hydroxy-pentadeca-9E-ene-11,13-diyn-2-one (26), and 8-hydroxypentadeca-9E,13Z-diene-11-yn-2-one (27), can be primarily found (Fig. 9), often with only small residual quantities of the native compounds. Hence, the roots of *E. pallida* are best stored in whole form.

Approximately 15 alkamides have been identified as important lipophilic constituents of *E. angustifolia* roots. They are mainly derived from undeca- and dodecanoic acid, and differ in the degree of unsaturation and the configuration of the double bonds (Fig. 7). The main structural type is a 2-monoene-8,10-diynoic acid isobutylamide, and some 2'-methyl-butylamides have also been found. In

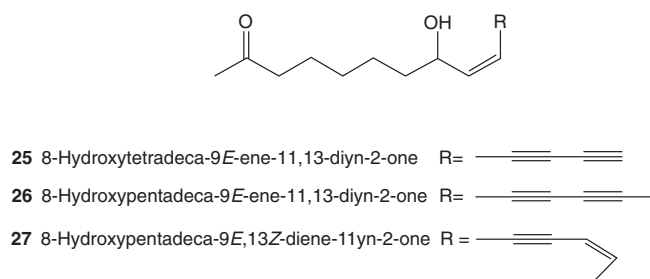


Figure 9 8-Hydroxy-ketoalkenyne formed via auto-oxidation in powdered *E. pallida* roots.

E. purpurea roots, 11 alkamides have been identified with the isomeric mixture of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (11, 12) as the major compounds (17).

The aerial parts of all three *Echinacea* species contain alkamides of the type found in *E. purpurea* roots, and also with dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (11, 12) as the main constituents (2). A series of pharmacological experiments have shown that *Echinacea* extracts containing alkamides have significant anti-inflammatory and immunomodulatory properties. Among the many pharmacological effects reported, modulation of macrophages and polymorphonucleocytes (PMN) immune cells and effects on cytokine/chemokine expression in human cells have been demonstrated most convincingly. Furthermore, inhibition of cyclooxygenase is known as an effective strategy to suppress pain and inflammation. Alkamides isolated from the roots of *E. angustifolia* inhibited cyclooxygenase-2 (COX-2)-dependent prostaglandin E₂ formation, but did not inhibit COX-2 expression at the transcriptional or translational level (5,8,9,40–42).

Determination of the alkamide content (dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides) in the different plant parts showed that it accumulates primarily in the roots and inflorescences, the highest being found in *E. angustifolia*. *E. pallida* roots contain only trace amounts, roots of *E. purpurea* 0.004% to 0.039%, and those of *E. angustifolia* 0.009% to 0.151%. The yield in the leaves is 0.001% to 0.03% (17). In a recent study, 62 commercial dried root and aerial samples of *E. purpurea* grown in eastern Australia were analyzed for the medicinally active constituents. Total concentration in root samples was 6.2 ± 2.4 mg/g, and in aerial samples was 1.0 ± 0.7 mg/g (43).

In 2001, Dietz et al. (44) reported the presence of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (11, 12) in human blood after oral administration of an ethanolic extract of *E. purpurea*. More recently, a study showed that the absorption maximum (C_{\max}) of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (11, 12) is reached 30 minutes after oral application. Due to these results, the mucous membrane of the mouth is most likely the major area of absorption (45). Jager et al. investigated the permeability of isobutylamides (11, 12) through Caco-2 monolayers. They found that the alkamides were almost completely transported from the apical to the basolateral side of the monolayer in six hours by passive diffusion and that no significant metabolism occurred (46). Matthias et al. (47) investigated the bioavailability of caffeic acid derivatives and alkamides also using Caco-2 monolayers. The caffeic acid conjugates (caftaric acid, echinacoside, and cichoric acid) permeated poorly through the Caco-2 monolayers although one potential metabolite, cinnamic acid, diffused readily with an apparent permeability (P_{app}) of 1×10^{-4} cm/sec, while alkamides were found to diffuse with P_{app} ranging from 3×10^{-6} to 3×10^{-4} cm/sec. Close monitoring of the transport for six hours revealed a nearly complete transfer to the basolateral side without significant metabolism. Transport experiments performed at 4°C showed only a slight decrease, which is a strong hint that dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (11, 12) cross biological membranes by passive diffusion. These data suggest that alkamides are more likely than caffeic acid conjugates to

pass through the intestinal barrier and thus be available for influencing an immune response (47).

Based on these in vitro studies and the performance of eight pharmacokinetic studies since 2005, evidence is given that alkamides are bioavailable in relevant concentrations after oral administration of *Echinacea* preparations or pure compounds (5). In all pharmacokinetic studies performed so far, the time to reach the mean concentration maximum after administration of liquid *Echinacea* preparations (*E. angustifolia* or *E. purpurea*) was approximately 30 minutes. T_{\max} after administration of *Echinacea* tablets, which has been investigated in two clinical studies, was varying within 45 minutes to 2.3 hours (5). As it has also been shown for *Echinacea* tinctures that more concentrated preparations needed longer to attain T_{\max} . Besides bioavailability, information concerning the distribution in tissues is an important issue in the evaluation of the systemic exposure and a prerequisite for the interpretation of in vitro pharmacological testing. The pharmacokinetic and tissue distribution of the dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (tetraenes), the main alkamides in *Echinacea* preparations, has been recently investigated and demonstrated a rapid absorption and distribution to the examined tissues (liver and four different brain regions) with a passage across the blood-brain barrier (BBB). The highest concentration was found in the striatum. The total amount of tetraenes in plasma was calculated as area under the curve ($AUC_{0-\infty}$) (794 min · ng/mL) and approximately 13%~45% of that found in different brain parts (1764–6192 min · ng/mL), and 63% of that in liver tissues (1254 min · ng/g). The C_{\max} in plasma was 26.4 ng/mL, while the C_{\max} in the different brain regions varied between 33.8 ng/g and 46.0 ng/g. The appearance in brain tissues offers new pharmacological options of alkamides containing *Echinacea* preparations with the emphasis on cannabinoid receptor interaction between the immune system and the central nervous system (48). Since alkamides show structural similarity with anandamide, an endogenous ligand of cannabinoid receptors, it was found that alkamides bind significantly to CB₂ receptors, which is now considered as a possible molecular mode of action of *Echinacea* alkamides as immunomodulatory agents (5,49). At the same time, Gertsch et al. demonstrated the modulation of TNF- α gene expression and multiple signal transduction pathways by *Echinacea* alkamides and postulated a mechanism related to cannabinoid receptors. To ascertain whether CB₂ was the receptor subtype involved in the observed effects, a CB₂ antagonist was used in combination with the dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides. The specific antagonist strongly abolished TNF- α transcription and thus indicated a strict peripheral cannabinoid-mediated process (40,41). Effects on CB₂ receptor-containing immune cells in humans were evaluated in a randomized, single dose, crossover ex vivo study with lipopolysaccharide (LPS)-stimulated blood cells after in vivo administration of two *E. purpurea* preparations standardized on 0.07 mg dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (EchinaforceTM tincture and tablets). Both forms of medication led to a significant decrease in production of proinflammatory cytokines (IL-8 and TNF- α), while changes in IL-6 concentration were not statistically significant (5). IL-8 and TNF-

α are proinflammatory cytokines. Therefore, the effect of the *Echinacea* preparations can be considered as an anti-inflammatory action and corresponds with the observed in vitro effects of alkamides. Also the direct effects of alkamides on T-lymphocytes, which are key mediators of antiviral immunity, have been investigated by testing inhibition of IL-2 production (50). This can explain why the symptoms of a common cold, like sore throat, can be reduced. In summary, a lot of recent pharmacological data suggest that *Echinacea* alkamides may not only have immunostimulatory but also anti-inflammatory and antiviral activity by a reduction in NO, TNF- α , IL-8, IL-2, and COX-dependent prostaglandin E₂ formation in different cell types and assays used.

From the above, it is obvious that not a single, but several constituents, like the alkamides, cichoric acid, glycoproteins, and polysaccharides, are responsible for the immunostimulatory activity of *Echinacea* extracts, and the application of extracts appears to be reasonable. However, conformity of these extracts is a must for generating consistent products and reproducible activity.

CLINICAL EFFICACY

Several dozen human experiments—including a number of blind randomized trials—have reported health benefits of *Echinacea* preparations. So far, clinical efficacy has been studied in randomized controlled clinical trials for cold pressed juice and hydroalcoholic tincture/extract of *E. purpurea* aerial parts, a standardized extract from *E. purpurea* roots (hydroalcoholic tincture/extract), a hydroalcoholic extract of *E. pallida* roots, and for *E. angustifolia* root tinctures. All of them have used a variety of different *Echinacea* preparations and study designs. Most importantly, the phytochemical profile of the preparations was not determined or not reported in most of the studies. Since different *Echinacea* preparations have varying phytochemical profiles due to the use of diverse species, plant parts, and extraction procedures, the variation in reported clinical effectiveness may be due to discrepancies in the chemical profile. In 16 randomized controlled trials summarized in the latest Cochrane database, only eight (50%) reported the exact characterization of the herbal remedy being used (10). In three experimental infection studies pooled by Schoop et al. (51), only one (33%) has been included without content details of the tested *Echinacea* product. In the 14 randomized clinical trials included in the meta-analysis by Shah et al. (11), five (36%) reported the chemical profiling of the herbal product used. Therefore, the newly published meta-analysis pooled results for all *Echinacea* preparations, although the original publication did not adequately describe the preparations tested. This lack of information needs to be taken into account when research on *Echinacea* is evaluated.

The most robust data come from trials testing *E. purpurea* extracts in the treatment for acute URI. A recent Cochrane review (10) summarized 16 randomized clinical trials of *Echinacea* to assess whether there is evidence that *Echinacea* preparations are (i) more effective than no treatment, (ii) more effective than placebo, (iii) similarly effective to other treatments in the prevention and treatment of the common cold. The authors concluded that there is

some evidence that preparations based on the aerial parts of *E. purpurea* might be effective for the early treatment of colds in adults.

Besides the Cochrane database review, two further meta-analyses have been published. In one meta-analysis, all performed clinical trials were pooled regardless of the product tested or the trial approach used (11), the second included only trials with experimentally induced infections (51). The meta-analysis by Shah et al. included two sets of studies: nine studies investigating whether *Echinacea* compounds prevent colds and seven studies investigating whether they shorten their duration. It has been stated that there is a significant improvement of common cold after taking *Echinacea*. In detail, *Echinacea* decreased the odds of developing the common cold by 58% and the duration of a cold by 1.4 days (11). The other meta-analysis of three preventative trials using an experimental virus challenge model showed that the odds of experiencing a clinical cold were 55% higher with placebo than with *Echinacea* (51). In this meta-analysis a study, published in the *New England Journal of Medicine*, evaluating the effect of chemically defined extracts from *E. angustifolia* roots on rhinovirus type 39 infection was included. Without pooling, the extracts did not have clinically significant effects on infection with a rhinovirus or on the clinical illness (52). These results and information should be a motivation to conduct larger prevention trials in the future.

As with most medications for the treatment of the common cold, the clinical data on *Echinacea* so far are not conclusive. There is a clear indication that preparations from the aerial parts and roots of *E. purpurea* may be effective. Preparations from *E. angustifolia* and *E. pallida* roots need further controlled clinical trials, in order to provide a better evidence for clinical efficacy (12).

CLINICAL PARTICULARS

Dosage Information

For internal use, the daily dose for aerial parts of *E. purpurea* for adults is 6 to 9 mL of pressed juice or equivalent preparations, 3 × 60 drops of a tincture (1:5, ethanol 55% v/v) or dried expressed juice equivalent to 8 to 18 g of the herbal substance, divided in two to four doses (13). For dried roots, the recommended daily dose is 3 × 300 mg one chewable tablet containing 40 mg extract (6.5:1) of *E. purpurea* roots and corresponding to 260 mg of herbal substance, every second hour (maximum nine tablets a day) (14). For external use, semisolid preparations with a minimum of 15% of pressed juice are recommended (16). For *E. pallida* roots, the recommended daily dosage of Commission E is tincture 1:5 with 50% (v/v) ethanol from native dry extract (50% ethanol, 7–11:1), corresponding to 900 mg herb (53), three times daily one tablet containing 30 mg dry extract (4–8:1) of *E. pallida* roots, four times daily two tablets containing 12 mg dry extract (4–8:1), or five times daily 25 drops containing 100% tincture (1:5) (15). The use in children younger than 1 year is contraindicated, and the use in children between 1 and 12 years of age is not recommended. The therapy should start at first signs of common cold. If the symptoms persist longer than 10 days during the use of the medicinal product, a doctor or a qualified health care practitioner should be consulted.

Adverse Effects and Herb–Drug Interactions

The safety profile of *Echinacea* has been based on the very few reported serious adverse events and reports from several clinical trials that showed a low frequency of side effects. Parnham (54) reported 13 adverse events that were possibly associated with *Echinacea* use in Germany between 1989 and 1995, and 4 were determined to be causal. All the adverse events were reported as allergic reactions. Taylor et al. (55) found an increased risk of rash when children of 2 to 11 years received *Echinacea* compared with those who received placebo. Mullins and Heddle (56) reported 51 possible cases of *Echinacea*-related allergy, of which 26 were suggestive of possible immunoglobulin E-mediated hypersensitivity. Allergic cross-reaction with daisy family members has been reported.

Some possible side effects from the German health authorities list (53):

- In rare cases, hypersensitivity reactions might occur. For drugs with preparations of *Echinacea*, rash, itching, rarely face swelling, shortness of breath, dizziness, and blood pressure drop have been reported.
- In case of diabetes, the metabolic status may worsen (53).

Perri et al. (57) reviewed the evidence for the use of *Echinacea* during pregnancy and lactation via seven electronic databases. They found good scientific evidence from a prospective cohort study that oral consumption of *Echinacea* during the first trimester does not increase the risk for major malformations. They reported that *Echinacea* is nonteratogenic when used during pregnancy. Caution should be maintained while using *Echinacea* during lactation until further high-quality human studies can determine its safety. In contrast, in 2007, there was the first study conducted to evaluate whether pharmaceuticals containing alcoholic extracts of *E. purpurea* given to pregnant mice influence angiogenic activity and may then lead to severe developmental disturbances. They found an increase in angiogenic activity of tissue homogenates in Esberitox group and a diminution in case of Immunol forte. The growth factor concentration was lower in all groups compared to the control. They concluded that there is some possibility that pharmaceuticals containing *E. purpurea* might influence fetal development in human also, because they may interfere with embryonal angiogenesis, and should not be recommended for pregnant women (58).

The German Commission E monograph cautions the use of *Echinacea* in persons who have autoimmune diseases, progressive systemic diseases, such as tuberculosis, multiple sclerosis, leukoses, collagenoses, and HIV infection. The warnings are based not on solid clinical evidence but on the theoretical possibility that the immune-mediated inflammatory mechanism of these diseases can be exacerbated by the immunostimulating properties of *Echinacea*.

No significant herb–drug interactions with *Echinacea* have been reported. Four studies were clinical trials and nine were in vitro assays, but most of the studies did not contain complete information about the concentration of extract used (59). No phytochemical analysis of the extracts was reported and limit the inferences that can be

drawn from these studies. A common problem of many herb–drug interaction reports is that investigators fail to report species, plant part used, preparation type, and concentrations assayed, and/or dose, each of which may affect the accuracy of reporting and the potential for herb–drug interactions.

Currently, there are no rational reports available on herb–drug interactions of any *Echinacea* product. Both *E. purpurea* aerial parts and roots appear to have a relatively low potential to generate cytochrome P450 (CYP 450) herb–drug interactions in vivo involving CYP1A2 and CYP3A4 (60). There is little if any effect of *E. purpurea* on systemic *p*-glycoprotein-mediated transport mechanism in vivo (61,62).

It can be concluded that there is a very low incidence of side effects associated with *E. purpurea*, since, in comparison with the large proportion of the population using *Echinacea* yearly (>10 million), there have been no known reported major outbreaks of side effects or death. Also in long-term treatment, the expressed juice of *E. purpurea* was shown to be well tolerated (54).

CONCLUSIONS

Echinacea, one of the most popular botanical supplements in North America, is employed as an immunomodulator, with antimicrobial and anti-inflammatory properties. So far, the overall therapeutic activity of *Echinacea* cannot be attributed to any single constituent. Pharmacological effects related to immune functions have been demonstrated for both high- and low-molecular-weight constituents. Compounds from the classes of caffeic acid derivatives, alkamides, polysaccharides, and glycoproteins are regarded as the most relevant constituents. Recent experiments have demonstrated that alkamides are detectable in human blood in relevant concentrations after oral administration of *Echinacea* preparations. Moreover, it has been shown that the main alkamides cross the BBB and bind to CB receptors, which is now considered as a possible molecular mode of action of *Echinacea* alkamides as immunomodulatory agents. Employing various species, plant parts, and extraction procedures results in different *Echinacea* preparations having distinguished phytochemical profiles. Clinical effectiveness may vary because of discrepancies in the chemical profile. Standardization of botanicals should guarantee that preparations contain therapeutically effective doses of active principles and should assure consistent batch-to-batch composition and stability of the active constituents. Also, clinical trials should be performed only with well-characterized *Echinacea* preparations.

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Elderberry

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INTRODUCTION

Sambucus nigra Linnaeus is a small deciduous tree, which grows widely in Europe, Western and Central Asia, and North Africa. It is often planted as a decorative tree in gardens and public parks. The English name Elder comes from the Anglo-Saxon word "aeld," which means fire. The hollow stems of the young branches were used for building a fire (1). The generic name *Sambucus* comes from the Greek word "Sambuca," an ancient musical instrument, made from the wood of this tree (1).

All parts of the tree have long been used in traditional medicine, that is, the bark, the fresh and dried leaves, the dried flowers, the fresh and dried fruits, and the dried roots. The tree is propagated by seeds sown in autumn, as well as by greenwood cuttings in early summer and by hardwood cuttings in winter. The flowers contain flavonoids, cyanogenic glycosides, triterpenes, phenolic acids, tannin, mucilage, pectin and sugar. They possess diuretic, diaphoretic, mild anti-inflammatory, and antiviral properties. The flowers are used to treat colds and catarrh of the upper respiratory tract. The fruits are rich in flavonoids, anthocyanin glycosides, and essential oils. They possess antiviral, immunostimulatory, and antioxidant activity. The fruits are used to treat flu and to boost the immune system. The standardized extract, Sambucol, has been shown to inhibit hemagglutination and replication of influenza viruses in vitro and to reduce the severity and duration of flu in clinical trials. The flowers and fruits, including the Sambucol extract, are safe, and can be administered to infants and children.

BACKGROUND

Sambucus nigra L. is a member of the family, Adoxaceae, and its pharmacopeial name is *Sambuci flos* or *Sambuci fructus*. Common names include Elderflower, European Elder flower, Black Elder flower, and Elderberry. The parts used are the flowers, fruits, and leaves.

The tree grows to a height of between three and seven meters. The bark is light brown to gray and fissured. The leaves are narrow, dark green, and serrated. The flowers are small, white, and fragrant (Fig. 1). They grow in large clusters in flat, umbrella-like umbels. Each flower has five petals, five stamens, and one inferior ovary. The flowering tops are gathered in the wild and dried and separated by sorting them into individual flowers. The peduncles are discarded (2,3). The flowers have a characteristic odor and a mild sweet taste.

The fruits are round like a ball, 4 to 7 mm long (Fig. 2). The young fruits are green and they change from dark purple to black when they ripen. Each fruit contains one to three brownish, egg-shaped seeds. The fruits are sweet (slightly bitter), mucilaginous, and acidic with a characteristic aroma (4,5).

In **traditional medicine**, the flowers are used as a diuretic, laxative, and diaphoretic, as well as a gentle astringent for the skin. They are used as an infusion, a gargle for mouthwash, and for respiratory disorders such as coughs, colds, laryngitis, flu, and shortness of breath. Infusions and lotions from the flowers are used for clearing the skin of freckles and sunburn (1). The aqueous extract of the flowers is used for washing hands and face, as well as to whiten and soften the skin. Externally, herbal pillows are used for subduing swelling and inflammation. The fresh flowers are used for the distillation of Elder Flower Water, which is prepared made from 100 parts of fresh flowers and 500 parts of water. The product is a mild astringent and a gentle stimulant. It is used as a vehicle for eye and skin lotions (1). Vinegar prepared from the flowers of Elder is a known old remedy for sore throat.

CHEMISTRY AND PREPARATION OF THE PRODUCTS

The flowers contain:

- Flavonoids (up to 3%) composed mainly of flavonoid glycosides (astragalin, hyperoside, isoquercetin, and rutin) and free aglycones (quercetin and kaempferol) (Fig. 3)
- Cyanogenic glycosides (glycosides of 2-hydroxy-2-phenylacetone, such as sambunigrin)
- Triterpenes approximately 1% including α - and β -amyrin, oleanolic, and ursolic acids
- Essential oils up to 0.3%, including 66% fatty acids (mainly linoleic, linolenic, and palmitic acids), alkanes (7%) and more than 60 other constituents including ethers, oxides, ketones, aldehydes, alcohols, and esters
- Minerals (8–9%), mainly potassium (6)
- Chlorogenic acid (3%), glucosides of caffeic and ferulic acids
- Tannin, mucilage, pectin, and sugar (7,8)

The highest amount of rutin (1.9%) is present in the buds together with pectin (0.8%) and sugars (2.5%) (9).

Elder leaves contain essential oils (0.02%), fats (4.8%), resins (4.3%), ketoses (3.5%), aldoses (0.9%), chlorophyll (0.8%), tannins (0.4%), pectins (0.2%), xanthophylls (0.1%), carotenes (0.05%), sambunigrin (0.04%),



Figure 1 Flowers of *Sambucus nigra* L.

and rutin (0.02%), as well as pentacyclic hydroxy triterpenoids—oleanoic and ursolic acids (9).

The fruits (Elderberries) are rich in flavonoids, thiamine, riboflavin, vitamins B6, C, and P-complex (10). Like the flowers, they contain flavonoid glycosides and anthocyanin glycosides (such as chrysanthemin, sambucin, and sambucyanin). They also contain approximately 0.01% of



Figure 2 Berries of *Sambucus nigra* L.

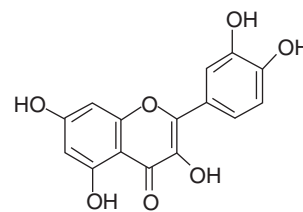


Figure 3 Quercetin chemical structure.

essential oils. Present in the seeds are the cyanogenic glycosides including holocalin, prunasin, sambunigrin, and zierin (10).

Quantitative Standards: Total flavonoids: not less than 0.8% (calculated as isoquercetin). Foreign matter: not more than 10% pedicles and not more than 1% other foreign matter. Loss on drying: not more than 10%. Ash: not more than 10% (8).

Preparations and Dosage

Infusion: 2 to 4 g of flowers in 150 mL of boiling water; strain after 10 minutes. Take three times daily. One teaspoon ~1.5 g. **Liquid Extract** (1:1 in 25% alcohol): 2 to 4 mL, three times daily.

Tincture (1:5, 25% alcohol): 10 to 25 mL per day (11–13). Another source (14) reports that the tincture is prepared in 45% alcohol and the dosage is 5 to 8 mL per day. According to WHO, the tincture should be prepared with 25% alcohol (11).

Topical Use: Infusion of the flowers is used as a wash or compress for improving oily skin or acne, or for treating injured tissues. Tincture of 3 to 5 mL diluted in 240 mL of water can be used for the same purpose (14). There are lotion and cream formulations of the flower extracts also.

Use as Food: The flowers are used as flavor in numerous food products, including alcoholic and non-alcoholic beverages, frozen dairy desserts, candy, baked goods, and gelatins and puddings. The highest concentrations of flowers are known to be in nonalcoholic beverages (0.05%) (15).

Phytomedicines: The flowers are added to a variety of tea mixtures. Flower extracts are also present in antitussives, for example, Sinupret for sinusitis (8).

Branded Preparation: Sambucol[®], liquid formula contains 38% standardized liquid Elderberry extract 2:1 (10 mL, 1–4 times a day). Lozenge formula contains 130 mg standardized Elderberry dry extract (1–2 lozenges, 2–3 times a day).

PRECLINICAL AND CLINICAL STUDIES

The extracts of the Elder have antiviral, anticatarrhal, diaphoretic, diuretic, and topical anti-inflammatory properties. Accordingly, Elderberry is used for colds, feverish catarrhal complaints, cough, and bronchitis, as well as an expectorant for treatment of mild inflammation of the upper respiratory tract (11).

Pharmacokinetics

The pharmacokinetics and the bioavailability of Elder ingredients are not fully understood. One of the main controversies is whether the anthocyanins are absorbed intact or after they are metabolized in the gastrointestinal tract. Pharmacokinetic studies of Elderberry extract show that anthocyanins are absorbed and excreted in an intact form (16,17). In the first study, six healthy volunteers were given a single oral dose of 30 mL Elderberry extract (147.3 mg total anthocyanins) resulting in a fast urinary excretion of intact anthocyanins; the $t(1/2)$ value was reported to be 1.74 hour (16). In another study, it was shown that Elderberry anthocyanins can be detected unchanged in the plasma, and most of them were excreted within four hours after consumption (17). A subsequent study with seven volunteers revealed similar results; however, it was concluded that the low urinary excretion of unchanged anthocyanins may indicate that a large portion of them are metabolized before entry into the circulation (18). All studies mentioned in this article (18) agree that oral administration of anthocyanins display first-order, one-compartment kinetics.

Activities and Indications

As already mentioned, the different parts of the plant are used for the treatment of a variety of ailments. The following discussion is therefore divided according to the individual plant parts and their respective indications.

Flowers

Human studies have not been documented for Elder flowers.

Anti-inflammatory Effects

Elder flowers alleviate inflammation and pain in carrageenan-induced rat paw edema. An alcohol extract of the flowers was administered intragastrically one hour before the administration of carrageenan (100 mg/kg) (19). The control drug, indomethacin (5 mg/kg body weight) inhibited carrageenan-induced rat paw edema by 45%, while the extract of Elder flowers inhibited inflammation by 27% (19).

Elder flowers were found to inhibit the proinflammatory activity of major virulence factors from the periodontal pathogens, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (20). Inhibitory effects on the biosynthesis of the inflammatory cytokines such as interleukin-1-alpha, interleukin-1-beta, and tumor necrosis factor alpha (TNF-alpha) were shown in vitro with an extract of flowers at a concentration of 30 µg/mL (21).

Antiviral Effects

An infusion made from flowers of *S. nigra*, aerial parts of *Hypericum perforatum*, and roots of *Saponaria officinalis* has shown antiviral activity against influenza types A and B both in mice and in vitro, as well as against herpes simplex virus type 1 (22).

Diuretic Effects

Intragastric administration of an infusion of the flowers (20 mL/kg body weight) or a potassium- and flavonoid-rich extract of the flowers had a diuretic effect in rats. Their

diuretic effect exceeded that of theophylline. The greatest activity was exhibited by extracts rich in potassium and flavonoids (23). It has also been stated that flavonols could also contribute to the diuretic action (12).

Diaphoretic Effects

Although the diaphoretic effect of the flowers is well known, the mode of action is not fully understood. It is possible that flavonoids and phenolic acids are involved (12). It has been suggested that Elder flowers increase the response of sweat glands to heat stimuli (diaphoresis) in healthy subjects (8,11).

Fruits (berries)

Antiviral Effects

Though there are far fewer studies on the berries than on the flowers, in recent years there has been an increase in the awareness of the benefits of the Elder fruit among scientists. Most of the scientific studies on Elderberries, including in vivo clinical studies, were performed using a standardized preparation called Sambucol. In vitro studies have shown a reduced hemagglutination and inhibition of replication of types A and B human influenza viruses including human H1N1 (Swine flu), as well as of animal strains including a type A turkey influenza virus (24). One study examined the effects of Sambucol against four strains of Herpes Simplex Virus-1 (HSV-1) (25), two of which were acyclovir-resistant. The viral replication was completely inhibited in all four strains, irrespective of whether the cells were preincubated with the extract, simultaneously incubated with extract, or the extract was added 30 minutes after viral adsorption to cells.

A direct binding assay established in vitro that flavonoids from the elderberry extract bind to H1N1 virions and, when bound, block the ability of the viruses to infect host cells. The flavonoids were identified as, 5,7, 3',4'-tetra-O-methylquercetin and 5,7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl)chroman-3-yl-3,4,5-trihydroxycyclohexanecarboxylate. This work was performed in 2007 on the H1N1 strain obtained from the American Type Culture Center and not on the novel 2009 H1N1 strain (26).

It is interesting to note that elderberry concentrates have no antibacterial effect and exhibited even a slight stimulatory effect on the growth of *Staphylococcus aureus* and *Saccharomyces cerevisiae* (27).

The above-standardized extract (Sambucol) was also tested in two double blind, placebo-controlled clinical trials. The first consisted of individuals with confirmed influenza B experiencing flu symptoms. Patients were randomized to receive either Sambucol or placebo (a liquid of the same color and texture as the Sambucol) daily for three days. A significant improvement in symptoms, including fever was experienced by 93.3% of the Elderberry-treated group within two days. In contrast, 91.7% of the placebo group showed a similar improvement only after day six. Complete cure was achieved within two to three days in approximately 90% of the Elderberry-treated group, and within six days in the placebo group. Higher levels of influenza antibodies were detected in patients receiving Elderberry than those receiving the placebo, suggesting

an enhanced immune activity (24). A second clinical trial (conducted by scientists from the University of Oslo) with 60 adults also demonstrated the safety and efficacy of the standardized Elderberry syrup in the treatment of influenza and its symptoms (28). Patients with either influenza type A or type B were given 15 mL of Sambucol or a placebo four times per day. Treatment was initiated within 48 hours of the onset of symptoms and continued for five days. The global evaluation scores (symptoms and overall wellness scores combined) for the Elderberry-treated group showed a pronounced improvement after a mean of 3.1 days as compared to 7.1 days for the placebo group. A larger number of patients in the control group resorted to a "rescue medication" such as paracetamol as compared to the treatment group. Both studies concluded that patients exhibiting flu symptoms and treated with Elderberry standardized extract recovered significantly faster than patients in the control group. As the authors note in their report, these findings need to be confirmed in a larger study.

Another placebo-controlled study indicated that Sambucol given as prophylactic to a group of chimpanzees accumulated over a period of six months reduced the flu-like symptoms to 12 days as compared to 39 days in the control group (total of ten chimpanzees randomized into two equal groups) (29).

Immune Enhancement

Results from two in vitro studies demonstrated that Sambucol upregulates cytokine release (30,31). Both studies used blood-derived monocytes and examined the concentration of four inflammatory cytokines (IL-1 β , IL-6, IL-8, TNF- α) and one anti-inflammatory cytokine (IL-10), in response to various Elderberry (Sambucol) formulations and control syrups (other branded preparations based on Echinacea and Propolis). A 1.3- to 6.2-fold increase in cytokine production was observed compared to control (31). It was concluded that the standardized Elderberry extract activates the healthy immune system and may possess immunoprotective or immunostimulatory effects when administered to patients with various diseases.

Antioxidant Activity

The berries are rich in anthocyanins, which serve as natural antioxidants. Ginsburg et al. (32) suggested that the unique composition of the constituents results in antioxidants, which are more resistant to oxidation. Because it has been observed that anthocyanin glycosides are indeed absorbed in humans (see pharmacokinetics), it is possible that Elderberry extracts containing anthocyanins may be a significant source of antioxidants.

Plasma antioxidant capacity and total phenolics were significantly increased one hour after ingestion of 400 mL of Elderberry juice (33), while heating over six hours at 95°C caused a decline of the trolox equivalent antioxidant capacity due to colorless degradation following thermal exposure (34). Another in vitro study demonstrated that Elderberry derived anthocyanins could be incorporated into the membrane and cytosol of endothelial cells (human aortic) and thus increase protection against oxidative stress (35).

Anticarcinogenic Activity

An aqueous acetone extract of the berries from cultivated *S. nigra* was fractionated and tested in a range of assays that gauge the anticarcinogenic potential. *S. nigra* fruits demonstrated significant chemopreventive potential through strong induction of quinone reductase and inhibition of cyclooxygenase-2, which is indicative of anti-initiation and antipromotion properties (36). Elderberry anthocyanins played a major role in chemoprotection and could inhibit the growth of a human colorectal adenocarcinoma (HT29) cell line (37).

Leaves

While the flowers and leaves are the plant parts mainly used, intraperitoneal administration of the unsaponifiable fraction (the fraction not soluble in water after saponification of total extract in KOH) of the leaves to mice moderately enhanced phagocytosis at a dose of 0.5 mL/animal (38).

Safety

The majority of safety issues refer to the presence of the cyanogenic glycoside sambunigrin, which may be hydrolyzed in various plant parts to free hydrogen cyanide. Various sources state that the ripe fruits are edible without causing harm and are frequently used as additives for various foods. The safety of the standardized Elderberry extract preparation, Sambucol was demonstrated in a clinical study (28), where it was concluded that this Elderberry extract is safe and can be administered to the whole population, including infants and children.

One study reported type I allergy to proteins of *S. nigra* (39) after inhalation of pollens, and the authors suggested that oral administration of flowers or berries could also induce allergies. On the contrary, Elderberry and Elder flowers were suggested to be beneficial for allergic symptoms and reduce nasal congestion and other upper respiratory discomforts.

Contraindications

To the best of our knowledge there are no published data showing that there are contraindications in the use of Elderberry.

Use During Pregnancy and Lactation: There are no known restrictions for pregnant or lactating women (6). Some authors propose that the use of Elder flowers during pregnancy should be avoided due to lack of toxicity data. However, there is no information available on impairment of fertility or teratogenic effects (7,40).

Drug/Herb Interactions

There are no reported drug/herb interactions with other plants or medications. Theoretically, since the Elderberry may exhibit a diuretic and a laxative effect, caution should be exercised when taken for a long time concomitantly with diuretics or with drugs that interact with diuretics and laxatives.

Side Effects

There have been no reported side effects from Elder flower preparations. The most common side effect for Elderberries quoted in the literature is the diuretic and laxative

effect (41). Elder preparations from unripe berries (as well as from other plant parts) can induce toxic effects in humans due to poisonous cyanogenic glycosides. Effects of cyanide, also known as hydrocyanic acid, on humans include nausea, vomiting, and diarrhea as well as central nervous system and respiratory depression, and general lethargy.

REGULATORY STATUS

Both flowers and berries are classified as GRAS (generally recognized as safe).

Elder is listed by the Council of Europe in Categories N1 (refers to the fruit and indicates that there are no restrictions on quantities used) and N2 (41). In the list of plants in the French official bulletin of drugs based on plants, instructions for the submission of registration (AMM allég), Elderberries belong to category one for which no toxicological studies are needed (42). The berries of Elder are rated by the American Herbal Products Association as Class 1, meaning "herbs which, when used appropriately, can be consumed safely without specific use restrictions" (43).

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Eleuthero

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INTRODUCTION

Eleuthero is obtained from *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim. [syn: *Acanthopanax senticosus* (Rupr. et Maxim.) Harms], a member of the ivy family (Araliaceae). Preparations of the dried root and rhizome or stems have an approximate 2,000-year history of use in Traditional Chinese Medicine (TCM), generally administered as a component of complex herbal mixtures prepared by aqueous decoction. For the related Japanese Kampo system of medicine, it is the rhizomes, often with root, that are specified. Dried aqueous-ethanolic extracts in tablet form and liquid extracts for intravenous drip are also used today as monopreparations in Oriental medical practice. Starting in the 1940s, scientists in the former Soviet Union began intensive pharmacological and clinical research on eleuthero preparations whereby the “adaptogen” concept was developed and tested over the following decades. It was proposed that an adaptogen is an innocuous substance, relatively free of side effects, has nonspecific action, increases resistance to a wide range of environmental or other physical stressors, and may have a normalizing action in the body irrespective of a diseased state. In 1962, the first eleuthero preparation received marketing authorization in the former Soviet Union. A quality standards monograph then became official in the *State Pharmacopoeia of the Union of Soviet Socialist Republics*. By 1975, eleuthero preparations entered European commerce and in 1991 the German health authority published a positive monograph for marketing authorization and labeling of eleuthero medicinal products. A corresponding quality standards monograph then entered the *German Pharmacopoeia* (now superseded by the *European Pharmacopoeia* eleuthero monograph). In 2008, on the basis of comprehensive expert committee reviews of the literature to date, the European Medicines Agency (EMA) and Health Canada each published their own final eleuthero monographs, which now have relevance for licensing or registration of eleuthero traditional herbal medicinal products (THMP) in EU Member States or eleuthero Natural Health Products (NHP) in Canada, respectively. EMA permits registration of eleuthero THMPs on the basis of evidence of traditional use only. Health Canada also permits licensing of eleuthero preparations on the basis of efficacy evidence for use in herbal medicine practice. As of 2009, the Canadian Licensed Natural Health Products Database listed details on over 100 eleuthero-containing NHPs. Although eleuthero is now regulated as an active substance for use in licensed, listed, or registered medicinal products in many

countries including Australia, Canada, China, European Community, Japan, and Russian Federation, since 1994 it has been regulated as a dietary supplement component in the United States. In the preamble of the Food and Drug Administration’s (FDA) final rule on statements made for dietary supplements concerning their effect on the structure or function of the body, the agency provided examples of potentially acceptable structure/function claims including “energizer,” “rejuvenative,” “revitalizer,” and “adaptogen,” all of which could apply to eleuthero dietary supplements depending on the level of evidence that the notifying firm compiles in its substantiation file. Although numerous preclinical and clinical studies have been carried out, aiming to prove the nonspecific adaptogen concept, the totality of evidence remains inconclusive to substantiate the efficacy of eleuthero preparations for any specific conditions. A significant amount of newer research involves preparations of eleuthero in combination with, or compared against, one or more other substances also proposed to exhibit adaptogenic action, among other combinations for treatment of various conditions ranging from upper respiratory tract infections to osteoarthritis.

BACKGROUND

The genus *Eleutherococcus* includes 38 species that occur in Eastern Asia, 18 of which occur in China (14 endemic). Known in Chinese medicine as *ci wu jia*, as *shi go ka* in Japanese medicine and as *eleuterokokk koljučij* in Russian, the species that is the subject of this article, *E. senticosus*, occurs in scrub fields, forests, roadsides, and valleys below 2000 m in China (Provinces of Hebei, Heilongjiang, Jilin, Liaoning, N Shaanxi, Shanxi, and Sichuan), Japan (Hokkaido Island), the Korean Peninsula, and the Russian Far East (Siberia). Other related species also used medicinally in China include *xi ci wu jia* [*E. setulosus* (Franchet) S.Y. Hu], *xi zhu wu jia* [*E. nodiflorus* (Dunn) S.Y. Hu], *wu geng wu jia* [*E. sessiliflorus* (Rupr. et Maxim.) S.Y. Hu], *lun san wu jia* [*E. verticillatus* (G. Hoo) H. Ohashi], *kang ding wu jia* [*E. lasiogyne* (Harms) S.Y. Hu], and *bai le* [*E. trifoliatus* (L.) S.Y. Hu] (1). Japanese researchers have determined that at least three different species are commercially traded interchangeably under the same common name. An analysis of the nuclear ribosomal DNA internal transcribed spacer sequence of eleuthero products available in the Japanese and Chinese markets found that only about

70% of all samples were made from the correct official species (*E. senticosus*). Quantitative determination of three marker compounds, eleutheroside B, syringaresinol diglucoside, and isofraxidin by ultra performance liquid chromatography/mass spectrometry showed that eleutheroside B and isofraxidin are specific to the correct source plant (2). Inaccurate identification of investigational products has implications for outcomes of laboratory and clinical research.

The eleuthero material of commerce is mainly collected from wild populations in the People's Republic of China and Russian Federation, with increasing commercial quantities being produced under regulations for organic certification according to a sustainable wild-crop harvesting practice standard. In China, some of the supply is also cultivated in fields and mountain terraces. Eleuthero is harvested in the spring or autumn, washed clean, dried and supplied as broken pieces of root and rhizome or stem. The rhizome is knotty, of irregular cylindrical shape, 15 to 30 cm long, and 1.4 to 4.2 cm in diameter. The root is cylindrical, mostly tortuous, 3.5 to 12 cm long, and 0.3 to 1.5 cm in diameter. The stems are long and cylindrical, varying in length, and 0.5 to 2 cm in diameter (3).

Listed in China's earliest materia medica published during the Han Dynasty (206 BC–AD 220), the *Shen Nong Ben Cao Jing* (Herbal Classic of the Divine Plowman), eleuthero continues to be used in TCM generally as a component of complex formulations but also as a monopreparation. In TCM theory, eleuthero acts to "replenish *qi* (vital energy) and invigorate the *spleen*" and "nourish the *kidney* and anchor the mind". The Chinese Pharmacopoeia Commission indicates the use of eleuthero preparations for treatment of lassitude, anorexia, aching pain in the lower back and knees, insomnia and dreamfulness due to *yang deficiency* in the *spleen* and *kidney*. *Yang deficiency* is a general term for lack of physiological energy of life with diminished functions (3).

In 1947, Russian pharmacologist Prof. Nikolai Vasilievich Lazarev, M.D., first proposed the term "adaptogen" to classify substances such as eleuthero that may act to increase "nonspecific" resistance to adverse influences to organism and stress (as a state of threatened homeostasis). In the following decades, antistress effects of eleuthero were investigated in animal experiments and human trials in the Soviet Union. A student of Dr. Lazarev, pharmacologist Prof. Israel I. Brekhman, M.D., defined the general pharmacodynamic characteristics of an adaptogenic substance and was responsible for introducing eleuthero preparations into Russian medical practice. The first eleuthero product was granted marketing authorization for clinical use in the former Soviet Union in 1962 (4,5). In 1978, the first eleuthero products were notified to the German health authority and in 1991, the German Commission E [now part of the Federal Institute for Drugs and Medical Devices (BfArM)] published a positive monograph. The approved indications were for use as a tonic for invigoration and strengthening in cases of fatigue and debility or declining capacity for work and concentration, as well as during convalescence. In 2003, BfArM published a more detailed monograph providing patient information text for labeling of registered eleuthero medicinal products in Germany (6). As a result of the European Union

Directive on Traditional Herbal Medicinal Products, which came into force in 2004, EMEA's Committee on Herbal Medicinal Products was charged with establishing Community Herbal Monographs for the registration and authorization of herbal medicinal products in EU Member States. The Committee on Herbal Medicinal Products conducted a comprehensive review of the scientific literature on eleuthero, assessing more than 300 papers, and in 2008 published a final monograph that indicates the therapeutic use of eleuthero as a THMP for symptoms of asthenia such as fatigue and weakness (7). The quality of eleuthero used as an active substance of a THMP must comply with the corresponding pharmacopeial quality standards monograph *Eleutherococci radix* PhEur (8).

In the United States, with the passage of the *Dietary Supplement Health and Education Act of 1994* (DSHEA), eleuthero preparations became regulated as dietary supplement products rather than as medicinal products. The FDA has provided examples of potentially acceptable structure/function statements that could be applicable to eleuthero dietary supplements (e.g., energizer, rejuvenative, revitalizer, or adaptogen) (9). In Canada, following the enactment of the *NHP Regulations*, which came into effect in 2004, the Natural Health Products Directorate (NHPD) was charged with developing the *NHPD Compendium of Monographs*, which applicants can reference in support of the safety and efficacy of an NHP as part of their product license application. In 2008, Health Canada's NHPD published its final monograph on eleuthero for compendial product license applications. The quality of eleuthero used as an active ingredient of an NHP may comply with the monograph of either the *European Pharmacopoeia* (*Eleutherococcus* PhEur) or *United States Pharmacopoeia* (*Eleuthero* USP) (10).

CHEMISTRY AND PREPARATION OF PRODUCTS

Over 35 compounds have been identified so far, but it is not yet clear which constituents or groups of constituents are most responsible for the effects of eleuthero. Because it appears that a complex mixture of constituents are responsible for the biological effects, the defined eleuthero preparation in its entirety must be regarded as the active substance. Certain constituents such as eleutherosides B and E are generally accepted to contribute to the therapeutic activity but are not responsible for the full therapeutic effect. As such, these constituents are used for control purposes and are defined as "active markers." Therefore it is important for researchers and product manufacturers to select eleuthero materials that test in conformance with all of the qualitative and quantitative standards of a specific pharmacopeial monograph for batch-to-batch uniformity and reproducible results. In any case, eleutherosides cannot be assigned to a particular compound class but instead belong to a variety of chemical groups (including lignans, phenylpropanes, coumarins, saponins, sterols, and sugars). Most eleutherosides were described under different names before they were isolated from *E. senticosus*. Although it has been suggested that use of the term "eleutherosides" should be discontinued, in order to prevent the impression that these substances belong to the

Table 1 Summarized Comparison of Eleuthero Pharmacopoeial Standards

Standard	JP XV	PhEur 6	PPRC 2005	USP 32-NF 27
Definition	Dried rhizomes often with root	Dried, whole or cut underground organs	Dried root and rhizome or stem	Dried rhizome and roots
Content	No standard (eleutheroside B present by HPLC)	Min 0.08% for the sum of eleutherosides B and E (by HPLC)	Min 0.05% of eleutheroside B (by HPLC)	Min 0.08% for the sum of eleutherosides B and E (by HPLC)
ID tests:	Macroscopic Microscopic Organoleptic HPLC test	Macroscopic Microscopic TLC test	Macroscopic Microscopic Organoleptic TLC test	Macroscopic Histology TLC test
Foreign matter	No standard	Max 3%	No standard	Max 3.0%
Loss on drying	Max 13.0%	Max 10.0%	Max 10.0%	Max 14.0%
Total ash	Max 6.0%	Max 8.0%	Max 9.0%	Max 8.0%
Acid-insoluble ash	Max 1.0%	No standard	No standard	No standard
Ethanol-soluble extractives	Min 2.5%	No standard	Min 3.0%	No standard
Water-soluble extractives	No standard	No standard	No standard	Min 4.0%

Abbreviations: JP XV, Japanese Pharmacopoeia, 15th edition 2007; PhEur 6.0, European Pharmacopoeia, 6th edition 2009; PPRC 2005, Pharmacopoeia of the People's Republic of China, 2005 English Edition; USP 32-NF 27, United States Pharmacopoeia 32nd revision National Formulary 27th edition 2009.

same compound group, use of this term continues in official compendia (11). Table 1 provides a summarized comparison of quality standards for eleuthero as published in currently valid editions of the *Japanese Pharmacopoeia* (JP) (12), *European Pharmacopoeia* (PhEur) (8), *Pharmacopoeia of the People's Republic of China* (PPRC) (3), and *United States Pharmacopoeia-National Formulary* (USP-NF) (13).

According to the 2008 EMEA assessment report, eleuthero root is characterized by the coexistence of pentacyclic and tetracyclic triterpenoidal saponins and their prosapogenins, lignans, coumarins, phenylcarbonic acids, and xanthenes. The main constituents are phenyl propane compounds (eleutheroside B: 0.5%, chlorogenic acid, coniferyl aldehyde and its glucoside, caffeic acid derivatives); lignans (eleutheroside E: 0.10%, eleutheroside B₄: 0.023%; coumarins (isofraxidin and its O-glucoside); triterpene saponins (2-protoprimulagenin A glycoside: 0.125%). Other constituents include steroids, carbohydrates, and immunologically active polysaccharides (heteroglycans and eleutherans) (14). As active markers, both the PhEur and USP, respectively, require that dried eleuthero root contain not less than 0.08% of the sum of eleutheroside B (C₁₇H₂₄O₉; M_r 372.37) and eleutheroside E (C₃₄H₄₆O₁₈; M_r 742.7), as determined by the High Performance Liquid Chromatography (HPLC) methods specified in the monographs. But according to the PPRC, the dried root and rhizome or stem must contain not less than 0.050% of "syringoside" (C₁₇H₂₄O₉; M_r 372.37) as determined by HPLC. Syringoside, a phenylpropanoid glycoside, is also referred to as "syringin" and/or "eleutheroside B" [chemical name: β-D-Glucopyranoside, 4-(3-hydroxy-1-propenyl)-2,6-dimethoxyphenyl]. Eleutheroside E is a lignan also referred to as "syringaresinol diglucoside" [chemical name: β-D-Glucopyranoside, (tetrahydro-1H,3H-furo(3,4-c)furan-1,4-diyl)bis(2,6-dimethoxy-4,1-phenylene)bis-].

Preparations of eleuthero are defined in official pharmacopoeial monographs and therapeutic compendia. Table 2 provides descriptions of compendial eleuthero preparations with details on their specified mode of administration and posology.

PRECLINICAL STUDIES

Numerous in vivo studies have been carried out designed to evaluate the adaptogenic response to a range of induced adverse conditions such as stress, immobilization, or chemical challenges in animal models using a 33% ethanolic extract of eleuthero root. For summaries of the extensive experimental pharmacology data, see eleuthero monographs in WHO Vol. 2 2002 (15) and ESCOP 2nd ed. 2003 (16).

Ethanolic extract of eleuthero stem bark administered with a feeding tube to Dawley rats (100 mg/kg body weight) caused c-Fos accumulation in both the supraoptic nuclei and paraventricular nuclei, which regulate stress response. Only the caudal regions in the nucleus of the solitary tract, a locus innervating both the supraoptic nuclei and paraventricular nuclei, were activated. This neuroanatomical pattern suggests a possible involvement of these stress-related brain loci in association with eleuthero ingestion (17). Liquid extract of eleuthero root combined with cadmium chloride given to mice in a drink solution led to a significant decrease of cadmium concentration in the blood and liver. Eleuthero decreased the cadmium-induced mitotic and apoptotic activity of liver cells (18). Intraperitoneal administration of eleuthero root extract prior to injection of lipopolysaccharide and D-galactosamine in mice significantly improved survival rate. Eleuthero pretreatment appeared to protect mice against lipopolysaccharide and D-galactosamine-induced endotoxic shock involving inhibition of NF-kappaB activation, which caused downregulation of TNF-α and involved upregulation of interleukin-10 (19).

CLINICAL STUDIES

This article discusses mainly the results of controlled clinical trials involving monopreparations of eleuthero published between 1990 and 2009 that are available in the English language literature. Many other trials published from the 1960s to the present are available only in the Chinese or Russian literature. English language abstracts

Table 2 Compendial Eleuthero Preparations, Mode of Administration and Posology

Preparation	Definition	Mode of administration	Posology
Eleuthero Dry Aqueous Extract EMA	Dry extract prepared by extracting with water from <i>Eleutherococcus</i> PhEur; drug-to-extract ratio range is between 15:1 and 17:1	Oral use in capsules or tablets	90–180 mg; may be taken in 1–3 doses daily
Eleuthero Dry Ethanolic Extract EMA	Dry extract prepared by extracting with ethanol and water from <i>Eleutherococcus</i> PhEur (ethanol 28–70% v/v)	Oral use in capsules or tablets	Daily dose corresponding to 0.5–4 g dried root; may be taken in 1–3 doses daily
Eleuthero Herbal Tea EMA	Comminuted <i>Eleutherococcus</i> PhEur supplied in bulk form or packed in filter sachets. Contains min. 0.08% sum of eleutherosides B and E	Oral use. Pour 150 mL boiling water over 0.5–4 g cut root. Infuse for 10–15 min	150 mL of tea infusion divided in 1–3 doses daily
Eleuthero Infusion NHPD	Comminuted Eleuthero USP or <i>Eleutherococcus</i> PhEur supplied in bulk form or packed in filter sachets. Contains min. 0.08% sum of eleutherosides B and E	Oral use. Pour 150 mL boiling water over 2–3 g cut root. Infuse for 10–15 min	150 mL of tea infusion daily
Eleuthero Liquid Extract EMA/NHPD	Hydroalcoholic liquid preparation prepared from <i>Eleutherococcus</i> PhEur; drug-to-extract ratio 1:1	Oral use liquid in dropper bottle	2–3 mL daily; may be taken in 1–3 doses
Eleuthero Solid Extract NHPD	See Powdered Eleuthero Extract USP	Oral use in capsules or tablets	0.065–0.195 g daily
Eleuthero Tincture EMA and/or NHPD	Alcoholic liquid preparation prepared by maceration or percolation from <i>Eleutherococcus</i> PhEur; drug-to-extract ratio 1:5	Oral use liquid in dropper bottle	10–15 mL; may be taken in 1–3 doses
Extractum Acanthopanax Senticosi PPRC	Soft extract prepared by extracting with water or ethanol from <i>Radix et Rhizoma seu Caulis Acanthopanax Senticosi</i> PPRC; drug-to-extract ratio 20:1. Contains min. 0.5% of syringoside, min. 45% water-soluble extractive, max. 30% water	Oral use in tablets; Tabellae Acanthopanax Senticosi PPRC	Extract: 0.3–0.45 g, 3 times daily Tablets: 2–3 twice daily
Powdered Eleuthero EMA	Powdered from <i>Eleutherococcus</i> PhEur. Contains min. 0.08% sum of eleutherosides B and E	Oral use in capsules or tablets	0.75–3 g daily; may be taken in 1–3 doses
Powdered Eleuthero Extract USP	Dry extract prepared from Eleuthero USP, extracted with hydroalcoholic mixtures; drug-to-extract ratio range is between 13:1 and 25:1. Contains min. 0.8% sum of eleutherosides B and E	Oral use in capsules or tablets	See Eleuthero Solid Extract NHPD

Abbreviations: EMA, European Medicines Agency; NHPD, Natural Health Products Directorate; PhEur, European Pharmacopoeia; PPRC, Pharmacopoeia of the People's Republic of China; USP, United States Pharmacopoeia.

for some of these are available but often lack essential details on study design and composition of investigational product. Most of the older studies were carried out without controls. Furthermore, the trend for much of the recent clinical research has been to evaluate the effects of polypreparations that contain eleuthero extract as just one of the active components or to evaluate the effects of concomitant administration of eleuthero with other herbal preparations. A review of clinical trials using eleuthero-containing complex mixtures is outside of the scope of this article. Table 3 summarizes recent (1990–2009) clinical studies involving monopreparations of eleuthero root or leaf. Table 4 provides a brief description of eleuthero-containing polypreparations that are undergoing clinical and laboratory research.

In a randomized controlled trial, Lee et al. (2008) investigated the effects of freeze-dried eleuthero leaf extract plus calcium versus calcium (control) on serum lipid profiles, biomarkers of oxidative stress, and lymphocyte DNA damage in postmenopausal women over a six-month period. There were significant decreases ($P < 0.001$) in serum LDL (127.54 ± 29.79 mg/dL versus 110.33 ± 22.26 mg/dL)

and the LDL/HDL ratio (2.40 ± 0.65 vs. 2.11 ± 0.58). Protein-carbonyl levels and lymphocyte DNA damage also decreased significantly ($P < 0.001$ and $P < 0.05$, respectively) (20). In a randomized, double-blind comparative trial, Weng et al. (2007) investigated the effects of an aqueous-ethanolic dry extract of eleuthero root plus lithium versus fluoxetine plus lithium in adolescents with bipolar disorder. No significant differences were seen. The response rate between the eleuthero and fluoxetine groups was similar (67.6% vs. 71.8%) as was the remission rate between both groups (51.4% vs. 48.7%) (21). Hartz et al. (2004) found no significant difference between eleuthero root and placebo after two months of treatment in volunteers with chronic fatigue syndrome (22). In a randomized, double-blind, placebo-controlled trial, Cicero et al. (2004) investigated the effects of eleuthero root dry extract in elderly hypotensive volunteers on health-related quality-of-life parameters. After four weeks, there were higher scores in social functioning ($P = 0.02$) scales but these differences did not persist to the eight-week period (23). Arushanian and Shikina (2004) investigated the effects of chronic administration of an ethanolic fluidextract

Table 3 Controlled Clinical Studies with Eleuthero Preparations 1990–2009

Reference	Duration	Number of patients	Preparation	Dosage	Comparison treatment
(20)	6 mo	40	Eleuthero leaf freeze-dried extract	2 × 500 mg t.i.d. + 500 mg calcium	Calcium
(21)	6 wk	76	Eleuthero root ethanolic dry extract	250 mg t.i.d. + 250 mg lithium b.i.d.	Fluoxetine or Lithium
(22)	2 mo	76	Eleuthero root powder	4 × 500 mg/day	Placebo
(23)	8 wk	20	Eleuthero root dry extract	300 mg/day	Placebo
(24)	1 mo	27	Eleuthero root liquid extract	20 drops b.i.d.	Placebo
(25)	30 days	45	Eleuthero root liquid extract	2 vials/day	Placebo
(26)	8 wk	47	Eleuthero root extract injection	Not known	Conventional treatment
(27)	6 wk	18	Eleuthero root 35% ethanolic extract	8 mL/day (4 g root equivalent)	Asian ginseng root extract or placebo
(28)	30 days	50	Eleuthero root fluidextract 1:1	25 drops t.i.d.	Echinacea herb juice preparation
(29)	7 days	9	Eleuthero root	1200 mg/day (for 7 days before trial)	Placebo
(30)	14 days	60	Eleuthero root extract injection	60–80 mL in 500 mL 0.9% saline by intravenous drip q.d.	Dextra-40 injection
(31)	3 mo each arm	24	Eleuthero root powder	1250 mg/day	Ginkgo leaf extract, vitamins or placebo
(32)	6 wk	20	Eleuthero root fluidextract	3.4 mL/day	Placebo
(33)	6 mo	93	Eleuthero root ethanolic dry extract	375 mg q.i.d.	Placebo
(34)	15 days	57	Eleuthero root extract injection	40 mL in 300 mL 5% dextrose injection by intravenous drip q.d.	Insulin and potassium chloride injection

of eleuthero root on light and color perception in healthy young adults without physical or ophthalmological disease. After one month, significant positive changes in eye sensitivity were observed in both morning and evening hours compared to placebo (24). In a randomized, double-blind, placebo-controlled trial, Facchinetti et al. (2002) concluded that 30 days of administration of eleuthero root liquid extract in healthy adults reduced some cardiovas-

cular responses to stress (25). In a randomized controlled trial, Ni et al. (2001), investigated the effects of eleuthero root extract injections on urinary albumin excretion and renal endothelin metabolism in type 2 diabetics with persistent microalbuminuria and normotension. After eight weeks of treatment, the levels of urinary albumin excretion as well as plasma and urinary endothelin lowered significantly ($P < 0.01$) in the eleuthero group with no

Table 4 Eleuthero-containing Polypreparations Undergoing Research

Eleuthero Combinations	Subject
With extract of andrographis herb [<i>Andrographis paniculata</i> (Burm. f.) Nees; Fam. Acanthaceae]	Treatment of upper respiratory tract infections
With extracts of andrographis herb, schisandra fruit [<i>Schisandra chinensis</i> (Turcz.) Baill.; Fam. Schisandraceae], and licorice root [<i>Glycyrrhiza glabra</i> L.; Fam. Fabaceae]	Treatment of familial Mediterranean fever
With extracts of Asian ginseng root [<i>Panax ginseng</i> C.A. Mey.; Fam. Araliaceae], Japanese aralia [<i>Aralia mandshurica</i> Rupr. & Maxim; Fam. Araliaceae], and rhodiola root [<i>Rhodiola rosea</i> L.; Fam. Crassulaceae]	Cardioprotective, inotropic, and antiarrhythmia properties
With extracts of Asian ginseng root, rhodiola root, and schisandra fruit	Impact on the level of energy, ability to work under stress, quality of life and wellbeing, in middle-aged working women
With extracts of echinacea [<i>Echinacea purpurea</i> (L.) Moench.; Fam. Asteraceae] and Malabar nut tree leaf [<i>Adhatoda vasica</i> Nees; Fam. Acanthaceae]	Treatment of acute upper respiratory tract infections
With tincture of maral root [<i>Rhaponticum carthamoides</i> (Willd.) Iljin; Fam. Asteraceae]	Effects on blood coagulation system during training in athletes
With aqueous-ethanolic dry extracts of maral root, rhodiola root, and schisandra fruit	Effects on cell-mediated and humoral immunity of patients with advanced ovarian cancer
With perilla fruit [<i>Perilla frutescens</i> (L.) Britt.; Fam. Lamiaceae]	Antiallergic effects
With rehmannia root [<i>Rehmannia glutinosa</i> Libosch. ex Fisch. & Mey.; Fam. Scrophulariaceae]	Postmenopausal osteoporosis
With aqueous extracts of rehmannia root and Tienchi ginseng [<i>Panax notoginseng</i> (Burk.) F.H. Chen; Fam. Araliaceae]	Knee osteoarthritis
With extracts of rhodiola root and schisandra fruit	Adjuvant in treatment of acute nonspecific pneumonia
With liquid extract of safflower [<i>Carthamus tinctorius</i> L.; Fam. Asteraceae]—as an injection	Treatment of traumatic intracranial hematoma

significant changes in the control group (26). In a comparative placebo-controlled study, Gaffney et al. (2001) investigated the effects of eleuthero root 35% ethanolic extract versus Asian ginseng root 35% ethanolic extract or placebo on competitive club-level endurance athletes engaged in normal in-season training. None of the immune system variables changed significantly nor showed any clear trend from pre- to post-test in any of the treatment groups. Furthermore, eleuthero increased rather than decreased hormonal indices of stress, suggesting a threshold of stress below which it increases stress response and above which it decreases stress response (27). Szolmicki et al. (2000) compared the effects of a eleuthero root fluidextract versus fresh pressed juice preparation of *Echinacea purpurea* herb [*Echinacea purpurea* (L.) Moench; Fam. Asteraceae] on cellular defense and physical fitness parameters. After one month, eleuthero influenced oxygen plateau by significantly increasing oxygen consumption during maximal physical exercise. Analysis of blood samples showed that the phagocytic activity of neutrocytes in the eleuthero group rose significantly and the number of neutrocytes actively participating in phagocytosis increased (28). Eschbach et al. (2000) found no significant differences in athletic endurance measurements between eleuthero and placebo groups at any steady-state time interval or during cycling time trials in highly trained male athletes (29). In a single-blind controlled trial, Han et al. (1998) evaluated the effect of eleuthero root liquid extract intravenous drip injection in treating acute cerebral infarction. After 14 days of treatment, the total effective rate of the eleuthero group (86%) was higher than the control group (50%). The nervous functional deficit score was significantly lower in the eleuthero group (9.96 ± 4.66) compared to control (13.56 ± 1.84). The proposed mechanism of action is ameliorating peroxidation in the brain and improving hypothalamic-pituitary-adrenocortical axis function (30). In a randomized, double-blind, placebo-controlled, crossover trial, Winther et al. (1997) compared the effects of eleuthero root, extract of ginkgo leaf [*Ginkgo biloba* L.; Fam. Ginkgoaceae], vitamins, and placebo on cognitive function in healthy middle-aged volunteers. After three months of treatment each (crossover) there were no changes in the D-2 concentration test results, whereas selective memory significantly improved during treatment with eleuthero ($P < 0.02$) versus placebo. The evaluation of 25 different visual analogue scales were also in favor of eleuthero ($P < 0.05$) versus placebo (31). In a six-week trial evaluating the effects of eleuthero root fluidextract on fatigue during submaximal and maximal aerobic exercise in athletes, Dowling et al. (1996) found no significant differences between eleuthero and placebo. It was concluded that ergogenic claims cannot be supported on the basis of these results (32). Williams (1995) investigated the effects of a standardized eleuthero root extract for immune protection against herpes simplex type 2 infection in patients with HSV-2. There were statistically significant results ($P = 0.0002$ to 0.0007) in the eleuthero group where 75% reported improvements in severity, duration, or frequency of herpes simplex type 2 attacks versus 34% in the placebo group. The investigator noted, however, that more than three months of treatment was required before optimum effects were observed, suggesting that eleuthero extract would be useful only for suppressive rather than episodic therapy (33). In a randomized controlled trial,

Shang et al. (1991) studied the effect of eleuthero root liquid extract intravenous drip injection in patients with coronary heart disease and myocarditis whose ventricular late potential (VLP) were positive. After 15 days of treatment, the rates of changing positive VLP into negative were 74.29% in the eleuthero group versus 34.6% in the insulin and potassium chloride control group ($P < 0.005$). The results appear to show that eleuthero injection may be an effective treatment in patients with positive VLP (34).

Efficacy

In a Cochrane Database of Systematic Reviews meta-analysis by Li et al. (2009) of West China Hospital, Sichuan University, the safety and efficacy of eleuthero preparations in patients with acute ischemic stroke was assessed. The researchers included randomized controlled trials comparing eleuthero with placebo or open control (no placebo) in patients with acute ischemic stroke. Of the 13 trials (962 participants) that met inclusion criteria, the outcome measure was the improvement of neurological deficit after treatment. Eleuthero was associated with a significant increase in the number of participants whose neurological impairment improved [risk ratio (RR) 1.22, 95% confidence interval (CI) 1.15 to 1.29]. The authors concluded, however, that the risk of bias in all the included trials was high, and therefore the data were not adequate to draw reliable conclusions about the efficacy of eleuthero in acute stroke. Much larger trials of greater methodological quality would be necessary (35).

Concerning efficacy evidence for an adaptogenic action, on the basis of a review of over 300 papers, the EMEA assessment report (2008) concluded that the principle of an adaptogenic action still needs further clarification and more preclinical and clinical studies. Although the EMEA is aware of the fact that numerous preclinical and clinical studies have been performed aiming to prove the adaptogenic concept, the clinical data have many limitations such as deficiencies in the description of inclusion and exclusion criteria, description of the investigational product, diagnosis, study design, and analysis etc. There is a wide range of clinical conditions that have been investigated and the number of patients has been very small in some studies. None of the studies would be sufficient to substantiate efficacy of eleuthero preparations in a clearly defined clinical condition. The total data available, however, are sufficient to justify further research into the adaptogenic concept (14). Individual researchers, however, continue to assert that good, albeit not strong, scientific evidence has been documented in trials in which eleuthero root preparations have been shown to increase endurance and mental performance in patients with mild fatigue and weakness (36).

Adverse Effects

According to the German BfArM (2003) and Canadian NHPD (2008), there are no known adverse reactions (6,10) although the EMEA assessment report (2008) states that there have been no systematic studies designed to detect adverse events. Individual case reports and other general evidence suggest that eleuthero preparations may cause insomnia in some people if taken too close to bedtime. Some cases of insomnia, as well as shifts in heart rhythm, tachycardia, extrasystoles, and hypertonia, were

reported in two clinical studies from the 1960s involving atherosclerotic patients. Two patients receiving high dosage eleuthero extract in a study involving 55 patients with rheumatic heart disease reported headaches, pericardial pain, palpitations, and elevated blood pressure. A small study involving 11 hypochondriac patients found that eleuthero liquid extract was well tolerated at a dosage range of 2.5 to 3.0 mL t.i.d. for 60 days, although some patients reported insomnia, irritability, melancholy and anxiety at dose levels of 4.5 to 6.0 mL (14). Thus, the EMEA final monograph (2008) lists the following potential undesirable effects: insomnia, irritability, tachycardia, and headaches with an unknown frequency of occurrence (7).

Interactions and Contraindications

There are no known or reported interactions with other substances. In a pharmacokinetic interaction study, Donovan et al. (2003) investigated the effects of standardized eleuthero root preparation (485 mg capsules containing 250 mg dry extract plus 235 mg powdered root) in 12 normal volunteers on the activity of cytochrome P450 CYP2D6 and CYP3A4. No significant differences were observed indicating that eleuthero at the generally recommended doses is unlikely to interact with coadministered medications that are primarily dependent on the CYP2D6 or CYP3A4 pathways for elimination (37).

Although the German BfArM monograph (2003) contraindicated the use of eleuthero preparations in cases of high blood pressure (6), according to a subsequent EMEA assessment report (2008) there is limited general evidence and very limited information from studies to support this contraindication. In two earlier clinical studies, it was recommended that the eleuthero extract investigational product should not be given to subjects with blood pressure in excess of 180/90 mm Hg. In any case, the EMEA final monograph included a contraindication of arterial hypertension (7,14). The final NHPD monograph (2008) also contraindicates eleuthero in cases of high blood pressure (10). Although there are no data suggesting the occurrence of general hypersensitivity to plants of the Araliaceae family, there is a theoretical potential of hypersensitivity reactions to eleuthero preparations. While there have been some studies involving pregnant women showing no teratogenic or embryotoxic effects, the general safety of using eleuthero preparations during pregnancy and lactation has not been fully established. Following the precautionary principle in the absence of sufficient data, a qualified health care practitioner should be consulted prior to use during pregnancy and lactation.

MECHANISM OF ACTION

Although the pharmacological effects of eleuthero preparations have been investigated extensively, the mechanism of action and the active compound(s) have not yet been fully identified (14). The general pharmacodynamic characteristics of an adaptogenic substance as originally defined by Dr. Brekhman are as follows:

- An adaptogen is almost nontoxic to the recipient.
- An adaptogen tends to be nonspecific in its pharmacological properties and acts by increasing the resistance of the organism to a broad spectrum of adverse biological, chemical, and physical factors.

- An adaptogen tends to be a regulator having a normalizing effect on the various organ systems of the recipient organism.
- The effect of an adaptogen is as pronounced as deeper are the pathologic changes in the organism.

According to a review paper by Panossian and Wikman (2009), the stress-protective effect of adaptogenic substances including eleuthero preparations is related to regulation of homeostasis via several mechanisms of action associated with the hypothalamic-pituitary-adrenal axis and the control of key mediators of stress response such as molecular chaperons (e.g., Hsp70), stress-activated c-Jun N-terminal protein kinase (JNK1), Forkhead Box O transcription factor DAF-16, cortisol, and nitric oxide (NO). According to this theory, the main point of action for an eleuthero preparation would appear to be upregulating and stress-mimetic effects on the "stress-sensor" protein Hsp70, which plays a role in cell survival and apoptosis. Hsp70 inhibits the expression of NO synthase II gene and interacts with glucocorticoid receptors directly and via the JNK pathway, thus affecting the levels of circulating cortisol and NO. Prevention of stress-induced increase in NO, and the associated decrease in ATP production, could result in increased performance and endurance. Adaptogen-induced upregulation of Hsp70 triggers stress-induced JNK-1 and DAF-16-mediated pathways regulating the resistance to stress, possibly resulting in enhanced mental and physical performance (36).

REGULATORY STATUS

Australia

Eleuthero root and preparations thereof are substances that may be used as active ingredients in Listed Medicines for supply in Australia (38). *Quality*: For active ingredients of listed medicines, the quality standard of the *British Pharmacopoeia* (BP) is the minimum standard that must be applied in its entirety. The PhEur and the USP, respectively, have also been adopted as additional default standards under the Therapeutic Goods Act. *Indications*: An adaptogenic herb traditionally used as a tonic to support the body during periods of exertion. Also useful during convalescence (39).

Canada

Eleuthero root is an NHP active ingredient, requiring premarketing authorization and product license issuance for over-the-counter human use. *Quality*: Must comply with the minimum specifications outlined in the current *NHPD Compendium of Monographs* (40). Pharmacopoeial standards currently accepted by the NHPD are the BP, PhEur, and USP (41). *Indications*: Dried root or preparations thereof (fluidextract, solid extract, tea infusion or tincture) used in Herbal Medicine as a tonic to help relieve general debility and/or to aid during convalescence, or to help improve mental and/or physical performance after periods of mental and/or physical exertion (10).

China

Eleuthero dried root and rhizome or stem (*Radix et Rhizoma seu Caulis Acanthopanax Senticosi*) is an active ingredient for use in Chinese medicinal preparations (e.g., aqueous

decoctions) and as starting material for the production of powdered ethanolic dry extract (*Extractum Acanthopanax Senticosi*) and tablets containing the extract (*Tabellae Acanthopanax Senticosi*). *Quality*: Eleuthero active ingredients of Chinese medicines must comply with the corresponding PPRC monograph. *Indications*: Hypofunction of the spleen and the kidney marked by general weakness, lassitude, anorexia, aching of the loins and knees, insomnia and dream-disturbed sleep (3).

European Community

Preparations of eleuthero (e.g., comminuted herbal substance for preparation of herbal tea, 1:1 liquid extract, 1:5 tincture, and dry extracts for solid dosage forms) are regulated as THMP requiring premarketing authorization and issuance of a traditional herbal registration for over-the-counter human use. *Quality*: Eleuthero for use in THMPs must comply with the PhEur monograph (*Eleutherococci radix*). *Indications*: Traditional herbal medicinal product for symptoms of asthenia such as fatigue and weakness (7).

Japan

Eleuthero rhizome is an active ingredient for use in Japanese Kampo medicine preparations. *Quality*: Eleuthero-active ingredients of Kampo medicines must comply with the JP monograph (*Eleutherococci senticosi Rhizoma*) (12).

Russian Federation

Eleuthero dried root and rhizome is an active ingredient for use in Russian medicinal products and as starting material for the production of a 33% ethanol extract (*Extractum Radicis et Rhizomatis Eleutherococcus*). *Quality*: Eleuthero-active ingredients must comply with the corresponding monograph of the *State Pharmacopoeia of the Union of Soviet Socialist Republics* (42).

United States

Preparations of eleuthero are regulated as dietary supplement products requiring FDA notification. The manufacturer of a eleuthero dietary supplement making a structure/function or general well-being claim must have substantiation demonstrating that the claim is truthful and not misleading (9). The dietary ingredient eleuthero or a dietary supplement containing it must identify the ingredient using the Latin binomial *E. senticosus* or the standardized common name "eleuthero." The term "Siberian ginseng" cannot be used (43). *Quality*: Dietary supplement quality standards monographs for eleuthero dried rhizome with roots, powdered eleuthero, and powdered eleuthero extract are official in the *United States Pharmacopoeia-National Formulary* (USP-NF) (13). The DSHEA amendments to the FD&C Act named *USP* and *NF* as the official compendia for dietary supplements. The amendments also provide that a dietary supplement may be deemed misbranded if it is covered by a monograph in an official compendium, is represented as conforming to this monograph, but fails to conform. Conformance to the *USP-NF* monograph is voluntary, however.

World Health Organization

A monograph for the oral use of powdered dried roots and rhizomes of *E. senticosus* (or equivalent preparations) is published in the *WHO Monographs on Selected Medicinal Plants* Volume 2. *Indications* (Medicinal Uses Supported by Clinical Data): As a prophylactic and restorative tonic for enhancement of mental and physical capacities in cases of weakness, exhaustion and tiredness, and during convalescence (15).

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Ephedra

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INTRODUCTION

Ephedra (Ma huang) is the common name for a herbal product used in traditional Chinese medicine. It comprises the aerial parts of three principal plant species: *Ephedra sinica* Stapf, *E. equisetina* Bunge, and *E. intermedia* var. *tibetica* Stapf (1–3). The plant is a natural source of the alkaloids (–)-ephedrine and (+)-pseudoephedrine (1).

GENERAL DESCRIPTION

Ephedra sinica Stapf is a low evergreen shrub with small scaly leaves. It flowers in June and July and produces fruit late in the summer (4). Approximately 45 to 50 species of ephedra have been described worldwide, including in the temperate and subtropical regions of Asia, Europe, and the Americas (2,5). Most of those native to North, South, and Central America, such as *E. nevadensis* (used to make Mormon tea), *E. trifurca*, and *E. antisiphilitica*, contain no alkaloids (5).

Most commercial uses of ephedra are based on its content of ephedrine, the main active constituent in the ephedra species known as *ma huang* (1). Alkaloid content increases as the plant matures, with peak concentrations in the fall (6). No ephedrine-type alkaloids are found in the roots, berries, or seeds of these plants, and the green upper parts of the stems contain significantly more alkaloids than the woody parts (6).

Traditionally, ephedra has been administered as a tea prepared by soaking 2 g dried aerial portions in 8 fl oz of boiling water for 10 minutes, ideally resulting in a content of 15 to 30 mg ephedrine. In commercial products, it is usually present in a formulation of powdered aerial portions or a dried extract (7). The ephedrine content of such products ranges from 12 to 80 mg per serving, with most being in the lower part of the range (8).

In the United States, ephedra was sold as a dietary supplement until April 2004 when the U.S. Food and Drug Administration (FDA) banned the sale of dietary supplements containing ephedrine alkaloids. As defined by Congress in the Dietary Supplement Health and Education Act, which became law in 1994, a dietary supplement is a product (other than tobacco) that is intended to supplement the diet; contains one or more dietary ingredients (including vitamins, minerals, herbs or other botanicals, amino acids, and other substances) or their constituents; is intended to be taken orally as a pill, capsule, tablet, or

liquid; and is labeled on the front panel as being a dietary supplement.

Ephedra-containing dietary supplements were most often promoted as weight loss aids or to improve energy levels and athletic performance, even though there is little or no evidence for their efficacy for these uses. Prior to the ban, a 1998 survey of more than 14,500 people, 18 years or older conducted in five states for the use of dietary supplements for weight loss reported that 7% used nonprescription products, while 1% consumed ephedra-containing preparations (9).

HISTORICAL USE

Ephedra has a history of more than 5000 years of medicinal use in China and India, where it has been used to treat cold, fever, flu, chills, headaches, edema caused by nephritis, nasal congestion, aching joints, coughing, and wheezing as well as to induce diuresis or perspiration (5,6). Evidence that its use predates even traditional Chinese and Indian medicine comes from the discovery of a species of ephedra found in a Neanderthal grave in Iraq dating from 60,000 B.C. (8). Analysis of the plants found in the grave indicated that they contained bioactive ingredients and were presumably used medicinally. There is documentation of its use by Discorides, the renowned Greek herbalist, in the 1st century and in Europe from the 15th to the 19th centuries (10). In the 1600s, Native Americans and Spaniards in the American Southwest used ephedra (the nonalkaloid-containing species) as a treatment for venereal disease (10).

Ephedrine, the principal active ingredient in most species, was first isolated in 1885 by Nagayoshi Nagai, a Japanese chemist trained in Germany (10). Other alkaloids, such as pseudoephedrine, norephedrine, and norpseudoephedrine, with similar but not identical properties, were subsequently found in various ephedra species (11).

Early studies on the pharmacological effects of ephedrine were conducted between 1888 and 1917, but it was not until the 1920s during studies of a variety of Chinese traditional herbs that ephedrine alkaloids became known to Western medicine. Ephedra, one of the herbs tested, gave significant results—an extract given intravenously to dogs resulted in a large increase in blood pressure (12). Subsequent publication of a series of studies on the pharmacological properties helped Western physicians understand its potential usefulness (11). These

experiments demonstrated that ephedrine had three advantages over epinephrine: it could be administered orally, was longer acting, and had a lower toxicity (3). As a result, the former alkaloid replaced the latter in the management of children with mild-to-moderate asthma. Subsequently, it became widely used as a nasal decongestant and central nervous system stimulant (4). Its ability to stimulate the central nervous system was recognized by the Japanese military, which administered it by injection to kamikaze pilots during World War II (8).

The herb ephedra was once recognized as an official drug in the United States, but widespread availability of synthetic ephedrine-type alkaloids virtually eliminated its clinical use (13).

CONSTITUENTS AND ACTIONS

Ephedra stems contain 0.5% to 2.5% (4) alkaloids, composed mainly of (–)-ephedrine and (+)-pseudoephedrine, with ephedrine content ranging from 30% to 90% of total alkaloid content depending on the source. The content of the various ephedra alkaloids varies in commercial preparations, but ephedrine and pseudoephedrine generally make up 90% to 100% (14). (–)-Ephedrine and five structurally related alkaloids, (+)-pseudoephedrine, (–)-*N*-methylephedrine, (+)-*N*-methylpseudoephedrine, (+)-norpseudoephedrine, and (–)-norephedrine, are responsible for the medicinal properties of these alkaloid-containing ephedra species (1). Ephedrine acts indirectly by stimulating α_1 -, α_2 -, β_1 -, and β_2 -adrenergic receptors to release norepinephrine from sympathetic nerve endings and directly as a β_1 -, β_2 -, and possibly β_3 -agonist (8,15,16). It promotes bronchodilation by stimulating β_2 -receptors in the lungs, which accounts for its effectiveness in treating bronchial asthma (12,16). However, its other adrenergic actions result in the generally undesirable effects of central nervous system stimulation including insomnia, irritability, hyperactivity (16), hypertension (14,17), and gastrointestinal symptoms (nausea and vomiting) (11). These side effects have led to the development of selective β_2 -agonists and the discontinuation of ephedrine use for bronchodilation.

Ephedrine increases heart rate and therefore cardiac output (16). It also causes peripheral vasoconstriction, which increases peripheral resistance and can lead to a sustained rise in blood pressure (16). In general, the increase in blood pressure is dose dependent (8), but doses under 50 mg do not always produce such an effect (14,17).

Chronic use of ephedrine for the management of asthma results in tachyphylaxis, making it an unreliable long-term therapeutic agent (16). Frequent doses become less effective as a result of the depletion of norepinephrine stores as well as the release of adenosine into synaptic junctions and increased cellular phosphodiesterase activity, both of which suppress catecholamine release (18). To potentiate the effects of ephedrine, methylxanthines (e.g., caffeine and theophylline), which interfere with the inhibitory effect of adenosine on both norepinephrine release and phosphodiesterase activity, are often combined with ephedra or ephedrine (19,20).

In the United States, ephedra-containing dietary supplements were widely promoted as weight loss aids because of their ephedrine content. The alkaloid has been postulated to do this by two mechanisms. First, by stimulating thermogenesis in laboratory animals (13,16,21) and humans (15,18), ephedrine increases energy expenditure. In rats, it has been shown to directly stimulate thermogenesis in brown adipose tissue via β -adrenergic receptors (16). However, studies in humans have provided evidence against the role of brown adipose tissue in ephedrine-induced thermogenesis (18). Although the site of action remains unclear, its thermogenic effect in humans has been widely confirmed and serves as the major rationale for its use as a weight loss aid. Second, the combination of ephedrine and caffeine acts as an appetite suppressant, reducing food intake. One study showed that about 75% of the effect of this combination on weight loss is the result of its anorectic properties, which may affect the satiety center in the hypothalamus (13,22). Evidence from animal studies and some human experiments shows that the combination reduces body fat but not lean body mass (13,23). However, because the human studies had few participants, larger studies would have to be carried out to confirm this finding.

The use of ephedrine as a nasal decongestant in over-the-counter preparations has been replaced for the most part by more selective β_2 -bronchodilators, but it is still included as a component of some pediatric prescription cold and cough medications and is found in a nonprescription medication for asthma (16). The ephedra constituent, pseudoephedrine is generally preferred as an oral decongestant because it is less potent and therefore less likely than ephedrine to cause central nervous system stimulation or hypertension (16).

Intravenous ephedrine is still widely used for the prevention and treatment of hypotension caused by spinal anesthesia, particularly during cesarean section (16). Other uses have included treatment of chronic urticaria, diabetic neuropathic edema, nocturnal enuresis, motion sickness, spastic or hypermotile bowel, and myasthenia gravis (24,25).

In addition to ephedrine alkaloids, some ephedra species include other nitrogen-containing secondary metabolites with known neuropharmacological activity (5). These include several cyclopropyl analogs of L-glutamate and methanoproline, and a cyclopropyl analog of L-proline, as well as common amino acids such as L-glutamate, L-glutamine, L-serine, and L-proline.

The stems of several ephedra species also contain kynurenates, derivatives of tryptophan catabolism that may help protect the plant from birds or rodents, but their physiological function is unknown (5). Kynurenates in ephedra stems exhibit antimicrobial activity against some gram-positive and gram-negative bacteria, although they are significantly less potent than the antibiotic ciprofloxacin.

Non-nitrogenous compounds found in some ephedra species include organic acids, phenolic compounds (flavonoids and tannins), and essential oils primarily composed of terpenoids (38.9%) (2,5). The tannin fractions have been shown to inhibit angiotensin converting enzyme activity, although to a much smaller degree than captopril.

Extracts of the roots contain the spermine-type alkaloids ephedradines A, B, C, and D, feruloylhistamine, and bisflavanols and cause hypotension when administered intravenously (2,26). The roots (*ma huang gen*) are not used in commercial dietary supplement products and are only a minor constituent of formulations used in traditional Chinese medicine.

PHARMACOKINETICS

Ephedrine is well absorbed after oral administration, is excreted largely unchanged in the urine, and has a serum half-life of 2 to 3 hours (14,27). When ingested in the form of *ma huang*, it has a t_{\max} (time of occurrence for peak drug concentration) of nearly four hours compared with only two hours for pure ephedrine (17).

Peak ephedrine blood levels are similar regardless of whether the alkaloid is taken as a herbal preparation or in the pure form. Ingestion of 400 mg of *ma huang* containing 20 mg ephedrine resulted in blood concentrations of 81 ng/mL, which is the same as the peak ephedrine levels observed after administering an equivalent amount of pure ephedrine (17).

PRECLINICAL STUDIES

Ephedra and its constituents have been studied in animals for a wide range of indications, including ulcer prevention, reduction of uremic toxins in renal failure, and immune modulation, as well as for their antimicrobial, antidiabetic, anti-inflammatory, antioxidant, and antitussive activities (2). Ephedrine has also been used in preclinical research to examine the efficacy and potential mechanisms of action of ephedra for weight loss (2,13).

CLINICAL TRIALS

A systematic review of the literature was conducted by RAND for published and unpublished sources of controlled clinical trials on ephedra and ephedrine used for weight loss and athletic performance in humans (6). Fifty-two controlled clinical trials of ephedrine or botanical ephedra used for weight loss or athletic performance enhancement in humans were included.

Efficacy for Weight Loss

Forty-four controlled trials were identified that assessed ephedra and ephedrine alkaloids used in combination with other compounds for weight loss, and 20 of these met inclusion criteria for the meta-analysis. Five pairs of treatment regimens were compared:

1. Ephedrine versus placebo (five studies): Ephedrine was associated with 1.3 lb/mo weight loss greater than placebo for up to four months of use.
2. Ephedrine plus caffeine versus placebo (12 studies): Ephedrine plus caffeine was associated with 2.2 lb/mo weight loss greater than placebo for up to four months of use.
3. Ephedrine plus caffeine versus ephedrine (three studies): Ephedrine plus caffeine was associated with 0.8 lb/mo weight loss greater than ephedrine alone.
4. Ephedrine plus herbs containing ephedra plus caffeine (three studies): Ephedra plus herbs containing caffeine was associated with 2.1 lb/mo weight loss greater than placebo for up to four months of use.
5. Ephedrine versus other active weight loss products (two studies): No conclusions could be drawn about ephedrine versus other active weight loss products because the sample size in these studies was too small.

Only one study compared an ephedra-containing product without caffeine but with other herbs and a placebo. This product was associated with a weight loss of 1.8 lb/mo, more than that associated with a placebo for up to three months of use.

None of the studies lasted longer than six months; hence, long-term weight loss and maintenance could not be assessed. The results indicate that the use of ephedrine, ephedrine plus caffeine, or dietary supplements containing ephedra plus herbs containing caffeine was associated with a statistically significant increase in weight loss for up to four months.

Efficacy for Athletic Performance Enhancement

There are no controlled clinical studies for athletic performance enhancement. RAND identified eight controlled trials of the effects of synthetic ephedrine for athletic performance enhancement, usually along with caffeine. The studies could not be pooled for meta-analysis because of the wide variety of interventions used. A few studies assessing the effect of ephedrine plus caffeine showed a modest improvement in very short-term athletic tasks such as weightlifting (1–2 hr after a single dose) and time to exhaustion for aerobic exercises. However, these trials were of very short duration and were done in small numbers of healthy young men, mostly military recruits. The results therefore cannot be generalized to the general public. Because all the studies were done in the same laboratory, the ability of other investigators to confirm the results has not been tested.

Published just before the FDA ban, a randomized trial of 15 healthy volunteers demonstrated that a single dose of a dietary supplement product containing ephedra and caffeine among other ingredients statistically significantly prolonged the QTc interval to longer than 30 milliseconds, a potentially dangerous effect (28).

Since the FDA's ban on the sale of dietary supplements containing ephedrine alkaloids, no clinical trials of significant size or duration have appeared in the scientific literature.

TOXICOLOGY

In Vitro Toxicity

The toxicity of eight water extracts of ephedra prepared from the entire plant using either ground or whole herb, boiled for 0.5 or 2 hours, and with either one or two extractions, was tested in a human hepatoblastoma cell line (HepG2) and a variety of animal cell lines (29). Of the cell lines tested, only a neuronal cell line (Neuro-2a)

showed significant sensitivity to the cytotoxic effects of the extracts. Grinding increased the toxicity of the resulting extracts. Normalizing the results for ephedrine content showed that the toxicity of the ephedra extracts could not be accounted for solely by ephedrine content, indicating the presence of other cytotoxic constituents. Kynurenates and cyclopropyl amino acids, both of which cause central nervous system toxicities and have been isolated from some species of ephedra, could be associated with the additional toxicity (5). However, neurotoxicity is eliminated during the boiling of extracts that are used in commercial products.

In studies performed by the National Toxicology Program, ephedrine was not mutagenic in four strains of *Salmonella typhimurium* and did not cause chromosomal aberrations in vitro in Chinese hamster ovary cells (30). Other in vitro researches of ephedrine or water or methanol extracts of ephedra have also not demonstrated any mutagenic effects (31,32).

In Vivo Toxicity

Several studies in mice have determined the oral LD₅₀ (median lethal dose) of water extracts of ephedra, which ranges from 4000 to 8000 mg/kg depending on the alkaloid content of the species used (2). On the basis of these measurements, the equivalent range of ephedrine was calculated to be 520 to 720 mg/kg.

The National Toxicology Program also evaluated the toxicity of ephedrine in B6C3F1 mice and F344 rats. During two-year studies, the animals were given diets containing 0, 125, or 250 ppm ephedrine per day. The mean body weight of the rats and mice receiving diets containing either dose of ephedrine was lower than those of controls, and survival was similar for the controls and exposed animals (30).

A teratogenicity study of ephedrine showed a frequency of 8% malformed chick embryo hearts with exposure to 0 or 0.5 μ mol ephedrine that increased to 26% for 5.0 μ mol (33).

A study in rats showed that the cardiotoxic effects of ephedra are significantly increased at doses at or above the ephedrine equivalent of 25 mg/kg, and that these effects are further increased with the addition of caffeine (34).

ADVERSE EVENTS

The safety of ephedra-containing dietary supplements has been a controversial subject, resulting in a high level of interest from regulators, manufacturers, and the public. MedWatch, the Adverse Reaction Monitoring System of the FDA, has recorded many reports of side effects concerning ephedra-containing products.

Ephedra-containing products were the subject of significant media coverage for quite a few years because of the deaths of several professional athletes who were allegedly taking these products (6). As a result of these safety concerns, ephedra was prohibited by the International Olympic Committee and the National Football League (6).

A review of the adverse effects reported in 50 controlled clinical trials of ephedra, ephedrine with or without caffeine, or botanicals containing caffeine concluded that

use of these substances was associated with 2 to 3 times the risk of nausea, vomiting, psychiatric symptoms, autonomic hyperactivity, and palpitations compared with placebo (35). No serious adverse events such as myocardial infarction, stroke, or death were reported in these studies, but the authors of the review noted that the small total number of patients in these experiments was not adequate to distinguish a serious adverse event rate of 1 in 1000 or higher.

To evaluate the incidence of serious side effects, several researchers reviewed ephedra-related adverse event reports in MedWatch as well as case reports in the published literature. The latter were evaluated in the RAND review because the total number of participants in the clinical trials was not sufficient to adequately assess the possibility of rare outcomes. Although such adverse event reports are not conclusive evidence of a cause-and-effect relationship, they can indicate the potential for such a relationship.

RAND searched the literature for published case reports and the MedWatch database for cases of serious adverse events that were idiopathic in etiology. If use of ephedra or ephedrine-containing products was well documented, then the possibility that ephedra or ephedrine caused the event was considered. Sentinel events were defined as adverse events associated with ingestion of an ephedra-containing product within 24 hours prior to the event and for which alternative explanations were excluded with reasonable certainty. Possible sentinel events were defined as adverse events that met the first two criteria for sentinel events but for which alternative explanations could not be excluded. RAND reviewed 71 cases reported in the published medical literature, 1820 case reports from the MedWatch database, and more than 18,000 consumer complaints reported to a manufacturer of ephedra-containing dietary supplements. The documentation for most reports was insufficient to support decisions about the relationship between the use of ephedra or ephedra-containing dietary supplements and the adverse event. Only 65 cases from the published literature, 241 from MedWatch, and 43 from a manufacturer of ephedra-containing dietary supplements had documentation sufficient for them to be included in the adverse event analysis. Among these, RAND identified 2 deaths, 3 myocardial infarctions, 9 cardiovascular accidents, 3 seizures, and 5 psychiatric events as sentinel events and 9 deaths, 6 myocardial infarctions, 10 cardiovascular accidents, 9 seizures, and 7 psychiatric events as possible sentinel events (35). About half of the sentinel and possible sentinel events occurred in individuals younger than 30 years.

Another study identified 140 MedWatch reports of adverse events concerning ephedra-containing products between June 1, 1997 and March 31, 1999 and concluded that 31% were definitely or probably related to use of these products and another 31% were possibly related (36). The events included death, stroke, hypertension, tachycardia, palpitations, and seizures.

A third study reviewed the MedWatch database for stroke, myocardial infarction, or sudden death associated with the use of ephedra-containing products from 1995 to 1997 (27). Of 926 cases of adverse event reports concerning the use of ephedra-containing products, 37 serious

cardiovascular events were identified as being temporally associated with the use of these products.

On the basis of this information, in April 2004, the FDA banned the sale of dietary supplements containing ephedrine alkaloids.

Subsequent to the FDA ban, other case reports of adverse events potentially associated with the use of dietary supplements containing ephedra have been published that include incidences of cardiomyopathy, stroke, coronary thrombosis, and serious psychiatric events (37–42).

CONTRAINDICATIONS

Because of the potentially dangerous effects of ephedrine on the heart and central nervous system, individuals with a history of cardiovascular disease; hypertension; hyperthyroidism; seizures; depression or other mental, emotional, or behavioral conditions; glaucoma; or difficulty in urinating because of benign prostatic hypertrophy should avoid taking ephedra-containing products (43).

Ephedrine is often used for intraoperative hypotension and bradycardia, raising concerns about preoperative use of ephedra-containing products. Such application puts people anesthetized with halothane at risk because halothane sensitizes the myocardium to ventricular arrhythmias (14).

The potential for serious side effects rises as serving size and frequency of use is increased (44). These risks may also be enhanced when ephedra-containing products are used with other sources of stimulants such as caffeinated beverages, over-the-counter drugs, and other dietary supplements containing stimulants.

DRUG INTERACTIONS

Ephedra-containing products should not be taken with, or for two weeks, after monoamine oxidase inhibitors or with drugs for Parkinson disease, obesity, or weight control; methyl dopa; or any product containing ephedrine, pseudoephedrine, or phenylpropanolamine (43).

FUTURE RESEARCH

To assess the safety of ephedra, a rigorous case-control study of ischemic vascular, cardiovascular, and heat stroke events should be given a high priority.

Basic research is needed on pharmacokinetic drug interaction assessments, including identification of interactions with agents such as anabolic steroids or sympathomimetics, and on physiological responses under conditions such as exercise and thermal stress.

Clinical trials will be necessary to assess the risk-benefit ratio of ephedra for weight loss among overweight and obese individuals. A phase II study could be used to evaluate adverse events, weight loss, physiological responses, and optimal dosing. A randomized clinical trial of adequate sample size could then be used to characterize side effects and evaluate efficacy with regard to moderate

or long-term weight loss and maintenance and relevant health outcomes.

The research portfolio described above would be time consuming and expensive but would answer the questions of ephedra safety and efficacy definitively. However, it is likely that as a result of the FDA's ban on dietary supplements containing ephedrine alkaloids this research will not be done.

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Evening Primrose

Fereidoon Shahidi and Homan Miraliakbari

INTRODUCTION

Intake of dietary fat, particularly essential fatty acids, is known to influence human health and disease status. Evening primrose oil (EPO), a source of γ -linolenic acid, has received much attention for its possible therapeutic effects on inflammatory and cardiovascular diseases, diabetes, and cancer, among others. The beneficial health effects attributed to the oil are thought to be mediated by the desaturated metabolite of γ -linolenic acid, namely dihomo- γ -linolenic acid, which is metabolized in the body to produce anti-inflammatory eicosanoids that may reduce the incidence or severity of human disease status and to promote health. EPO is also a source of antioxidative tocopherols. This entry attempts to summarize the effects of EPO in health promotion and disease risk reduction.

BACKGROUND

Evening primrose (*Oenothera biennis*) is a biennial herb with erect stems reaching 3 ft in height and has fragrant, yellow flowers that bloom at nightfall (evenings). The plant is native to North America but has been naturalized in Europe and parts of the Southern Hemisphere. Following pollination (usually performed by moths), short and cylindrical capsules containing many small seeds are formed (1). The oil extract of evening primrose seeds (evening primrose oil or EPO) is a rich source of the essential polyunsaturated omega-6 (n -6) fatty acid, linoleic acid (65–80% of total fatty acids), and its desaturated metabolite γ -linolenic acid (8–14% of total fatty acids) (2). EPO is also a good source of α -tocopherol (3). Several reports show that EPO is beneficial in the promotion of human health and in the treatment of several diseases, including heart diseases, cancer, inflammatory diseases, diabetes, and diseases related to women's health (4). However, the U.S. Food and Drug Administration has not approved the use of EPO for any of the health claims attributed to its use (5). Among others, the oil is available as a dietary supplement in North America and Europe. Several moderately to highly refined dietary oil supplements containing EPO have been developed and marketed for specific uses including Efamast[®] for benign breast pain and Epogam[®] for atopic eczema, although no health agencies support these claims.

Essential Fatty Acids

Humans are unable to synthesize polyunsaturated fatty acids (PUFAs), but are able to elongate and desaturate

their 18-carbon (C_{18}) precursors obtained through the diet (6). In humans, two essential PUFA families, namely, the omega-6 (n -6) and the omega-3 (n -3) families, are recognized with linoleic acid (18:2, n -6) and α -linolenic acid (18:3, n -3), serving as their parent compounds, respectively. The designation n -6 and n -3 indicates whether the sixth or the third carbon from the methyl terminus is unsaturated. EPO is a rich source of the n -6 fatty acid, γ -linolenic acid (18:3, n -6), which can be elongated in vivo to produce dihomo- γ -linolenic acid (20:3, n -6) and then desaturated to produce arachidonic acid (20:4, n -6). Several putative health benefits of EPO have been attributed to γ -linolenic acid and its metabolites (4).

Metabolic Functions of Omega-6 Fatty Acids

In humans, linoleic acid (18:2, n -6) is the essential fatty acid required in the highest amount, which is estimated to be approximately 1% to 2% of total caloric intake in adults (7). Dietary linoleic acid can be elongated and desaturated in the body to produce other long-chain n -6 fatty acids. If dietary linoleic acid intake is deficient, γ -linolenic acid (18:3, n -6), dihomo- γ -linolenic acid (20:3, n -6), arachidonic acid (20:4, n -6), and docosapentaenoic acid (22:5, n -6) become essential. Hence, these fatty acids are referred to as conditionally essential (Fig. 1) (8). Both n -6 and n -3 fatty acids are chain elongated through the same biosynthetic pathways. Thus, elongation and desaturation of long-chain n -3 and n -6 PUFAs are proportional to the dietary intake of their C_{18} precursors (9). The efficiency of fatty acid elongation pathways in humans is estimated to be 5% to 7% under optimal conditions when adequate dietary C_{18} essential fatty acids are present (9).

The C_{18} n -6 and n -3 fatty acids are essential partly because they are the precursors of C_{20} and C_{22} lipid-based cytokines or eicosanoids [prostaglandins (PG), thromboxanes (TX), and leukotrienes (LT)]. These lipid mediators play crucial roles in vascular physiology and inflammatory responses, among others (10). The long-chain n -6 and n -3 fatty acids have also been beneficial in many diseases, for example, cancer (11), cardiovascular diseases, including stroke (12). Therapeutic health effects of EPO have often been attributed to its high content of γ -linolenic acid, which is elongated in vivo to dihomo- γ -linolenic acid (13), which can then be metabolized via the cyclo-oxygenase or lipoxygenase enzyme families to yield series-1 PG and TX or series-3 LT, respectively. The cyclo-oxygenase and lipoxygenase products of dihomo- γ -linolenic acid and hence EPO have been shown to exert anti-inflammatory (14), anti-proliferative (15), and anticarcinogenic (16) effects. However, the desaturase product of

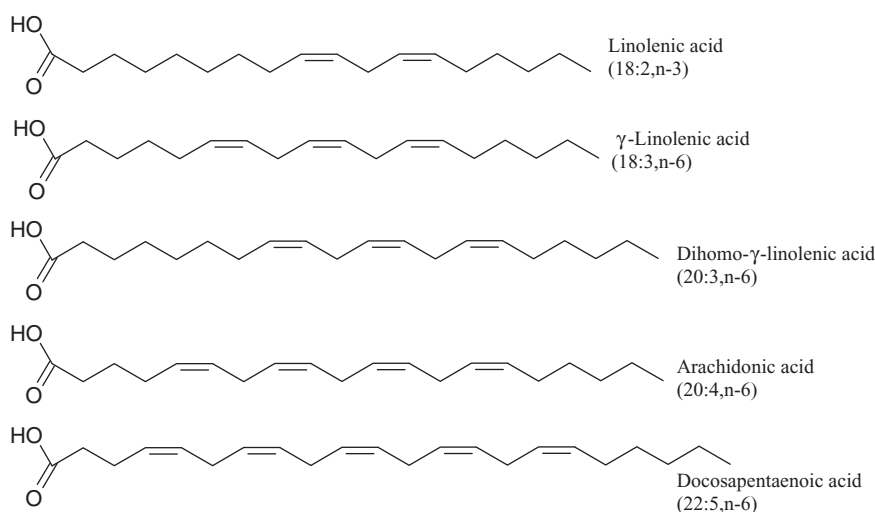


Figure 1 Essential and conditionally essential fatty acids of the omega-6 (*n*-6) family.

dihomo- γ -linolenic acid, arachidonic acid (20:4, *n*-6), is metabolized to produce proinflammatory PG and LT that may negate the anti-inflammatory potential of dihomo- γ -linolenic acid in vivo (17).

The long-chain *n*-6 and *n*-3 fatty acids play important roles in maintaining the fluidity or optimal state of cell membranes and are key components in the membranes of highly specialized cells such as neurons, erythrocytes, cardiomyocytes, retinocytes, germ cells, and immune cells (18). Linoleic acid and elongation products thereof have a role in maintaining epidermal integrity. They are essential for the development and maintenance of the skin's water-impermeable barrier, the stratum corneum (19). The elongation products of *n*-6 and *n*-3 fatty acids are also required for proper growth and development of the brain and retina during gestation and infant life (20), and for normal brain function and vision in adults (21). More recently, these products have been shown to affect gene expression, leading to pronounced changes in metabolism, cellular differentiation, and growth (22).

EPO AND INFLAMMATORY DISEASES

Chronic inflammation associated with diseases such as inflammatory bowel disease, psoriasis, arteriosclerosis, and rheumatoid arthritis may be caused or attenuated by alterations of normal eicosanoid pathways resulting in overproduction of inflammatory cytokines. Many anti-inflammatory drugs act as inhibitors of proinflammatory cytokine production. For example, nonsteroidal anti-inflammatory drugs (NSAIDs) (acetylsalicylic acid or aspirin) irreversibly inactivate cyclo-oxygenase activity, thereby inhibiting proinflammatory PG and TX biosynthesis. EPO may be of use in the treatment and/or management of some inflammatory diseases because it has been shown to reduce proinflammatory eicosanoid biosyn-

thesis, while concomitantly increasing anti-inflammatory biosynthesis both in vitro and in vivo (23). Currently, there is much research investigating their therapeutic potential for inflammatory diseases.

Several research groups have performed feeding studies with EPO to unravel its effects on proinflammatory cytokine production in cell-based in vitro model systems (24). In one recent study (25), 3-week-old male Wistar rats were fed diets supplemented with oils including EPO for eight weeks. Experimental diets contained 15% EPO, sardine oil, or virgin olive oil by weight, while control diets contained 2% corn oil. After the eight-week feeding period, lymphocytes and other blood cells were obtained from the peritoneal region of animals from all four groups and were purified to yield a suspension containing approximately 85% polymorphonuclear leukocytes and 15% mononuclear lymphocytes. The leukocyte suspensions were stimulated with the calcium ionophore (A23187) to induce the production of cyclo-oxygenase-derived eicosanoids, which were quantified by radioimmunoassay. In these studies, leukocytes from EPO-fed animals had reduced the production of proinflammatory prostaglandin E₂ (PGE₂) and thromboxane B₂ (TXB₂) compared to those from control animals. However, the difference reached significance for PGE₂ only (25). Larger and more important differences were observed for leukocytes from the fish oil-fed animals but not for those with virgin olive oil. These results show that EPO may be an effective anti-inflammatory agent because its long-term dietary presence reduces lymphocyte reactivity, possibly because of its γ -linolenic acid constituent (25).

EPO and Atopic Dermatitis

Atopic dermatitis, also known as atopic eczema, is an immune-mediated inflammatory skin disorder characterized by redness, itching, and oozing vesicular lesions that become scaly, crusted, or hardened (26). Corticosteroid

and antipruritic treatments are common for addressing atopic dermatitis, but concerns regarding the side effects of these drugs have prompted search for alternative natural and less toxic treatments (27). The effects of EPO supplementation on atopic dermatitis have received much attention because the fatty acid composition of the oil may interfere with the production of proinflammatory cytokines, which could potentially reduce the symptoms of this disease (28). Furthermore, low Δ -6-desaturase activity leading to γ -linolenic acid deficiency has previously been reported as a contributing factor to atopic dermatitis (29), making EPO (with its high γ -linolenic acid content) the subject of much interest in eczema therapy. Morse et al. (30) performed a meta-analysis on nine placebo-controlled studies investigating the efficacy of EPO supplementation in the treatment of atopic dermatitis; the individual studies were performed in eight different centers (30). This analysis included two of the earliest large-scale studies on EPO and eczema as well as seven small ones (14–47 participants). In these studies, patients and doctors assessed the severity of eczema in experimental and placebo groups by scoring measures for skin symptoms including itchiness, dryness, and scaliness. Subjects in the experimental groups were provided with Epogam EPO capsules containing 10% γ -linolenic acid. Results from all studies were pooled together to give a global clinical score for each assessment point. This early meta-analysis showed that the clinical scores for patients receiving Epogam supplements were significantly better than those for placebo groups, particularly for the symptom of itch ($P < 0.0001$). Furthermore, there was a positive correlation between plasma dihomo- γ -linolenic acid levels and clinical score improvement (30). However, the results of this meta-analysis have been questioned by experts for two main reasons: First, it did not consider the relatively large study by Bamford, Gibson, and Renier (31), which included 123 participants and did not demonstrate any therapeutic effect of EPO on eczema. Second, this meta-analysis as well as seven of the studies referenced by it was sponsored by Scotia Pharmaceuticals, the manufacturer of Epogam. Some recent studies have failed to reflect the efficacy of EPO as treatment for atopic eczema, as exhibited in the placebo-controlled trial conducted by Hederos and Berg (32) that included 60 eczemic children supplemented with Epogam for 16 weeks as well as in the larger placebo-based trial by Berth-Jones and Graham Brown (33) that included 123 patients. It has been postulated that EPO treatment may modify immunological parameters associated with atopic dermatitis such as plasma interferon- γ (INF- γ) and immunoglobulin-E (IgE) levels (34,35). Recently, Yoon, Leg and Lee (35) investigated this possibility in 14 children with atopic dermatitis. A group of six children without the disorder were used as normal controls. EPO was administered to participants with atopic dermatitis until their symptom scores remained below one for two weeks. EPO treatments lasted 75 ± 58 days; all participants receiving EPO exhibited clinical improvements and $>42\%$ of this group were completely cleared of symptoms of atopic dermatitis. Before EPO treatments, the mean plasma INF- γ levels in the experimental group were significantly lower than that of the control group ($P < 0.01$). But after treatments, INF- γ levels in the experimental group increased

to a level equal to that of the control group ($P < 0.01$) (35). Plasma IgE levels of the experimental group were significantly greater than that of the control group both before and after the treatment. The results of this study imply that EPO is therapeutic in children with atopic dermatitis, and the observed clinical improvements are likely due to the normalization of INF- γ levels and perhaps other immunological parameters (35).

Thus, no consensus exists among the results of different studies on the effects of EPO supplementation for atopic dermatitis. The heterogeneous nature of the patients, who can exhibit slight to very serious symptoms, may explain the observed inconsistencies (36). Recent reviews show that the current body of literature is too inconsistent, making it impossible to conclusively link EPO supplementation to improvements in eczema symptoms (37,38). Larger scale, placebo-controlled studies are needed to provide firm evidence about the postulated beneficial effects of EPO on atopic dermatitis symptoms.

EPO and Rheumatoid Arthritis

Arthritis is a degenerative chronic disease that can affect any of the body's joints. The term arthritis refers to the inflammation of a joint, but not all arthritic diseases involve inflammation. One very common type of the disease is rheumatoid arthritis, which is a chronic inflammatory disease involving the body's immune system. According to Health Canada, four million Canadians suffer from arthritis. Rheumatoid arthritis is an autoimmune condition that occurs in younger adults and sometimes in children. It is due to the release of inflammatory cytokines into the fluid space between the bones of a joint (synovium), which causes chronic inflammation of the cartilage covering the ends of the bones. The most common symptoms are pain, stiffness, and, if left untreated, bone deformity (15). The Raynaud phenomenon and Sjögren syndrome are two inflammatory diseases commonly seen in rheumatoid arthritis patients (39). Raynaud phenomenon is an immunological syndrome characterized by vascular spasms and enhanced blood cell aggregation that leads to ischemia of the fingers, toes, ears, and nose and causes severe pain and pallor in the affected extremities (40). Sjögren syndrome is an immunological disease affecting predominantly women in their 30s and leads to the destruction of exocrine glands. The main symptoms include persistent cough and dry eyes and mouth (41). Ingestion of EPO enhances dihomo- γ -linoleic acid levels (42) and may promote the production of anti-inflammatory series-1 PG or reduce proinflammatory series-2 PG production, which may be of benefit to rheumatoid arthritis patients. To investigate this possibility, research has been performed on the effects of EPO on rheumatologic conditions.

Two early studies investigating the effect of EPO supplementation on rheumatoid arthritis symptoms were conducted by Brown et al. (43) and Hansen et al. (44). Both studies involved only 20 or less participants who were supplemented with low daily doses of EPO (< 1 g) for three to four months. The tests showed no significant improvements, although a trend toward amelioration was observed in rheumatoid arthritis sufferers in the study by

Hansen et al. (44). Jantti et al. (45) studied the effects of EPO or olive oil administration (approximately 1.4 g/day) for 12 weeks on sufferers. This research was unique because participants were required to abstain from NSAID use for the duration of the study, which could potentially negate the possible therapeutic effects of EPO if they are mediated by the anti-inflammatory PG and TX products of dihomo- γ -linolenic acid (45). Compared to baseline, neither the olive oil nor EPO group exhibited any clinical improvements in rheumatoid arthritis symptoms (45). Although these early studies did not provide evidence of EPO's therapeutic effects, it is possible that the supplementation regimes were insufficient and that higher doses of the oil given over a longer period may improve rheumatoid arthritis symptoms. More recently, Brzeski, Madhok, and Capel (46) examined the effects of supplementation of 6 g of EPO or olive oil per day for six months on symptom management in 40 rheumatoid arthritis sufferers. While olive oil supplementation was supposed to act as a placebo, a significant improvement was, however, noted at the end of the six-month evaluation period compared to baseline, similar to that observed for participants in the EPO group. Thus, both olive oil and EPO supplementations were equally effective in improving the clinical scores of rheumatoid arthritis sufferers, and selection of an appropriate placebo must be carefully exercised in such studies so that the possible therapeutic effects of EPO are not masked. Belch et al. (47) studied the effects of EPO and EPO plus fish oil (approximately 6 g/day) on rheumatoid arthritis symptoms in 49 participants over a 12-month supplementation period. In this study, 16 patients were given EPO, 15 EPO plus fish oil, and 18 liquid paraffin as placebo (47). Measures of disease activity and NSAID use were used to assess the effectiveness of the treatments. After the supplementation period, NSAID use was lower in both the EPO and EPO plus fish oil groups. However, no clinical improvements were observed in either treatment group implying that EPO did not serve as a disease-modifying agent for rheumatoid arthritis (47). The results of a similar, but larger, study including 402 rheumatoid arthritis patients were reported by Darlington and Stone (48). This was a multicenter, double-blind, and placebo-controlled trial. Participants were randomized into one of two groups receiving between 2 and 3 g/day of EPO (experimental group) or sunflower oil (placebo); the duration of the treatment period may not have been consistent among the centers. The results of this study did not reveal any therapeutic effects of EPO in sufferers of rheumatoid arthritis (48).

The current body of literature regarding EPO supplementation and rheumatoid arthritis does not provide any clear understanding regarding its therapeutic effects; few studies, however, show a clear improvement in symptoms, but many refute its therapeutic potential, because of the use of faulty designs. Some of these studies have used inappropriate placebos, others have recruited participants exhibiting a wide range of disease symptoms, and many have used treatment doses that were too small to promote clinical improvements in sufferers of rheumatoid arthritis. Larger scale, placebo-based trials are needed to conclusively evaluate the effects of EPO on rheumatoid arthritis.

EVENING PRIMROSE AND CARDIOVASCULAR DISEASE

Cardiovascular disease is the common term for all diseases that affect the heart and circulatory system, including ischemic, nonischemic, hypertensive, and valvular heart disease. It is the leading cause of death in the Western societies (49) and has been linked to the high fat intake, particularly saturated fat, common in the Western diet (50). Besides high saturated and trans fat intake, other risk factors include diabetes mellitus, smoking, stress, physical inactivity, high sodium intake, and genetic predisposition. The hallmark of cardiovascular disease is cardiac dysfunction, which in most cases is caused by hypertension due to the narrowing of large arteries with atheromatous plaques or the total occlusion of coronary arteries (thrombus) caused by atheromatous blockages leading to myocardial tissue necrosis. Both conditions reduce the heart's ability to pump blood and can result in either chronic or sudden heart failure.

Several reports indicate that ingestion of dietary PUFA reduces the likelihood of cardiovascular disease. Much attention has been paid to the antithrombotic and antiatherogenic effects of *n*-3 fatty acids of fish oils, while oils rich in *n*-6 fatty acids have evoked relatively less interest (51). Arachidonic acid is the major metabolite produced from dietary linoleic acid, and eicosanoids produced from arachidonic acid are known as being proaggregatory. Thus, *n*-6 fatty acids have been speculated to promote thrombosis and atherogenesis (52). However, not all dietary *n*-6 fatty acids become elongated and desaturated into arachidonic acid. For example, dietary γ -linolenic acid is rapidly converted into dihomo- γ -linolenic, which is the precursor of the antiaggregatory vasodilator, PGE₁ (52). EPO is a source of antioxidative compounds such as α -tocopherol (53), which may protect highly oxidizable biomacromolecules such as low-density lipoproteins (LDL) from oxidation *in vivo*, which is known to precede some aspects of cardiovascular disease, most notably blood vessel damage (54). The fact that EPO is a source of γ -linolenic acid and antioxidative compounds has led researchers to investigate its possible cardioprotective effects. Most of these studies have been conducted using animal models of cardiovascular disease.

Singer et al. (55,56) using spontaneously hypertensive rats, revealed that dietary EPO intake alters blood lipid profiles and systolic pressures, thereby improving cardiovascular risk factors (55,56). The dietary proportions and types of fats consumed can alter platelet–blood vessel interactions. Long-chain *n*-3 fatty acids and dihomo- γ -linolenic acid may exert antithrombotic activity because their eicosanoid metabolites reduce platelet aggregability, a known cardiovascular risk factor (57). De la Cruz et al. (58) investigated the ability of EPO to reduce blood platelet aggregation in response to inducers (adenosine diphosphate or collagen) using a total of 40 male white New Zealand rabbits fed normal and atherogenic diets. These researchers also measured platelet thromboxane synthesis and malondialdehyde (MDA) production to assess blood platelet reactivity. Two control groups were used in this study: one group received a low-fat diet (2.1%, w/w) with normal lipid composition (normal diet group), while the

other was fed a high-fat diet (12.2%, w/w) containing primarily saturated fatty acids (atherogenic diet group). The diets of experimental animals were supplemented with EPO to get a total fat content between 19.8% and 20.3%. These feeding trials lasted six weeks; on the last day, the animals were anesthetized and blood samples were obtained for subsequent analysis. Compared to the normal control diet, the atherogenic control diet resulted in hyperlipidemia (hypercholesterolemia and hypertriglyceridemia) with a concomitant decrease in high-density lipoprotein (HDL) levels. Platelet aggregation and platelet production of thromboxane B₂ (TXB₂) and MDA were several times greater in the atherogenic diet group compared to controls. Supplementation of EPO in normal diets did not alter blood lipid and platelet parameters compared to normal controls. However, EPO significantly reduced total blood cholesterol and triacylglycerols, while increasing HDL levels in animals fed atherogenic diets, and reducing blood lipid aggregation level to that of normal control values ($P < 0.05$ – $P < 0.01$). Treatment also reduced TXB₂ and MDA production by platelets from animals fed atherogenic diets, although these values were still significantly higher than those of the control animals fed on a normal control diet ($P < 0.05$ – $P < 0.01$). Diets rich in saturates promoted the onset of cardiovascular risk factors such as platelet reactivity and aggregation, and EPO treatment normalized these parameters substantially in male New Zealand white rabbits (58). More recently, De la Cruz et al. (59) assessed the antioxidant potential of EPO in 40 male white New Zealand rabbits that were fed atherogenic diets (50% fat, w/w) or normolipidic diets (20% fat, w/w) containing 15% EPO (w/w); controls were fed either atherogenic or normolipidic diets without EPO enrichment. The duration of the feeding period was six weeks, after which all animals were sacrificed, and samples of liver, brain, heart, aorta, and blood platelets from each animal were collected to test MDA and glutathione levels. The results of this study showed that in normolipidic diets, EPO treatment did not alter MDA production or glutathione levels. However, atherogenic diets promoted MDA production (both induced and noninduced TBARS) and glutathione oxidation in all tissues and platelets. Meanwhile, tissues of animals receiving atherogenic diets plus EPO exhibited a lesser induction of MDA production and glutathione oxidation ($P < 0.05$ – $P < 0.01$). Thus, EPO supplementation may reduce the production of lipid oxidation products in tissues, which are implicated in the onset of atherosclerosis (59). The cardioprotective effects of EPO may be due to the presence of antioxidative components. Charnock (60) recently evaluated the effects of dietary EPO, sunflower oil, borage oil, and saturated fat against ventricular fibrillation in male Hooded-Wistar rats fed normal chow diets supplemented with 6% EPO (w/w) for 32 weeks. At the end of the feeding period, the animals were anesthetized and ventilated, followed by surgical ligation (occlusion) of the left descending coronary artery to induce ventricular fibrillation and cardiac arrhythmia. In this study, incidence and duration of ventricular fibrillation were lowest in the EPO group. Interestingly, EPO exerted a protective effect against ventricular fibrillation, which exceeded that of borage oil (containing three times more γ -linolenic acid than EPO). This implies that γ -linolenic acid may not

be the therapeutic component of EPO; some other compounds present in EPO may exert cardioprotective effects in this animal model (60).

Very few investigations on the cardioprotective effects of EPO have been carried out in humans. Abraham et al. (61) examined changes in the fatty acid composition of adipose tissue, serum triacylglycerol and cholesterol ester levels, and lipoprotein profiles in men with low dihomo- γ -linolenic acid levels supplemented with safflower oil or EPO for four months. Participants in this study were split into four groups (6–9 men per group) receiving 10, 20, or 30 mL/day of EPO or 20 mL/day of safflower oil. Adipose and blood samples were obtained from participants before and after the treatment period. Treatment with EPO at levels ≥ 20 mL/day increased adipose dihomo- γ -linolenic acid levels ($P < 0.01$); safflower oil did not increase adipose dihomo- γ -linolenic acid content. Within individual participants, similar trends were observed between adipose fatty acid compositions and serum triacylglycerol and cholesterol ester fatty acid compositions. Safflower oil and EPO treatments did not significantly lower LDL or raise HDL levels, implying that EPO may not exert strong antiatherogenic effects in humans, contrary to the findings from the animal models (61). However, Abraham et al. (61) used a highly select group of men most likely possessing low Δ -6-desaturase activity, which may have affected the outcome of this study. More recently, Khan et al. (62) performed a double-blind placebo-based study to examine the effects of an eight-month treatment with food oils on vascular tone and endothelial function in 173 healthy volunteers (118 males and 55 females). The placebo oil used was 25:75 (w/w) mixture of soybean oil and coconut oil, administered at 10 g/day to the placebo group. Participants in the EPO group received 5 g EPO plus 5 g placebo oil per day. The results for the EPO group showed that it did not alter the vasodilator responses to acetylcholine (general endothelial function) or sodium nitroprusside (endothelium-independent vasodilation) in healthy volunteers (62).

The majority of results from human and animal studies exhibit different findings with respect to the cardioprotective effects of EPO, because certain invasive tests cannot be performed on humans. In animal models, specific cardiovascular conditions can be induced, thereby allowing researchers to more accurately measure specific responses to EPO. More research involving human subjects and possibly trials involving recovering cardiovascular disease patients already taking medication need to be done in order to assess whether EPO exerts any cardioprotective effects in medicated patients.

EPO AND CANCER

Cancer is a general term for more than 100 diseases that are characterized by uncontrolled and abnormal growth of cells (neoplasia) derived from normal tissues. Cancerous cells may develop into tumors that could produce toxins or spread locally or through the bloodstream and lymphatic system to other parts of the body (metastasis). The initial steps that lead to the transformation of normal cells into cancerous cells are not well understood.

But many lines of evidence implicate the involvement of mutated genes in cells progressing toward cancer. Cancer cells are incapable of apoptosis, a property that makes them potentially "immortal" (63). Two recognized treatments for cancer are chemotherapy, which is the administration of drugs (alkylating agents, e.g., vinblastine and chlorambucil) and radiation therapy in which cancerous tumors are subjected to ionizing radiation. Both treatments cause irreparable damage to the DNA of cancer cells, thereby promoting cell apoptosis. Currently, much research is being conducted to identify new cancer therapies with less adverse side effects and greater specificity toward cancer cells. Experimental and epidemiological studies have demonstrated that the composition of dietary fat affects the incidence and progression of some cancers (64).

Several cancerous cell lines have been developed from animal and human malignant tumors. They serve as excellent *in vitro* models for cancer studies because the biochemical processes that occur within these cells are remarkably similar to those within their parent tumors. Early reports using cancerous cell lines have shown that γ -linolenic acid and metabolites of this compound suppress cell proliferation (65). The fact that EPO is a dietary source of γ -linolenic acid has prompted several research groups to investigate its anticancer potential. Gardiner and Duncan (66) examined the effects of EPO and safflower oil on the growth of precultured BL6 melanoma tumors implanted in mice. After implantation, the mice were fed diets containing 6% EPO or safflower oil for four weeks after which tumor growth and Δ -6-desaturase activity were measured in each animal. Results showed that EPO promoted *in vivo* tumor growth to a greater extent than safflower oil and that the Δ -6-desaturase activities of the *in vivo* BL6 tumors were lower than that of *in vitro* BL6 tumors. These results collectively imply that BL6 tumor growth *in vivo* is enhanced in the presence of dietary γ -linolenic acid, which may ameliorate the antigrowth effects of low Δ -6-desaturase activity because γ -linolenic acid is a product of this enzyme's activity (66). Ramesh and Das (67) examined the effects of topical EPO or fish oil application on skin carcinogenesis in male Swiss albino mice. This study employed a multistage skin carcinogenesis model involving two clearly defined steps: initiation and promotion using noxious chemicals topically applied on the skin of mice. Treatments of 10 mg EPO or fish oil were used twice daily for 14 weeks, after which papilloma (benign skin tumors) counts and histopathological assessments of excised skin samples were performed. Both fish oil and EPO significantly reduced papilloma formation compared to the control animals receiving no topical EPO or fish oil treatment ($P < 0.05$). However, neither EPO nor fish oil could influence skin cell proliferation in this model. These results imply that EPO may interfere with the development of papillomas but cannot influence the metastatic properties of skin tumors in this model (67). Booyens et al. (65) studied liver cancer cell proliferation in six patients taking EPO supplements and reported a decrease in tumor sizes in three patients as well as considerable reductions in plasma levels of cancer markers (65). More recently, Kollias et al. (68) studied the effects of EPO (4 g/day) on breast fibroadenoma (benign solid

growth) in 20 female subjects. The treatment period lasted six months, after which clinical and ultrasound measurements of previously diagnosed fibroadenomas were compared. Results of these measurements showed that EPO treatment did not alter fibroadenoma size compared to matched controls (68).

Data from animal models imply that EPO may exert anticarcinogenic effects when consumed as a dietary component, and some human studies support these findings. However, the subset of studies involving human subjects has been small scale and is quite prone to errors due to interpatient variability. Larger scale human studies with subject populations that exhibit similar disease symptoms and severity are needed to provide strong support for the anticarcinogenic potential of EPO.

EPO AND WOMEN'S HEALTH

Several adverse health effects have consistently been observed in menopausal and postmenopausal women. Menopausal hot flashes, postmenopausal osteoporosis, and chronic breast mastalgia (chronic breast pain) are examples of adverse health states affecting women (69). The etiologies of these conditions are not well understood, but one common factor has been associated with their declining estrogen level (70). It has been speculated that alterations in hypothalamic activity may underline the clinical aspects of adverse menopausal conditions; however, this view is not accepted widely (71). For most women, estrogen replacement therapy is an effective treatment for adverse menopausal conditions. But several concerns regarding the long-term safety of estrogen replacement therapy have prompted the search for natural, nonhormonal substitutes, and EPO in this regard has received some positive attention (2).

Chenoy et al. (72) performed a randomized, double-blind, placebo-based trial to evaluate the effect of oral EPO consumption or paraffin supplementation (4 g/day for 6 months) in the treatment of hot flashes among 56 menopausal women. Results were based on reports given in diaries of all 56 participants, which showed no significant difference between EPO and placebo treatments despite numerous claims of its efficacy (72). Menopause is known to precede bone mineral density loss leading to osteoporosis that significantly weakens bones making them prone to fractures after light traumas. Adequate premenopausal calcium and vitamin D intakes are negative risk factors for osteoporosis, while smoking and back-to-back pregnancies are promotional risk factors. Bassey et al. (73) recently performed a randomized placebo-controlled trial to investigate changes in bone density markers in response to Efascal[®], an EPO plus eicosapentaenoic acid (EPA), and calcium supplement. This study included 43 pre- and postmenopausal women supplemented with either 4 g Efascal, 1 g calcium per day, or placebo. Final assessments were made after 12 months. Completed food frequency questionnaires were used to assess background calcium intake. The results of this study showed that Efascal treatments did not significantly alter bone mineral density or any other osteoporosis markers, which may indicate the lack of therapeutic efficacy of Efascal in

osteoporosis treatment or the already optimal nutritional state of participants in this study (73). Breast mastalgia is the most commonly diagnosed breast problem and can lead to severe pain that interferes with daily life. Effective medications are available, but the high frequency of side effect occurrence with these drugs has stemmed interest in alternative remedies for breast mastalgia (2). Recently, Blommers et al. (74) investigated the effects of fish oil and EPO on breast mastalgia in 120 premenopausal women. The participants were divided into four groups receiving either 3 g EPO plus 3 g fish oil per day, 3 g EPO plus 3 g corn oil per day, 3 g fish oil plus 3 g corn oil per day, or 6 g corn oil per day (the control group). The treatments were given for six months and symptom questionnaires were sent to participants three and six months into the trial and were compiled for subsequent symptom analysis. The results of this study showed no significant differences between the three experimental groups and the control group with respect to the number of days with active mastalgia, indicating that EPO supplementation may not be an effective treatment for breast mastalgia (74).

Premenstrual syndrome affects millions of premenopausal women and exerts a wide variety of symptoms including abdominal pain, breast tenderness, headache, depression, fatigue, and mood swings that usually subside within 72 hours after the onset of menstrual flow (75). The syndrome occurs more frequently in women with atopic allergies (76) and in those with a high saturated fat intake (77), which implicates abnormal fatty acid metabolism or dietary PUFA deficiency as promotional factors for premenstrual syndrome. Some reports have indicated that EPO may be effective in the management of premenstrual syndrome symptoms. However, few placebo-controlled trials have been conducted to investigate this possibility. Khoo, Munro, and Battistutta (78) examined the therapeutic effectiveness of EPO (Efamol®) in the relief of symptoms of premenstrual syndrome. This was a prospective, randomized, double-blind, placebo-controlled trial involving 38 women supplemented with 4 g/day of EPO or placebo for six months (six menstrual cycles). No significant differences in premenstrual syndrome symptoms were observed between the EPO and placebo groups throughout this study. Yet both groups did exhibit improvements in overall symptoms (psychological, fluid retention, and breast pain) implying that the improvement observed in the EPO group was due to the placebo effect (78). Budeiri, Li-Wan-Po, and Dornan (79) analyzed the results of seven placebo-controlled trials that investigated the effects of EPO on premenstrual syndrome symptoms (meta-analysis). The differences that existed between these studies made a rigorous meta-analysis impossible. EPO was shown to exert moderate beneficial effects compared to placebo for breast pain associated with premenstrual syndrome. Yet, it displayed no overall evidence of effectiveness in the management of premenstrual syndrome (79).

A link between pregnancy complications and low maternal PUFA status has been established. Marine diets rich in *n*-3 PUFA are known to reduce the incidence of gestation-induced hypertension (80), a condition characterized by increased platelet aggregation and vasoconstriction leading to thrombosis in nonhypertensive

women. If in addition to hypertension high urinary protein levels develop (proteinuria), the condition is called pre-eclampsia. Zielinski et al. (81) studied the effects of EPO against hypertension development in pregnant rabbits. In this study, pregnant and nonpregnant rabbits were supplemented with 50 mg/kg per day EPO for 10 days. Pregnant and nonpregnant rabbits on regular diets were used as controls. After the supplementation period, angiotensin II, a blood clotting factor, was administered to animals to induce hypertension, and the pressor response (increase in blood pressure) was measured. Results showed that EPO-supplemented pregnant rabbits exhibited a significantly lower systolic and diastolic response to angiotensin II compared to the control pregnant rabbits. No significant difference in the pressor response to angiotensin II existed between nonpregnant rabbits of both groups. These results may be attributable to the high levels of γ -linolenic acid that may have enhanced PGE₁ (antiaggregatory) synthesis. Coadministration of PGE₁ with angiotensin II reduces vascular responsiveness to angiotensin II (81). Moodley and Norman (82) performed a randomized placebo-based trial to study the effects of 4 g/day EPO administration in 47 women with established pre-eclampsia, showing that no difference in pre-eclampsia symptoms (blood pressure, and blood aggregability) existed between the two groups (82). One of the largest clinical trials investigating the effect of EPO treatment on women with pre-eclampsia was performed by D'Almeida et al. (83). A total of 150 women in their first trimester of pregnancy were supplemented with a mixture of EPO, EPA, and docosahexaenoic acid (DHA) or placebo for six months. Some were treated with magnesium oxide. Participants supplemented with the EPO mixture exhibited significantly better cardiovascular function (reduced incidence of edema, $P < 0.004$) and reduced pre-eclampsia, implying that EPO in combination with EPA and DHA may be helpful in the prevention of pre-eclampsia (83).

Early studies have shown that EPO is an effective natural remedy for some female specific adverse health conditions (2). But more recent studies disagree with some of these findings, showing that EPO exerts no significant change beyond a placebo effect in controlled human trials. More investigations are needed in order to conclusively resolve these discrepancies. In addition, patient assessments should be based on clinical evaluations and not on symptom frequency questionnaires in order to reduce the level of uncertainty in results and increase the significance of experimental findings.

EPO AND DIABETES MELLITUS-INDUCED NEUROPATHY

Insulin is a peptide-based pancreatic hormone that stimulates most body cells to absorb plasma glucose necessary for energy and cellular metabolism. Several mammalian glucose transporters require insulin for activation and cell membrane incorporation (receptor requirement). Diabetes mellitus is a disease caused by a relative or complete lack of insulin action that leads to detrimental alterations in carbohydrate metabolism. "Diabetes mellitus" is Latin for "to flow sweet," which refers to the large volume of urine produced daily with high glucose content. There are two

forms of diabetes mellitus: juvenile onset and adult onset diabetes. Juvenile onset diabetes (Type-I diabetes) is a genetic disease believed to be caused by an autoimmune reaction early in life in which the pancreatic β -cells that secrete insulin into the bloodstream become nonfunctional, thereby causing insulin deficiency that can only be treated with insulin injections; hence, the term insulin-dependent diabetes mellitus (IDDM). Adult onset diabetes (Type-II diabetes), by far the most prominent form, is characterized by normal-to-high β -cell activity and insulin levels but markedly decreased insulin sensitivity and tends to affect obese individuals (84). Both Type-I and Type-II diabetes result in high plasma glucose levels that can alter plasma proteins such as hemoglobin and disrupt microvascular circulation by increasing blood coagulation that may require the amputation of limbs and can lead to neuropathy. Diabetics have been shown to have diminished Δ -5- and Δ -6-desaturase activity, causing alterations in tissue fatty acid profiles and deficiencies in some long-chain PUFA (85). Membrane fatty acid composition is known to affect the cellular response to insulin. Increases in membrane PUFA levels enhance cellular insulin sensitivity and receptor recruitment, while decreasing the levels has the opposite effect, decreased insulin responsiveness (86). Because essential PUFA are known to improve insulin responsiveness at the cellular level, several studies have been done to investigate whether oils rich in PUFA, such as EPO, can improve diabetes management. Most of the investigations on the effects of EPO on diabetes mellitus have been carried out using animal models in which the administration of diabetogenic agents or pancreatectomy is done to induce the disorder. These models closely resemble Type-I diabetes and are particularly useful in the study of diabetic neuropathy.

Vascular changes that occur during diabetes mellitus may cause reductions in nerve perfusion leading to impaired nerve function and neuropathy. Cameron et al. (87) examined whether EPO supplementation could help overcome diabetic nerve conduction deficits in diabetic rats. The animals were treated with streptozotocin (a diabetogenic agent) and fed normal diets for one month to allow for the accumulation of nerve conduction deficits. After this initial feeding period, experimental animals were fed diets supplemented with 10% EPO for one month to assess whether this treatment could reduce the extent of diabetes-induced abnormalities. Control diabetic and nondiabetic rats were fed normal diets for two months. Results of this study showed that after the initial 1 mo feeding period, diabetic rats exhibited deficits in motor and sensory nerve conduction velocity, which were maintained over a two-month period. In EPO-treated diabetic rats, deficits in nerve conduction velocity were restored to the level of nondiabetic control rats (87). These findings suggest that EPO is an effective treatment for diabetes-induced neuropathy in this animal model. More recently, the effects of sunflower oil, EPO, and two structured γ -linolenic acid-containing triacylglycerols were examined on nerve conduction deficits in streptozotocin-induced diabetic rats (88). Animals were fed diets supplemented with sunflower oil, EPO, dilinolein mono- γ -linoleate, or tri- γ -linoleate for six weeks after diabetes induction to examine whether the stereospecific distribution of γ -linolenic acid in triacylglycerols could influence

diabetes-induced neuropathy. All diets were prepared to contain equal amounts of γ -linolenic acid, except for sunflower oil-supplemented diets that served as control diets. Results showed that all three γ -linolenic acid-containing diets restored nerve conduction velocity, while sunflower oil-treated controls showed no such improvements, implying that the stereospecific distribution of γ -linolenic acid in dietary triacylglycerols did not influence its therapeutic effects in this animal model (88). Ford et al. (89) examined effects of treatments with EPO, α -lipoic acid, or sunflower oil on peripheral nerve conduction and vascular parameters in streptozotocin-induced diabetic rats. For six weeks after the induction of diabetes mellitus, the animals were fed normal diets; normal untreated rats were placed on the same diet for use as controls. After the initial feeding period, control and diabetic animals were treated daily with 300 mg/kg body weight α -lipoic acid, 10 g/kg body weight EPO, or 10 g/kg body weight sunflower oil for 27 weeks. EPO treatments for two weeks significantly improved both motor and sensory nerve conduction velocity in diabetic rats ($P < 0.05$ or less) and resulted in the restoration of normal nondiabetic control values in these animals. However, no significant vascular improvements were observed in the EPO-treated diabetic group. α -Lipoic acid treatments resulted in significant improvements in both nerve conduction velocities and vascular parameters in diabetic rats ($P < 0.05$ or less). Sunflower oil-treated diabetic rats showed no significant differences in vascular and neuronal conduction parameters compared to control diabetic rats (89).

Findings from animal models show that EPO is an antineuropathic agent in streptozotocin-induced diabetes and may be of benefit in the prevention of neuropathy in diabetic humans. To date, however, no published trials have investigated the effects of EPO on diabetic neuropathy in humans.

OTHER HEALTH EFFECTS OF EPO

Animal and human studies have investigated the effects of EPO on mental health (90), inflammatory bowel disease (91), and renal health (92). Joy, Mumby, and Joy (90) systematically searched several scientific databases for randomized trials investigating the effects of EPO on schizophrenia. In some trials, EPO supplementation improved symptoms, while others showed no such effects (90). Greenfield et al. (93) showed that EPO treatment improved stool frequency in ulcerative colitis patients after three months of supplementation; no such effects were observed for patients treated with Max-EPA fish oil. Animal studies show that EPO treatments can significantly reduce cyclosporin-A-mediated nephrotoxicity (94,95). Yoshimoto et al. (96) showed that EPO treatments significantly improved skin symptoms and abnormal plasma fatty acid compositions of hemodialysis patients. These health effects of EPO seem promising. However, the amount of existing literature is small making it hard to draw clear conclusions about these effects. Furthermore, most of these studies have been small scale with few having more than 40 subjects; large-scale trials involving humans are needed (Table 1).

Table 1 Summary of Research Findings on the Effects of EPO in Health and Disease

Disease/condition studied	Type of evidence (References)	Overall findings
Atopic dermatitis	Small-scale human trials (30–38)	EPO may be effective treatment for some clinical symptoms
Rheumatoid arthritis	Small- and medium-scale human trials (43–48)	Inconclusive; several trials possess faults in their study protocol
Cardiovascular disease	Animal studies (55–60) and small-scale human studies (61,62)	EPO is effective in animals; no evidence suggests effectiveness in humans
Cancer	In vitro/animal studies (65–68) and small-scale human trials (68)	Animal studies suggest EPO is antiproliferative; some human studies agree
Menopausal hot flashes	Small-scale human trials (72)	Inconclusive findings
Osteoporosis	Small-scale human studies (73)	Ineffective in reducing bone density loss and other markers of osteoporosis
Breast mastalgia	Small-scale human studies (74)	Few studies indicate therapeutic effectiveness
Premenstrual syndrome	Small-scale human trials (78,79)	No clinical evidence of effectiveness
Pregnancy-induced hypertension/pre-eclampsia	Animal studies (81) and small-scale human trials (82,83)	Both animal and human studies implicate EPO as therapeutic
Diabetes mellitus-induced neuropathy	Animal studies (87–89)	EPO is an antineuropathic agent in diabetic animal models

CONCLUSIONS

EPO might modify several health factors that could in turn modify the onset of disease states. It may exert therapeutic effects in inflammatory and immune-mediated diseases, cardiovascular diseases, cancer, and some conditions associated with menopause. These effects may in part be due to the oil's γ -linolenic acid constituent that could promote the production of anti-inflammatory dihomogamma-linolenic acid-derived eicosanoids and/or reduce proinflammatory arachidonic acid-derived eicosanoid production, thus affecting several aspects of cellular biology and physiology such as immune hyper-reactivity, vascular function, and cellular proliferation. However, it is quite possible that some other components or group of compounds present in EPO may be responsible for its health effects. Incorporation into normal human diets would provide adequate levels of γ -linolenic acid and vitamin E, thereby reducing the likelihood of their deficiency. Thus, EPO may exert several beneficial effects on human health and disease states. Nonetheless, further studies are needed before any casual relationships between health/disease states and EPO can be made.

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Feverfew

Dennis V. C. Awang

INTRODUCTION

Feverfew (*Tanacetum parthenium*) has been used since antiquity for a variety of medicinal purposes, prominent among them being alleviation of fever, headache, and women's ailments. Claims of efficacy are almost invariably anecdotal. However, over the past two decades, randomized controlled trials have been conducted in the prophylaxis of migraine and treatment of rheumatoid arthritis. While the quality of these trials is varied, there is good evidence of feverfew's potential in migraine: statistically significant reduction in frequency and severity of attacks and degree of nausea and vomiting has been observed following administration of feverfew leaf. While it is said that more people in the U.K. currently self-medicate with feverfew for arthritis than for migraine, no clear benefit has been demonstrated in the single clinical trial conducted with rheumatoid arthritis patients. Neither the constituent(s) of feverfew nor the mechanism(s) of action is/are yet known. Parthenolide, the dominant sesquiterpene lactone (STL) constituent of the clinically tested sesquiterpene chemotype, and long considered the active antimigraine principle, is no longer considered to be a significant contributor in that respect. Also, the latest trial, using a supercritical carbon dioxide extract of feverfew leaf, lends promise to the development of a reliably consistent and effective standardized preparation. No serious adverse reactions have been recorded, although the development of mouth ulcers has caused a small percentage of consumers to discontinue treatment. No drug interactions have been observed so far.

BACKGROUND

Botany

Nomenclature

Following residence in a number of genera after its original assignment to *Rudbeckia* and then *Matricaria* by Linnaeus, feverfew, a member of the plant family Asteraceae (Compositae), is currently recognized as *Tanacetum parthenium* (L.) Schultz Bip. Today, the only previous synonym occasionally encountered in commerce and the scientific literature is *Chrysanthemum parthenium* (L.) Bernh (1).

The most popular common names of this plant are: bachelor's buttons, featherfew, featherfoil (federfoy), flirwort, midsummer daisy, nosebleed, *matricaire*, *grande*

camomille (French), *mutterkraut* (German), *altamisa*, *Santa Maria*, *manzanilla* (Spanish) (2) (Fig. 1).

The generic term *Tanacetum* is claimed to be derived from the Greek word *athanatos*, meaning immortal (*thanatos*, death), which alludes to the ever-lasting nature of the plant's dried flowers. The specific epithet *parthenium* likely originates from the Greek *parthenos*, meaning virgin, apparently in reference to the traditional use by women for menstrual difficulties (3). The common name feverfew is widely held to be a translation of the Latin *febrifugia*, an agent that dispels fever.

Physical Description

The feverfew plant is a strongly aromatic bushy perennial that can grow to 90 cm high. Its crushed leaves are bitter and have a distinctly camphorous odor. The stems (up to 5 mm in diameter) have yellow-green, deeply divided leaves, 2 to 5 cm long, that bear characteristic glandular and covering trichomes (hairs). The wild-type feverfew, traditionally used as medicine, has daisy-like flower heads composed of 5 to 30 functional male and female yellow disc florets, generally 1 to 2 cm in diameter, surrounded by a single row of female ray florets, each with a white corolla 3 to 8 mm long, with a 2 to 7 mm long strap.

Several taxonomic varieties and forms have been recognized on the basis of differences in floral and leaf morphologies. Some of these have two or more rows of ray florets, and at least one is devoid of ray florets (4); two prominent cultivars, *crispum* and *aureum*, have curled leaf edges and decidedly yellow leaves, respectively (5).

Native to the mountains of the Balkan Peninsula, feverfew has spread throughout Europe, to North, Central, and South America, and is now widely cultivated commercially both as a medicinal and as an ornamental plant (6).

Medicinal feverfew is derived mainly from the leaves of the plant, but some countries, such as France, allow inclusion of stalk and flowers (7). Commercial dried feverfew leaf usually contains about 10% of stalk (8). Feverfew leaf extracts are also available.

History

Ancient Uses

The ancient medicinal applications of feverfew have been categorized broadly into three main groups: Treatment for fever and headache; use in cases of difficulties in labor, threatened miscarriage, and regulation of menstruation; and relief of stomachache, toothache, and insect bites (9).



Figure 1 *Tanacetum parthenium* (L.) Schultz Bip. Source: Courtesy of J.S. Peterson, USDA-NRCS PLANTS Database.

Since the time of Dioscorides, the first century Greek physician, feverfew has been used to treat intermittent fevers and has been grown in monastic gardens for that purpose (9). John Gerard(e)'s book, *The Herball*, originally published in 1597 (10), claimed that "(Feverfew) is very effectual for all pains in the head coming of a cold cause, the herb being bruised and applied to the crown of the head." Gerard further added that "Feverfew dried and made into powder, and two drams of it taken with hony or sweet wine, purgeth by siege melancholy and flegme; wherefore it is very good for them that are giddie in the head, or which have the turning called vertigo, that is a swimming and turning in the head. Also it is good for such as be melancholic, sad, pensive, and without speech." Also, Gerard claimed that feverfew was "profitable applied to Saint Antonies fire, to all inflammation and hot swellings." Feverfew has been termed "the aspirin of the 18th century (11)." Culpeper's *Complete Herbal*, originally printed in 1649 (12), recommended feverfew for "ague" (fever with chills): "the decoction drank warm, and the herb bruised, with a few corns of bay-salt, and applied to the wrists before the coming of the ague fits, does take them away." In support of effectiveness against stomachache and insect bites, a very early herbal, Bancke's (1525), is quoted by Berry (9): "This is named Federfoy. His virtue is to conforte a mannus stomake. It is good to asswage the axes cotydan, ye crampe, and to tempre it that cometh of colde stomackes. Also it is good to lay to a soore that is byten with venymous beestes; it will hele it shortly on it be layde thereto." Regarding toothache, Charles Estienne declares: "Stampd and applied unto the teeth or eare of the side that aketh, it wholly asswageth the paine of the teethe (13)."

As noted earlier, the third major area of traditional medicinal application of feverfew deals with "female complaints." Regarding its usefulness as an emmenagogue, assisting and promoting menstrual flow, Gerard (10) states: "... it procureth womens sickness with speed, it bringeth forth the afterbirth and the dead child..." According to

Culpeper (12), "Venus commands this herb, and has commended it to succour her sisters to be a general strengthener of their wombs, and to remedy such infirmities as a careless midwife has there caused; if they will be pleased to make use of her herb boiled in white wine, and drink the decoction, it cleanses the womb, expels the after-birth, and does a woman all the good she can desire of a herb."

As a final note on the traditional use of feverfew for headache and claims by some authors as to its ancient use in migraine (9) and specifically "for the prevention of migraine" since the time of Dioscorides (AD 50) (14), the often cited opinion of John Hill, M.D., recorded in 1772 in his book *The Family Herbal* ("In the worst headache this herb exceeds whatever else is known") (11), has been interpreted as a reference to migraine. However, it is highly questionable whether the characteristic features of migraine were appreciated then and whether feverfew's migraine prophylactic potential was recognized. Feverfew's antimigraine effect was discovered in modern times only in 1973 (15), and it has been widely held that feverfew's influence is entirely prophylactic and not in the realm of symptomatic relief. However, a prominent researcher in the field has reported that the housekeeper of a retired British professor of physiology experienced dramatic relief from a migraine attack: within 20 to 30 minutes of chewing five leaves, "her pain had vanished (16)." Of course, any such anecdotal claim of efficacy, whether ancient or modern, cannot be taken as proof of medicinal value.

Modern Uses

Feverfew is currently widely used to mitigate migraine attacks, as a palliative in arthritis, and for the treatment of psoriasis (17). However, while it is claimed that, since the 16th century, feverfew has been used by more people in the U.K. for arthritis than for migraine (11), most of the research attention has been focused on the latter condition (see clinical studies). The dramatic upsurge of interest in feverfew for migraine occurred following newspaper accounts of favorable responses in sufferers whose condition was resistant to conventional medication (11). The stimulus for this burgeoning attention was the experience of Ann Jenkins, a Welsh doctor's wife, who in 1973 at age 68, upon the suggestion of the elderly father of a friend of her sister's, had begun experimenting with feverfew; the old gentleman, interestingly enough, had found feverfew helpful in treating his arthritis. After increasing the dose from one small leaf to three, after five months, the vomiting associated with the migraine attacks stopped. She also required less of her conventional migraine treatment, ergotamine. After 6 months, she went for an entire month without a migraine attack, and after 10 months, the attacks ceased (15).

The Questionnaire

With the assistance of Mrs. Jenkins, Dr. E. Stewart Johnson, associated with the City of London Migraine Clinic, solicited participants in an epidemiological survey on the use and value of feverfew. A questionnaire was provided inquiring as to whether headaches during use of the herb were less frequent, more frequent, less painful,

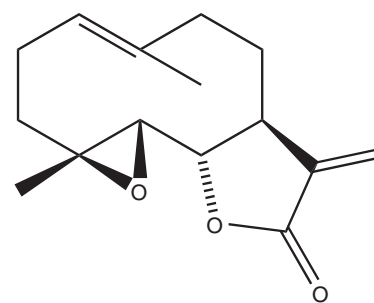
more painful, or unchanged. Of the roughly 300 responders, 253 were judged to be suffering from true migraine, 93% having been diagnosed by a doctor. Of these, 72% reported reduced frequency of migraine attacks, while 26% felt that their headaches worsened. This success rate was virtually identical for participants whether or not they were suffering from other ailments or taking other medications. A further assessment of 242 patients for actual numbers of attacks each month before and during feverfew treatment revealed that 33% no longer suffered any migraine attacks while taking the herb and 76% had fewer monthly episodes. Eighty percent of those who stopped taking feverfew reported recurrence of severe migraine within a week or two. A polling of feverfew users on their experience with conventional migraine-preventive drugs produced very interesting results. Clonidine, the pharmaceutical most commonly tried, was deemed ineffective by 72% of feverfew users, while ergotamine, the most helpful of the other drugs, was judged helpful by 62%. Most of the other drugs were found less than 50% effective. Curiously, about 40% of feverfew users attributed pleasant side effects to the plant: relief from the symptoms of coexisting arthritis, less muscular tension, more restful sleep, and the like. Responders to the questionnaire had been taking feverfew for an average of two-and-a-half years (15). Traditionally, feverfew users take two to four small or one to two large leaves per day, often in a bread and butter sandwich, sometimes mixing honey with the crushed leaves to further mask the bitter taste (11).

CHEMISTRY

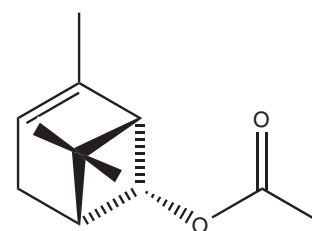
The main chemical constituents that have received attention regarding biological activity fall into three categories, namely, essential oil, STLs, and flavonoids. The flavonoids of feverfew are the lipophilic flavonols di- and trimethylethers of 6-hydroxykaempferol and quercetagenin, and the hydrophilic flavone glycosides apigenin-7-glucuronide, luteolin-7-glucuronide, and chrysoeriol-7-glucuronide (18). The trimethylether of 6-hydroxykaempferol, originally named tanetin, and characterized as 3,7,4'-substituted, was later determined to be the known flavonol santin, a 3,6,4'-trimethylether. Santin, 6-hydroxykaempferol-3,6-dimethylether, and quercetagenin-3,6-dimethylether (axillarin) are the three main flavonol constituents. Two further minor lipophilic flavonols have been identified unequivocally: quercetagenin-3,6,3'-trimethylether (jaceidin) and quercetagenin-3,6,4'-trimethylether (centaureidin) (19).

The volatile essential oil is dominated by camphor (43–44%) and chrysanthemyl acetate (24%), accompanied by lesser amounts of spiroketal enol ether diynes, camphene, germacrene-D, *p*-cymene and terpinen-4-ol. The dominant STL, parthenolide, is also released in the volatile oil, as are the ubiquitous phytosterols, sitosterol, and stigmasterol (20–22). No significant infraspecific variation has been observed in the composition of the volatile oil (23) (Fig. 2).

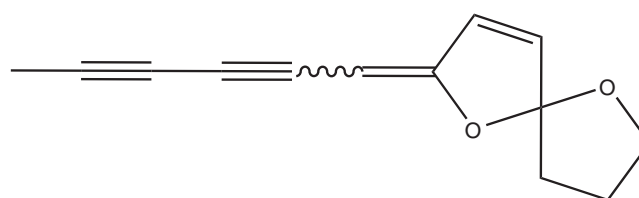
STLs have until recently been the prime focus of chemical and biological attention among feverfew constituents. The germacranolide (10-membered carbon ring), parthenolide, dominates the STL chemotype that has been



Parthenolide



trans-Chrysanthemyl acetate



Isomeric spiroketal enol diynes

Figure 2 Some bioactive constituents of *Tanacetum parthenium*.

subjected to clinical evaluation. The most comprehensive analysis of the chemical content of feverfew leaf has been conducted by Bohlmann and Zdero (24). Parthenolide comprises more than 80% of the total STL content of this chemotype, in concentrations as high as 2% of dry weight in individual plants (25). At least two other STL chemotypes of feverfew have been recognized, devoid of parthenolide (26). The most prominent STLs accompanying parthenolide in the clinically efficacious STL chemical profile are 3 β -hydroxy parthenolide, the isomeric guaianolide (5/7-carbocyclic) *bis*-epoxides, canin (α) and artemcanin (β), the endoperoxide precursor of canin, tanaparthin- α -peroxide, and the cyclopentenone *seco*-tanaparthinolide A, all containing an α -methylene- γ -butyrolactone moiety (16,27). Canin and artemcanin also occur in a Mexican chemotype devoid of parthenolide, in which the eudesmanolides reynosin and santamarin are prominent (16).

Respecting the historical identification of feverfew STLs, it should be noted that there is a serious question

concerning the presence of unusually structured compounds such as chrysanthemonin, chrysanthemolide, and partholide (28), which are not identified elsewhere as feverfew constituents and are likely the result of degradation during protracted refluxing for one week and subsequent processing; chrysanthemonin, a trisesquiterpene species, has been described as (29) "a novel dimeric germacranolide nucleus, esterified at C-8 by a related esterified sesquiterpenic acid." Likewise probably artifactual are the chlorine-containing STLs reported from *T. parthenium* extracted from the same material with chlorinated solvent; the two isomeric chlorohydrins in question were not detected by Hylands, at the University of London, who provided the feverfew leaf, and almost certainly resulted from epoxide ring opening by hydrogen chloride present in the chloroform used for extraction (30). One of the two chlorides, characterized by X-ray analysis, is formally an adduct chlorohydrin formed by β -chloride opening of the α -3,4-epoxy function of canin. The structure of the other isomer, uncrystallized, has not been confirmed, but the proposed C-10 epimerized configuration seems extremely unlikely, not having ever been observed in any feverfew STL.

CLINICAL STUDIES

Migraine

Of the six clinical trials reported, results on one (31) are available only by way of an abstract, which provides neither information on the nature of the tested material nor adequate details about outcome measures. Nonetheless, a systematic review of these trials has accorded it a rating on the Jadad (32) scale of judging quality (33). Of the other five trials, three with dried whole leaf preparations and two with extracts, four are positive. One of the studies that used extract provided a negative result of singular importance (34).

The first of the positive trials, a randomized double-blind and placebo-controlled study (35), involved only 17 patients and has been properly criticized not only on the basis of its small size, but also because the subjects were self-selected, being convinced of the efficacy of feverfew from their history of beneficial self-medication for roughly three to four years and the corresponding expectation of relapse attendant on deprivation of the medicine. In this four-month-long study, eight and nine subjects, respectively, received a daily dose of 50 mg of freeze-dried feverfew leaf powder or placebo. Those who took placebo had a significant increase in both frequency and severity of migraine attacks as well as of nausea and vomiting during the early months of feverfew withdrawal. Two subsequent trials, conducted in the U.K. (36) and Israel (37), used encapsulated air-dried feverfew leaf powder. The British trial was of a randomized, double-blind, placebo-controlled crossover design. After a one-month single-blind placebo run in, 60 patients were randomly allocated to a daily capsule of the treatment (70–114 mg; mean 82 mg) or matching placebo (dried cabbage leaves) for four months and then switched for a further four months. Results on 59 patients were analyzed and revealed feverfew to be associated with a reduction in the mean number and severity of attacks as well as in the degree of vomiting. In the

Israeli trial, 57 patients were divided into groups of 30 and 27 after a preliminary two-month treatment with feverfew (100 mg daily), following which a double-blind placebo-controlled crossover study was conducted in two phases over two months each. As with the earlier British trial, a significant reduction in pain intensity was observed as compared with placebo—as was a "profound" reduction in typical migraine symptoms such as vomiting, nausea, and sensitivity to noise and light.

Two trials have been conducted with extracts of feverfew leaf, one a failure (34) and the other a limited success (38). The earlier trial with 90% ethanol extract was judged to be methodologically of superior quality (33) but revealed no difference between placebo and the treatment, which contained roughly twice the level of parthenolide as the feverfew treatment used in the successful Israeli trial (37). These observations impose the unavoidable inference that parthenolide cannot be directly responsible for feverfew's antimigraine activity. The ineffectiveness of this extract preparation was likely due to loss or degradation of the active principle(s) as a result of the protracted extraction process, involving stirring in solvent for 19 days at ambient room temperature (34). The final trial employed a proprietary supercritical carbon dioxide extract of feverfew leaf in a randomized, double-blind, placebo-controlled multicenter trial with four parallel groups receiving daily doses of 2.08, 6.25, and 18.75 mg for 12 weeks. While the proprietary preparation failed to exert a significant migraine prophylactic effect in general, it was safe and effective at 6.25 mg thrice daily in a small subgroup of patients with at least four migraine attacks per month. The authors of this last study cautioned that their findings should be regarded with reservation on account of the small number of subjects. These German researchers have completed another trial, which supported the findings of the initial study (39).

Arthritis

A single clinical trial in rheumatoid arthritis failed to show any beneficial effect in 40 women treated with 70 to 86 mg of dried feverfew leaf or placebo for six weeks (40). However, considering the continued popularity of the plant for treating the symptoms of this condition, it has been suggested that it may well be of benefit in milder cases of arthritis than that afflicting the women in this trial, who were extremely refractory cases and unresponsive to all conventional arthritis drugs. The authors of the failed trial suggested further that feverfew may be of benefit in osteoarthritis and for soft tissue lesions (40).

Mechanism of Action

The simplistic mechanism of feverfew action in migraine, involving Michael addition of systemic nucleophiles to α,β -unsaturated lactones such as parthenolide and inhibition of the release of serotonin (5-hydroxytryptamine, or 5-HT) from blood platelets, is now totally discredited (41). It seems likely that the feverfew constituent(s) responsible for its antimigraine activity will be found in the volatile fraction of the plant's leaf. However, the relevance of any of the plethora of *in vitro* pharmacological activities noted in both aqueous and organic extracts of feverfew leaf (16,17) has not been established. Nonetheless,

inhibition of the release of damaging substances from white blood cells in inflamed joints and skin could account for the claimed benefit of feverfew in arthritis and psoriasis (17).

Migraine Prophylaxis

Of the three main categories of feverfew chemical constituents demonstrated to possess biological activity, most attention has been directed toward STLs, especially parthenolide. However, as noted above, the ineluctable inference from the failed Dutch trial would seem to be that parthenolide is not a significant contributor to the antimigraine effects of feverfew leaf. No flavonoids have been reported in the efficacious antimigraine supercritical carbon dioxide fluid extract of feverfew leaf (38,39). The Dutch scientists who conducted the unsuccessful trial of a 90% alcoholic extract of feverfew leaf (34) noted that the content of an essential oil component, *trans*-chrysanthenyl acetate, had declined from 0.25% in the leaf raw material, to 0.017% in the phytotherapeutic preparation, suggesting to them a possible relationship to the observed inefficacy of their preparation. However, though evaporation of volatile components of the essential oil may have been somewhat of a contributing factor to loss of activity, degradation of active principle(s) over the protracted period of digestion of the leaf material, is more likely to be the main basis of inactivation (42).

Feverfew essential oil contains, in addition to parthenolide, two other dominant constituents, namely camphor and *trans*-chrysanthenyl acetate, along with lesser amounts of isomeric spiroketal enol ether diynes (42). These last isomers have been identified both in the German proprietary carbon dioxide extract (percentage unspecified) (22) and in ether extracts of feverfew flower heads (*trans*: 6.1%) and root (*cis*: 57.5%, *trans*: 5.1%, and a pyran analog of *trans*: 7.5%) (21). In the flower head extract, parthenolide, camphor, and *trans*-chrysanthenyl acetate represent 28.4%, 18.9%, and 15.5% of total extract content, respectively; though these compounds were present in the leaf extract at 6.1%, 20.1%, and 4.7%, respectively, they were not detected in the root extract. Other prominent components of the root extract are a pair of triterpenoids (19.9%), one an alcohol and the other tentatively identified as D-friedoolean-14-en-3-ol (5.3%). Chrysanthenyl acetate, like parthenolide, is known to inhibit prostaglandin synthetase and to have analgesic properties (19). Testing of an ether extract of feverfew root for antimigraine activity should be revealing since the extract is free of chrysanthenyl acetate, parthenolide, and flavonoids.

Though the antimigraine activity of feverfew was originally thought to be due to inhibition of production of inflammatory platelet-aggregating prostaglandins, later studies revealed a prostaglandin-independent effect different from that obtained with other inhibitors of platelet aggregation, such as nonsteroidal anti-inflammatory drugs, such as aspirin (43).

The spiroketal enol ether diynes are potent insect and antifeedant compounds, and are also present in *Chrysanthemum segetum* (corn marigold) (44) and *Matricaria recutita* (German chamomile), of which the *cis* isomer was shown to exert a spasmolytic action at least 10

times as great as that of papaverine (45). Enol ethers in contrast to saturated ethers, are very susceptible to acid-catalyzed hydrolysis. The aqueous alcoholic Dutch extraction medium (34), rendered acidic by the presence of long-chain fatty acids, such as arachidic, linolenic myristic, oleic, palmitic, and stearic acids, could provide an environment conducive to such degradation—disruption of the extended diyne-diene conjugation by conversion of spiroketal enol ether to hydroxyketone can be expected to have a significant effect on extract activity.

SAFETY

Adverse Effects

No serious adverse effects have been reported for feverfew consumption. The side effect that has received the most attention is mouth ulceration, the formation of recurrent, so-called aphthous ulcers (commonly referred to as “canker sores”). Interestingly, in the University of Nottingham trial (36), more patients (16) in the placebo group reported mouth ulceration than those in the verum group (10). Johnson (15) had previously noted that 11.3% of the 253 patients participating in a questionnaire survey admitted to experiencing mouth ulceration when asked but only 6.4% volunteered such information; a parallel situation was obtained for indigestion, with 6.5% and 3.9%, respectively. It is also interesting to note that mouth ulceration from feverfew appears to be a systematic effect that resolves within a week or so of discontinuation of the treatment, but returned on rechallenge. It has been claimed that the mouth ulceration can be alleviated by treatment with tincture of myrrh (normally derived from *Commiphora* species, especially *Commiphora molmol*) (46). Nonsteroidal anti-inflammatory drugs (NSAIDs), increasingly used for migraine prophylaxis and arthritis, also produce recurrent aphthous ulceration (47). Feverfew sometimes induces a more generalized inflammation of the oral mucosa and tongue, with attendant swelling of lips and loss of taste. This soreness is likely caused by direct contact with leaves during chewing and probably due to the interaction of STLs known to cause contact dermatitis (48).

Feverfew leaf does not appear to affect blood pressure, heart rate, body weight, or the results of hematological and biochemical tests, but rare cases of transient palpitations, colicky abdominal pain, and heavier menstruation have been reported (35).

Also, a “post-feverfew syndrome” has been identified in long-term feverfew users who stopped taking the herb. About one-tenth experienced moderate-to-severe aches, pains, and stiffness in joints and muscles, along with CNS symptoms of anxiety and poor sleep (35). It has been speculated that such sleep disturbances may be due to withdrawal of melatonin, present in significant quantities in the leaf (2.45 µg/g in fresh; 2.19 µg/g in dried) (49).

Toxicology

While no formal studies have been conducted to assess chronic toxicity in animals, it has been argued that such tests are now superfluous since feverfew has been used by large numbers of people continuously for many years,

some for more than 10 years, without apparent ill effect (35).

A study involving 30 females who had been consuming feverfew for more than 11 months revealed no differences in the frequency of chromosomal aberrations or the frequency of sister chromatid exchanges compared with a matched set of nonusers (50).

Contraindications

In view of feverfew's traditional reputation as an emmenagogue, its capacity to induce uterine contraction in full-term women, and its ability to cause abortion in cattle, it would seem prudent for pregnant women to avoid its use. It should also be noted that when feverfew was used to promote menstruation, it was taken in much higher doses than those currently employed for treating migraine and arthritis. Finally, little is known of the effects of feverfew on migraine and arthritis in pregnancy (15).

Feverfew is also contraindicated in persons with recognized hypersensitivity to other members of the Asteraceae, since crossreactivity is common among plants in this family (51).

Drug Interactions

Feverfew is often indicated by certain scientists as an anti-coagulant herb, based on the ability of parthenolide, only one of its numerous biologically active constituents, to inhibit platelet aggregation. Yet, neither bleeding episodes nor abnormal coagulation tests have been reported from feverfew use (52).

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Flaxseed

Lilian U. Thompson and Julie K. Mason

INTRODUCTION

Flax (*Linum usitatissimum*) or linseed is an ancient crop, which has been grown for fiber (linen) and nutritional and industrial use. Although flax is used for these purposes belong to the same *Linum* species, they differ in varieties. In Europe, “flax” is the term often used to describe the crop used for fiber and “linseed” is the term for nutritional and industrial use (1). In North America, the terms “linseed” and “flax” are used interchangeably, however, there is preference for the use of “flaxseed” term when referring to the seed for human consumption. In this chapter, the term “flaxseed” will be used to refer to the seed and “flax” to refer to the plant.

Flaxseed is a dietary supplement or ingredient increasingly being used by consumers for its perceived health benefits. This chapter will provide information on flaxseed—its production, composition, health beneficial effects including in cardiovascular disease (CVD), breast, prostate, and colon cancer, bone health and menopausal symptoms, and safety, as observed in animal, clinical, and epidemiological studies, and mechanisms of action. The physiological roles of its major bioactive components, n-3 fatty acid-rich flaxseed oil and lignans, taken as dietary supplement or as part of flaxseed, will be discussed. Regulations in the use of flaxseed are also described.

BACKGROUND

As previously reviewed, flax is an ancient crop dating at least 8000 years ago in Syria, Turkey, and Iran (1). Domestication started around 7000 BC and, based on archeological findings, linseed oil appears to have been used for embalming and the linen for fabric for Egyptian cloth. Around 1000 BC, flaxseed was used in breads in Jordan and Greece and later on as laxative. In Ethiopia, flaxseed was used in stews, porridge, and drinks, sometimes roasted and mixed with pulses. It is believed that French immigrants brought flax to North America around AD 1600. Early use of flax in North America was for fiber in the linen industry and for oil in paints and linoleum industries. Interest in the use of flaxseed as a food started around 1980s and as a functional food in the 1990s.

While the origin of flax is near the Middle East, flax is currently grown in over 30 countries with the main producers being Argentina, Canada, China, India, Poland, Romania, Russia, Uruguay, and USA; Canada is the leading producer and exporter. In temperate climate areas, flax

is a summer annual crop although winter annual crop has also been grown in some European countries with mild winters. Flax varieties grown for linen are different from those used for food.

There are anecdotal evidence of efficacy and safety of flaxseed. Around 500 BC, Hippocrates wrote about its use in reducing abdominal distress, as a laxative and poultice (1). Raw flaxseed was boiled in water and the solution was drunk to serve as a stool softener. Around the eighth century AD, the emperor of the West (Charlemagne) considered flaxseed as healthful and he passed laws and regulations requiring flaxseed consumption by his subjects. Flaxseed-containing drink was used in Ethiopia to reduce itching with the effect attributed to its oil content. In North America, pioneers made flaxseed poultices for treating cuts and burns. Certain flaxseed varieties were used as well for eyewash, coughs, gallstones, lung and digestive disorders including constipation. Although flaxseed has been used for various illnesses and disorders in the past, it is only in recent years that scientific studies demonstrating the efficacy of flaxseed have been conducted.

CHEMISTRY AND PRODUCTS

Flaxseed

Cultivated flaxseed varies in composition depending on cultivar, growth location, and environmental conditions but its typical composition is shown in Table 1 (2–4). Of interest and the main reason for its use as dietary supplement or ingredient for health benefits, however, is its high amount of oil rich in the n-3 fatty acid, α -linolenic acid (ALA), the high amount of dietary fiber, and the phytoestrogen called lignans, in addition to its high-quality protein. Flaxseed also contains tocopherols and many phenolic acids including ferulic, coumaric, caffeic, chlorogenic, gallic, protocatechuic; *p*-hydroxybenzoic acid, sinapic acid, and vanillic acid. Since phenolics have antioxidant properties, they may also contribute to the potential health benefits of flaxseed. A concern is the presence of antinutritional factors such as the cyanogenic glucosides primarily linamarin, linustatin, and neolinstatin, which produce toxic hydrogen cyanide when hydrolyzed. It can be removed from the seed by solvent extraction (e.g., alkanol-ammonia-water-hexane) or heating (e.g., microwave, autoclaving, oven heating, extrusion, water boiling). Other antinutritional factors include phytic acid, which can bind minerals and make them less bioavailable, and vitamin B6 antagonist (linatine), which

Table 1 Composition of Flaxseed (2–4)

Macronutrient components		Micronutrient/phytochemical components	
Component	Per 100 g	Component	Per 100 g
Sugars (g)	<1.0–2.0	Lignans (mg)	
Dietary fiber (g)	30.5–36.8	Secoisolariciresinol	375.32
Soluble (g)	9.2–11.0	Matairesinol	0.15
Insoluble (g)	21.3–25.8	Lariciresinol	2.81
Fat (g)	39.8–45.6	Pinoresinol	0.73
Fatty acid (% of total)		Cyanogenic glycosides (mg)	
Lauric (C16:0)	4.8	Linamarin	13.8–31.9
Stearic (C18:0)	3.6	Linustatin	213–352
Oleic (C18:1)	21.0	Neolinustatin	91–203
Linoleic (C18:2)	22.8	Phytic acid (mg)	23–33
α -Linolenic (C18:3)	57.4	Tocopherols (mg)	
Protein (g)	17.4–24.1	α -Tocopherol	0.0–1.2
Amino acids (g)		β -Tocopherol	2.4
Alanine	0.99–1.05	γ -Tocopherol	8.5–39.5
Arginine	1.88–2.04	δ -Tocopherol	0.2–1.1
Aspartate	2.06–2.22	Minerals (mg)	
Cystine	0.36–0.40	Iron	3.67–16.4
Glutamate	3.86–4.37	Zinc	3.82–9.36
Glycine	1.12–1.38	Manganese	1.30–4.28
Histidine	0.48–0.57	Copper	0.79–1.71
Isoleucine	0.97–1.03	Calcium	200–440
Leucine	1.29–1.37	Magnesium	320–410
Lysine	0.87–0.96	Vitamins (mg)	
Methionine	0.36–0.43	Thiamine (B1)	0.03–0.60
Phenylalanine	0.97–1.04	Riboflavin (B2)	0.10–0.30
Proline	0.78–0.82	Niacin (B3)	1.4–5.5
Serine	0.88–1.07	Pantothenic acid	1.5–7
Threonine	0.82–0.88	Pyridoxine (B6)	0.4–10
Tryptophan	0.29–0.32	Ascorbate (C)	1.3
Tyrosine	0.51–0.55	Folate	278
Valine	1.17–1.25	Cyanocobalamine (B12)	0–0.5

can lead to vitamin B6 deficiency and poor growth but can be removed by alcohol extraction. Flax absorbs cadmium, which can translocate to the seed and the amount of which varies with varieties and growth location. Accumulation of cadmium in the human kidney can result in renal dysfunction.

Most flaxseed varieties are brown in color except one variety (Omega), which has yellow color; these varieties have very similar macronutrient composition. Solin variety is also yellow colored but is genetically developed to contain low ALA (2–3%) and should not be confused with the high ALA (>50%) Omega variety.

Flaxseed structure consists of the embryo or germ, a thin endosperm and two cotyledons encased in a seed coat called the spermoderm (3). Approximately 55% of the hand-dissected seed is the cotyledon, 36% is the hull (seed coat and endosperm), and 4% is embryo. Most of the oil in the seed (75%) is present in the cotyledon, with 22% in the seed coat and 3% in the embryo. In contrast, a greater proportion of the lignans and dietary fiber are found in the hull.

Flaxseed is commercially available for food as either whole or ground (milled) seed or partially defatted meal. In recent years, flaxseed has also been processed by abrasive milling to separate the hull from the cotyledon fraction. The hull fraction is now being sold as a lignan concentrate containing approximately 4% to 5% lignan for

use as dietary supplement or ingredient, while the cotyledon fraction is sold as a low fiber, high n-3 fatty acid oil-containing ingredient. Whole or ground flaxseed has been incorporated in food products including bakery, pastas, breakfast cereals, nutribars, and dairy products (2).

Flaxseed Oil

Flaxseed oil is comprised primarily of neutral lipids (acylglycerols and fatty acids) (96%) and some polar lipids (glycolipids and phospholipids) with more than 50% of its fatty acid as ALA, an essential n-3 polyunsaturated fatty acid (PUFA) (Table 1) (2,3). Flaxseed is the richest plant source of ALA. ALA cannot be synthesized de novo in mammals and therefore is available only through dietary sources, such as flaxseed and flaxseed oil, perilla oil, canola oil, and soybean oil. ALA is metabolized to the longer chain n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) through an alternating series of elongation (addition of two carbons) and desaturation (double bond insertion) reactions via $\Delta 6$ and $\Delta 5$ desaturases (5) (Fig. 1). Both EPA and DHA can be metabolized by lipoxygenase, cyclooxygenase, and cytochrome P450 enzymes to produce anti-inflammatory mediators including neuroprotectin D1, E and D series resolvins, and 3-series prostaglandins. The n-6 PUFA, that is, linoleic acid (LA) is metabolized by the same enzymes as n-3 PUFA to form arachidonic acid (AA), a precursor of

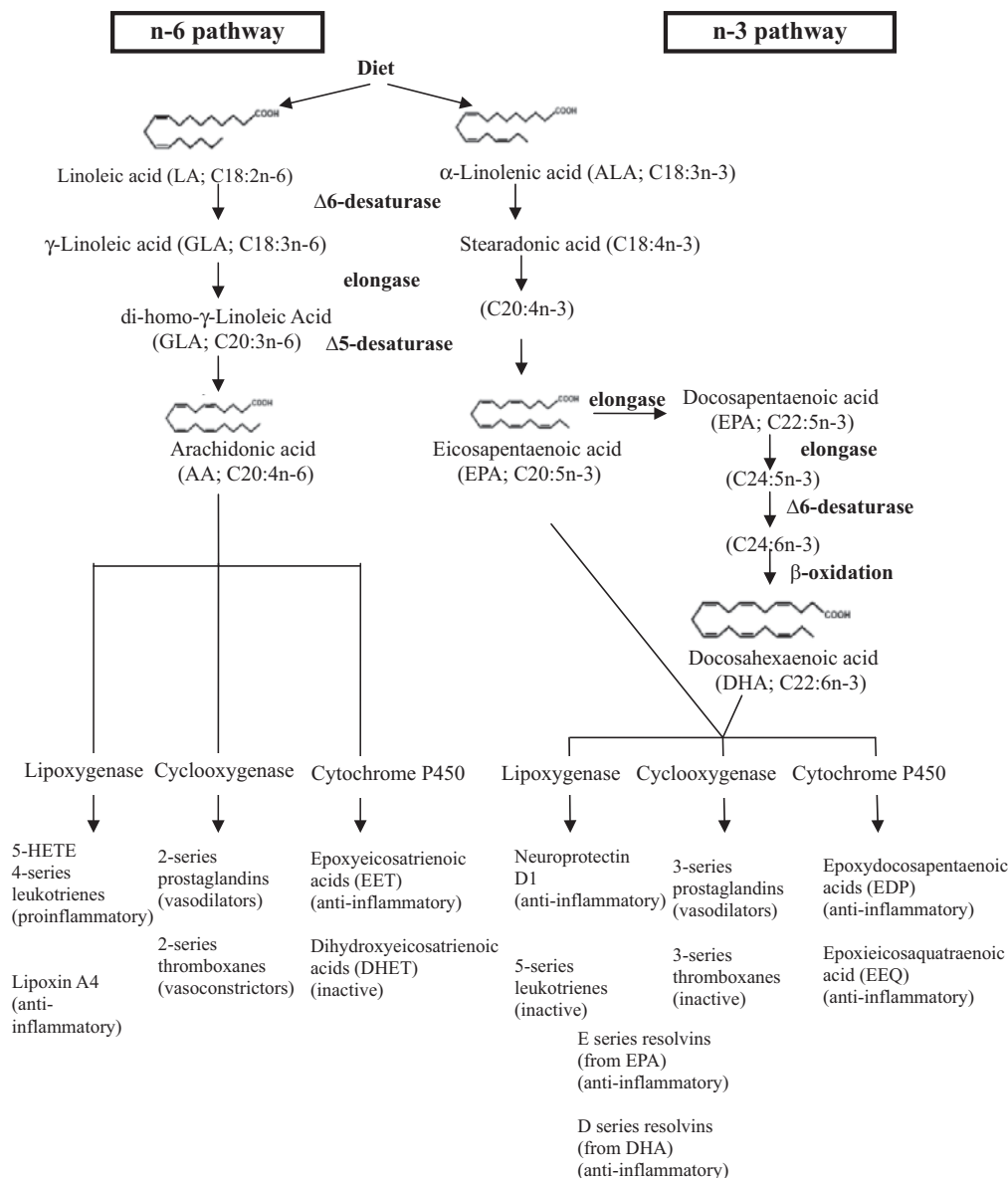


Figure 1 Metabolism of n-3 fatty acid (α -linolenic acid) compared with n-6 fatty acid (linoleic acid).

inflammatory eicosanoids including the 4-series leukotrienes and 5-hydroxyeicosatetraenoic acid (HETE) (Fig. 1). Since n-3 and n-6 PUFA metabolism occur in a competitive fashion, a high intake of n-6 fatty acid reduces the conversion of ALA to EPA and DHA. Therefore, for beneficial health effects of n-3 fatty acid-rich diets, reduction of AA synthesis and increasing the n-3/n-6 fatty acid ratio are desirable.

Recent reviews on ALA conversion to longer chain n-3 PUFA consistently showed an increase in plasma EPA with ALA supplementation and no significant increase in DHA levels (6,7). Studies have shown a <0.3% to 8% and up to 21% conversion of ALA to EPA in men and women, respectively. The conversion through to DHA is even lower at <4% but often undetectable in men and up to 9% in women (8). This variability may stem from

the influence of factors such as energy balance and the presence of other dietary factors such as n-6 fatty acids and cofactors influencing desaturation-chain elongation. The physiological action of ALA is not yet fully established but since most of it is catabolized for energy (8), its action may be due to its direct effect or through its longer-chain metabolites.

Lignans

Lignans are compounds derived from the coupling of cinnamic alcohol residues. They are phytoestrogens and those found in foods include secoisolariciresinol, matairesinol, pinoresinol, syringoresinol, lariciresinol, hydroxymatairesinol, medioresinol, and sesamin. Lignans are present in most plant foods but flaxseed is the richest source with the level more than 100 times higher than in

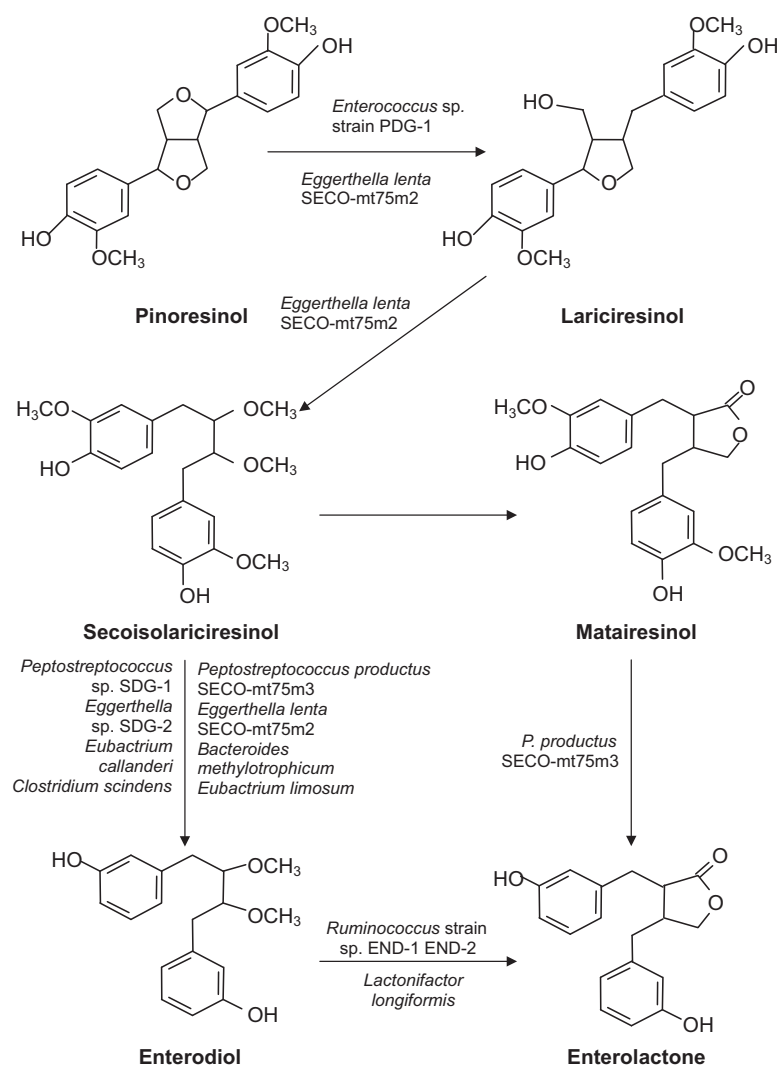


Figure 2 Metabolism of lignans.

other plant foods (2–4,9). Approximately 95% of the lignan in flaxseed is secoisolariciresinol diglucoside (SDG) with matairesinol, pinoresinol, and lariciresinol present in small amounts. In flaxseed, SDG is esterified with 3-hydroxy-3-methylglutaric acid forming an oligomer referred to as lignan complex or polymer. It is also linked to other phenolic acids, such as glucosylated coumaric, caffeic, and ferulic acids. SDG can be extracted from flaxseed using aqueous alcohol and methods are available for producing products containing approximately 40% SDG (2,3,10). Some of these extracts are now commercially sold for use as dietary supplements or food ingredients.

The lignans are metabolized by the colon microbiota into the mammalian lignans (enterolignans) called enterolactone and enterodiol (3,11,12) (Fig. 2). Pinoresinol is metabolized to lariciresinol then to secoisolariciresinol and finally to enterodiol and enterolactone. Matairesinol, which can also be formed from secoisolariciresinol, is metabolized directly to enterolactone. Some of the intestinal bacteria responsible for the conversions have been identified (3,12) (Fig. 2). After the formation, enterolignans are absorbed in the colon into the bloodstream or excreted in the

feces. Enterolignans undergo enterohepatic circulation, that is, they are excreted through the bile duct to the intestines, further metabolized in the colon and reabsorbed. A portion of the enterolignans is excreted in the urine primarily as glucuronide conjugates with small amounts as sulfate conjugate or free. Nine hydroxylated metabolites of enterolactone and enterodiol, produced in the liver, have been reported in the urine after the intake of flaxseed but these accounted for only <5% of the total urinary lignan excretion. The serum and urinary levels of lignans are related to plant lignan intake and thus have been used in studies to indicate plant lignan or flaxseed intake. The conversion of plant lignans is dependent on microbiota activity, thus the conversion to enterolignans differs with the study participant's dietary habits and lifestyle, and is reduced by antibiotic intake (11). Urinary lignan excretion is directly related to flaxseed intake up to 25 g/day (13).

Because of its hard seed coating, whole flaxseed has low lignan availability that is increased two- to fourfold when flaxseed is crushed or milled (14). However, the availability of lignans in flaxseed nutrition bars was similar to those observed with ground seed because of the

extensive chewing of the flaxseed bar before swallowing (15). Based on urinary lignan excretion after the intake of flaxseed, processing of ground flaxseed into bread or muffins does not appear to affect the lignan availability (13). However, based on SDG analysis, recovery of added SDG in breads was reported to be only 73% to 75% after baking (2).

HEALTH BENEFITS

Cardiovascular Disease

Flaxseed has high ALA, lignan, and soluble fiber contents that can potentially protect against CVD. They may play a role through their effect on blood lipid levels, oxidative stress, inflammation, endothelial function, arrhythmia, and atherosclerosis, which have been demonstrated in animal and human studies (Table 2).

Animal Studies

As previously reviewed, dietary flaxseed's effect on atherosclerosis and hence CVD has been explored using animal models including the hypercholesterolemic rabbit and the cholesterol fed, low-density lipoprotein receptor (LDLR^{-/-}) deficient mouse (10). In both animal models, flaxseed feeding has been shown to suppress the development of atherosclerosis, but there is less clarity in the effect on blood lipids. Dietary flaxseed prevented the increase in triglycerides (TGs) in the cholesterol-fed rabbits; however, it had no effect on TG levels in the mouse model. On the other hand, flaxseed showed no effect on cholesterol levels in the rabbit model yet showed a strong protective effect in the mouse model.

Studies have looked into which component of flaxseed may be responsible for its antiatherogenic and lipid lowering effects (10). CDC flaxseed (Crop Development Center, type II flaxseed) with similar composition as ordinary flaxseed but with reduced ALA content (2–3%) fed to hypercholesterolemic rabbits caused a 69% suppression in the development of atherosclerosis with no effect on serum lipids. Flaxseed oil-rich diets (5% in diet, equivalent to 7.5 g/kg/day flaxseed) did not suppress the development of hypercholesterolemic atherosclerosis in rab-

bits nor did it cause any differences in serum lipids. SDG supplementation (15 mg/kg body weight/day) for eight weeks significantly reduced total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and TC/high-density lipoprotein (HDL-C). Overall, these suggest that flaxseed can reduce blood lipid and cholesterol levels and atherosclerosis in hypercholesterolemic animals and this effect may be attributed to the lignan component or other components such as the dietary fiber and not to the flaxseed oil.

Clinical Trials

The animal study findings are in agreement with the effect of flaxseed intake on blood lipids in clinical trials recently reviewed (10). Flaxseed consumption (10–50 g/day) in both healthy and hypercholesterolemic individuals have been found to reduce serum TC and LDL-C levels by 1.6% to 18% with no significant effect on levels of HDL-C or TG. There was, however, variability as there are studies that also showed no effect on TC or LDL-C. Meta-analysis of 28 clinical trials of flaxseed or flaxseed component effect on blood lipid profile also showed a reduction in TC and LDL-C with flaxseed intervention and no effect on HDL-C or TG concentrations (16). The effects depend on the form of flaxseed used, study quality, sex, and initial lipid profile, with greater effects in hypercholesterolemic and less or no effect in normocholesterolemic subjects. Further analysis showed that both whole (milled) flaxseed and lignan extract supplementation significantly improved TC and LDL-C levels while flaxseed oil intervention caused no significant change. ALA, on the other hand, has been shown in a clinical trial to have beneficial effects in coronary heart disease (CHD). The Lyon Diet-Heart Study, a secondary prevention trial, found that the ALA-rich Mediterranean type diet caused 70% reduction in subsequent adverse cardiac events in patients who previously suffered myocardial infarction compared with the prudent diet resembling the American Heart Association diet (17).

Epidemiological Studies

A review of several large epidemiological studies has suggested the beneficial effects of ALA in CHD (3). Three large prospective studies have shown a protective role of ALA

Table 2 Biomarkers Indicating Potential Mechanisms Whereby Flaxseed and Its Components Modulate Cardiovascular Disease

Biomarkers	Component					
	Flaxseed		Lignan		Flaxseed oil	
	In vitro/animal	Human	In vitro/animal	Human	In vitro/animal	Human
Blood Lipids						
• TC	↓↕↔(10)	↓↔(10,16)	↓↔(10)	↓(10,16)	↔(10)	↔(10,16)
• LDL-C	↓(10)	↓↔(10,16)	↓↔(10)	↓(10,16)	↔(10)	↔(10,16)
• HDL-C	↔(10)	↔(10,16)	↑↔(10)	↔(10,16)	↔(10)	↔(10,16)
• TG	↓↔(10)	↔(10,16)	↔(10)	↔(10,16)	↔(10)	↔(10,16)
Atherosclerosis	↓(10)	—	↓(10)	—	↔(10)	—
Oxidative stress	↓(10)	↔(19)	↓(10)	—	—	—
Inflammation	↓(10)	—	—	↓(10)	↓(6)	↓↔(6,10)
Endothelial function	↑(10)	—	—	↔(26)	—	↑(25)
Arrhythmia	—	—	—	—	↓(6)	↓↔(10)
Platelet aggregation	—	↓(10)	—	—	↓(10)	↓↔(10)

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TGs, triglycerides.

Results of studies: ↓ decrease; ↑ increase; ↔ no change.

in CHD event. The Health Professionals study and the Nurses Health study each followed subjects over six years and found that ALA was the only fatty acid associated with the reduction in nonfatal and fatal CHD, respectively (18,19). Interestingly, EPA and DHA were not associated with heart attack risk indicating that their effects on CVD may differ from that of ALA. These results are strengthened by results seen in other prospective studies such as the Multiple Risk Factor Intervention Trial, which showed a significant reduction in CHD fatality across quintiles of ALA intake; 0.56 g/day difference in ALA intake resulted in a 42% reduction in fatal CHD (20). Conversely, fewer epidemiological studies have looked specifically at lignan intake and CHD. The Kuopio Ischaemic Heart Disease Risk Factor Study, a 7.7-year follow-up study on men who suffered a CHD found that plasma enterolactone levels were significantly lower in cases versus control (21). A Dutch prospective study, however, showed no association between plasma enterolignan levels and the risk of non-fatal myocardial infarction (22). Conflicting evidence can be related to several factors including methods of lignan analysis, small range of plasma lignan levels, and the food sources of the lignans. Nonetheless, these epidemiological studies suggest that flaxseed has components that may be potentially effective in reducing the risk of CHD.

Mechanisms of Action

As mentioned previously, flaxseed has consistently been shown to reduce atherosclerosis in two animal models; however, there is a disagreement in its effect on blood lipids depending on the model and therefore other mechanisms may also be responsible for the antiatherogenic effect of flaxseed.

Other mechanisms that have been suggested for flaxseed's protective effect against CVD are summarized in Table 2 and include: (a) Reducing oxidative stress, which is associated with the development of atherosclerosis: Lignans have been shown to have anti-oxidant activities in *in vitro* studies (23); however, flaxseed intake did not ameliorate oxidative stress in clinical trials (24); (b) Anti-inflammatory action: n-3 fatty acids have demonstrated anti-inflammatory effects (7) and infectious disease and inflammation contribute to the development of atherosclerosis (25). Flaxseed supplementation has been shown to reduce markers of inflammation in mice [e.g., interleukin (IL)-3, vascular cell adhesion molecule-1 (VCAM-1)] (26) and ALA supplementation from flaxseed oil has been shown to reduce inflammatory markers [e.g., C-reactive peptide (CRP), serum amyloid A, IL-6, and soluble VCAM-1] in dyslipidemic patients (27). Flax lignan complex (dose providing 500 mg SDG/day for six weeks) effect on inflammation was studied in healthy postmenopausal women and was found to reduce plasma levels of CRP but had no effect on other markers including IL-6 and sVCAM-1 (28). This indicates that lignans may have a small anti-inflammatory effect; however, more studies are needed. (c) Endothelial effect: Animal studies have shown that flaxseed can protect against the dysfunctional contraction and relaxation of the aorta that accompanies high dietary cholesterol-induced atherosclerosis (29). Clinical studies have shown that flaxseed oil supplementation in obese adults increased systemic arterial compliance, a marker of endothelial function (30). On

the other hand, dietary lignan complex (500 mg of SDG) in postmenopausal women did not result in any benefit in endothelial function (31). These results indicate that it may be the oil component that plays a role in flaxseed's endothelial effect. (d) Antiarrhythmic effect: n-3 fatty acids have been shown using rat, nonhuman primate, and dog models to have antiarrhythmic properties (7). Studies comparing the effect of ALA to DHA have shown that ALA can reduce the heart rate to the same extent as DHA although it is a delayed effect. An intervention study conducted in humans was unable to confirm the antiarrhythmic effect of ALA. Prospective studies have, however, suggested a link between ALA intake and decreased risk for abnormally prolonged repolarization of the heart muscle (32), between ALA content of the adipose tissue and improved heart rate variability score indicative of reduced arrhythmia risk (33). Further research is needed to determine the effect of ALA on cardiac rhythm. (e) Platelet aggregation: In the rabbit model, flaxseed oil treatment inhibited thrombin and fibrinogen-induced platelet aggregation (34). Furthermore in apolipoprotein E^{-/-} LDLR^{-/-} mice, an n-6/n-3 ratio of 0.29:8 provided by flaxseed oil and safflower oil caused a dose-dependent antithrombotic effect (35). Results of these animal studies have not been consistently confirmed in human studies. One small study showed that 40 g/day of flaxseed oil for 23 days reduced platelet aggregation in healthy men (36); however, a larger study showed no anti-platelet effect of flaxseed oil (37). Two clinical studies looked at the effect of milled flaxseed feeding and platelet aggregation and both have shown a beneficial effect (38,39). More research is needed to elucidate the mechanisms responsible for these effects.

Breast Cancer

Animal Studies

Because of the potential hormonal effects of flaxseed and its lignans, more work has been done on their effect on hormone-related cancer such as breast and prostate cancer than the other types of cancer, although much of the work has been conducted using animal models. As previously reviewed (2,3,40), flaxseed (5%) fed before (preinitiation stage) or after carcinogen administration (promotion stage) in rats reduced the incidence and number of mammary tumors compared with the control. When flaxseed was fed nine weeks after carcinogen treatment (progression stage), tumors regressed by >50%. Similar effects were observed when SDG, its enterolactone metabolite, or flaxseed oil was fed, indicating that both the lignans and the flaxseed oil play a role in the flaxseed effect. Flaxseed at 10% level also reduced the tumor growth and metastasis incidence in athymic mice with human estrogen receptor negative (ER-) breast cancer, particularly in the lung and lymph node. SDG and flaxseed oil at levels present in 10% flaxseed similarly reduced the metastasis incidence, demonstrating that the lignan and oil components of flaxseed contribute to the antimetastatic effect of flaxseed (41). Flaxseed can reduce metastasis even after tumor is excised, but is more effective in reducing tumor recurrence when the excised tumor is still small (42). In ovariectomized (OVX) athymic mice with established human ER+ breast tumors (MCF-7) at low circulating estrogen level to simulate postmenopausal situation, 10%

flaxseed diet regressed the tumor size to a greater extent than the control and this effect was the same as that of pure SDG at levels equivalent to that in the 10% flaxseed diet (43). However, the effect of the SDG-rich flaxseed hull fraction equivalent to the amounts present in 10% flaxseed did not reduce the tumor growth to the same extent as the pure SDG, indicating that the hull fraction may need a higher level of intake to produce the same effect (43). The SDG is more effective at regressing the tumor growth than the flaxseed oil (44). Flaxseed, SDG, and flaxseed oil did not interfere with, but rather increased the effectiveness of tamoxifen, a primary breast cancer drug, in reducing human tumor growth in mice, at both low and high levels of circulating estrogen (45,46). Although SDG and flaxseed oil were responsible for the flaxseed effect, flaxseed oil was more effective than the SDG when fed in combination with tamoxifen treatment (46).

Clinical Trial

To test whether the effect seen in the animal models can be reproduced in humans, a randomized, double-blind, placebo-controlled clinical trial was conducted in postmenopausal women with newly diagnosed breast cancer (47). The patients consumed 25 g flaxseed in muffin formulation or placebo muffin daily for a mean duration of 32 and 39 days, respectively. Tumor biopsies taken before and after the feeding periods showed 34% lower rate of cell proliferation, 71% lower expression of c-erbB2 (neu, HER2), and 31% increase in apoptosis in the flaxseed group while no significant change was observed in the placebo group. These changes, which suggest slower tumor growth and better prognosis in the flaxseed group, were significantly correlated with the urinary lignan excretion and the amount of flaxseed consumed, in agreement with observations in the animal models. Overall, therefore, the animal studies and this clinical trial are in agreement that flaxseed and its lignans and oil components do not promote tumor growth and may even be protective against breast cancer.

Epidemiological Studies

Breast cancer risk has not been related to the intake of flaxseed in epidemiological studies but has been related to the intake of lignans or ALA. A recent meta-analysis that included 6 cohort, 6 nested case control, and 10 case-control studies showed no association between plant lignan intake and breast cancer risk in all women but a significant 15% reduction in risk was observed in postmenopausal but not in premenopausal women (48). In contrast, blood enterolactone level was not associated with breast cancer regardless of menopausal status. The significant effect of plant lignan intake in postmenopausal women indicates that the lignans may be more effective only at low estrogen level but the reason for this is still not very clear. In a recent prospective study of 51,823 women with average 8.3 years follow-up, a significant inverse association between lignan intake and breast cancer risk was observed especially among postmenopausal hormone users but there was no heterogeneity across ER/progesterone receptor (PR) subtypes (49). Others have shown, however, that the inverse association between plasma enterolactone and breast cancer risk was

significantly different between ER α and ER β tumors and most evident in ER α but not in ER β tumors (50).

Epidemiological studies on the relationship between ALA and breast cancer are conflicting (3). While significant relationship between high levels of ALA in the adipose tissue of breast cancer patients and the lower incidence of breast cancer metastasis has been observed, no tendency to decrease breast cancer risk was associated with increased serum or erythrocyte ALA levels. In a recent study of 56,007 French women with eight years follow-up, breast cancer risk was inversely associated with the intake of ALA from fruits and vegetables but was positively related to ALA intake from nut mixes and processed foods (51). ALA intake was also positively associated in women in the highest quintile of dietary vitamin E, indicating the need to consider not only food sources but also interaction of the fatty acids with anti-oxidants when determining the association of fatty acids with breast cancer.

Mechanisms of Action

The mechanisms of flaxseed effect on breast cancer have been attributed to the activities of its lignans and ALA-rich oil components. Regarding the lignans, suggested mechanisms as previously reviewed include the following (3,11,40,52): (a) Lignans may interfere with the binding of endogenous estrogen to ER α and ER β thus altering its action. They have been shown to bind weakly to ER α and ER β . (b) They can reduce the availability of estrogens by inhibiting the activities of enzymes involved in the synthesis and regulation of estrogen synthesis: aromatase, 17-hydroxysteroid dehydrogenase, and 5 α -reductase. However, while serum estradiol was reduced after the intake of 5 g or 10 g flaxseed, a study showed that consumption of ground flaxseed at 7.5 g/day for six weeks and then 15 g/day for six weeks did not significantly reduce estradiol, estrone, and testosterone in postmenopausal women although it significantly reduced estrone in overweight/obese women (53). (c) They can also stimulate the production of sex hormone-binding globulin that binds estrogen and testosterone and thus reduce their free concentration and availability in the plasma; however, results on this are conflicting with some showing this effect while others do not. (d) They can alter the metabolism of estrogen that leads to the production of 2-hydroxyestrone (2OHE1) and 16-hydroxyestrone (16OHE1). Since 2OHE1 has low biological (estrogenic) activity while 16OHE1 has estrogenic activity, an increase in 2OHE1 to 16OHE1 has been suggested to be associated with reduced breast cancer risk. Flaxseed (10–25 g/day) has been shown to increase the ratio of 2OHE1 to 16OHE1 in pre- and postmenopausal women indicating a protective effect. (e) They may modify estrogen and growth factor action through several mechanistic pathways. Flaxseed and its lignans have been shown to reduce cell proliferation, increase apoptosis, decrease angiogenesis, and the expressions of ER α , ER β , pS2, Bcl2, cyclin D1, insulin-like growth factor-1 (IGF-1), IGF-I receptor (IGF-IR), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), mitogen-activated protein kinase (MAPK), and Akt, indicating that their effects include the downregulation of the ER and growth factor-mediated signaling pathways (43–46,54). (f) Nonhormone-related mechanisms may also be

involved. In vitro studies using various test models have shown that lignans (10–100 μ M) have anti-oxidant activities (23,55). However, feeding flaxseed to postmenopausal women did not show significant changes in various anti-oxidant biomarkers in the human plasma (24), although flaxseed and SDG reduced oxidative stress in the hypercholesterolemic rabbits (10).

Regarding ALA-rich oil, its mechanisms have been related in part to the inhibition of the formation of prostaglandin E₂ (PGE₂), leukotriene B₄ (LTB₄), and LTC₄ from the n-6 fatty acids, which have been shown to directly stimulate the growth of malignant cells in addition to their effects on humoral and cell-mediated immunity. Other suggested mechanisms include alteration of the lipid composition of tumor cell membranes, hence affecting membrane fluidity and growth factor expression and signal transduction that influence tumor growth. It can also form cytotoxic peroxidation products (56).

Prostate Cancer

Animal Studies

The role of lignans on prostate cancer has been reviewed (3,11). Flaxseed-derived lignans reduced the growth in vitro of LNCaP, DU-145, and PC-3 prostate cancer cell lines. Flaxseed diet inhibited the prostate tumor growth and development in the transgenic adenocarcinoma mouse prostate model (57). A plant lignan hydroxymatairesinol, which like SDG can be metabolized to the enterolignans, increased the proportion of nongrowing tumors, decreased cell proliferation, and increased cell apoptotic index in nude mice transplanted with LNCaP human prostate cancer cells compared with control (58).

Clinical Trials

In a pilot study, men scheduled for prostatectomy surgery consumed a low-fat diet supplemented with 30 g ground flaxseed/day (59). After an average 34 days, the cell proliferation index was significantly lower and apoptotic index was higher when compared with historical matched controls. A follow-up study determined the effect on prostate cancer patients of low-fat diet, and 30 g flaxseed diet, alone and in combination, for an average 30 days (60). A significant reduction in tumor cell proliferation index in the flaxseed arms but no effect on apoptotic index and other biomarkers were observed. These clinical trials support the hypothesis that flaxseed maybe protective against prostate cancer.

Epidemiological Studies

In contrast, some epidemiological studies (meta-analysis) showed an increased risk of prostate cancer associated with high intake or blood level of ALA, with the association stronger in case-control than in prospective studies (61). In one systematic review and meta-analysis of eight case-control and eight prospective studies, high ALA intake or tissue concentration was weakly positively associated with prostate cancer risk (62). However, the authors qualified this conclusion because of significant heterogeneity across studies and evidence of publication bias. Furthermore, food sources of ALA in these studies were predominantly meat, dairy products, and salad dressings and not flaxseed. A more recent meta-analysis of five

prospective studies support a weak protective association between prostate cancer risk and ALA intake (63). Those that consume >1.5 g ALA/day has lower prostate cancer risk than those who consumed <1.5 g ALA/day. Obviously, the conflicting effect of ALA on prostate cancer needs to be resolved. Epidemiological studies have not shown a significant association between plasma enterolactone and prostate cancer (64).

Mechanisms of Action

Because flaxseed has both the ALA and lignans and yet clinical trials showed protective effect of flaxseed against prostate cancer, it is possible that any potential adverse effect caused by ALA may have been modified by the lignans in flaxseed where they are together. The mechanism of any ALA effect on prostate cancer is not clear. In contrast, in in vitro studies, it was shown that the inhibitory effect of the lignan enterolactone on the growth of PC-3 prostate cancer cells is in part through inhibition of IGF-1/IGF-IR signaling; it inhibited the activation of IGF-IR and its downstream AKT and MAPK and their phosphorylation (65). Cyclin D1 expression was also downregulated. Some of the mechanisms mentioned under breast cancer may likewise be possible in prostate cancer, it being a hormone-sensitive cancer.

Colon Cancer

The effect of flaxseed and its lignans and oil on colon cancer is conflicting. In vitro studies showed that enterolactone and enterodiol can reduce the cell proliferation and induce cell-cycle arrest of colon cancer cells (11). In carcinogen-treated rats, aberrant crypts (precursors of adenoma and colon cancer) were reduced by 2.5% to 10% flaxseed diet and purified SDG (66). Lower number and incidence of colon tumors with flaxseed oil feeding have also been observed (67). However, 5% flaxseed (68) or pure secoisolariciresinol or matairesinol at 0.02% in diet (69) did not reduce tumor growth in Apc Min mouse, a mice model for adenomatous polyposis coli multiple intestinal neoplasia, although another study using the same mice model showed reduced colon tumorigenesis (70). Any discrepancy was thought to be related in part to the fact that enterolignans are formed from the plant lignans in the colon, while in the APC Min mouse the tumors are formed primarily in the small intestine.

Regarding epidemiological studies, case-control studies showed an inverse association between colorectal cancer risk and plasma lignan levels (71) or lignan intake (72). However, a prospective Dutch study of 35,000 subjects with average 7.5 years follow-up did not support the association of high plasma enterolignan concentration with a reduction in colorectal cancer after adjustment for colorectal cancer risk factors (73).

The effect of flaxseed or its oil on colon cancer development has been attributed to the increase in the n-3 fatty acids and reduction in cyclooxygenase 1 and 2 (COX1 and COX2) levels in the serum and colon tissues (67,70) and reduction in IGF-1 signaling (74).

Bone Health

Mammalian lignans and n-3 fatty acids have been shown to exert direct effects on bone cells in in vitro studies through modulation of cell viability, DNA content, and

biomarkers of bone formation (75,76). Flaxseed is a rich source of both components indicating its potential to improve bone health.

Animal Studies

In animal models, dietary flaxseed has transient effect on bone composition and strength. For example, female rats fed 5% or 10% flaxseed or 6.2% defatted flaxseed resulted in decreased plasma alkaline phosphatase, an indicator of bone formation (77); this also indicates that the flaxseed effect may be due to the defatted portion (i.e., lignan, dietary fiber). Dietary lignan, at levels present in 5% and 10% flaxseed, fed during suckling alone or through to different developmental stages (i.e., adolescence, adulthood) increased femur strength at adolescence without a change in bone mineral content (BMC) (78). The effect was no longer seen at adulthood indicating that female rat bone is more sensitive to lignans during early life when endogenous levels of sex hormones are low (i.e., adolescence). In male rats, flaxseed, but not SDG, fed through to adolescence reduced biomechanical strength but again this was not seen in adulthood (79). These studies indicate that flaxseed and SDG may have gender-specific effects on bone, which do not last into adulthood.

The effect of flaxseed feeding was studied in OVX rodents, which models the postmenopausal, osteoporotic situation. A 10% flaxseed diet fed for 12 weeks resulted in no change in bone strength or composition (80) but fed for 25 weeks, increased biomarkers of bone strength (81). In one study, no benefit of treatment with enterolactone or enterodiol was observed in the bone of OVX mice with human breast cancer (82). However, flaxseed feeding enhanced the effectiveness of low-dose estrogen therapy on bone properties indicating that flaxseed may provide a benefit for patients in treating the bone loss that accompanies menopause (80). Evidently, controversy remains in the effect of flaxseed and its components on bone health in animal models and a further study of functional measures of bone strength are needed.

Clinical Trials

Regarding clinical studies, three randomized, double-blinded, placebo-controlled trials [(two short term, 3–4 months) (83,84) and one long term (12 months) (85)] in healthy postmenopausal women showed no significant effect of dietary flaxseed between baseline to end of study and placebo versus treatment on bone mineral density (BMD) and biomarkers of bone metabolism. A randomized placebo-controlled trial conducted to determine the effect of flaxseed lignan complex supplementation (543 mg lignan complex/day in a tablet form, 32.9% SDG) during an exercise program on bone health in men and women older than 50 years showed no significant changes in BMD and BMC (86). In a double-blinded crossover trial, a high ALA diet (LA/ALA = 1.6/1) provided by flaxseed oil and walnut oil fed for six weeks to hypercholesterolemic and overweight men and women significantly reduced serum N-telopeptides of type 1 collagen, tumor necrosis factor- α (TNF- α) (proinflammatory cytokine and osteoclastogenic factor) compared with a low ALA diet (LA/ALA = 9.5/1) without affecting bone-specific alkaline phosphatase (87). No differences were seen in BMD between groups but this may be attributed to short study duration. These results

suggest that ALA-rich flaxseed oil may be beneficial in bone health by decreasing bone breakdown through an attenuation of osteoclastic activity.

Epidemiological Studies

Regarding epidemiological studies, greater urinary enterolactone levels were correlated with higher BMD in Korean postmenopausal women with existing osteoporosis (88). However, in a study of healthy Dutch postmenopausal women with low ($\leq 0.5\%$) or high ($\geq 2.5\%$) rates of cortical bone loss at the radius, greater urinary enterolactone level was correlated with greater rates of bone loss (89). There are many possibilities for the discrepancies in these results including variations in subject populations (osteoporotic vs. healthy) and methods of enterolactone analyses. ALA's role in bone loss prevention was seen in a study showing that higher LA to ALA ratio was associated with a lower hip BMD in healthy older men, menopausal women not using hormone replacement therapy (HRT), and postmenopausal women using HRT (90).

Although clinical studies showed that flaxseed and its lignan component may have little effect in the prevention of osteoporosis, it is important to note that no adverse effects were seen. ALA may be beneficial in the prevention of bone loss; however, further studies of longer duration are necessary to determine whether this effect will translate into increased BMD and bone strength.

Menopausal Symptoms

Since flaxseed is one of the richest dietary sources of the phytoestrogen SDG, the precursor of enterodiol and enterolactone that may mimic the activity of estrogen, studies have assessed its potential to attenuate symptoms that come with the loss of endogenous estrogen production of menopause. Many menopausal women suffer from hot flashes, night sweats, and vaginal dryness and although HRT has been proven to prevent and relieve these symptoms, it is associated with an increased risk of specific diseases such as CVD and breast cancer.

A randomized crossover clinical study compared the effectiveness of flaxseed supplementation to HRT and showed that flaxseed feeding for two months attenuated symptoms as effectively as two months of HRT (91). However, in a randomized, double-blind, wheat germ placebo-controlled clinical trial of 190 women, both flaxseed (40 g/day) and the wheat germ placebo for one year significantly reduced severity scores of menopausal symptoms from baseline but there was no difference between these groups (85). No significant effect of flaxseed was also observed in another randomized placebo-controlled trial (92) indicating the need for placebo when conducting studies on menopausal symptoms. In one study, flaxseed supplementation to postmenopausal women suffering from hot flashes showed a greater than 50% reduction in hot flush scores compared with baseline (93). There was no placebo control group in this study but they concluded that the reduction in hot flush activity was greater than what would have been seen with a placebo. Other clinical studies that have been reviewed (3) showed varied results with some showing no effect and some showing benefit in only specific symptoms (hot flashes and vaginal dryness). Flaxseed supplementation showed no adverse

effect; however, in one study where soy was also used as an intervention, an increase in vaginal cytology maturation index was seen (94).

Other Potential Health Benefits

Several animal models have shown that flaxseed and in particular SDG can reduce the development of diabetes and this effect was associated with the ability of SDG to reduce oxidative stress (95). Flaxseed decreased plasma glucose and insulin levels in hypercholesterolemic women (91). In a randomized placebo-controlled crossover trial of type 2 diabetics with hypercholesterolemia, supplementation with flaxseed-derived lignan capsules (360 mg/day) for 12 weeks improved glycemic control (96) and also modulated the CRP levels in type 2 diabetics although it was observed more in women and not in men (97). Daily consumption of 5 g flaxseed gum (dietary fiber) by type 2 diabetics has also been shown to reduce blood glucose response as well as total and LDL cholesterol (98). However, flaxseed oil (10 g/day) has no effect on glycemic control in type 2 diabetes (99).

Flaxseed, cold pressed flax meal or its isolated dietary fiber (gum) has been shown to improve laxation or reduce constipation, a well-known effect of high fiber diet (100,101). Other suggested beneficial effects of flaxseed include treatment improvements in kidney disease (3); however, studies in this area are still very limited.

SAFETY

The presence of cyanogenic glycosides is a concern but considering that adults can detoxify 30 to 100 mg of cyanide/day, one needs to consume large amounts of flaxseed to show cyanide toxicity (10). An intake of >10 tablespoons/day (~100 g) of raw milled flaxseed is estimated to raise the cyanogen levels above 50 to 60 mg. Heat treatment destroys cyanogens in flaxseed and the amounts present in baked products or cereals may not pose problems. Cadmium is also present in flaxseed but the risk associated with it has been suggested to be less than that from rice or wheat. However, flaxseed with very high levels of cadmium may have estrogenic effect and may be a concern when consumed during pregnancy and lactation. Flaxseed contains vitamin B6 antagonist (linatine) but this has not been considered a problem because the diet normally contains sufficient amounts of vitamin B6. Although very rare, some individuals may have anaphylactic or allergic reaction to flaxseed so one has to test for it before consuming large amounts of flaxseed. Gastrointestinal discomfort associated with intake of large amounts of dietary fiber is often reported with flaxseed intake.

Recently, a meta-analysis of 174 randomized controlled trials determined the side effects (broadly categorized as gynecological, gastrointestinal musculoskeletal, neurological) of phytoestrogens including the lignans and isoflavones although more on the latter (102). It was concluded that the phytoestrogens caused moderately elevated rates of gastrointestinal side effects but did not significantly increase rates of vaginal bleeding, endometrial hyperplasia, endometrial cancer, and breast cancer and have generally safe side effect profile.

Safety of flaxseed interaction with drugs has to be taken into consideration when consuming flaxseed or its lignan or oil components (103). For example, flaxseed decreases platelet aggregation and increases bleeding time; therefore it may increase the risk of bleeding when taken with anti-platelet or anti-coagulant drugs. Because of the hormonal action of lignans, it is surmised that flaxseed intake may enhance tumor growth and interfere with the action of hormone-related treatments, for example, tamoxifen, HRT. However, as mentioned earlier, most animal studies and clinical trials have shown that flaxseed, or its lignans or oil do not promote tumor growth and does not interfere but rather enhance the effectiveness of tamoxifen. Furthermore, animal studies have shown that flaxseed combined with low-dose estrogen therapy lead to a greater BMD and peak load at the tibia in OVX rats. Further studies need to be conducted to assess the potential harmful or beneficial interactions between flaxseed and other hormone-related therapies.

REGULATORY STATUS

Every country has its own regulations for the use of flaxseed. There is currently no regulation regarding the level of flaxseed that can be added to foods but there is no objection to the use of flaxseed at 8% level in baked products or at 4% in dry cereal in Canada, and at 12% level in the United States. Recently, however, the U.S. Food and Drug Administration (FDA) gave a no objection decision to the request of Flax 2015 and Flax Council of Canada to consider whole and milled flaxseed as GRAS (generally recognized as safe) (104). GRAS is an FDA designation that means the substance is considered safe by experts and therefore is exempted from the Federal Food Drug and Cosmetic Act food tolerance requirements. The decision was based on observations that (a) flaxseed products have long history of use and regarded as safe and nutritious, (b) animal and human research has confirmed its safety, and (c) the composition and purity of whole and milled flaxseed have been adequately characterized to assure a wholesome food grade product. The addition of flaxseed to conventional foods is considered safe and limited only by current Good Manufacturing Practices. In Canada, food manufacturers are allowed to state in the Nutrition facts label the n-3 fatty acid (ALA) content per serving. In the United States, the Nutrition label can include the percent of the Daily Value for ALA per serving.

CONCLUSION

Flaxseed is an ancient crop that in recent years increased in popularity as food because of its suggested health benefits. The benefits have been attributed to its exceptionally high levels of n-3 fatty acid (ALA)-rich oil, phytoestrogen lignans especially SDG, and dietary fiber, components that have also been isolated as fractions or extracts to be used as dietary supplements. Whole flaxseed and the lignan have been shown to improve plasma TC and LDL-C with no effect on plasma HDL-C and TG particularly in hyperlipidemic individuals. The ALA-rich flaxseed oil has little effect on blood lipids but has also been linked to reduced

risk of CVD events perhaps through lipid-lowering independent mechanisms. There is evidence suggesting that flaxseed and its SDG or oil components have anti-oxidant, anti-inflammatory, antiarrhythmic, anti-platelet aggregation, and good endothelial function effects, which can help reduce CVD risk, but more research is needed to further establish mechanisms. Extensive animal studies are consistent in showing that flaxseed can reduce breast tumor development at various stages of carcinogenesis including preinitiation, promotion, progression, and metastasis. A clinical trial with breast cancer patients agreed with the observation that flaxseed does not promote but rather reduces breast tumor growth. However, this needs further confirmation in trials with large number of subjects. Both the lignan and the oil appear to contribute to the breast cancer protective effect involving both hormone and nonhormone-related mechanisms. Limited evidence suggests that flaxseed and lignans are protective against prostate cancer but the role of ALA is less clear. There is conflicting evidence for the protective role of flaxseed on colon cancer. Flaxseed does not seem to have strong effect, positive or negative, on bone health; if any, flaxseed oil may have a better effect. However, animal studies suggest that flaxseed may enhance the effectiveness of low-dose estrogen therapy. There is little effect of flaxseed consumption in reducing menopausal symptoms and those that have been observed may be attributed to a placebo effect. Potential health benefits of flaxseed and SDG in reducing diabetes risk have been suggested in some animal and human studies. As an excellent source of dietary fiber, flaxseed is well known to exert a laxative effect.

Whole or milled flaxseed is considered safe for consumption; hence the U.S. FDA has indicated no objection in considering flaxseed as GRAS. ALA is an essential PUFA with the Adequate Intake established by the National Academies' Institute of Medicine at 1.6 g/day for adult males and 1.1 g/day for adult females. This can be met with the daily intake of less than 10 g flaxseed or 4 g flaxseed oil. In clinical studies, the daily consumption levels tested for reduction in disease risks ranged from 10 to 50 g flaxseed, 1 to 40 g flaxseed oil, and 50 to 600 mg flaxseed lignan. However, more research is needed to find the optimal dose for these components to exert the desired health benefit.

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Folate

Pamela Bagley and Barry Shane

INTRODUCTION

Folate is a dietary essential B-complex vitamin required for human health. It functions as a cofactor in the synthesis of nucleotides required for DNA synthesis and in amino acid metabolism. The classic symptom of folate deficiency is megaloblastic (large cell) anemia, which is a reflection of defective DNA synthesis. Poor folate status is also a risk factor for cancer. The finding that periconceptional folate supplementation greatly reduces the incidence of birth defects in humans has led to fortification of the food supply with folic acid in the United States and other countries.

STRUCTURE AND CHEMISTRY

Folate is a generic term for a family of chemically and functionally related compounds based on the folic acid structure (Fig. 1). Folic acid (molecular weight 441.4), also known as pteroylmonoglutamate, is an oxidized, synthetic form of the vitamin and consists of a pterin (2-amino-4-hydroxy-pteridine) ring linked to para-aminobenzoic acid, which is conjugated to a molecule of L-glutamic acid. Folic acid is reduced within the cell to the metabolically active 5,6,7,8-tetrahydrofolate form. Folates in tissues act as donors and acceptors of one-carbon units in metabolic reactions known as one-carbon metabolism. These one-carbon units can be at the oxidation level of methanol (5-methyl-tetrahydrofolate), formaldehyde (5,10-methylene-tetrahydrofolate), or formate (5- or 10-formyl-tetrahydrofolate or 5,10-methenyl-tetrahydrofolate). The predominant coenzyme forms are listed in Table 1.

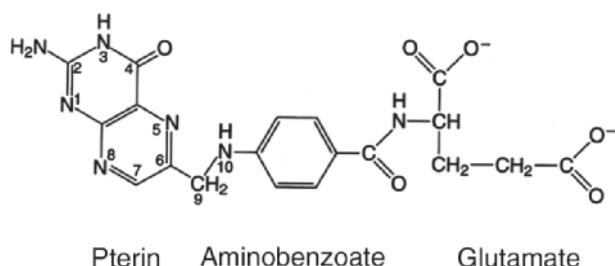


Figure 1 Structure of folic acid (pteroylglutamate). One-carbon substituents can be at the N-5 and/or N-10 positions of the reduced tetrahydrofolate molecule.

Table 1 Metabolically Active Forms of Folate^a

Unsubstituted folates	
7,8-Dihydrofolate (reduced at positions 7 and 8)	
5,6,7,8-Tetrahydrofolate	
Substituted folates	One-carbon group
5-Methyl-tetrahydrofolate	-CH ₃
5-Formyl-tetrahydrofolate (folinic acid, leucovorin)	-CHO
5-Formimino-tetrahydrofolic acid	-CHNH
10-Formyl-tetrahydrofolate	-CHO
5,10-Methylene-tetrahydrofolate	-CH ₂ -
5,10-Methenyl-tetrahydrofolate	-CH-

^aActive forms are polyglutamate derivatives.

Practically all tissue folates are polyglutamate forms in which the glutamate tail is extended via an unusual peptide bond via the γ -carboxyl of glutamate. Glutamate chain lengths can vary from approximately 4 to 10 in human tissues. Metabolism of folates to polyglutamate forms is required for their biological activity, as the polyglutamate forms are much more effective substrates for folate-dependent enzymes than are the monoglutamate derivatives, which are the transport forms of the vitamin (1). Conversion of folates to polyglutamates of chain length greater than three or more is also required for effective retention of folate by tissues (2), and allows tissues to maintain much higher folate levels than in plasma.

BIOCHEMISTRY AND FUNCTIONS

Folate coenzymes are involved in three major inter-related metabolic cycles in the cytosol of cells. These cycles are required for the synthesis of thymidylate and purines, precursors for DNA and RNA synthesis, and for the synthesis of methionine from homocysteine and the interconversion of serine and glycine (Fig. 2).

5,10-Methylene-tetrahydrofolate plays a central role in these cycles as it can be used directly for thymidylate synthesis or it can be reduced to 5-methyl-tetrahydrofolate in the methionine synthesis cycle or can be oxidized to 10-formyl-tetrahydrofolate to be used in purine synthesis. Although the enzymes involved in these synthetic cycles are normally located in the cytosol, during the S phase of the cell cycle the enzymes involved in thymidylate synthesis translocate to the nucleus, which also contains a folate pool (3). Mammalian cells also contain a large, separate mitochondrial folate pool that is involved in serine and choline metabolism and in the provision of one-carbon precursors for cytosolic one-carbon metabolism (4,5).

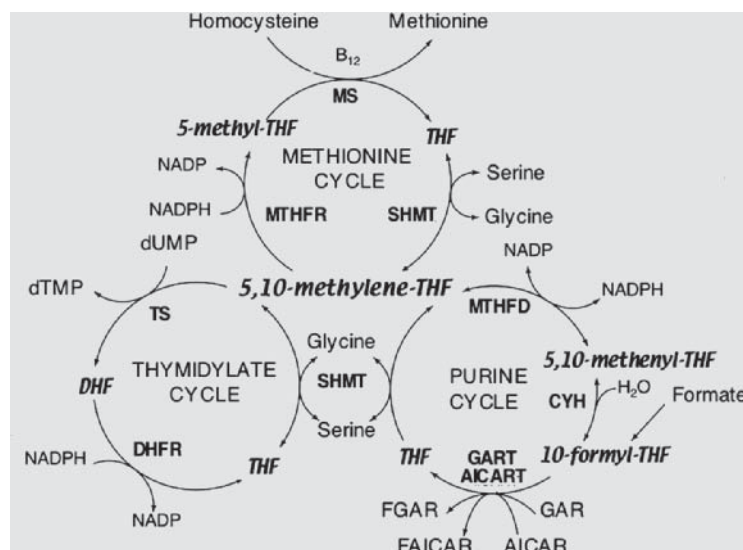


Figure 2 The major metabolic cycles of folate-dependent one-carbon metabolism in the cytoplasm of cells. The cycles use reduced tetrahydrofolate (THF) polyglutamates as substrates. **Abbreviations:** SHMT, serine hydroxymethyltransferase; MTHFR, methylene-tetrahydrofolate reductase; MS, methionine synthase; TS, thymidylate synthase; DHFR, dihydrofolate reductase; MTHFD, methylene-tetrahydrofolate dehydrogenase; CYH, methenyl-tetrahydrofolate cyclohydrolase; GART, GAR formyltransferase; AICART, AICAR formyltransferase; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; FAICAR, formyl-aminoimidazole carboxamide ribonucleotide; AICAR, aminoimidazole carboxamide ribonucleotide; GAR, glycylamide ribonucleotide; FGAR, formyl-glycylamide ribonucleotide; DHF, dihydrofolate; NADP, nicotinamide adenine dinucleotide phosphate; B₁₂, vitamin B₁₂ (cobalamin).

Serine–Glycine Interconversion

Serine is the major provider of one-carbon units for folate-dependent one-carbon metabolism (1). It donates its β -carbon to tetrahydrofolate to generate glycine and 5,10-methylene-tetrahydrofolate. The reaction, which is catalyzed by the pyridoxal phosphate (PLP)-dependent enzyme serine hydroxymethyltransferase (SHMT), is at near equilibrium and can be easily reversed. Mammalian tissues contain two distinct isozymes of SHMT, one cytosolic and one mitochondrial, encoded by different genes (6). The mitochondrial isozyme, which is expressed in all tissues, may be responsible for the generation of the majority of one-carbon units required for cytosolic one-carbon metabolism (7). 5,10-Methylene-tetrahydrofolate generated via the mitochondrial SHMT reaction can be oxidized to 10-formyl-tetrahydrofolate and then hydrolyzed to formate. The formate produced exits the mitochondria and the one carbon is reincorporated into the cytosolic one-carbon pool as 10-formyl-tetrahydrofolate. 10-Formyl-tetrahydrofolate can be reduced to 5,10-methylene-tetrahydrofolate in the cytosol via the action of a reversible trifunctional C1 synthase enzyme. The tissue distribution of the cytosolic isozyme of SHMT is more limited than the mitochondrial enzyme. The cytosolic isozyme is highly expressed in the liver and kidney and in rapidly replicating cells. A major role of the hepatic cytosolic SHMT enzyme may be to synthesize serine from glycine for use in gluconeogenesis. Mammalian cell mutants that lack mitochondrial SHMT but express cytosolic SHMT are glycine auxotrophs (8).

Nucleotide Synthesis

Folate is required for the synthesis of thymidylate, a nucleotide required specifically for DNA synthesis and repair. Thymidylate synthase catalyzes the transfer of the one-carbon group from 5,10-methylene-tetrahydrofolate to the 5'-position of deoxyuridine monophosphate (dUMP) and its reduction to a methyl group to generate deoxythymidine monophosphate (dTMP). The folate

molecule also provides the reducing component in this reaction and the tetrahydrofolate is oxidized to dihydrofolate. The dihydrofolate generated has to be reduced back to tetrahydrofolate before it can be reutilized in one-carbon metabolism in a reaction catalyzed by dihydrofolate reductase (DHFR). Thymidylate synthetase activity is primarily expressed in replicating cells during the S phase of the cell cycle and is highest in fast growing cells. Consequently, drugs targeted to DHFR, such as methotrexate, have proven to be effective chemotherapeutic agents as they are selective inhibitors of rapidly growing cells (9,10). Some of the cytosolic folate enzymes involved in provision of thymidylate are sumoylated and translocate to the nucleus during the S phase of the cell cycle (3). These include thymidylate synthase, DHFR, and cytosolic SHMT. In addition, a variant form of mitochondrial SHMT, which is expressed from a second promoter on the mitochondrial SHMT gene and contains a different exon 1 lacking a mitochondrial leader sequence, is also found in the cytosol of cells and also translocates to the nucleus during the S phase of the cell cycle (11).

Cytosolic folate coenzymes are also used in two steps of de novo purine biosynthesis. The C-8 and C-2 positions of the purine ring are derived from 10-formyl-tetrahydrofolate in reactions catalyzed by glycylamide ribonucleotide (GAR) transformylase and 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) transformylase. The enzymes involved in de novo purine are multifunctional and form a complex in the cytosol called the purinosome (12). This complex remains in the cytosol during the S phase of the cell cycle.

Methionine Synthesis

The methylation of homocysteine to produce methionine uses 5-methyl-tetrahydrofolate as the methyl donor in a reaction catalyzed by methionine synthase, one of only two vitamin B₁₂-dependent enzymes in mammals (Figs. 2 and 3) (13). 5-Methyl-tetrahydrofolate is generated from 5,10-methylene-tetrahydrofolate in a reaction

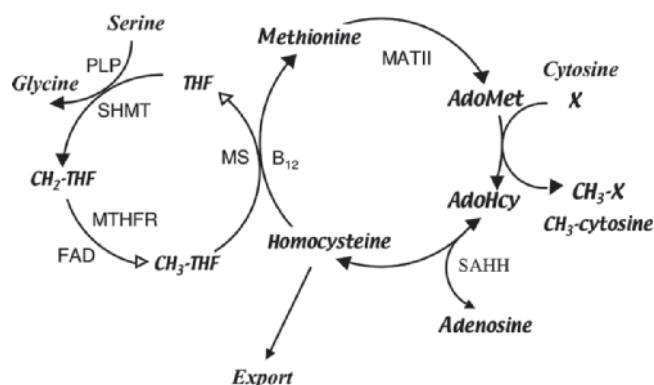


Figure 3 The folate-dependent methionine synthesis cycle and the transmethylation cycle. Abbreviations: THF, tetrahydrofolate; FAD, flavin adenine dinucleotide; PLP, pyridoxal phosphate; B₁₂, vitamin B₁₂; AdoMet, adenosylmethionine; AdoHcy, adenosylhomocysteine; SHMT, serine hydroxymethyltransferase; MTHFR, methylene-tetrahydrofolate reductase; MS, methionine synthase; MATII, methionine adenosyltransferase; SAHH, AdoHcy hydrolase.

catalyzed by the flavoprotein methylenetetrahydrofolate reductase (MTHFR). Methionine can be metabolized to S-adenosylmethionine, which acts as the methyl donor in many reactions, including the methylation of DNA, histones and other proteins, neurotransmitters and phospholipids, and the synthesis of creatine. These methylation reactions play important roles in development, gene expression, and genomic stability. S-Adenosylhomocysteine, the product of methylation reactions, is a potent inhibitor of many methyltransferases and is catabolized by hydrolysis to adenosine and homocysteine. In liver and kidney, homocysteine can be further metabolized to cysteine via the transsulfuration pathway utilizing the PLP-dependent enzymes cystathionine- β -synthase (CBS) and cystathioninase. In most other tissues, homocysteine is exported to the circulation or is reconverted back to methionine via the folate-dependent methionine synthase reaction.

Homocysteine can also be remethylated to methionine by cytosolic betaine homocysteine methyltransferase (BHMT), a folate-independent enzyme that is expressed in high levels in liver and in some species, including humans, in kidney (14). Betaine (trimethylglycine) is derived from mitochondrial oxidation of choline. Dimethylglycine, the product of the BHMT reaction, is oxidized in the mitochondria to sarcosine (methylglycine) and then to glycine by the folate-dependent flavoproteins dimethylglycine dehydrogenase and sarcosine dehydrogenase. Each oxidation results in the formation of a molecule of 5,10-methylene-tetrahydrofolate from tetrahydrofolate (1).

Methionine is a dietary essential amino acid as mammals lack the ability to synthesize homocysteine, its carbon skeleton, and homocysteine is not normally present in the diet. It is often the most limiting amino acid in human diets. Methylation reactions account for a large proportion of the methyl group intake in humans and the methionine synthase and BHMT reactions allows salvage of its backbone after its use for methylation.

The folate-dependent methionine cycle is sensitive to inadequate folate status. When folate status is poor,

the decreased ability to remethylate cellular homocysteine results in an increased plasma homocysteine level, and the plasma total homocysteine level is an indirect non-specific indicator of folate insufficiency. Fasting homocysteine levels are also influenced by vitamin B₁₂ status and nonfasting levels by vitamin B₆ status and methionine intake.

PHYSIOLOGY

Absorption, Transport, and Bioavailability

Folic acid does not occur in nature and is rarely found in unfortified foods and is not an active form of the coenzyme. However, it is the most common form of folate used in supplements and in fortified food products because it is highly bioavailable and chemically stable and normally is readily reduced to tetrahydrofolate, the active coenzyme form of folate. Stabilized forms of reduced folates such as 5-methyl-tetrahydrofolate are now available and are being used in some supplements. Food folates typically occur in a reduced, polyglutamyl form. Before absorption, they are cleaved to their monoglutamyl forms by a brush border glutamylhydrolase (glutamate carboxypeptidase II), sometimes called intestinal folate conjugase (15). Folates are absorbed in the proximal small intestine via the proton coupled folate transporter (PCFT) (16), an apically expressed transmembrane, pH-sensitive carrier that transports both oxidized and reduced folates with similar efficacy under the slightly acidic conditions of the gut wall. The affinities of folic acid and 5-methyl-tetrahydrofolate for the PCFT carrier are around 1 μ M (17). Transport across the basolateral membrane of the mucosal cell is via low affinity, high capacity, general anionic carriers known as multidrug resistance associated proteins (MRPs), as these proteins were originally identified as being responsible for multidrug resistance of cells to chemotherapeutic agents (17). Most dietary folate is metabolized to 5-methyl-tetrahydrofolate during its passage across the intestinal mucosa into the portal circulation and/or by first pass metabolism in the liver.

Most of the folic acid from supplements or fortified foods is also metabolized to reduced folates, primarily 5-methyltetrahydrofolate, during its passage across the intestinal mucosa and liver, and behaves similarly to reduced folates. However, low levels of free folic acid are found in fasted plasma when the diet contains folic acid and higher levels are present in nonfasted samples (18). Folic acid has to be reduced to dihydrofolate to enter the naturally occurring folate pool. This reduction, and its further reduction to tetrahydrofolate, is catalyzed by DHFR. The initial reduction of folic acid to dihydrofolate is very slow compared to its further reduction to tetrahydrofolate. Wide variations in DHFR activity have been reported for human liver (19) and it has been suggested that folic acid utilization may be impaired in individuals expressing low levels of DHFR. When high doses of folic acid or other folate forms are consumed, some are absorbed by a nonsaturable process and appear in the peripheral circulation unchanged.

The bioavailability of folic acid and reduced folate monoglutamates are usually similar and are close to 100% when consumed on an empty stomach and approximately

85% when added to, or given with, food. The bioavailability of food folate is lower and depends on the food matrix. On typical diets, food folate bioavailability is approximately 50% (20). Consequently, dietary reference intake (DRI) values are expressed in dietary folate equivalents (DFE), as folate added to food or in supplements is approximately 1.7-fold more bioavailable than food folate.

Very large doses of folic acid and other folates are well absorbed and can result in very high plasma vitamin levels. However, these fall quite rapidly as the renal threshold is exceeded and much of the dose is excreted within 24 hours. Although very high plasma folate levels can be achieved, tissue folate levels increase only marginally, often less than twofold due to the limited ability of tissues to metabolize these large doses to the polyglutamate forms required for folate retention.

Folate monoglutamate in plasma is transported into peripheral tissues via the reduced folate carrier (RFC) (21). This transmembrane carrier is specific for reduced folates with affinities in the low micromolar range while the affinity for folic acid is approximately 100-fold lower (K_t approximately 200 μ M). The liver carrier transports both folic acid and reduced folates and is thought to be PCFT. Plasma folate is normally in the 10 to 30 nM range, so RFC and PCFT would not be saturated under any physiological condition, and folate transport rates into tissues should be responsive to any change in plasma folate concentration.

A second family of closely related folate transporters known as folate-binding proteins or folate receptors (α , β , and γ) are expressed in a more limited range of epithelial tissues (17). High levels of folate-binding protein are expressed in the choroid plexus, kidney proximal tubes, and placenta and in a number of human tumors, while lower levels have been found in a variety of other tissues. The affinities of reduced folate monoglutamates for these receptors vary by receptor but are very high, usually in the low nanomolar range, while their affinity for folic acid is even higher (approximately 1 nM). This transporter is responsible for reabsorption of folate in the kidney by a receptor-mediated endocytotic process and is believed to play a similar role in folate transport in other tissues (22). Tissues that express this receptor also express PCFT, which is thought to mediate folate transport across the acidified endosomal membrane. Patients with hereditary folate malabsorption (HFM) disease, a rare autosomal recessive disorder caused by mutations in the *PCFT* gene, have a severe systemic folate deficiency and very low folate levels in the cerebrospinal fluid (CSF) (23).

An additional distinct cellular transporter responsible for the transport of reduced folate monoglutamates into the mitochondrion has been identified (24). Folic acid is not transported into the mitochondria.

Tissue Retention and Turnover

Folate monoglutamate transported into cells is metabolized to polyglutamate forms by the enzyme folylpolyglutamate synthetase. Mammalian cells contain mitochondrial and cytosolic isozymes encoded by a single gene (25). In the cytosol, much of the entering folate is metabolized to the 5-methyl-tetrahydrofolate derivative, which is a poor substrate for folylpolyglutamate synthetase (25). This has to be metabolized to tetrahydrofolate via the methion-

ine synthase reaction before effective polyglutamylation and tissue retention is achieved. As the flux of 5-methyl-tetrahydrofolate through the methionine synthetase reaction is quite limiting, particularly when the cell contains high levels of 5-methyl-tetrahydrofolate polyglutamate, much of the newly absorbed folate would not be retained by the tissue and would appear in the circulation predominantly as 5-methyl-tetrahydrofolate.

Under normal conditions of dietary intake and status, whole body folate turns over quite slowly with a half-life in excess of 100 days (26). Urinary excretion of intact folate accounts for only a very small proportion of this turnover. Over 99% of tissue folate is in the polyglutamate form. The actual mechanism of catabolism is poorly understood but primarily involves cleavage at the C9-N10 bond to generate *p*-aminobenzoylpolyglutamates and a pterin moiety (27). The *p*-aminobenzoylpolyglutamates are hydrolyzed to the monoglutamate by a lysosomal glutamylhydrolase and acetylated, and then excreted in urine as *N*-acetyl-aminobenzoylmonoglutamate. The pterin moiety is excreted in bile and appears in the feces.

Feces contain very high levels of folate but most, if not all, of this arises from bacterial synthesis in the lower gut. Studies in rodents have shown that some of this bacterially synthesized folate is bioavailable (28). Whether folate synthesized by gut bacteria in humans is also bioavailable has not been determined.

FOLATE DEFICIENCY

Megaloblastic Anemia

Folate deficiency is usually due to a dietary insufficiency, although it can arise from other causes such as malabsorption syndromes. The classical symptom of folate insufficiency is megaloblastic anemia, a condition reflecting deranged DNA synthesis in the erythropoietic cells. Folate deficiency resulting in megaloblastic anemia is a fairly common condition in pregnancy. Blood cells are enlarged and often multinucleated. Megaloblastic changes occur in all fast growing tissues such as the marrow and the gut epithelia. Megaloblastic cells contain close to twice the normal DNA content and the DNA is partially fragmented. Many cells are arrested in the G2 phase just prior to mitosis. Cells that divide often undergo apoptosis. The defect in DNA synthesis in folate deficiency has been ascribed to defective thymidylate synthesis under these conditions with a resulting increase in uracil misincorporation into DNA. Removal of uracil by the repair enzyme uracil DNA glycosylase, and a decreased repair of the gaps produced by this enzyme, would lead to an increase in double-stranded DNA breaks under these conditions (29).

Megaloblastic anemia or pernicious anemia is also a classical symptom of impaired B₁₂ status. This condition, which is quite prevalent in the elderly, is seldom due to a dietary deficiency but usually results from malabsorption of B₁₂. However, the anemia that results is identical to that of folate deficiency. In B₁₂ deficiency, the B₁₂-dependent methionine synthase enzyme is inactive and cytosolic folate is "trapped" as 5-methyl-tetrahydrofolate at the expense of other folate coenzyme forms required for one-carbon metabolism such as thymidylate synthesis, leading to a functional folate deficiency in the cell

(13). As 5-methyl-tetrahydrofolate is a poor substrate for folylpolyglutamate synthetase, the ability of tissues to accumulate folate is reduced and the functional folate deficiency is compounded by a drop in cellular folate levels. As the defective DNA synthesis in pernicious anemia is caused by an induced secondary folate deficiency, high levels of folate can cause a hematological response in patients with megaloblastic anemia due to B₁₂ deficiency, but folate is ineffective in preventing the severe neurological pathologies associated with B₁₂ deficiency.

Vascular Disease

Severe genetic conditions that result in very marked hyperhomocysteinemia are associated with a variety of clinical symptoms including early onset occlusive cardiovascular and cerebrovascular disease. These genetic diseases include MTHFR deficiency and CBS deficiency, enzymes involved in the homocysteine remethylation and transsulfuration pathways, respectively. Lowering of homocysteine with high doses of folate and betaine improves the clinical picture in these patients, strongly suggesting that homocysteine is the causative agent of these conditions, although the actual mechanism has not been definitively established (30).

Many epidemiological studies have suggested that chronic *mild* hyperhomocysteinemia is a risk factor for occlusive vascular disease. In many case-control studies, plasma homocysteine concentrations in patients with vascular disease were higher than in matched controls. However, this relationship has not been found consistently in prospective studies. Fasting homocysteine levels have been inversely correlated with both plasma folate and food folate intake and increased folate intake lowers the mean homocysteine of groups, with the greatest effect on those with the highest initial plasma homocysteine levels. If mildly elevated homocysteine is a causative risk factor for vascular disease, then it may be that a simple dietary intervention can lower the risk. However, recent randomized controlled trials with folic acid (and vitamins B₁₂ and B₆) have not found any benefit in reducing recurrence rates of cardiovascular events (31), although some benefit has been reported for stroke recurrence (32). Homocysteine levels were, as expected, significantly reduced in the B vitamin-treated group in these trials. Although results from recurrence rate trials do not support a role for B vitamin supplements in reducing the risk of cardiovascular events, the populations under study were quite elderly and had a previous event, and the studies may have been underpowered to observe a small effect. It may be that B vitamin supplements could have a benefit earlier in life before any putative damage caused by elevated homocysteine has occurred.

A common polymorphism (677 C→T, Ala to Val) in the flavoprotein MTHFR that results in a "heat-labile" enzyme and decreased enzyme activity in tissues has been implicated as one reason for the folate responsiveness of a subset of hyperhomocysteinemic subjects (33). The incidence of the Val/Val homozygote (around 10% in the U.S. population) is significantly increased in subjects with the highest deciles of homocysteine levels. The valine substitution reduces the affinity of the enzyme for its flavin

adenine dinucleotide (FAD) cofactor, and the loss of FAD leads to decreased protein stability. Folate binding "locks" the FAD cofactor in place on the enzyme and stabilizes the protein (34). Comparisons of Val/Val individuals with Ala/Ala subjects have demonstrated that differences between these groups are most obvious in subjects with both poor folate and riboflavin status and that increased dietary folate not only lowers homocysteine in both groups but also eliminates the differences between the genotypes (35). Many case-control studies have suggested that the 677 T allele in MTHFR is associated with vascular disease risk although prospective studies have been equivocal. There is a large variation in penetrance of this polymorphism among different ethnic groups and this may have confounded some of the case-control studies.

Neural Tube Defects

In the early 1990s, double-blind randomized trials confirmed that supplemental folic acid given prior to conception and during early pregnancy significantly reduced by over 70% the occurrence and recurrence of neural tube defects (NTDs), the most common birth defects in humans (36–38). This was most likely not due to correction of a simple folate deficiency. Instead, it is thought that some women and/or their offspring have a higher requirement for folate due to genetic reasons. Later a very large population-based study in China showed that folic acid supplements (400 µg/day) reduced NTD occurrence by 79% in a high incidence region and 41% in a low incidence region, and the underlying incidence refractory to folate supplementation was similar between regions (39). The current fortification of the food supply with folic acid in the United States and some other countries arose because those individuals at risk for having an NTD child cannot be identified at the moment.

A large number of folate candidate genes have been screened as risk factors for NTDs. Homozygosity for offspring with the 677 T (Val) allele of MTHFR has been shown to be a risk factor in a number of studies with the risk increasing very significantly in comparisons of offspring of mothers with poor folate status. However, carrying this particular allele can only account for approximately 15% of the population-based attributable derived risk (40), and the vast majority of offspring that are homozygous for the 677 T allele do not develop NTDs.

Cancer

Epidemiological studies have shown that poor folate status is associated with an increased risk for certain types of cancer including colon cancer (41). The mechanism behind this is not known, but uracil misincorporation arising from defective thymidylate synthesis has been suggested as one possibility, as this would influence both DNA synthesis and repair. As changes in folate status influence the remethylation of homocysteine to methionine and alter adenosylmethionine to adenosylhomocysteine ratios, it has also been proposed that the increased cancer risk in folate deficiency may be due to hypomethylation of DNA and/or histones. Changes in DNA and histone methylation have been observed in many tumors and it has been demonstrated that methionine deficiency causes hypomethylation of DNA.

Subjects homozygous for the MTHFR 677 T allele demonstrate a decreased cancer risk that is most pronounced in comparisons of subjects of good folate status (41). It has been shown that this polymorphism can direct more of the one-carbon flux into thymidylate synthesis and incorporation into DNA, and away from methionine synthesis (42). Normally, 5-methyl-tetrahydrofolate is the only form of folate found in plasma and the red cell. However, significant amounts of 10-formyl-tetrahydrofolate are seen in red cells of some MTHFR 677 T subjects (42), also indicating some redirection of the one-carbon metabolic flux. The reduced flux into methionine synthesis in MTHFR 677 T subjects may argue against hypomethylation in folate deficiency being responsible for the increased cancer risk.

INDICATIONS AND USAGE

Supplementation to Achieve Recommended Intake Levels

In 1998, the United States Food and Nutrition Board of the National Academy of Sciences and a joint FAO/WHO Expert Consultation on Human Vitamin and Mineral Requirements reviewed their recommendations for folate intake (20). Both groups used plasma and red blood cell folate concentrations as primary indicators of folate adequacy, and plasma total homocysteine levels as an additional indicator of folate status. Their conclusions appear in Table 2.

The suggested levels represent the daily dietary folate required to ensure adequate nutrition in 97.5% of the population and are an overestimation of the level needed for most people in any given group. Individuals who do not routinely consume the suggested level of folate from

foods should be encouraged to supplement their diets with folate.

Treatment of Folate Deficiency

Folate deficiency has traditionally been diagnosed as a low serum or red blood cell folate level [<3 ng/mL (7 nM) and <160 ng/mL (360 nM) respectively] in the setting of macrocytic anemia. However, anemia reflects a late stage of folate depletion; earlier stages are often evidenced solely by low blood folate levels and/or elevations in homocysteine levels. Vitamin B₁₂ deficiency artifactually increases serum folate levels and decreases red blood cell folate levels, and also increases plasma homocysteine levels, which can confound the diagnosis.

After ruling out a B₁₂ deficiency, rapid restoration of a folate-replete state can be achieved by administering 500 to 1000 μ g of folic acid orally per day. There is little evidence that doses greater than 1000 μ g convey additional benefit. Once the folate deficiency is resolved, daily intake of folate based on the levels found in Table 2 should be maintained. In cases of severe intestinal malabsorption or an inability to use the gastrointestinal tract, it may be necessary to administer 1 mg of folic acid parenterally via an intravenous or intramuscular route. This dose appears suitable for initial repletion of a folate-deficient state.

Prevention of Neural Tube Defects

Because the neural tube closes during the fourth week of embryonic life before many women realize they are pregnant, and the population at risk for this condition cannot be identified, it is recommended that all women planning a pregnancy or are capable of becoming pregnant take 400 μ g folic acid, either as a supplement and/or in fortified foods in addition to normal dietary intake (20). This recommendation is based on the intervention trials and it is not known whether lower levels of supplementation would be as effective. It should be noted that this recommendation is distinct from the DRIs for pregnancy. Few pregnant women would receive the recommended dietary allowances (RDA) of 600 μ g from their diet and it would be practically impossible to increase dietary folate intake by the additional 680 μ g that would be equivalent to the 400 μ g folic acid supplement recommended for NTD protection. Women with a family history of NTDs or who have had a previous NTD-affected pregnancy should be encouraged to take a daily supplement containing 4 mg folic acid during the periconceptual period.

Beginning in 1998, the FDA mandated fortification of the U.S. food supply with folic acid to reduce the incidence of NTDs. Food fortification rather than targeting supplements to women planning pregnancies was deemed necessary because of a perceived failure of public health efforts to influence those most at risk. Fortification was planned at a level expected to achieve an average extra daily folic acid intake of 100 μ g, which would be equivalent to approximately 170 μ g of extra food folate because of its higher bioavailability, and with few individuals expected to receive over 1 mg, to limit the possibility of masking of B₁₂ deficiency. Because of "overage" by food manufacturers, the average increased folic acid intake in the U.S. population was initially closer to 200 μ g

Table 2 RDIs for Folate

RDA		^a RNI	
Age	μ g DFE ^b /day	Age	μ g DFE/day
0-5 mo	65	0-6 mo	80
6-11 mo	80	7-12 mo	80
1-3 yr	150	1-3 yr	160
4-8 yr	200	4-6 yr	200
9-13 yr	300	7-9 yr	330
14 yr and above	400 ^c	10 yr and above	400
Pregnancy	600	Pregnancy	600
Lactation	500	Lactation	500

Recommended Dietary Allowances (RDA, 1998 United States Food and Nutrition Board of the Institute of Medicine) and Recommended Nutrient Intakes (RNI, 1998 Joint FAO/WHO Expert Consultation on Human Vitamin and Mineral Requirements). Values given for the FAO/WHO Expert Consultation are provisional values published in a preliminary report.

^aValues for males and females in all age groups were combined as they did not differ, and age groups with the same RNI were combined.

^bValues are given as dietary folate equivalents (DFE). 1 DFE = 1 μ g food folate = 0.6 μ g synthetic folic acid taken with food = 0.5 μ g synthetic folic acid taken on an empty stomach.

^cIn view of evidence linking increased folate intake with reduction of neural tube defects in the fetus, it is recommended that all women capable of becoming pregnant consume 400 μ g synthetic folic acid from supplements and/or fortified foods in addition to intake of food folate from a varied diet. The RDA value for pregnancy does not include this additional folate.

and is now approximately 140 μg (equivalent to approximately 240 μg food folate). Postfortification NTD rates have fallen by approximately 26% in the United States (43). Some argue that increased fortification is required to further decrease NTD rates and point to the 70% reduction observed in the initial intervention trials. However, NTD rates had been falling prior to fortification and the underlying rate in the United States was quite low compared to some other countries. The folate intervention studies in China demonstrated a 70% drop in NTD rates in areas of high prevalence and a much smaller drop in areas of lower prevalence (39). In Canada, which fortifies at a level similar to the United States, but has better NTD ascertainment, the estimated reduction was approximately 46% with more dramatic reductions in provinces with higher prefortification prevalence rates (44). It is likely that the current level of fortification has already prevented most, but not all, folate-responsive NTDs. Food fortification has exposed the whole population to a very significant increase in folate intake, not just the individuals at risk for NTDs. Concerns have been raised about the possibility of unanticipated side effects, as limited studies have been carried out on the effects of high doses of folate (see later).

Although fortification was initiated to reduce NTD incidence, there may have been other benefits. Plasma and red cell folate levels have increased and the proportion of the population with deficient levels has fallen dramatically. The proportion of the population with elevated plasma homocysteine levels has also fallen by approximately 50% (45), and vitamin B₁₂ status rather than folate status is now the major determinant of fasting homocysteine levels in the U.S. population.

Adverse Effects of Drugs on Folate Status

Treatment with certain drugs may indicate the need for administration of supplemental folate. For instance, many physicians prescribe methotrexate (7.5–15 mg/week) for the treatment of rheumatoid arthritis, asthma, and ulcerative colitis. Side effects of this treatment, including alopecia, stomatitis, pancytopenia, and interstitial pulmonary fibrosis, are thought to be inversely related to serum folate levels at the start of treatment. Daily administration of 1 mg of folic acid to people who take methotrexate appears to significantly reduce the development of side effects, and does not interfere with the efficacy of treatment (46).

Long-term use of sulfasalazine, an anti-inflammatory that is often administered for the treatment of inflammatory bowel disease or rheumatoid arthritis, is often associated with folate deficiency. Therefore, prophylactic coadministration of folic acid at 400 to 1000 μg /day is indicated.

Phenytoin, phenobarbital, and primidone have repeatedly been associated with either low serum folate levels or frank folate deficiency. Therefore, chronic administration of these drugs should also prompt prophylactic administration of 400 to 1000 μg of folic acid per day.

Chronic alcoholism is associated with a considerable risk of folate deficiency. A large part of this effect can be explained by the fact that the diet of a chronic alcoholic is often folate poor. However, other mechanisms

have also been described, including impaired intestinal deconjugation of polyglutamated food folates, increased metabolic turnover or urinary excretion, and cleavage of the folate molecule by acetaldehyde. Since deficiencies of other B vitamins commonly accompany folate deficiency in this setting, administration of a multivitamin containing 400 μg of folic acid is probably the best approach to avoid development of deficiency.

Hyperhomocysteinemia

Although very high levels of homocysteine can cause vascular disease, the evidence that modest elevations in plasma homocysteine increase risk is not that compelling. Because the cost of treating hyperhomocysteinemia with vitamins is exceedingly low, hyperhomocysteinemia remains a reasonable indication for B vitamin supplementation. Some contend that this is particularly true for the secondary prevention of cardiovascular disease when other reversible risk factors cannot be identified. Some potential adverse effects of B vitamin supplement have been reported in the vascular disease recurrence trials with folate, B₁₂, and B₆, primarily due to the B₆ component (31). While this remains to be confirmed, it is probably prudent to limit supplements to RDA levels, which for most people would be sufficient for maximal reduction of homocysteine.

In healthy ambulatory populations, low dietary intake and low blood levels of folic acid, B₁₂, and B₆ are the primary determinants of blood homocysteine levels (47). It is clear that daily administration of these three vitamins reduces blood homocysteine levels to levels associated with considerably lower cardiovascular risk, at least in some studies. Administration of a daily dose of 400 μg of folic acid often suffices, with two notable exceptions. If hyperhomocysteinemia is due to a deficiency of B₁₂ or B₆, folate supplementation will not help. Secondly, the hyperhomocysteinemia that commonly develops in individuals with chronic renal insufficiency and in postrenal transplant recipients is particularly resistant to low doses of folic acid supplementation (48). It appears that doses of 2.5 mg of folic acid per day are necessary for optimal homocysteine reduction in this population. Normalization of homocysteine levels in individuals with end-stage renal disease may never occur, even with supraphysiological doses of folic acid.

Cancer Prevention

Observational studies continue to indicate an inverse relationship between folate intake or folate blood levels and the risk of developing certain common cancers, and data from animal experiments support a true causal relationship. Ecological studies with folate supplement users have also indicated a protective effect. However, limited clinical intervention trials on colon polyp recurrence have failed to show any benefit of a 1 mg/day supplement of folic acid. Some adverse effects on other cancers were noted, but the evidence for this is weak (49). Additional clinical intervention trials are in progress but results are not currently available.

A study of colon cancer rates in the United States and Canada suggested a slight increase or blip during the period 1996 to 1999, and it was hypothesized that this

may have been due to the introduction of folic acid fortification in these countries (50). While possible, these data could also be explained by confounders such as the introduction of routine colonoscopies around this time. Approximately one-third of the U.S. population take vitamin supplements, usually containing 400 μg folic acid, which is much higher than the extra folic acid intake obtained from food fortification. It is difficult to reconcile the hypothesis that food fortification has increased cancer risk with the many other studies indicating that folic acid supplements reduce cancer risk.

The appropriate setting for prophylaxis with folic acid for cancer prevention remains inconclusive, in part due to this concern about potential risks. In large doses, folate is known to accelerate the growth of existing cancers. Therefore, there is concern that supplemental folate may inadvertently accelerate the growth of a precancerous or cancerous polyp in the colon. However, it might well be that normal folate status is sufficient to promote growth of cancerous lesions, and the epidemiological data are best explained by poor folate status being a risk for cancer development. It is prudent to await further research on this topic before folic acid is used for cancer prevention in individuals at increased risk.

CONTRAINDICATIONS

Vitamin B₁₂ Deficiency

Vitamin B₁₂ deficiency is often undiagnosed and may affect a substantial percentage of the population, particularly the elderly. It may be associated with hematologic symptoms (megaloblastic anemia) and/or neurologic symptoms (dementia, paresthesia, and ataxia). People who have, or are at risk for, vitamin B₁₂ deficiency should not be given folic acid without concomitant monitoring for and treatment of vitamin B₁₂ deficiency. One risk of folic acid supplementation is that high doses can prevent (mask) the hematologic manifestations of B₁₂ deficiency, and thus delay diagnosis, while allowing the associated neurologic complications to progress. Furthermore, there is anecdotal evidence suggesting that folic acid supplementation may precipitate or exacerbate the neurologic damage of vitamin B₁₂ deficiency. This was also suggested by a recent population-based study comparing functional markers of B₁₂ deficiency (such as methylmalonic acid) in subjects with low serum B₁₂, which showed that a subset of this population with the highest plasma folate levels had increased levels of these indicators of B₁₂ deficiency (51). While consistent with exacerbation of B₁₂ deficiency by high folate, it should be noted that the subgroup with the highest folate levels were almost certainly supplement users and these supplements would also have contained vitamin B₁₂. Selecting for those with high folate and low B₁₂ would probably be selecting for a group with severe malabsorption of B₁₂.

PRECAUTIONS AND ADVERSE REACTIONS

Drug Interactions

In large amounts, folic acid has been reported to counteract the antiepileptic effect of phenobarbital, phenytoin,

Table 3 Upper Limits for Folic Acid Set by the 1998 Food and Nutrition Board of the National Academy of Sciences

Age (years)	Upper limit ($\mu\text{g}/\text{day}$)
1-3	300
4-8	400
9-13	600
14-18	800
>19	1000

and primidone and increase the frequency of seizures in susceptible individuals. Because of the drug-nutrient interaction between these anticonvulsant drugs and folate, people taking these three drugs are also at risk of folate deficiency.

Overdosage

Folic acid doses of up to 15,000 μg in healthy adults without convulsive disorders have not been associated with any reported serious adverse effects. The Food and Nutrition Board of the National Academy of Sciences recommended 1000 μg as an upper limit for folic acid for adults 19 years and older, including pregnant and lactating women (20). This upper limit, which refers to supplemental folic acid and not food folate, was not related to any known toxicity of folate per se. Instead, the concern was the possible masking of B₁₂ deficiency anemia and limited information, primarily anecdotal, that folate might exacerbate the neurological manifestations of B₁₂ deficiency. The Food and Nutrition Board set upper limits for children and adolescents by adjusting the adult limit on the basis of relative body weight. Table 3 gives the upper limits for folic acid by age group. No upper limit was set for infants due to lack of adequate data. The Food and Nutrition Board also recommended that food (or maternal milk) be the only source of folate for infants.

Circulating Folic Acid

Fortification of the food supply with folic acid and supplement use has resulted in the appearance of low levels of free folic acid in the circulation. Concerns have been raised about the presence of this unnatural form of the vitamin in the circulation, although there is no credible evidence currently available to suggest that there are any harmful effects unique to folic acid itself as opposed to other forms of the vitamin. It is possible that the extremely tight binding of folic acid to the folate receptor could interfere with transport of other folates that utilize the folate receptor for entry into tissues. The fate of plasma folic acid would differ from reduced folates such as 5-methyltetrahydrofolate in that its removal from the circulation would primarily involve tissues that express the folate receptor and/or PCFT as opposed to most peripheral tissues that express the RFC.

One study has related circulating folic acid to a reduction in natural killer cell number although the significance of this is not clear (52). Another has related circulating folic acid and high folate to impaired cognitive function (53), although this is at odds with other studies reporting that folic acid supplements improve cognitive function in the elderly (54).

COMPENDIAL/REGULATORY STATUS

Not applicable.

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French Maritime Pine

Peter J. Rohdewald

INTRODUCTION

Pycnogenol® is the registered trade name for a special standardized extract from the bark of the French maritime pine (*Pinus pinaster* ssp. *atlantica*), distributed exclusively by Horphag Research, U.K. The spray-dried, powdered extract is marketed worldwide as a food supplement, as herbal medicine, as an active cosmetic ingredient, as well as in functional foods.

The traditional use of pine bark was related to treatment of scurvy and wound healing. Currently, Pycnogenol is used to restore capillary integrity in cases of gingivitis, retinopathy, edema, and hemorrhoids. Other fields of application are connected to improved endothelial function with corresponding improved blood flow, relieving high blood pressure, and prevention of platelet aggregation. The anti-oxidant activity was found to improve attention-deficit/hyperactivity disorder (ADHD) symptoms in children and cognitive function in elderly citizens. Human pharmacologic studies point to significant anti-inflammatory activity and Pycnogenol was shown to be effective in diverse pathologies, such as osteoarthritis, dysmenorrhea, endometriosis, asthma, and sunburned skin. More recently, a potent inhibition of α -glucosidase by the high-molecular-weight procyanidin species was identified. Several clinical studies have shown a glucose-lowering effect of Pycnogenol, also when taken in addition to oral anti-diabetic medication. Safety of Pycnogenol is well documented, corroborated by experience with 7000 patients in numerous clinical trials carried out to date. Pycnogenol has GRAS (generally recognized as safe) status in the United States. Unwanted effects are mild and transient, and no interactions with drugs have been reported.

BACKGROUND

Raw material:

- Family: Pinaceae
- Genus: *Pinus*
- Species: *Pinus pinaster* Aiton, ssp. *atlantica* D. del Villar
- Part used: Outer bark

P. pinaster ssp. *atlantica*, the Atlantic maritime pine, is cultivated in large monocultures in South Western France in the Biscay area (Fig. 1). It is distinguished from other species by the thick, deeply fissured, reddish bark, representing a geographic race adapted to harsh climate and sandy soil. Trees are cut for timber production after cultivation for 30 to 50 years, and the fresh outer bark is used



Figure 1 Forest of French maritime pine trees.

for extraction throughout the year. The bark pieces are 1 to 3 cm thick, and they are formed from up to 50 mussel-shaped, deep red or light brown layers. The inner side of the bark is slightly concave and plane, while the outer part is irregular with deep cut V-shaped fissures.

Traditionally, preparations from pine bark had been used in the Middle Ages for wound healing, as referred to in the *Thesaurus Medicaminum* of the Zurich pharmacist H. Minner (1479). Also, in North America, Native Americans used bark from conifers for wound healing, and to treat scurvy (1).

CHEMISTRY AND PRODUCTION

Composition

Pycnogenol represents a concentrate of phenolic compounds, consisting of phenolic acids, catechin, taxifolin, and procyanidins (2).

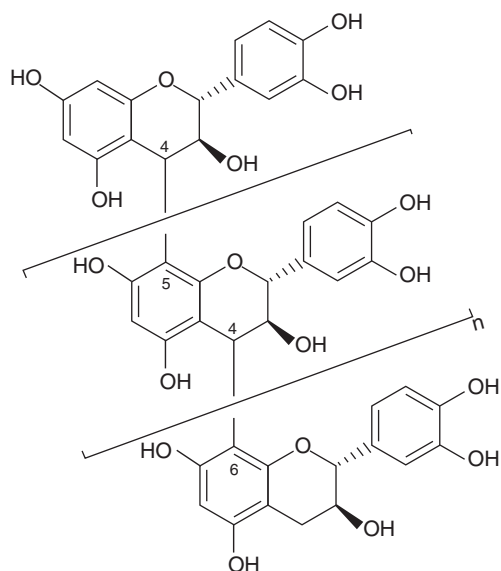


Figure 2 Procyanidin with type 4–8 bonds. Monomers could be catechin or epicatechin units.

Pycnogenol consists predominantly of procyanidins, condensed catechin, and epicatechin in various chain lengths up to dodecamers (Fig. 2). The catechin–epicatechin units can be linked by C4–C8 bonds or by C4–C6 bonds, with the C4–C8 linked isomers predominating. The extract is standardized to contain $70\% \pm 5\%$ procyanidins in accordance with the USP for maritime pine bark extract.

Further to the procyanidins, monomeric flavan-3-ol species are present in Pycnogenol with catechin as the main monomeric procyanidin and only traces of epicatechin. Taxifolin is available in free form and as taxifolin glucoside.

Furthermore, the extract bears several phenolic acid species, which are derivatives of benzoic acid—*p*-hydroxybenzoic acid, protocatechic acid, vanillic acid, and gallic acid—or of cinnamic acid, *p*-cumaric acid, caffeic acid, and ferulic acid. Glycosides and glucose esters of these phenolic acids have been identified in the extract.

As inorganic ions, calcium, potassium, and iron are present, together with traces of manganese, zinc, and copper (3).

Production

The extract is prepared from fresh sorted, cleaned, and crushed bark. The patented extraction process chain uses ethanol and water as solvents in a multistep process. The purified aqueous extract is spray dried and represents a very fine, red to brownish-colored powder with an aromatic smell and astringent taste. It is soluble in water and short-chained alcohols and ketones (i.e., ethanol, glycerol, acetone) and insoluble or sparingly soluble in oils. One thousand kilograms of bark is needed to produce 1 kg Pycnogenol.

Formulations

Pycnogenol is available as tablets or capsules of 20 to 100 mg. For oral health care, a mouth spray delivering 2 mg per actuation and a chewing gum containing 5 mg Pycnogenol are available. As Pycnogenol is water soluble, it is available in functional drinks. A wide range of cosmetic products containing the extract is sold worldwide.

Analysis

The quality of the bark of *P. pinaster* is controlled according to the monograph “Maritime Pine Extract” of the *United States Pharmacopoeia* and *The National Formulary* (4).

The standardized extract, Pycnogenol, corresponds to the monograph: “Maritime Pine Extract” of the *National Formulary of the US Pharmacopoeia* (4) in terms of identity and purity using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The content of procyanidins is standardized to $70\% \pm 5\%$, quantified spectrophotometrically after oxidative hydrolysis.

Stability

Pycnogenol, stored and protected from light and humidity in well-closed containers at room temperature, is stable over a period of 3 years.

PRECLINICAL STUDIES

Circulatory Function

Stimulation of e-NOS

Pycnogenol stimulates the activity of endothelial nitric oxide synthase (e-NOS) in vitro and in vivo (Fig. 3). In isolated aortic rings from rats (2) as well as in human spermatozoa (R Stanislavov MD and V Nikolova PhD, 2007, unpublished results), it stimulated endothelial NO production from natural substrate L-arginine by e-NOS. Nitric oxide (NO) initiates the release of cyclic 3',5'-guanosine monophosphate (cGMP) in smooth muscle cells and leads to vasorelaxation. Furthermore, NO reacts with blood platelets and prevents their aggregation.

Inhibition of Adrenaline-Induced Vasoconstriction

Both adrenaline as well as noradrenaline are very potent vasoconstrictors. In experiments with isolated aortic rings

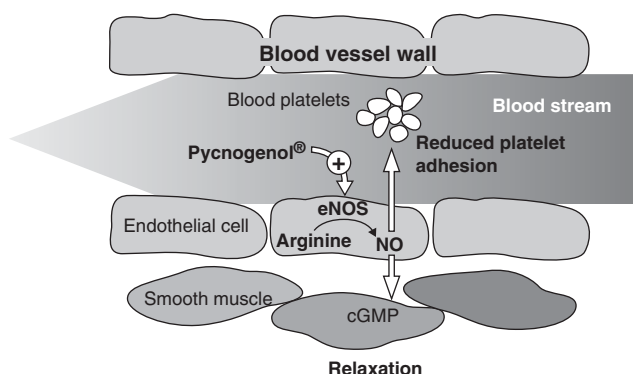


Figure 3 Scheme for enhanced NO production by increasing synthesis of endothelial NOS.

from rats, Pycnogenol inhibited the vasoconstriction induced by these stress hormones. The effect was dose dependent and could not be observed after removal of the endothelium (2).

Antihypertensive Effect

In vitro, Pycnogenol inhibits the angiotensin-converting enzyme (ACE), while in rats, blood pressure could be reduced significantly after i.v. injection (2).

Capillary Integrity

Protein Binding

Procyanidins, the main constituents of Pycnogenol, belong to the class of nonhydrolyzable tannins and have a high affinity to proteins. Pycnogenol binds selectively to collagen, elastin, and skin powder, whereas binding to egg albumin is low (5). Also, the interaction with enzymes is specific, as a result of the differing IC_{50} values (3) for inhibition of various enzymes. Stabilization of membranes of erythrocytes via protein binding may be the cause of prevention of hemolytic injury in glucose-6-phosphate dehydrogenase-deficient human erythrocytes (6).

Capillary Sealing

Spontaneous hypertensive rats show a pathologically high leakage of capillaries. Feeding with Pycnogenol produced a long-lasting, dose-dependent increase of capillary resistance against a topically applied vacuum (2).

Anti-inflammatory Activity

Radical Scavenging Activity

In several in vitro models, Pycnogenol inactivated superoxide and hydroxy radicals as well as inhibited the formation of singlet oxygen and nitric oxide radicals (2,3). The superior capacity of procyanidins in radical scavenging is based on their ability to retain scavenging activity by intramolecular rearrangements (2). The lifetime of the ascorbyl radical is prolonged by Pycnogenol to a greater extent than by other bioflavonoids (3), and the oxidation of low-density lipoprotein (LDL) was inhibited in vitro (3). DNA was protected against iron/ascorbate-induced strand breaks by Pycnogenol (3). Toxicity of free radical producing anti-tumor drugs was reduced by pretreatment of mice with Pycnogenol without reducing the anti-cancer activity of doxorubicin and cyclophosphamide (7).

Antioxidative Effects in Biological Systems

Incubation with Pycnogenol protected α -tocopherol in endothelial cells against oxidation by peroxynitrite (3), protected nerve cells against β -amyloid or glutamate-induced toxicity (2,3), and inhibited peroxidation of retinal lipids more efficiently than vitamins E and C (2). Neurons were protected from amyloid- β -peptide-induced apoptosis (8).

Stimulation of Synthesis of Antioxidative Substances

Pycnogenol incubation doubles the concentrations of antioxidative enzymes in vascular endothelial cells (2). Synthesis of proteins in macrophages is increased, and activity of antioxidative enzymes, like catalase or superoxide dismutase, is dose dependently enhanced (2).

Interaction with NF- κ B

In a murine macrophage cell line, preincubation with Pycnogenol blocked the activation of nuclear factor kappa B (NF- κ B) and the activator protein (AP-1), major transcription factors centrally involved in inflammatory processes (9). In a human lymphocyte cell line, the extract inhibited the transcription factors nuclear factor for activated T-cells (NF-AT) and AP-1 (9). It also prevented the UV-induced activation of transcriptional factors NF- κ B and AP-1 in human cell lines from fibroblasts and keratinocytes (2). In human endothelial cells, pretreatment with Pycnogenol suppressed the activation of NF- κ B by tumor necrosis factor- α (TNF- α) (2). These results indicate that proinflammatory responses can be inhibited by Pycnogenol early in the biochemical reaction chain at the transcriptional level.

Inhibition of Inflammatory Mediators

At the level of cytokines, Pycnogenol blocks production of interleukins 1, 2 (9), 6, and 10 (2). Synthesis of inducible NOS is blocked by preincubation of macrophages with Pycnogenol (3), and the release of histamine from mast cells is inhibited in vitro (2).

Inhibition of Adhesion Molecules

Intercellular adhesion molecules are necessary for tissue invasion of leukocytes in inflammatory processes. Pycnogenol pretreatment downregulates expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (2).

Inhibition of Matrix Metalloproteases

Matrix metalloproteases (MMPs) destroy collagen and elastin. Pycnogenol and its metabolites inhibit MMP1, MMP2, and MMP9, and furthermore prevent the release of MMP9 from human monocytes (6).

Inhibition of UV-Induced Damage

Topical application of a Pycnogenol-containing gel significantly prevented erythema formation after UV radiation (2) and inhibited photocarcinogenesis (10). Wound healing was accelerated and scar formation reduced following application of 1% to 2% Pycnogenol in gels (11).

Age-Related Degenerative Processes

Stimulation of Immune System

Incubation with Pycnogenol augmented phagocytosis in macrophages (12). Feeding of the extract to immunosuppressed mice provided protection against protozoal infection (13), and to senescence-accelerated mice restored levels of progenitor cells and β - and T-lymphocytes in a dose-dependent manner (2). Oral administration of Pycnogenol to retrovirus infected mice (2) or mice inoculated with cancer cells (14) showed enhanced natural killer cell activity.

Enhancement of Cognitive Function

Administration of Pycnogenol to senescence-accelerated mice significantly enhanced the memory retention rate in a dose-dependent manner compared to nonsupplemented mice in step-through and step-down tests. The treatment also significantly improved the cognitive behavior in the shuttle box test (2).

Antiaging Effects

The life span of mice was prolonged after oral administration of a Pycnogenol-containing combination of antioxidants (15). That of drosophila was increased after feeding the extract (16).

Spasmolytic Activity

Phenolic constituents of Pycnogenol, ferulic acid, and caffeic acid, possess spasmolytic activity as demonstrated in vivo on the isolated rat uterus (2) as well as in vivo in experiments with rats (2).

Blood Glucose Lowering

In streptozotocin-induced diabetic rats, Pycnogenol feeding lowered blood glucose concentrations and enhanced concentrations of antioxidative substances in blood (17).

Proposed Mechanisms of Action

Based on the support of NO production, Pycnogenol offers a range of pharmacological effects on the vascular system, such as increased microcirculation, improvement of erectile function, antihypertensive effects, and inhibition of platelet aggregation. The enhancement of capillary integrity results in an anti-edema effect and prevents microbleedings. Its radical scavenging potency may contribute to the anti-inflammatory actions and beneficial effects in degenerative diseases. The spasmolytic activity of phenolic acids contained in Pycnogenol is probably related to its activity in reducing premenstrual cramps and pain. The lowering of blood glucose in streptozotocin-induced diabetic rats points to an anti-diabetic effect.

Safety Studies (On File at Horphag Research Ltd., U.K.)

Animal Toxicology

The *absence of mutagenic* effects has been shown using the Ames test, the chromosome aberration assay in human lymphocytes and the micronucleus test in mice.

Acute toxicity is very low. Fourteen acute toxicity tests had been performed using three different species and oral, subcutaneous, intraperitoneal, and intravenous routes of administration. LD50 data varied after oral administration from 1000 to 4000 mg/kg.

Chronic toxicity had been tested in three species after oral administration. The no observed adverse effects level (NOAEL) was established as 100 mg/kg/day.

In six *reproduction toxicity* studies with three species no teratogenic effects were detected, no signs of perinatal toxicity, or negative effects on fertility were noted.

Tolerance after topical application was tested in several models. Skin and eye irritation tests in rabbits and contact hypersensitivity test in guinea pigs showed that Pycnogenol is nonirritating. In human volunteers, no skin irritation was found with the patch-occlusion test.

Bioavailability, Absorption, Metabolism, and Excretion

Healthy volunteers took a single dose of 300 mg Pycnogenol or 200 mg daily for five days to reach steady state conditions in a pharmacokinetic investigation (18). Plasma samples taken at defined time points were analyzed by HPLC. Monomeric molecules, such as catechin, caffeic acid, ferulic acid, and taxifolin were found in plasma within 30 minutes. The primary metabolite devel-

oping from procyanidins was δ -(3,4-dihydroxy-phenyl)- γ -valerolactone, which appeared in the blood stream four hours after intake. Another 10 unidentified metabolites were found in plasma.

Urinary excretion of Pycnogenol constituents and metabolites was investigated in human volunteers at different time point post consumption. After oral intake, four metabolites could be found in the urine of a human volunteer. Taxifolin and ferulic acid were excreted as glucuronides or sulfates after 1 to 4 hours (2). Another investigation found a maximum excretion of ferulic acid after intake of Pycnogenol at 17 hours (3). Procyanidins had been metabolized to valerolactones, such as δ -(3,4-dihydroxy-phenyl)- γ -valerolactone, which are excreted as glucuronides after 8 to 15 hours (2).

CLINICAL STUDIES

Anti-oxidant Activity

Pharmacologic Investigations

Healthy volunteers were supplemented with 150 mg Pycnogenol daily for six weeks. The oxygen radical absorbance capacity (ORAC) of blood plasma increased significantly by 40% after three and six weeks, as compared to baseline. After a four-week washout period, the ORAC value dropped to almost baseline values (19).

Participants diagnosed with erectile dysfunction were supplemented with 120 mg Pycnogenol a day, a control group received placebo. After three weeks, the Pycnogenol group showed a statistical significant increase of the "ferric reduction activity of plasma" (FRAP) values as compared to placebo (20).

A decrease of markers indicative for oxidative stress was identified in some studies. Plasma F2-isoprostanes, metabolites developed from oxidized lipids, decreased significantly in the elderly in a double-blind placebo-controlled study after three months intake of 150 mg Pycnogenol a day, while placebo had no effect (21). Oxidative DNA damage was evaluated in children treated with Pycnogenol for improving symptoms related to ADHD (22). Blood lymphocyte were analyzed for 8-oxo-7,8-dihydroguanine (8-oxoG) and their presence decreased significantly after one-month treatment with Pycnogenol, while the placebo did not show any change. The decreased 8-oxoG values returned to baseline values after one-month discontinuation of Pycnogenol.

Improvement of Spermatozoa Quality

Lack of anti-oxidants has been connected with malformed spermatozoa. Quality of spermatozoa of 19 subfertile men was significantly improved in terms of morphology and mobility after supplementation with 200 mg Pycnogenol for 90 days (23). Supplementation with Pycnogenol + L-arginine aspartate and vitamin E or testosterone improved dramatically motility, concentration, and quality of spermatozoa (R Stanislavov and V Nikolova, unpublished results).

Cognitive Function in the Elderly

A double-blind placebo-controlled study with healthy volunteer elderly participants (aged 60–85 years) who were not cognitively impaired found a significant

improvement of memory-based cognitive variables after three months treatment with 150 mg Pycnogenol a day (21). The authors found a correlation of memory improvement with decreased plasma F2-isoprostanes, metabolites developing from oxidized membrane lipids.

Attention-Deficit/Hyperactivity Disorder in Children

ADHD symptoms were shown to significantly decrease during treatment with Pycnogenol (1 mg/kg body weight) after one-month treatment, as compared to a placebo group (24). Symptoms were evaluated independently by parents and teachers using Connor's rating scale. One month after cessation a complete relapse of symptoms was found. The authors attribute the reduced ADHD symptoms to the anti-oxidant effect of Pycnogenol (22), but also point to decreased stress hormone levels of adrenaline and noradrenaline (25).

Circulatory Function

Improved Endothelial Function

Endothelium-dependent vasodilatation was investigated in healthy volunteers in double-blind placebo-controlled fashion (26). Forearm blood flow was evaluated by strain-gauge plethysmography following injection of acetylcholine. After consumption of 180 mg Pycnogenol daily for two weeks, vasodilatation significantly augmented forearm blood flow as compared to placebo. Administration of N^G -monomethyl-L-arginine, an e-NOS inhibitor, completely abolished the Pycnogenol[®]-induced augmentation of forearm blood flow response to acetylcholine, suggesting that Pycnogenol[®] improves endothelial function with increased NO production.

Improved Microcirculation

By microscopic observation of blood capillaries through fingernails it was found that the capillary diameter was increased significantly compared to placebo following intake of Pycnogenol. Supplementation of 60 cardiovascular patients with the extract for one month improved microcirculation significantly as a result of increased vasodilatation. The rate of cardiovascular events diminished significantly in the Pycnogenol group compared to placebo (2).

Antihypertensive Effect

In a double-blind placebo-controlled crossover study with 11 patients, supplementation with Pycnogenol lowered systolic blood pressure of patients from average 140 to 133 mm Hg and lowered thromboxane levels (27). In another double-blind placebo-controlled trial with 58 subjects, intake of 100 mg allowed to reduce significantly the dosage of the calcium channel blocker nifedipine, required for treatment of patients with hypertension. Plasma levels of endothelin-1 were reduced, and concentrations of prostacyclin were elevated (28). In a study of hypertensive type II diabetes patients on ACE-inhibitor medication, the intake of Pycnogenol over an investigational period of three months allowed 58.3% of patients to achieve a systolic blood pressure below 130 mm Hg. Half of the patients were able to reach healthy blood pressure with decreased ACE-inhibitor treatment when taking Pycnogenol. An unpublished study with hypotensive subjects (systolic pressure $\geq 90 \leq 115$ mm Hg) showed

that intake of 100 mg Pycnogenol daily over a period of 30 days did not significantly lower blood pressure (Daniel Pella MD, PhD.; written personal communication, 2005).

Muscular Cramps

Pycnogenol was described to help individuals suffering from common muscular cramps and pain. These individuals, as well as a group of athletes, suffered muscular cramps as a result of circulatory disorders related either to venous insufficiency, intermittent claudication, or diabetes (29). In all groups, 200 mg Pycnogenol taken a day for a period of four weeks significantly lowered the frequency of cramps and pain severity on a visual analog scale. Discontinuation for one week led to a partial relapse of symptoms without reaching pretreatment severity.

Inhibition of Platelet Aggregation

Smoking produces an activation and aggregation of blood platelets. This platelet aggregation was inhibited in smokers dose dependently by Pycnogenol (2), and the effect persisted over several days. In another group of smokers, thromboxane levels were decreased in blood in addition to the inhibition of platelet aggregation (2). Also, in cardiovascular patients, platelet aggregation was inhibited following intake of the extract (2).

Prevention of Thrombosis

In a double-blind, placebo-controlled, randomized trial with 198 passengers, 400 mg Pycnogenol prevented thrombus formation after long-haul flights in the verum group. In the placebo group, five cases of thrombosis were observed, and none in the verum group (30).

Cholesterol Lowering

In an open controlled study with 25 volunteers, LDL was lowered significantly after four weeks supplementation with 150 mg Pycnogenol, while high-density lipoprotein (HDL) as well as overall radical absorbance capacity of blood was increased (19). A double-blind placebo-controlled study with 21 patients showed significant reduction of total cholesterol and LDL after intake of 120 mg Pycnogenol (20). In a comparative controlled study, total cholesterol and LDL were significantly lowered (31).

Capillary Integrity

Gingival Bleeding

In a placebo-controlled study with dental students, a Pycnogenol-containing chewing gum (5 mg per piece) reduced gingival bleeding and plaque formation compared to regular sugar-free chewing gum (32).

Diabetic Retinopathy

Diabetic microangiopathy causes leakage of retinal capillaries. Two open-case experiments, two double-blind, placebo-controlled trials, and a multicenter field study, the latter with 1169 patients showed unequivocally that Pycnogenol in dosages of 20 to 160 mg a day retained progression of retinopathy and partly improved visual acuity. It restored capillary integrity and reduced leakage of blood into the retina (33).

An investigation of early stage retinopathy characterized by retinal edema with less pronounced bleedings showed that Pycnogenol (150 mg a day) significantly

improves retinal edema and visual acuity during three months treatment (34).

Inhibition of Edema Formation

Chronic venous insufficiency is associated with edema formation in the lower legs, leading to the feeling of heavy legs, swelling, cramps, and pain. Pycnogenol was described to improve venous microangiopathy in patients with severe chronic venous insufficiency. Skin microcirculation increased and capillary filtration decreased after eight weeks of treatment (35). Transcutaneous respiration was found to be improved as judged by decreased PCO_2 and increased PO_2 .

In five placebo-controlled, double-blind studies and three double-blind, controlled experiments, these symptoms were significantly reduced (2). Findings could be objectivated by measuring the circumference of lower limbs (31) and demonstrated superior activity compared to a commercial horse chestnut seed extract, a remedy for venous disorders (31). A comparative study of Pycnogenol versus a commercially available diosmin/hesperidin combination showed that Pycnogenol counteracted edema and symptoms significantly faster and was more pronounced (36). Another study compared the efficacy of troxerutin alone with a combination of troxerutin and Pycnogenol for relieving symptoms of chronic venous insufficiency. The combination was more effective for improving edema and symptoms and after discontinuation less relapse of symptoms occurred (37). Pycnogenol was shown to lower edema occurring as side effect in hypertensive patients medicated with nifedipine or ACE inhibitors (38). In healthy travelers on international flights, the intake of Pycnogenol prior to departure counteracted the development of ankle swellings (39).

The healing process of venous ulcers was found to be significantly accelerated with Pycnogenol as compared to an untreated control group (40). Skin microcirculation as well as transcutaneous respiration was found to be improved.

Jetlag Symptoms

Pycnogenol (150 mg/day for one week) was found to decrease typical jetlag symptoms when taken two days prior to departure for intercontinental travel (41). Symptoms such as fatigue, headache, disorientation, nausea, insomnia, and irritability were found to be less frequent and less pronounced than in an untreated control group. CT scans of passengers were found to show subliminal brain edema after arrival, which were less pronounced in the Pycnogenol-treated group.

Hemorrhoids

Pycnogenol has been investigated in a trial for patients with acute hemorrhoids (42). Within less than 48 hours after an acute attack, patients were treated with Pycnogenol that was found to significantly counteract bleedings, pain, and further symptoms as compared to a placebo-treated group. A third group received both oral and topical Pycnogenol that was more effective than oral treatment alone.

Anti-inflammatory Activity

Human Pharmacologic Investigations

Peripheral blood monocytes derived from healthy subjects before and after supplementation with Pycnogenol (200 mg daily for five days) were investigated to elucidate anti-inflammatory mechanisms (43). Ex vivo stimulation of monocytes in the presence of volunteers blood serum showed a decreased activation of key inflammatory mediator NF- κ B by in average 15%. A linear correlation between NF- κ B inhibition and release of matrix metalloproteinase-9 (MMP-9) was found. MMP-9 expression is well known to be one of many proteins under control of NF- κ B.

Another human pharmacologic study was carried out investigating neutrophils from humans who took 150 mg Pycnogenol for five days (44). Following ex vivo stimulation, expression of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) was significantly inhibited as judged by real-time PCR, when volunteers had taken Pycnogenol.

Treatment of Osteoarthritis

Pycnogenol has been investigated in three clinical studies for lowering pain and improving flexibility in patients with mild to moderate osteoarthritis. The Western Ontario McMasters Questionnaire (WOMAC) was employed in these studies for evaluating the improvement of symptoms. Pycnogenol gradually improved pain, joint stiffness, and joint function scores, respectively, from one month to the next until completion of three months. Pain scores decreased by 43%, 40%, and 55%, respectively, after three months treatment in these studies (45–47). All three studies found that patients required significantly less pain management with nonsteroidal anti-inflammatory drugs (NSAIDs) when taking Pycnogenol, while this was not the case in the respective placebo-control groups. One of the three studies with 156 patients pointed to a better mobility of patients as judged from their walking distance on a treadmill, which increased from baseline average 68 m to 198 m after three months treatment with Pycnogenol. In that study, a subset of patients presented with elevated serum C-reactive protein levels at baseline, which was found to be significantly decreased from average 3.9 to 1.1 mg/L after three months treatment with Pycnogenol.

Improvements of Dysmenorrhea

Initial exploratory trials found that administration of 30 to 60 mg Pycnogenol helped women suffering from menstrual pain in dysmenorrhea and endometriosis (2). Supplementation with 60 mg Pycnogenol reduced intake of analgesics, number of days with pain, and intensity of low back pain and abdominal pain in 42 women suffering from menstrual pain (48). A multicenter field study showed that women taking Pycnogenol for two months required significantly less NSAID analgesic medication than a placebo-treated group. Interestingly, cessation of Pycnogenol intake for one month did not lead to a relapse to higher analgesic intake (49).

Pycnogenol was shown to gradually decrease pain in endometriosis over an investigation period of 48 weeks. On a visual analog scale, the average pain of 32 women

decreased from severe to moderate and five women got pregnant (50).

Reduction of Asthma Symptoms

The anti-inflammatory effects of Pycnogenol also contribute to its beneficial action on asthma patients. In a placebo-controlled, double-blind, crossover study, asthma symptom scores of 22 patients were significantly lower and lung function parameters higher in the Pycnogenol-treated group, while leukotriene levels decreased (2). In a double-blind placebo-controlled study with 60 children, the extract improved pulmonary function and asthma symptoms and reduced use of rescue inhalations. Leukotriene levels in urine were significantly lowered (51).

Inhibition of UV-Induced Inflammation

Oral intake of Pycnogenol lowers the inflammatory response to UV radiation. The minimum erythema doses were significantly increased after intake of 1.11 mg/kg body weight of Pycnogenol for four weeks in 21 healthy volunteers and further enhanced after a larger dose of 1.66 mg/kg likewise for four weeks (2). In a study with 30 women, taking 75 mg Pycnogenol for one month (52), the UV-induced discoloration of sun-exposed skin areas, melasma, could be reduced with respect to size of the affected area and intensity of discoloration.

Decrease of Side Effects in Cancer Chemotherapy and Radiotherapy

In a pilot trial, a daily dosage of 150 mg Pycnogenol was taken by cancer patients during their course of chemoradiotherapy (53). Pycnogenol was found to decrease the incidence and severity of a broad range of symptoms to some extent, as compared to a control group taking placebo.

Anti-diabetic Effects

In patients with freshly diagnosed type II diabetes, Pycnogenol was found to dose dependently lower both fasting and postprandial blood glucose levels (54). A dosage of 50 mg Pycnogenol significantly lowered postprandial blood glucose, no effect was found on insulin levels. The effect is suggested to result from α -glucosidase inhibition by large-chain procyanidins (55).

Results were confirmed in a placebo-controlled double-blind study with type II diabetic patients on medication with metformin and sulfonylurea (56). Pycnogenol taken in addition to antidiabetic medication significantly further lowered fasting blood glucose and, furthermore, decreased endothelin-1 and increased prostacyclin. Another double-blind placebo-controlled study with type II diabetic patients confirmed significant reduction of fasting glucose and HbA_{1c} levels in patients receiving metformin, sulfonylurea, thiazolidinediones, or combinations of these (57). Moreover, 125 mg Pycnogenol a day significantly lowered systolic blood pressure in these hypertensive diabetic patients who were medicated with ACE inhibitors. This study found significantly decreased urinary albumin.

Pycnogenol was found to improve signs and symptoms of diabetic microangiopathy (58). Microcirculation

of the lower legs in patients with diabetic ulcers was increased and capillary filtration decreased in response to four weeks treatment with 150 mg Pycnogenol a day. Pycnogenol improves the healing process of diabetic ulcers, which coincides with increased transcutaneous PO₂ and decreased PCO₂ (59).

Efficacy

Pycnogenol has demonstrated anti-edema effect in several clinical studies as well as efficacy in inhibiting the progression of retinopathy. Other clinical investigations support the application of the extract to protect the circulation by inhibition of platelet aggregation, lowering of cholesterol, and an antihypertensive effect. An enhanced radical absorbing capacity after intake of Pycnogenol is probably related to protection against UV radiation and inflammatory diseases. The anti-diabetic effect and anti-spasmodic activity has to be confirmed by further clinical and mechanistic studies.

Optimum Intake

Clinical studies suggest an optimum dose range between 40 and 100 mg Pycnogenol/day or 1 mg/kg body weight. Pycnogenol should be taken together with breakfast to minimize gastrointestinal troubles.

Side Effects

The evaluation of clinical studies with more than 7000 patients to date (end of year 2009) revealed no serious adverse events related to intake of Pycnogenol. The rate of mild side effects is low, and unwanted effects, such as gastrointestinal troubles, dizziness, nausea, headache, or skin sensations were mild and transient in most cases.

Contraindications

To date, no contraindications have been seen.

Observed Drug Interactions

No drug interactions have been reported until now.

Use in Pregnancy and for Children

Despite the fact that teratogenicity tests showed no teratogenic effects, the intake of Pycnogenol during the first three months of pregnancy and during breast feeding should be avoided as a general precaution. Children younger than 12 years should not take Pycnogenol because no clinical experience is available with young children.

REGULATORY STATUS

In most countries, for example, in the United States, Australia, the United Kingdom, Belgium, the Netherlands, Finland, Italy, Thailand, Taiwan, P.R. China, and Japan, Pycnogenol is used as a food supplement. In the United States, Pycnogenol has the status of GRAS. In Greece, Switzerland, Colombia, and Venezuela, it is a nonprescriptional herbal drug.

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Garcinia

Frank Greenway

INTRODUCTION

Garcinia cambogia is the Latin name for a plant that bears the tamarind fruit and thrives on the Indian subcontinent and in western Sri Lanka. The dried rind of the tamarind fruit, Malabar tamarind, contains (–)-hydroxycitric acid (HCA), the one isomer of citric acid that inhibits adenosine triphosphate (ATP) citrate oxaloacetate lyase (EC 4.1.3.8) (CLy) also known as citrate lyase or citrate cleavage enzyme. Inhibition of this enzyme limits the generation of acetyl CoA, which is needed for fatty acid and cholesterol synthesis. HCA reduces food intake and causes weight and fat loss in rodents. It has also been a component of dietary herbal supplements for the treatment of obesity. The results of human trials have been mixed. This appears to be due to the bioavailability of HCA varying according to its method of preparation. This chapter will examine the safety and efficacy of *G. cambogia* for weight loss through animal trials, clinical trials, and case reports.

BACKGROUND

Garcinia is from the family called Guttiferae, which is a large genus of polygamous trees or shrubs distributed in tropical Asia, Africa, and Polynesia. Garcinia consists of 180 species, of which 30 are found in India. An isomer of citric acid, HCA, is found in the fruit rinds of three species of Garcinia: *cambogia*, *indica*, and *atroviridis*. These species thrive prolifically on the Indian subcontinent and in western Sri Lanka.

G. cambogia is a small or medium-sized tree with a rounded crown and horizontal or drooping branches. The leaves are dark green, elliptical, and shiny, 2 to 5 in. long and 1 to 3 in. broad. The fruits are ovoid, red, or yellow when ripe, contain six to eight seeds and are 2 in. in diameter with six to eight grooves. The tree is found in evergreen forests up to an altitude of 6000 ft. It flowers during the hot season and the fruits ripen during the rainy season.

G. indica is a slender evergreen tree with drooping branches and ovate leaves 2.5 to 3.5 in. long and 1 to 1.5 in. broad. The fruit is dark purple and has five to eight seeds. It flowers in November to February and the fruits ripen in April to May. *G. atroviridis* is a moderate-sized graceful tree growing 30 to 50 ft high with leaves that are 6 to 9 in. long and 2 to 3 in. broad. Its fruits are orange-yellow, 3 to 4 in. across and fluted.

The fruits of *G. cambogia* are too acidic to be eaten raw. A tea made from this fruit has been used in folk

medicine for rheumatism, bowel complaints, and in veterinary medicine to treat mouth pathology in cattle. The fruit of *G. indica* has an agreeable flavor and a sweet acidic taste. It is used as a garnish to give an acidic flavor to curries and for preparing syrups in the summer months. The fruit has also been used in folk medicine as a treatment for parasitic worms, dysentery, hemorrhoids, tumors, pain, and heart complaints. The dried rinds of both species are used to flavor curries. The fruit rinds of *G. atroviridis* are stewed with sugar and eaten or used as a sour relish in curries and as a dressing for fish (1).

The dried rind of the fruit of *G. cambogia* is known as Malabar tamarind and is used extensively for culinary purposes and commercially for Columbo curing of fish. It contains 30% citric acid as dry weight and acts like a pickling media with bacteriostatic properties due to its low pH. In fact, this curing process has been shown to prevent the formation of histamine in dark meat fish, such as skipjack, and prevent scombroid fish poisoning (2).

More recently, other uses of *G. cambogia* fruit have been explored. In experiments that screened for dose-dependent tumoricidal effects of a wide variety of natural extracts using an immortalized neuroblastoma cell line of spontaneous malignant origin, *G. cambogia* was found to have tumoricidal activity (3). Garcinol and guttiferone K isolated from *G. cambogia* were shown to protect lipids and proteins from oxidation (4). An extract from the fruit rind of *G. cambogia* was shown to improve the histology of Crohn disease and ulcerative colitis in rodents (5). Attempts have been made to isolate the bioactive components that may be responsible for these effects, but more work is needed. The fresh biomass of *G. cambogia* has been demonstrated to remove trivalent arsenic from contaminated ground water and has the capability of being reused for five cycles (6). Despite these potential uses of *G. cambogia*, the predominant use commercially has been in dietary supplements to treat obesity. The rest of this chapter will concentrate on the use of the extract from the rind of *G. cambogia* and its active component HCA for the treatment of obesity.

CHEMISTRY AND PREPARATION

HCA, one of the isomers of citric acid, was shown by Watson and Lowenstein in the late 1960s to be an inhibitor of the extra mitochondrial enzyme, CLy (7). Aqueous extractions of the fruit rind from *G. cambogia* contain both HCA and its lactone. When the extract is saponified with excess alkali and passed through an ion exchange resin it

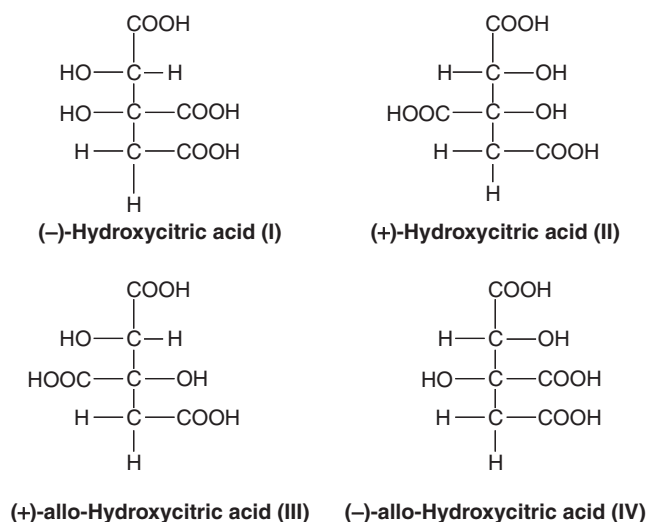


Figure 1 Structures of hydroxycitric acid isomers. Source: From Ref. 1.

is converted to its free HCA but, by concentrating the water extract, all of the HCA is converted to the lactone. One method of isolating HCA from the *G. cambogia* involves extracting the dried fruit rinds with ether, evaporating the ether to give the lactone, and passing the lactone through an anion exchange column to recover HCA after elution with sodium and potassium hydroxide.

Citric acid has four carbon atoms, so it has two isomers around each of the inside carbons for a total of four isomers (Fig. 1). Free acid leads to the formation of HCA lactone during concentration and evaporation due to its hygroscopic nature (Fig. 2). Thus, for commercial purposes, stable derivatives have been prepared using the lactone and the sodium, potassium, or calcium salts. All the original animal studies were performed by using the sodium salt.

HCA was demonstrated to be the only isomer of citric acid to competitively inhibit CLy, and the salt of HCA was shown to be much more active than its lactone. CLy exists in the cell outside the mitochondria. Inside the mitochondria, acetyl CoA, water, and oxaloacetate form citrate and CoA. The citrate crosses the mitochondrial membrane and enters the cytoplasm of the cell. In the cytoplasm of the cell outside of the mitochondria, citrate plus ATP plus CoA

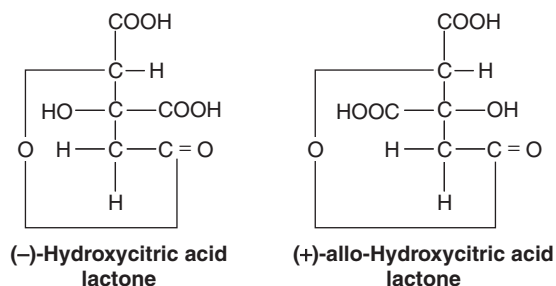


Figure 2 Structures of hydroxycitric acid lactones. Source: From Ref. 1.

form acetyl CoA and oxaloacetate. The acetyl CoA formed in this manner forms the basis for fatty acid and cholesterol synthesis (Fig. 3). Thus, by inhibiting CLy, HCA inhibits the synthesis of cholesterol and fatty acids. Inhibition of cholesterol and triglyceride synthesis by HCA has been suggested as a treatment for hyperlipidemia, since the elevation of triglycerides in obesity is driven by an increased production of very low-density lipoproteins in the liver. Triglycerides in rodents treated with HCA were decreased by 20% and cholesterol was reduced by 30% compared to controls. HCA not only depresses lipogenic rates in the liver, fat, and intestine, but also decreases food intake, reduces body weight, and body fat without reducing body protein levels (8). This reduction in body weight in rodents treated with HCA was entirely driven by the reduction in food intake, as demonstrated by a similar reduction of body weight in pair-fed controls (9). The reduction in food intake by HCA did not seem to be due to conditioned aversions.

PRECLINICAL STUDIES

Studies with 3T3L1 cells, murine preadipocytes, treated with HCA showed that lipid accumulation and the differentiation of preadipocytes was inhibited (10). A gene expression study demonstrated that HCA increased the RNA expression of genes involved in lipolysis while decreasing those involved in the differentiation of fat cells (11). A gene expression study also revealed an upregulation of genes expressing the serotonin receptor in the abdominal fat cells of rats (12). In a study using rat cortical slices, serotonin reuptake was inhibited and serotonin availability was increased in a manner similar to that seen with serotonin reuptake inhibitors, which are known to affect body weight (13). Three tests for genotoxicity were performed with HCA—a bacterial reverse mutation assay (Ames test), an in vitro chromosomal aberration test, and an in vivo micronucleus test. Only the in vivo micronucleus test showed any abnormalities (14), and it demonstrated a dose-dependent increase in micronucleated polychromatic erythrocytes per thousand polychromatic erythrocytes. Although this test showed that HCA preferentially induced micronuclei, another study using the same assay gave a normal result (15).

The original studies showing body fat reduction in rats without a reduction in body protein, a reduction in triglyceride synthesis, a reduction in cholesterol synthesis, a reduction of serum levels of cholesterol and triglyceride, in addition to a reduction in food intake were performed using the sodium salt of HCA. Most of the recent studies with HCA have not been specific regarding the exact composition of the HCA (the lactone, a monovalent salt, or a divalent salt) making interpretation of the results of these studies more difficult.

There were three preclinical studies, using *G. cambogia*, performed in mice that bear mentioning. One demonstrated that *G. cambogia* extract reduced visceral adiposity (16). Another showed that *G. cambogia* reduced insulin and insulin resistance (17). The third study showed that mice-fed chow containing 10% by weight of sucrose and 3.3% as an herbal extract of *G. cambogia* showed no skin pathology over four weeks (18). A rat study using

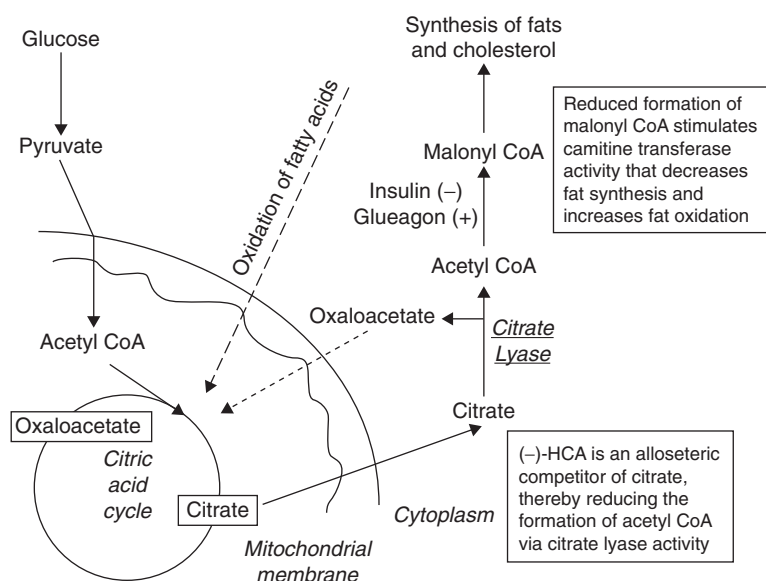


Figure 3 Mechanism of action of (7)-hydroxycitric acid [(7)-HCA] on fatty acid (FA) metabolism. (7)-HCA is a strong allosteric competitor of citrate, decreasing the formation of cytosolic acetyl coenzyme A (CoA). The decreased availability of acetyl CoA would lead to a reduced formation of malonyl CoA which, in turn, would stimulate the activity of carnitine transferase (CAT), the enzyme responsible for the transfer of fats back into the mitochondria for oxidation. Insulin indirectly makes CAT more sensitive to inhibition by malonyl CoA, whereas glucagon activates CAT and promotes lipolysis. Source: From Ref. 39.

G. cambogia deserves mention as well. Rats given dexamethasone 10 mg/kg/day or dexamethasone with *G. cambogia* fruit extract 1 g/kg/day for eight days were compared in terms of their serum lipids. The *G. cambogia* extract blocked the rise in LDL cholesterol and very low-density lipoprotein (VLDL) triglycerides seen with dexamethasone in the control rats (19). In addition to rodents, *G. cambogia* has been shown to reduce fat mass in cats as well (20).

The other preclinical studies used HCA. Mice were treated with 10 mg of HCA or a water control for 25 days. Free fatty acid levels were significantly higher, glycogen content of the gastrocnemius muscle was higher, maximal swimming time to fatigue was longer, and the respiratory quotient was lower at the end of the study in the HCA group showing that HCA promotes lipid oxidation and spares carbohydrate utilization at rest and during exercise compared to the untreated control group (21). A study in rats demonstrated that HCA stimulated erythropoiesis in addition to promoting weight loss (22). Another study in rats showed that HCA reduced the respiratory quotient (RQ) (23). Rats that lost weight on food restriction regained weight at a slower rate when treated with HCA compared to rats in the control group (24). A study of a commercial calcium and potassium salt of HCA mixed in a 1:1 ratio reduced food intake and attenuated inflammation, oxidative stress, and insulin resistance in Zucker rats compared to controls (25). The same calcium-potassium salt mixture of HCA reduced body weight, triglycerides, cholesterol, and increased high-density lipoprotein (HDL) cholesterol (25).

Safety studies were also carried out using the same calcium-potassium salt mixture. In studies lasting 90 days, rats were treated with up to 5% of their diet with a product that was 60% potassium salt and 40% calcium salt of HCA. The expected reduction in body weight and food intake was observed, but there was no hepatic DNA fragmentation, no difference in hematology testing, clinical chem-

istry testing, or histopathological findings. In addition, there was no change in testicular lipid peroxidation (26). Another study in Zucker rats showed testicular pathology in those rats treated with high doses of a different HCA preparation (27). The no adverse effect level in the study that demonstrated testicular toxicity was 389 mg of HCA per kilogram of body weight per day that is equivalent of 6 g/day in an obese human and above the doses sold for weight loss. The study showing testicular atrophy has not been repeated.

CLINICAL STUDIES

Manufacturers of dietary herbal supplements often combine several supplements in one product. This makes determining very difficult which component of the supplement may be responsible for the effects seen in a trial. For that reason, this review will concentrate on studies that look at *G. cambogia* or HCA in isolation or with a minimum of additional ingredients.

All the original animal studies conducted by the Roche Pharmaceutical company used sodium salt of HCA. Due to the hygroscopic nature of the sodium salt and the tendency of HCA to form a lactone, which is ineffective in inhibiting citrate cleavage enzyme, the original commercial forms of HCA were made from the calcium salt that is more stable. Since the calcium salt is poorly bioavailable, the products that are now being sold usually contain the potassium salt or a combination of the potassium salt with the calcium salt. Louter-van de Haar et al. (28) performed an interesting study in which they compared three products—the first was 97% potassium salt, the second was 50% potassium salt, and the third was a 1:1 mixture of a potassium and a calcium salt (Fig. 4). As one might expect, the rodents receiving these products by gavage had the greatest reduction in food intake caused by the product containing almost entirely the potassium salt, and almost

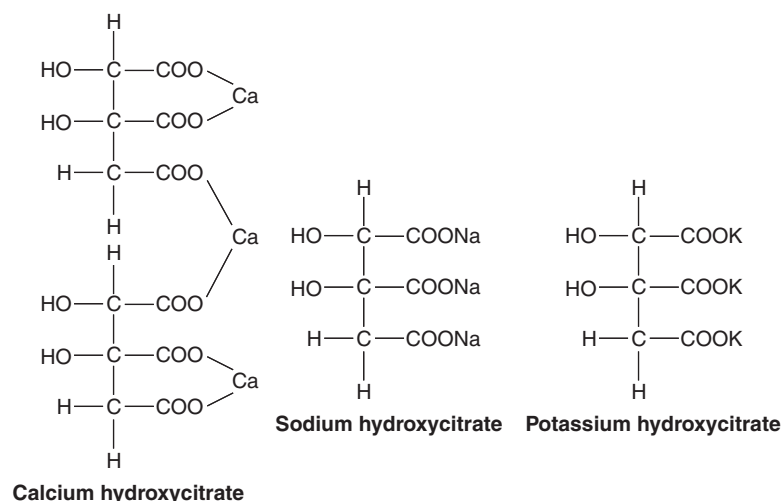


Figure 4 Structures of hydroxycitric acid derivatives. Source: From Ref. 1.

no food intake reduction with the mixture containing the potassium and calcium salts, with the lower concentration potassium salt alone in between the other two in decreasing food intake. These findings are consistent with the larger doses of the potassium and calcium salts needed to achieve blood levels and efficacy in clinical trials. It also is consistent with the lack of efficacy of some of the earlier clinical trials that were performed at a time when most of the commercial products were made entirely with the calcium salt. Unfortunately, the papers describing HCA studies often do not describe the form of HCA they used. The form of HCA used in the clinical trials to be described in this section will be included when available, since the

form of HCA may explain the divergent results seen in the various clinical trials (Table 1).

Although the doses of HCA in the clinical studies vary widely from 110 to 4400 mg/day, most of the studies report a significant weight loss compared to placebo in studies lasting from 2 to 12 weeks. The study by Heymsfield et al. (29) did not show weight loss, and it was one of the longer and larger studies in addition to being well designed. This study did not confirm that blood levels of HCA increased during administration of the oral supplement. At the time this study was performed, most of the commercial products used the calcium salt of HCA. Thus, it is possible that some, if not all, of the negative

Table 1 Clinical Trials of *Garcinia cambogia* Extract (GCE) Containing Hydroxycitrate in Overweight Humans

References	Number ^a	Study design	Dose ^b (mg/day)	Kg loss ^c	Comments
29	40/35	R-D-P 8 wk	1500	4.1/1.3	Lipid reduction
29	77/55	O 8 wk	1500	5.2%	With chromium
30	54/39	R-D-P 8 wk	1500	5.1/1.9	With chromium
29	200/186	R-D-P 4 wk	1500	1.3/0.6	With chromium and carnitine
31	60/-	R-D-P 8 wk	1320	6.4/3.8	
32	50/48	R-D-P 6 wk	2400	NS	Fat loss w/caffeine-chromium
33	150/-	R-D-P 4 wk	110	12.5/7.9	With chromium and chitosan
29	135/84	R-D-P 12 wk	1500	3.2/4.1	Difference not significant
34	10-10	R-D-P 3 day	3000	N/A	No difference in RQ
35	10-10	R-D-P 1 day	4400	N/A	No Δ in RQ, + blood HCA levels
36	89/-	R-D-P 12 wk	1200	3.7/2.4	No difference in appetite
37	21/crossover	R-D-P 2 wk	500	NS	No Δ in appetite/food intake
38	11/crossover	R-D-P 2 wk	500	NS	No Δ in RQ, EE, or satiety
39	24/crossover	R-D-P 2 wk	900	NS	Food intake down 15-30%
40	6/crossover	R-D-P 5 days	250	N/A	Exercise time \uparrow , RQ \downarrow , athlete
41	6/crossover	R-D-P 5 days	250	N/A	Exercise RQ \downarrow , untrained men
42	6/crossover	R-D-P 5 days	250	N/A	Exercise time \uparrow , RQ \downarrow , women
43	90/82	R-D-P 8 wk	2800	4.9/1.5	\downarrow LDL, Trig, EI, RQ, \uparrow HDL, 5HT
44	10/-	R-D-P 10 days	1500	N/A	HCA \downarrow de novo lipogenesis, RQ

^aSubjects enrolled/completed the trial.

^bDose of *Garcinia cambogia* rind extract or hydroxycitrate used per day in the trials expressed as hydroxycitrate when possible.

^cWeight loss in kilograms with exception of one study expressed in percent weight loss and so labeled.

R-D-P, randomized double-blind and placebo-controlled; O, open label trial with a single arm; RQ, respiratory quotient; HCA, hydroxycitric acid; +, positive; EE, energy expenditure; Δ , change; \downarrow , decreased; \uparrow , increased; LDL, low-density lipoprotein cholesterol; Trig, triglycerides; EI, energy intake; HDL, high-density lipoprotein cholesterol; 5HT, serotonin; NS, not significant; N/A, not applicable.

studies of HCA may be due to the poor bioavailability of the calcium salt. Although, like with weight loss, the results of different studies conflict, HCA has been shown in clinical trials to reduce LDL cholesterol, triglycerides, and raise HDL cholesterol. Food intake and appetite have been reduced with HCA, and HCA decreased de novo lipogenesis. HCA increases exercise time to exhaustion, spares glycogen, and increases fat oxidation in trained athletes as well as in untrained men and women.

Moreover, two safety studies in humans bear mentioning. Due to the concern raised by the study in Zucker rats cited earlier that demonstrated testicular toxicity, Hayamizu et al. explored the effect of 1000 mg/day of HCA over 12 weeks in 44 men and women on serum testosterone, estrone, and estradiol. No significant effects were seen (45). Another study evaluated electrocardiograms in 20 healthy volunteers given a commercial product, Metabolife 356 ephedra-free, which contained *G. cambogia* in unspecified amounts in addition to a large number of other dietary herbal supplement ingredients. The QTc and other electrocardiographic variables were measured over five hours with no evidence of any toxicity (46). The safety of Super CitriMax[®], a potassium and calcium salt of HCA, was reviewed (47). The animal studies cited were reviewed earlier in the preclinical section. Several placebo-controlled double-blind trials using up to 2800 mg/day of HCA were cited with no adverse events reported. The authors concluded that animal and human data support the safety of HCA in humans up to a level of 2800 mg/day.

Several case reports document adverse events in participants taking products containing HCA. Many, if not most, commercial dietary herbal supplement products containing *G. cambogia* or HCA contain many other ingredients as well. This, of course, makes interpreting these case reports more difficult. One case report describes a 54-year-old woman who developed rhabdomyolysis with a peak creatine phosphokinase (CPK) of 1028 IU/L three hours after taking a dietary herbal supplement. Her chest pain lasted two hours and did not return with discontinuation of the supplement. The supplement contained ephedra, guarana, chitosan, *Gymnema sylvestre*, and *G. cambogia*. This case report, since it is the only one referencing rhabdomyolysis, is less concerning than those reporting hepatotoxicity of which there are several reports.

Two subjects taking Hydroxycut, a product made by MuscleTech in Ontario, Canada, were the subjects of a case report describing severe hepatotoxicity. Both subjects were healthy males between 25 and 30 years of age. One had a cholestatic picture with an alkaline phosphatase over 500 U/L, a bilirubin of 7.8 mg/dL, and a liver biopsy showing cholestasis. The other subject had liver enzymes in the 4000 to 6000 U/L range and a bilirubin of 7.8 mg/dL suggesting a more hepatitic picture. Hydroxycut contains *G. cambogia*, *Gymnema sylvestre*, glucomannan, α -lipoic acid, willow bark extract, L-carnitine, green tea extract, and guarana extract. Four other cases of reversible hepatic toxicity in healthy people between the age of 20 to 40 years taking Hydroxycut were also reported. A 45-year-old asthma patient being treated with montelukast was reported to die of liver failure and was taking supplements containing *G. cambogia* among many other ingredients. Due to the association of liver toxicity with Hydroxycut,

it was postulated that *G. cambogia* extract was playing a role, but montelukast has also been associated with hepatic failure. Since animal toxicity studies do not support a role for *G. cambogia* in hepatic toxicity, and since the cases of hepatic toxicity seem to be primarily associated with Hydroxycut, it seems likely that the Hydroxycut product may have contained a hepatotoxic contaminant. Since dietary herbal supplements are considered to be foods, the burden of proof that a supplement is toxic or unsafe rests with the Food and Drug Administration (FDA) in contrast to drugs where proof of safety is the responsibility of the pharmaceutical manufacturer. Case reports with no denominator are not convincing proof of toxicity when no similar findings are seen in controlled clinical trials. When the FDA has attempted to restrict dietary herbal supplements due to concern about safety, their decisions have usually been overturned due to a lack of scientific proof showing toxicity. This was the case with ephedra until the most recent decision to declare ephedra as an adulterant, which was not challenged by industry.

MECHANISM OF ACTION

Pair feeding experiments in rodents demonstrated that a reduction in food intake was responsible for the weight loss seen with HCA treatment (9). Attention has been given to the mechanism by which HCA decreases food intake. It has been shown that HCA inhibits the synthesis of fatty acids in the brain, and it has been postulated that this might have a role in signaling food intake. HCA has also been shown to release serotonin from cortical brain slices of a rat, and an increase in central serotonin has been associated with weight loss. HCA also limits acetyl CoA availability for the synthesis of acetylcholine, and this, too, has been postulated to alter the control of food intake. Removing the afferent fibers from the subdiaphragmatic vagus nerve did not block the anorectic effect of HCA, suggesting that the reduction in food intake was not mediated by vagal afferents. The increase of glycogen storage in the liver and muscle has been pointed out as another possible explanation for the decrease in food intake, but Hellerstein and Xie (48) demonstrated that neither hepatic glycogen nor hexose phosphates were involved in the food intake suppression. It appears that the most likely explanation for the reduction in food intake is the reduction of malonyl CoA caused by the lack of acetyl CoA substrate from HCA inhibition of CLy. The reduction in malonyl CoA removes the suppression of carnitine palmitoyl transferase 1 (CPT 1) by malonyl CoA, which would result in stimulating fat oxidation. HCA appears to act peripherally, since there are negligible amounts of HCA in the brains of treated rats, and HCA suppresses food intake in rodents with ventral medial hypothalamic lesions. Recently, studies were performed with human preadipocytes differentiated in culture. The addition of a potassium and calcium salt of HCA increased lipolysis induced by isoproterenol and by iso-butyl-methylxanthine. HCA also induced leptin expression and dispersed fat droplets suggesting an increase in lipolysis. Of 54,676 genes screened, 348 were downregulated and 366 were upregulated by HCA. This gene expression work supports the antiadipogenic properties of HCA and generates hypotheses regarding the

mechanism of action of HCA that can be explored in future research (49).

REGULATORY STATUS

The rinds from the *G. cambogia* fruit have a long history of use in foods in India. They are used in folk medicine, to preserve fish, and to flavor curries and syrups. Thus, *G. cambogia* is recognized as a food product. The discovery that *G. cambogia* extract contains high levels of HCA that inhibits CLy and reduces food intake led to its use in dietary herbal supplements for the treatment of obesity. Dietary herbal supplements are also considered food from a regulatory perspective in the United States.

SUMMARY AND CONCLUSIONS

The rinds of the *G. cambogia* fruit have a long history of culinary use in India to flavor curries, preserve fish, and for use in folk medicine. In the late 1960s, HCA, one of the four isomers of citric acid, was found to be in abundance in the rind of the *G. cambogia* fruit, and it was discovered that this isomer uniquely inhibits CLy. CLy is the first step in fatty acid synthesis outside the mitochondrion. Inhibiting this enzyme reduces the supply of acetyl CoA and reduces malonyl CoA production. This removes the inhibition on CPT1 and may be a mechanism by which HCA decreases appetite. The reduction in food intake by HCA is responsible for its reduction in body weight, but other mechanisms like the increase in serotonin that has been demonstrated may also be involved in the mechanism of the food intake reduction. Studies of gene transcription have shown a variety of genes that are differentially regulated by HCA and these findings may suggest further research into the mechanisms by which HCA reduces food intake.

Preclinical data suggest that the use of HCA is safe at very high levels and it consistently demonstrates weight loss with a reduction in cholesterol and triglycerides. Preclinical studies also show a reduction in visceral fat, a reduction in insulin resistance, an increase in exercise time to exhaustion, an increase in fat oxidation, and a sparing of carbohydrate substrate during exercise. One study raised a concern about testicular damage from high dose HCA, but this finding has not been replicated. Clinical studies are much less consistent, but one can find studies supporting the preclinical findings. Safety concerns were raised about liver toxicity, but this appears to be due to a single product and may be due to a contaminant in the manufacturing process.

In conclusion, an extract from the rinds of the *G. cambogia* fruit contains a specific isomer of citric acid, HCA, which inhibits CLy, reduces food intake, and causes weight loss in animal and man. The preclinical and clinical studies suggest that the extracts made from *G. cambogia* fruit rinds have a wide margin of safety. It now appears that the monovalent salts of HCA are most effective in causing weight loss, reducing cholesterol and triglycerides, increasing exercise endurance, and increasing fat oxidation during exercise. The confusion regarding the efficacy of *G. cambogia* extract in the treatment of obesity

appears to be due to the poor bioavailability or poor inhibition of CLy by the lactone of HCA or its divalent calcium salt. This suggests that future trials should consider using the monovalent salts of HCA acid.

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Garlic

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INTRODUCTION

Garlic (*Allium sativum*) is revered throughout the world as part of a healthful diet (1,2). Reference to garlic was made about 5000 years ago in Egyptian and Indian writings. According to early transcripts from Egypt, Greece, Rome, China, and India, garlic was prescribed for multiple medical purposes, including treatment of infections, improved physical performance, and protection against toxins. These medicinal attributes, coupled with its savory characteristics, have made garlic a true cultural icon. This chapter provides a critical synopsis of the evidence relating garlic to health and how its source, quantity, and duration of exposure can influence the body's response.

USAGE

Garlic is not simply a spice, herb, or vegetable but a combination of all three, which translates to its use in various ways in meal preparations. It is a member of the Alliaceae family of plants and thus has characteristics similar to those of onions, leeks, and chives (Fig. 1). This family has over 2500 species, pointing to its size and diversity.

Knowledge about garlic's intake is qualitative, rather than quantitative, because it is not traditionally considered in dietary assessment surveys. Therefore, consumption patterns must be viewed with caution because personal preferences, and therefore intakes, vary considerably. Nevertheless, consumption patterns are recognized to vary from region to region and from individual to individual within region (3,4). According to United States Department of Agriculture (USDA) reports in the late 1990s, approximately 18% of Americans consume at least one food containing garlic on a typical day, which is comparable to the consumption pattern of foods such as French fries (16%) (5). Garlic also continues to be one of the top-selling dietary supplements in the United States and in several other parts of the world. In 2008, according to Information Resources, Inc., the top-selling herbal singles in the food, drug, and mass-market channels were cranberry (*Vaccinium macrocarpon*), soy (*Glycine max*), garlic (*A. sativum*), saw palmetto (*Serenoa repens*), and ginkgo (*Ginkgo biloba*).

Knowledge about garlic usage is typically qualitative, rather than quantitative, because it is not traditionally monitored by dietary assessment surveys, and standardized databases for the many varieties of garlic and supplement preparations are not readily available. Regardless, its average intake in the United States has been estimated to



Figure 1 Garlic has been revered for its medicinal properties for centuries, as evident by ancient writings from Egypt, Greece, China, and India. Today 300 to 400 varieties of garlic cultivated worldwide. While garlic is more than a source of sulfur, a variety of compounds that may promote health are known to arise when it is peeled and crushed.

be approximately 0.6 g/wk or less (3), whereas in some parts of China, it may be as much as 20 g/day (4,6). Average intakes may have limited meaning because personal preferences, and therefore usage, can vary considerably. It is not clear whether the response is dose dependent, although there is evidence from China suggesting that a reduction in prostate cancer risk occurs in subjects consuming higher amounts (>10 g/day) of garlic compared with those consuming less (≤ 2.2 g/day) (7). While several biological processes may respond to garlic, it remains unclear who benefits, what factors determine the magnitude of the response, and the minimum quantity and duration needed to bring about a response.

Claims about the health benefits of garlic likely contributed to its clinical usage, especially by individuals flirting with alternative health care strategies. Peng et al. (8) found that approximately 43% of veteran outpatients were taking at least one dietary supplement along with

Table 1 Structures of Some Biologically Active Lipid- and Water-Compounds Isolated from Garlic

Chemical	Structure
Allicin	$\text{CH}_2=\text{CH}-\text{CH}_2-\overset{\text{O}^-}{\underset{ }{\text{S}^+}}-\text{CH}_2-\text{CH}=\text{CH}_2$
Ajoene	$\text{CH}_2=\text{CH}-\text{CH}_2-\overset{\text{O}^-}{\underset{ }{\text{S}^+}}-\text{CH}_2-\text{CH}=\text{CH}-\text{S}-\text{S}-\text{CH}_2-\text{CH}=\text{CH}_2$
Diallyl sulfide (DAS)	$\text{CH}_2=\text{CH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}=\text{CH}_2$
Diallyl disulfide (DADS)	$\text{CH}_2=\text{CH}-\text{CH}_2-\text{S}-\text{S}-\text{CH}_2-\text{CH}=\text{CH}_2$
Diallyl trisulfide (DATS)	$\text{CH}_2=\text{CH}-\text{CH}_2-\text{S}-\text{S}-\text{S}-\text{CH}_2-\text{CH}=\text{CH}_2$
(S)-Allylcysteine (SAC)	$\text{CH}_2=\text{CH}-\text{CH}_2-\text{S}-\text{CH}_2-\overset{\text{NH}_2}{\underset{ }{\text{CH}}}-\text{COOH}$
(S)-Allylmercaptocysteine (SAMC)	$\text{CH}_2=\text{CH}-\text{CH}_2-\text{S}-\text{S}-\text{CH}_2-\overset{\text{NH}_2}{\underset{ }{\text{CH}}}-\text{COOH}$

their prescription medication(s). Several botanicals are commonly consumed by patients with cancer and include black cohosh, Echinacea, garlic, ginkgo, green tea, kava, milk thistle, and St John's wort (9). Adusumilli et al. (10) found that approximately 57% of patients undergoing elective surgery had used herbal medicine at some point in their life. Echinacea, aloe vera, ginseng, garlic, and ginkgo were among the most common. Interestingly, in this study, one in six patients used herbal supplements during the month of surgery. Stys et al. (11) reported that patients with a history of myocardial infarction, coronary revascularization, and hyperlipidemia and a family history of coronary artery disease were more likely to use supplements, including multivitamins, vitamin E, vitamin C, vitamin B, folate, garlic, calcium, coenzyme Q10, and ginkgo, than were those without comparable health concerns.

While some individuals tolerate rather large quantities of garlic, for example, 20 g/day, some may not be as tolerant (12–14). A spectrum of adverse reactions has been observed, admittedly rather infrequently, including contact dermatitis, respiratory distress, gastrointestinal disturbances, bleeding abnormalities, and anaphylactic shock. The dearth of case reports highlighting these events points to the low frequency but also that complications can occur as a result of garlic over indulgence.

CHEMISTRY OF GARLIC

Garlic's distinctive characteristics arise from sulfur, which constitutes almost 1% of its dry weight (15). While garlic does not typically serve as a major source of essential nutrients, it may contribute to several dietary factors with potential health benefits. While carbohydrates constitute only approximately 33% of garlic's weight, there are a significant proportion of oligosaccharides, which may influence gastrointestinal flora or gastrointestinal function (16,17). While garlic has a moderate amount of protein, it is a relatively rich source of the amino acid arginine. Antioxidant properties associated with carbohydrate-arginine polymers may contribute to some of garlic's proposed health benefits (18). The presence of several other constituents, including selenium and

flavonoids, may influence the magnitude of the body's response to garlic (19–21).

Most studies about garlic constituents focus on its sulfur components (22–24) (Table 1). The primary sulfur-containing constituents in garlic bulbs are γ -glutamyl-(S)-alk(en)yl-L-cysteines and (S)-alk(en)yl-L-cysteine sulfoxides. Considerable variation in (S)-alk(en)ylcysteine sulfoxide content of garlic has been reported, ranging between 0.53% and 1.3% of the fresh weight, with allicin [(S)-allylcysteine sulfoxide] being the largest contributor (22). Allicin concentrations can increase during storage because of the transformation of γ -glutamyl-L-cysteines. In addition to allicin, garlic bulbs contain small amounts of (+)-(S)-methyl-L-cysteine sulfoxide (methiin) and (+)-(S)-(trans-1-propenyl)-L-cysteine sulfoxide, (S)-(2-carboxypropyl)glutathione, γ -glutamyl-(S)-allyl-L-cysteine, γ -glutamyl-(S)-(trans-1-propenyl)-L-cysteine, and γ -glutamyl-(S)-allyl-mercapto-L-cysteine (4,15,22–24).

Allicin is the major thiosulfinate compound (allyl-2-propenethiosulfinate or diallyl thiosulfinate) occurring in garlic and its aqueous extracts. When garlic is chopped or crushed, allinase enzyme, present in garlic, is activated and acts on alliin (found in the intact garlic) to produce allicin [thio-2-propene-1-sulfinic acid (S)-allyl ester]. Because allicin is relatively unstable, it further decomposes to sulfides, ajoene, and dithiins (24,25). Garlic's characteristic odor arises largely from allicin and its oil-soluble metabolites. Heating garlic is associated with a denaturing of allinase and a reduction in allyl mercaptan, methyl mercaptan, and allyl methyl sulfide. The decreased formation of these metabolites is associated with a reduction in smell (25) and with its anticarcinogenic potential (26). Overall, the method used to process garlic can dramatically influence the sulfur compounds that predominate and its biological response (4,26,27).

While the pharmacokinetics of allyl sulfur compounds within mammals has not been adequately examined, it is unlikely that allicin occurs in a significant proportion once garlic is consumed. If it does, the liver should quickly transform it to diallyl disulfide (DADS) and allyl mercaptan (28). Germain et al. (29) provided evidence that DADS is absorbed and transformed into allyl mercaptan, allyl methyl sulfide, allyl methyl sulfoxide, and

allyl methyl sulfone. Thus, hosts of compounds likely arise from ingestion of the parent compounds found in garlic. While allyl methyl sulfone predominated in tissues, both the sulfoxide and the sulfone have been identified in urine (29).

IMPLICATIONS IN HEALTH PROMOTION

Garlic has been reported to alter several physiological processes that may influence health, including those associated with heart disease and cancer (30–34). While these results generally support earlier views about garlic's medicinal properties, the clinical findings pointing to the potential benefits of garlic are often mixed, suggesting that not all individuals will respond identically, if at all, or that there are inadequate clinical studies to make firm recommendations about the quantity or duration needed to bring about a response. The strength of the evidence can also be inadequate. Such is the case for the relationship between garlic intake and the proposed reduced occurrence of the common cold, which appears to rely largely on poor-quality evidence (35). Nevertheless, scientists, legislators, and consumers worldwide continue to be intrigued with garlic's potential health benefits. This passion may come from the mounting, and rather compelling, preclinical evidence that points to the influence of garlic and its related sulfur components on a host of biological processes associated with health (21).

Some of the health benefits attributed to the consumption of garlic and its associated allyl sulfur components are

- Antibacterial
- Antifungal
- Antiviral
- Anticarcinogenic
- Antioxidant
- Anti-inflammatory
- Antithrombotic

Antimicrobial Effects and Cancer Prevention

Historical documents reveal that garlic has been used for centuries to preserve foods (36,37). Garlic extracts have been demonstrated to suppress the proliferation of microbes including *Salmonella*, *Escherichia coli* O157:H7, and *Listeria*. Lee et al. (38) found that garlic is very active against a spectrum of pathogens, including clinical antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis*, vancomycin-resistant enterococci, and ciprofloxacin-resistant *Pseudomonas aeruginosa*. Most recently, it was found that the antibacterial activity of allicin was completely eliminated by cysteine, glutathione, and coenzyme A but not by non-SH-compounds. The oxygen in the structure ($-S(=O)-S-$) of allicin therefore functions to liberate the (S)-allyl moiety, which might be an offensive tool against bacteria (39).

Chronic bacterial prostatitis is a relatively common cause of relapsing urinary tract infections in men. Sohn et al. (40), in a rat model, examined the antimicrobial and anti-inflammatory effects of garlic [9 mg of garlic concentrate per kilogram of body weight (Uiseong

nonghyup, Uiseong, South Korea) diluted in 1 mL of distilled water and administered through an oral gavage twice a day for three weeks] in the presence or absence of ciprofloxacin on chronic bacterial (*E. coli* Z17, O2:K1:H–)-induced prostatitis. The garlic group showed decreased bacterial growth and reduced prostatic inflammation compared with the control group. Furthermore, the use of garlic plus ciprofloxacin showed a significant decrease in bacterial growth as well as improvement in prostatic health compared with the use of ciprofloxacin alone. These results suggest that garlic can work in concert with other antimicrobial/anti-inflammatory agents.

Garlic and its components can also serve as potent antifungal agents (41). Lemar et al. (42) reported that fresh garlic extract was more effective in retarding *Candida albicans* than was a garlic powder extract. The antiyeast activity of garlic oil appears to be relative stable. Incorporating the allyl sulfur compounds in nanoparticles enhances their antifungal properties (43). Most recently, Ledezma and Apitz-Castro (44) suggested that ajoene might be one of the most potent allyl sulfur compounds in garlic that has antifungal properties.

It should be noted that all microbes are not equally sensitive to garlic or its constituents. Sivam et al. (45) reported that while 40 µg of thiosulfinate per milliliter inhibited *Helicobacter pylori*, it did not influence *S. aureus*. Nevertheless, it should also be noted that in some cases the garlic extract was more effective than classical antibiotics (36). At this point, it is unclear what accounts for the variation in antimicrobial response, although differences in uptake and/or metabolism of the bioactive component are most likely involved, and therefore source of the preparation becomes an important determinant of the response.

In addition to allicin, compounds including diallyl sulfide (DAS), DADS, (E)-ajoene, (Z)-ajoene, (E)-4,5,9-trithiadeca-1,6-diene-9-oxide [(E)-10-devinylajoene, (E)-10-DA], and (E)-4,5,9-trithiadeca-1,7-diene-9-oxide [iso-(E)-10-devinylajoene, iso-(E)-10-DA] have been reported to influence microbial growth (46–48). Although there are clear differences in the efficiency with which these compounds alter proliferation, relatively small amounts appear to be effective deterrents. The target(s) accounting for the antimicrobial effects of allyl sulfur compounds remain to be determined. However, it is logical to assume that the response reflects alterations in membrane protein sulfhydryls and/or a change in the redox state. Support for this comes from studies showing that allyl sulfur compounds can influence the fluidity of cellular membranes (49).

Heating is recognized to blunt the antimicrobial effectiveness of garlic, suggesting that alliin breakdown is needed to bring about a response. Lee et al. (38) found that cooked garlic and commercial garlic pills exhibited no antimicrobial activity against a spectrum of pathogens, again suggesting that alliinase inactivation prevents the formation of the actual active component. Thus, it will not be surprising that garlic preparations vary in their antimicrobial properties. Unfortunately, because most preparations are not standardized to an active component or to a biological/biomarker response, it is impossible to draw firm conclusions about commercial preparations in the marketplace. Likewise, because few clinical studies have examined the antimicrobial response to garlic or its specific allyl sulfides, it is difficult to draw firm conclusions

about the physiological significance of findings in this area, although the results are tantalizing.

Coronary Effects of Garlic

Garlic has received considerable attention for its possible cardiovascular benefits (30–32). While a number of studies have reported that garlic lowers cholesterol and several other factors linked with heart disease, numerous inconsistencies in the literature are also noted. It is certainly possible that garlic may influence the genesis and progression of cardiovascular disease through several biological effects including a decrease in total and low-density lipoprotein (LDL) cholesterol, an increase in high-density lipoprotein (HDL) cholesterol, a reduction in serum triglyceride and fibrinogen concentrations, a lowered arterial blood pressure, and/or an inhibited platelet aggregation. A recent meta-analysis (50) revealed that garlic therapy did not produce any statistically significant reduction in serum total cholesterol level (mean difference: -0.04 mmol/L, 95% CI: -0.15 to 0.07 mmol/L), LDL-cholesterol level (mean difference: 0.01 mmol/L, 95% CI: -0.10 to 0.11 mmol/L), triglycerides level (mean difference: -0.05 mmol/L, 95% CI: -0.17 to 0.06 mmol/L), or apolipoprotein B level (mean difference: -0.02 g/L, 95% CI: -0.03 to 0.001 g/L), although moderate-to-high heterogeneity was noted (50). The contradictory results may be due to several factors, including a lack of consistency in the dosage of garlic employed, the standardization of garlic preparations in terms of active component(s), and the duration of intervention and the degree of insult (variation in degree of hypercholesterolemia). The large variation in the quantity of garlic used, that is, 7 to 28 cloves/day, makes direct comparisons difficult. Several years ago, Thomson and Ali (51) reported that consuming 3 g of fresh garlic daily for 16 weeks decreased blood cholesterol by approximately 21%. Because a significant decrease was not detected before four weeks, there may be minimal exposure time before a response occurs. Thus, not only quantity but also duration of exposure must be considered when evaluating results from garlic intervention studies.

In a recent meta-analysis (52) involving 29 trials, garlic was correlated with significantly reduced total cholesterol (-0.04 ; 95% CI: -0.15 to -0.07 mmol/L) and triglyceride levels (-0.05 ; 95% CI: -0.17 to -0.06 mmol/L) but did not significantly affect LDL or HDL levels. Again, it is unclear how important quantity and duration are in determining the outcome.

LDL oxidation is recognized as one of several factors involved with the initiation and progression of atherosclerosis (53). Oxidation of LDL occurs when exposed to free radicals released by surrounding cells, such as smooth muscles cells, or monocytes/macrophages, both of which might be influenced by garlic intake (54). Several years ago, Munday et al. (55) provided evidence that oxidation of LDL particles by Cu^{2+} from subjects was reduced in subjects given 2.4 g aged garlic extract (AGE) daily for seven days compared with those who did not receive the supplement. A similar response was not observed when subjects were given raw garlic (6 g), suggesting that not all preparations are comparable in bringing about a physiological change. Most recently, Ou et al. (56) compared the abilities of four allyl sulfur compounds (DAS, DADS,

(S)-ethylcysteine, *N*-acetylcysteine) for their ability to alter LDL oxidation. While all were found effective, there were clear differences in their efficacy. It should be noted that water-soluble allyl sulfur compounds such as those found in deodorized preparations have also been reported to reduce LDL oxidation (57). Overall, it is unclear whether the literature discrepancies about garlic and LDL oxidation relate to the subjects examined, the preparations used, and/or the quantity and duration of exposure.

The uptake of oxidized LDL (ox-LDL) by vascular endothelial cells is recognized as a critical step in the initiation and development of atherosclerosis. Adhesion molecules are known to be upregulated by ox-LDL and numerous inflammatory cytokines likely play a pivotal role in the overall response. Recently, Lei et al. (58) demonstrated that DAS, DADS, and DATS (diallyl trisulfide) reduced adhesion molecule expression induced by ox-LDL and cell surface expression of (*E*)-selectin and vascular cell adhesion molecule 1 (VCAM-1). The protein kinase A (PKA) inhibitor H89 reversed the suppression of VCAM-1 by DADS and DATS, but the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin had no effect. In contrast, wortmannin abolished DADS- and DATS-induced suppression of ox-LDL-induced (*E*)-selectin expression. These results suggest that the suppression of ox-LDL-induced (*E*)-selectin and VCAM-1 expression by DADS and DATS, and thus monocyte adhesion to endothelial cells, is likely dependent on the PI3K/PKB or PKA/CREB signaling pathway in an adhesion molecule-specific manner.

Aortic stiffening is another risk factor in cardiovascular morbidity and mortality. This stiffness coincides with a high systolic blood pressure and augmented pulse pressure. Reuter et al. (59) provided evidence that garlic reduced blood pressure, increased fibrinolytic activity, and inhibited platelet aggregation in humans. These findings are supported recently by findings in the rabbit that garlic can reduce atherosclerotic plaque (60). However, Isaacsohn et al. (61) using another garlic preparation (Kwai) found no change in blood pressure. The dearth of studies, coupled with the wide variation in experimental designs, makes it virtually impossible to evaluate garlic as a modifier of blood pressure (62). Interpreting results with inadequate study designs, methodological deficiencies with few details about blood pressure measurement, makes meta-analysis of questionable value. Furthermore, the particular insult (systolic or diastolic) may be a decisive factor in determining the response because a meta-analysis suggests that those with elevated systolic blood pressure are more responsive to garlic (63).

Preclinical evidence does suggest that a reduction in blood pressure is plausible. Specifically, using a Goldblatt model for hypertension, Al-Qattan et al. (64) reported that garlic was effective in exerting a sustained depression in arterial blood pressure possibly by regulating sodium homeostasis. Garlic treatment has also been found to lead to a dose-dependent vasorelaxation in both endothelium-intact and mechanically endothelium-disrupted pulmonary arterial rings *in vitro* mode (65). The likelihood that the response depends on nitric oxide (NO) synthase activity was suggested by the ability of NG-nitro-L-arginine methyl ester, a NO synthase inhibitor, to block the vasorelaxation.

The ability of garlic to influence blood NO concentrations in human beings was recently examined (66). Ingesting 2 g fresh, but not boiled, garlic was found to increase the basal plasma level of NO from 2.7 to 8.8 μ M. Interestingly, basal plasma IFN- α level increased from 9.5 to 46.3 nM. Thus, consumption of garlic appears to stimulate synthesis of NO and, in turn, IFN- α in humans.

The influence of garlic on NO status appears to relate to reducing the complications associated with a biological insult. Allicin and ajoene have been reported to cause a dose-dependent inhibition of the iNOS (inducible nitric oxide synthase) system in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages (67). iNOS is recognized to occur in human atherosclerotic lesions and is thought to promote the formation of peroxynitrites. Evidence exists that allicin may inhibit iNOS activity through a dose-dependent decrease in iNOS mRNA levels and by reducing arginine transport through a downregulation of CAT-2 mRNA (68). Kim et al. (69) provide evidence that both garlic extracts and (S)-allyl cysteine have similar behaviors in reducing iNOS and NO concentrations in macrophages and endothelial cells.

Acute coronary syndromes can occur when an unstable atherosclerotic plaque erodes or ruptures, thereby exposing the highly thrombogenic material inside the plaque to the circulating blood (70). This exposure triggers a rapid formation of a thrombus that occludes the artery. Campbell et al. (71) noted that feeding a deodorized garlic preparation (Kyolic) reduced the fatty streak development and vessel wall cholesterol accumulation in rabbits fed a cholesterol-fortified diet. Similarly, garlic consumption for 48 weeks in a randomized trial was found to reduce arteriosclerotic plaque volumes in both the carotid and femoral arteries by 5% to 18% (72). Experiments by Siegel et al. (73) prove evidence that garlic extracts can inhibit Ca^{2+} binding to heparan sulfate proteoglycan. Because the ternary proteoglycan receptor/LDL cholesterol/calcium complex is critical for the "nanoplaque" composition, and ultimately for the arteriosclerotic plaque, the observed reduction caused by garlic provides a biological basis for why some individuals may benefit more than others.

Aggregates of activated platelets also likely have a pivotal role in coronary syndromes. Garlic and some of its organosulfur components have been found to be potent inhibitors of platelet aggregation (74). Boiling garlic retards its ability to inhibit platelet aggregation, again suggesting allyl sulfur compounds likely need to be formed by alliin lyase (alliinase) action (75). Induction of aggregation also influences the response to garlic. Hiyasat et al. (76) found that garlic extracts inhibited platelet aggregation induced via the adenosine diphosphate (ADP) pathway and to a lesser extent aggregation induced by epinephrine, whereas arachidonic acid- and collagen-induced aggregation was not affected. Unfortunately, few studies have documented that garlic can modify platelet aggregation in vivo. Several years ago, Steiner and Li (77) provided evidence that consumption of AGE in humans reduced epinephrine- and collagen-induced platelet aggregation, although it failed to influence ADP-induced aggregation. Their studies also provided evidence that platelet adhesion to fibrinogen could be suppressed by consumption of this garlic supplement. It should be noted

that the effectiveness of allyl sulfur compounds tends to disappear rapidly, suggesting that genetic differences in its metabolism/catabolism may determine overall response.

Overall, garlic's ability to reduce hyperlipidemia, hypertension, and thrombus formation under some circumstances makes it a likely candidate for lowering the risk of heart disease and stroke. Undeniably, the enormous variability observed in the literature indicates that global benefits are unlikely. It remains unclear why these discrepancies exist, but total exposures, genetics, and the degree of abnormality are likely key variables. Additional studies will surely clarify who might benefit most from added garlic more than social intake.

Influence on Multiple Tissues and Processes Related to Cancer

Preclinical models provide rather compelling evidence that garlic and its associated components can reduce the incidence of breast, colon, skin, uterine, esophagus, and lung cancers (21,33,78,79). However, evidence in human investigations is less compelling (34). *It is unclear whether the varied diet of humans or the quantity of garlic typically consumed accounts for differences between human and animal investigations.* The ability of garlic to inhibit tumors arising from different inducing agents and in different tissues indicates that a generalized cellular event is likely responsible for the change in tumor incidence and that the response is highly dependent on environmental or other types of biological insults. Fluctuations in several processes associated with cancer, including carcinogen formation, carcinogen bioactivation, DNA repair, tumor cell proliferation, and/or apoptosis, may account for these observations (Fig. 2). It is likely that several of these processes are modified simultaneously. Their specificity in terms of the dose of allyl sulfur needed to bring about a response

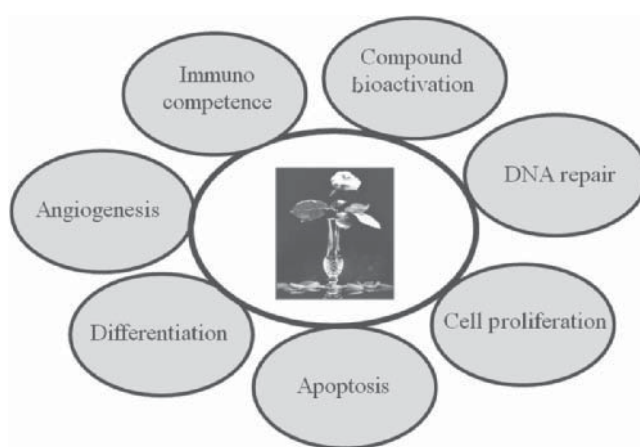


Figure 2 The anticancer benefits ascribed to garlic may relate to alterations in one or more processes. Each of these processes has been reported to be altered by one or more allyl sulfur compounds that occur in processed garlic. Garlic is sometimes referred to as the stinking rose because of its benefits often being linked to one or more of its sulfur compounds it can supply.

and the temporality of the change needed to bring about a phenotypic change needs additional clarification.

Nitrosamine Formation and Metabolism

Suppressed nitrosamine formation continues to surface as one of the most likely mechanisms by which garlic may block cancer. Several studies (21) suggest that allyl sulfur compounds can retard the spontaneous and bacterial-mediated formation of nitrosamines. Because many of the nitrosamines are considered suspect carcinogens in various tissues, this block may be particularly important. Dion et al. (36) demonstrated that not all allyl sulfur compounds were equal in retarding nitrosamine formation. The ability of (S)-allyl cysteine (SAC) and its non-allyl analog (S)-propyl cysteine to retard NOC formation, but not DADS, DPDS (dipropyl disulfide), and DAS, reveals the critical role that the cysteine residue plays in the inhibition. However, lipid-soluble allyl sulfur compounds are effective in retarding microbial growth and thus may indirectly influence the formation of nitrosamines.

The reduction in nitrosamine formation may arise secondarily to increase the formation of nitrosothiols. Williams (80) suggested, almost 20 years ago, that several sulfur compounds might reduce nitrite availability for nitrosamine formation by enhancing the formation of nitrosothiols. Because the allyl sulfur content among garlic preparations can vary enormously, it is reasonable that not all commercial preparations will be equivalent in their ability to retard nitrosamine formation. While S-nitrosylation is known to influence health and disease, it is unclear how garlic influences this process across various cell types.

Some of the most compelling evidence that garlic depresses nitrosamine formation in humans comes from studies by Mei et al. (81). Their studies demonstrated that ingesting 5 g of garlic per day blocked the enhanced urinary excretion of nitrosoproline resulting from exaggerated nitrate and proline intake. Most recent evidence suggests that as little as 1 g of garlic may be sufficient to suppress nitrosoproline formation (82). The significance of this observation comes from the predictive value that nitrosoproline has as an indicator for the synthesis of other potential carcinogenic nitrosamines (83).

The anticancer benefits attributed to garlic are also associated with suppressed nitrosamine bioactivation. Evidence from multiple sources point to the effectiveness of garlic to block DNA alkylation, an initial step in nitrosamine carcinogenesis (84,85). In agreement with this reduction in bioactivation, Dion et al. [46] reported that both water-soluble (S)-allyl sulfide and lipid-soluble DADS retarded nitrosomorpholine mutagenicity in *Salmonella typhimurium* TA100. Aqueous garlic extracts have also been shown to reduce the mutagenicity of ionizing radiation, peroxides, Adriamycin, and *N*-methyl-*N'*-nitro-nitrosoguanidine (86).

A block in nitrosamine bioactivation may arise from inactivation of cytochrome P450 2E1 (CYP2E1) (87,88). An autocatalytic destruction of CYP2E1 may account for some of the chemoprotective effects of DAS and possibly other allyl sulfur compounds against nitrosamine carcinogenesis (88). Fluctuations in the content and overall activity of P450 2E1 may be a key variable in determining the mag-

nitude of the protection provided by garlic and associated allyl sulfur components.

Bioactivation and Response to Other Carcinogens

Garlic and several of its allyl sulfur compounds can also effectively block the bioactivation and carcinogenicity of a host of carcinogenic compounds (21). This protection, which traverses a diverse array of compounds and cancers occurring in several tissues, again suggests an overarching biological response. Because metabolic activation is required for many of these carcinogens, there is likelihood that either phase I or II enzymes are altered (89,90). Interestingly, little change in cytochrome P-450 1A1, 1A2, 2B1, or 3A4 activities has been detected following treatment with garlic or related sulfur compounds. However, this lack of responsiveness may relate to the quantity and duration of exposure, the quantity of carcinogen administered, or the methods used to assess cytochrome content or activity. Wu et al. (91), using immunoblot assays, found that the protein content of cytochrome P450 1A1, 2B1, and 3A1 was increased by garlic oil and each of several isolated disulfide compounds. Their data demonstrated that the number of sulfur atoms in the allyl compound is inversely proportional to the depression in these cytochromes. Thus, phase I enzyme activity changes may account for some of the anticancer properties attributed to garlic.

Changes in bioactivation resulting from a block in cyclooxygenase and lipoxygenase may also partially account for the reduction in chemically induced tumors (92). Ajoene has been shown to interfere with the COX-2 pathway in LPS-activated RAW 264.7 cells (93). While limited, there is also evidence that garlic and associated sulfur components can inhibit lipoxygenase activity (94,95). Collectively, these studies pose interesting questions about the role of both cyclooxygenase and lipoxygenase in not only forming prostaglandins, and therefore modulating tumor cell proliferation and immunocompetence, but also their involvement in the bioactivation of carcinogens. Additional attention is needed to clarify what role, if any, these bioactivation enzymes have in determining the biological response to dietary garlic and its allyl sulfur components.

Detoxification and Allyl Sulfur Specificity

Increased activity of several detoxification enzymes, including NAD(P)H:quinone oxidoreductase and glutathione (S)-transferase (GST), may also partially account for the experimental anticancer properties provided by garlic (96). Not all GST isozymes are influenced equally by organosulfur compounds (97). Bose et al. (98) demonstrated that mGSTP1 mRNA expression was either unaltered in liver or moderately increased in forestomach following treatment with DPDS, indicating that the allyl group is critical for the mGSTP1-inducing activity of DADS. The induction of GST also depends on the presence of the enhancer element GPE I (99). Electromobility gel shift assay have revealed that the DADS and DATS induction of GST is accompanied by a concentration-dependent increase in DNA-binding activity of nuclear activator protein-1 (AP-1). The phosphorylation of c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), but not of p38, was also stimulated in the

presence of both garlic allyl sulfides. Pretreatment with JNK and ERK inhibitors prevented the increase in AP-1-DNA-binding activity and also the induction of GSTP protein by either allyl sulfide. These results indicate that the effectiveness of DADS and DATS on GSTP expression is likely related to the JNK-AP-1 and ERK-AP-1 signaling pathways and, thus, that DADS and DATS enhance the binding of AP-1 to GPE I (99).

Rarely have water- and oil-soluble allyl sulfur compounds been compared within the same study. Nevertheless, available evidence suggests that major differences in efficacy among extracts are not of paramount importance, at least for blocking the initiation phase of carcinogenesis (100). While subtle differences among garlic preparations are likely to occur, quantity, rather than source, appears to be a key factor influencing the degree of protection. Differences that do occur between preparations likely relate to the content and effectiveness of individual sulfur compounds. Nevertheless, the number of sulfur atoms present in the molecule seems to influence the degree of protection, in the order DATS > DADS > DAS (101). Likewise, the presence of the allyl group generally enhances protection over that provided by the propyl moiety (98,102).

Cell Proliferation and Apoptosis

Several lipid- and water-soluble organosulfur compounds have been examined for their antiproliferative efficacy (21,32,103). Some of the more commonly used lipid-soluble allyl sulfur compounds in tumorigenesis research are ajoene, DAS, DADS, and DATS. A breakdown of allicin appears to be necessary for achieving maximum tumor inhibition. Previous studies reported that lipid-soluble DAS, DADS, and DATS (100 μ M) were more effective in suppressing canine tumor cell proliferation than isomolar water-soluble SAC, (S)-ethyl cysteine, and (S)-propyl cysteine (103). While treating human colon tumor cells (HCT-15) with 100 μ M DADS completely blocks growth, approximately 200 μ M SAMC [(S)-allylmercaptocysteine] is required to lead to a similar depression (104). No changes in growth were observed with concentrations of SAC up to 500 μ M. Undeniably, not all allyl sulfur compounds from garlic are equally effective in retarding tumor proliferation. While the quantities used in the investigations are relatively high, these do appear to be close to what might happen with more physiological garlic intake.

Evidence exists that these allyl sulfur compounds preferentially suppress neoplastic over nonneoplastic cells. Adding DATS (10 μ M) in vitro to cultures of A549 lung tumor cells inhibited their proliferation by 47%, whereas it did not influence nonneoplastic MRC-5 lung cells (101). The antiproliferative effects of allyl sulfides are generally reversible, assuming that apoptosis has not occurred (104). Similarly, Kim et al. (105) found not only that DATS triggers mitochondria-mediated apoptosis program in human prostate cancer cells (LNCaP, LNCaP-C81, LNCaP-C4-2) irrespective of their androgen responsiveness and also that a normal prostate epithelial cell (PrEC) line was significantly more resistant to apoptosis induction.

SAMC, DAS, and DADS have also been reported to increase the percentage of cells blocked within the G₂/M phase (106,107). p34(cdc2) kinase is a complex that governs the progression of cells from the G₂ into the M phase

of the cell cycle. Activation of this complex promotes chromosomal condensation and cytoskeletal reorganization through the phosphorylation of multiple substrates, including histone H1. The G₂/M phase arrest induced by DADS has been found to coincide with the suppression in p34(cdc2) kinase activity (106). DADS appears to inhibit p34(cdc2) kinase activation through a decrease in the p34(cdc2)/cyclin B(1) complex and modest change in p34(cdc2) hyperphosphorylation (106). Using LNCaP and HCT-116 human cancer cells, Xiao et al. (107) demonstrated that checkpoint kinase 1-mediated mitotic arrest resulting from DATS is key to apoptosis induction. The G(2) arrest was accompanied by downregulation of cyclin-dependent kinase 1, cell division cycle (Cdc) 25B, and Cdc25C, leading to Tyr15 phosphorylation of Cdk1 (inactivation). The DATS-mediated mitotic arrest correlated with inactivation of anaphase-promoting complex/cyclosome as evidenced by accumulation of its substrates cyclinB1 and securin. Transfection with Chk1-targeted siRNA conferred significant protection against DATS-induced mitotic arrest (107).

Scavenging of DADS-induced reactive oxygen species (ROS) by N-acetyl cysteine or reduced glutathione has also been reported to inhibit cell cycle arrest, apoptosis, and p53 activation caused by DADS treatment (108). These results suggest that ROS trigger the DADS-induced cell cycle arrest and apoptosis and that ROS are involved in stress-induced signaling upstream of p53 activation. Transfecting p53 small interfering RNA prevented the accumulation of cleaved poly(ADP-ribose) polymerase and sub-G1 cell population by 65% and 35%, respectively. Moreover, DADS-induced apoptosis was prevented by treatment with oligomycin, known to prevent p53-dependent apoptosis by reducing ROS levels in mitochondria (108).

Several of the allyl sulfur compounds from garlic have also been reported to induce apoptosis (109,110). Activation of caspase-3 appears to be at least partially responsible for the induction of apoptosis (111,112). The induction of apoptosis DATS, and likely other allyl sulfur compounds, appears to relate to an induction of proapoptotic Bax protein and upregulation and relocation of p53 (110). Knockdown of Bax and Bak proteins conferred significant protection against DATS-induced apoptotic cytoplasmic histone-associated DNA fragmentation (113).

DADS has also been reported to inhibit the growth of Hras oncogene transformed tumors in nude mice (114), thus suggesting that other mechanisms may account for a reduction in tumor proliferation and increase in apoptosis. Allicin has been reported to induce activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) in human peripheral mononuclear cells and also in wild-type Jurkat T-cells (115). Allicin failed to activate ERK1/2 in Jurkat T cells that express p21(ras), in which Cys118 was replaced by Ser. Because these cells are not susceptible to redox-stress modification and activation, the authors suggested that immunomodulation may be involved with the redox-sensitive signaling such as activation of p21(ras) (115).

Cell Differentiation and Angiogenesis

Lea et al. (116) suggest that at least part of the ability of DADS to induce differentiation in DS19 mouse

erythroleukemic cells relates to its ability to increase histone acetylation. DADS caused a marked increase in the acetylation of H4 and H3 histones in DS19 and K562 human leukemic cells, and more recently in HL 60 cells (117). Allyl mercaptan is a particularly potent inhibitor of histone deacetylase (HDAC) (118). Histone deacetylase inhibition has the potential to derepress epigenetically silenced genes in cancer cells, leading to cell cycle arrest and apoptosis. Interestingly, DADS inhibition of HDAC has also been reported to be sufficient to stimulate adipogenesis (119). Dashwood and colleagues have screened several garlic-derived small organosulfur compounds for their ability to inhibit HDAC activity in vitro (118,120). Chromatin immunoprecipitation assays identified hyperacetylated histone H3 on the P21WAF1 gene promoter within four hours after allyl mercaptan exposure, which correlated with increased binding of the transcription factor Sp3 (120). Their findings suggest a primary role for Sp3 in driving P21 gene expression after HDAC inhibition by allyl sulfur compounds and coincided with cell cycle arrest. In contrast to the effect on histone acetylation, there was a decrease in the incorporation of phosphate into histones when DS19 cells were incubated with 25 μ M (S)-allylmercaptocysteine (121).

Alliin has been reported to cause a dose-dependent inhibition of fibroblast growth factor-2 (FGF2)-induced human endothelial cell tube formation and angiogenesis in the chick chorioallantoic membrane (CAM) model (122). Both vitamins C and E significantly ($p < 0.001$) enhanced the inhibitory efficacy of alliin on angiogenesis (122). The water-soluble allyl compounds in AGEs have also been reported to inhibit the growth of colorectal cancer cells and their angiogenic properties (123). Xiao et al. (124) suggest that the antiangiogenic characteristics of DADS relate to its ability to downregulate vascular endothelial growth factor (VEGF) secretion and VEGF receptor-2 protein level and inactivation of Akt kinase.

Dietary Modifiers of Garlic and Allyl Sulfur Efficacy

The influence of garlic on the cancer process cannot be considered in isolation, because several dietary components and the intake of drugs can markedly influence its overall impact (40,92,125,126). Garlic may enhance the pharmacological effect of anticoagulants (e.g., warfarin, flutindione) and reduce the efficacy of anti-AIDS drugs (i.e., saquinavir), although it is difficult to predict how important these interactions truly are for the general public (127,128). Among the dietary factors recognized to influence the response to garlic are total fat, selenium, methionine, and vitamin A (125). Selenium, supplied either as a component of the diet or as a constituent of the garlic, has been reported to enhance the protection against 7,12 dimethylbenz(a)anthracene mammary carcinogenesis over that provided by garlic alone (125,129). Suppression in carcinogen bioactivation, as indicated by a reduction in DNA adducts, may partially account for this combined benefit of garlic and selenium; both selenium and allyl sulfur compounds are recognized to alter cell proliferation and induce apoptosis. Other food components bring about enhanced efficacy by reducing the amounts of allyl sulfur required to bring about a response or by influencing

an additional molecular target and thereby bringing about a synergist response.

SUMMARY AND CONCLUSIONS

Garlic is a plant within the genus *Allium* that appears to have multiple characteristics that may significantly promote health and disease prevention. While it is possible that all allium foods possess similar health benefits, there is a dearth of comparative studies. Because garlic has few side effects, there are few disadvantages associated with its enhanced use, except for its lingering odor. However, odor does not appear to be a prerequisite for many of the health benefits because preclinical studies indicate that water-soluble (S)-allyl cysteine (predominates in deodorized preparations) sometimes provides benefits comparable to those provided by compounds that are linked to odor. It is probable that garlic and its associated water- and lipid-allyl sulfur compounds influence multiple and key molecular targets associated with cancer prevention. While most can savor the culinary experiences identified with garlic, some individuals because of their gene profile and/or environmental exposures may be particularly responsive to more exaggerated intakes. While there continues to be considerable interest in the health benefits of garlic, the number of controlled clinical studies limits the conclusions that can be drawn. Likewise, the dearth of studies that have contrasted the importance of source and/or dose of specific garlic preparations makes interpretation of existing data particularly challenging. At this point, the most prudent advice may be to continue to enjoy the savory characteristics of garlic as frequently as possible.

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Ginger

Tieraona Low Dog

INTRODUCTION

Ginger is a popular spice and its world production is estimated at 100,000 tons annually, of which 80% is grown in China (1). In addition to its long history of use as a spice, references to ginger as a medicinal agent can be found in ancient Chinese, Indian, Arabic, and Greco-Roman texts. Ginger has been used for a variety of conditions, but it is chiefly known as an antiemetic, anti-inflammatory, digestive aid, diaphoretic, and warming agent. In the year 2005, ginger sales ranked 20th among those of all herbal supplements sold in U.S. mainstream retail stores (2).

NAME AND GENERAL DESCRIPTION

The Zingiberaceae family consists of 49 genera and 1300 species, of which there are 80 to 90 species of *Zingiber* and 250 species of *Alpinia*. This entry will focus primarily upon the scraped or unscraped rhizome of common ginger, *Zingiber officinale* Roscoe, a reed-like plant grown in numerous subtropical areas of the world, including Jamaica, India, China, and Africa (3).

CONSTITUENTS

Ginger rhizome contains 4% to 10% oleoresin composed of nonvolatile, pungent constituents (phenols such as gingerols and their related dehydration products, shogaols); nonpungent fats and waxes; 1.0 to 3.3% volatile oils of which 30% to 70% are sesquiterpenes, mainly β -bisabolene, (–) zingiberene, β -sesquiphellandrene, and (+) arcurcumene; monoterpenes, mainly geranial and neral; 40 to 60% carbohydrates, mainly starch; 9 to 10% proteins and free amino acids; 6 to 10% lipids composed of triglycerides, phosphatidic acid, lecithins, and free fatty acids; vitamin A; niacin; and minerals (4).

Gingerols are the most abundant pungent compounds in fresh rhizome, the most abundant being 6-gingerol. Shogaols, the dehydrated form of gingerols, are present only in small quantities in the fresh rhizome and are mainly found in the dried and thermally treated roots, with 6-shogaol being the most abundant (5).

PHARMACOKINETICS

A clinical trial was conducted to evaluate the pharmacokinetic profile of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol and their conjugate metabolites at six-dose levels of ginger extract standardized to 5% gingerols, 100, 250,

500, 1000, 1500, and 2000 mg, administered orally to 27 healthy human volunteers (6). Researchers found that the main pungent constituents of ginger root, 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol, are quickly absorbed and detected in the serum as glucuronide and sulfate conjugates, with the majority detected as glucuronide metabolites. These constituents, at concentrations normally found in ginger root (0.5%–2.5%), are detectable in the serum starting at a 1-g dose with the exception of 6-gingerol, which is detectable at a 250-mg dose with maximum concentrations ranging from 0.1 $\mu\text{g/mL}$ to 1.7 $\mu\text{g/mL}$. Ginger conjugates begin to appear 30 minutes after oral dosing, reaching their T_{max} between 45 to 120 minutes, with elimination half-lives ranging from 75 to 120 minutes at the 2-g dose.

As there are more clinical trials evaluating the effectiveness of ginger for a variety of conditions, pharmacokinetic studies using multidose modeling are essential to optimize dosing regimens and delivery systems.

PHARMACODYNAMICS

Antiemetic Activity

Numerous human clinical trials have addressed the antiemetic effects of dried ginger root in the treatment of hyperemesis gravidarum (7), motion sickness (8), postoperative nausea (9), and chemotherapy-induced nausea and vomiting (10). The mechanism of action and constituent(s) responsible for the antiemetic activity of ginger are not completely understood. A class of antiemetics found to be clinically effective in the treatment of chemotherapy-induced and postoperative nausea and vomiting are the 5-hydroxytryptamine (5-HT) antagonists, specifically 5-HT₃. Several components of ginger, viz., 6-gingerol, 6-shogaol, and galanolactone, have shown anti-5-HT activity in isolated guinea pig ileum. Galanolactone is a competitive antagonist predominantly at ileal 5-HT₃ receptors (11). A study in rats found that an acetone extract of ginger and ginger juice effectively reversed the cisplatin-induced delay in gastric emptying typically seen when the drug is administered. The reversal produced by the ginger acetone extract was similar to the effect seen with the 5-HT₃-receptor antagonist ondansetron; ginger juice, at doses of 2 and 4 mL/kg orally (p.o.) was superior to the drug (12). Other researchers have demonstrated that ginger increases gastrointestinal (GI) motility, reducing the feedback from the GI tract to central chemoreceptors (12), though a double-blind crossover trial of 16 healthy volunteers who were randomly allocated to receive either

1 g of dried ginger or placebo found no effect on gastric emptying (13).

Motion Sickness

Human studies evaluating the effects of ginger on experimentally induced motion sickness (14) and four human clinical trials evaluating the use of ginger for motion sickness have been published. The first randomized, double-blind, placebo-controlled study was published in 1988. Eighty Danish naval cadets (ages 16–19 yr) were randomized to receive either 1 g of dried ginger powder or placebo. Symptoms of seasickness were evaluated over the following four hours. Participants who received ginger powder experienced less seasickness than those in the control group ($P < 0.05$). No power calculation was included in the report.

A 1994 randomized, double-blind, nonplacebo-controlled study of 1475 volunteers (age 16–65 yr) traveling by sea compared the efficacy of seven antiemetic medications: Touristil[®] (cinnarizine 20 mg, clomperidone 15 mg), Marzine[®] (cyclizine 50 mg), Dramamine[®] (dimenhydrinate 50 mg, caffeine 50 mg), Permesin[®] (meclizine 25 mg, caffeine 20 mg), Stugeron[®] (cinnarizine 20 mg), Scopoderm TTS[®] (scopolamine 0.5 mg), and Zintona[®] (product standardized to minimum 1.4% volatile oils and minimum 2.0 mg gingerols and shogaols in capsule containing 250 mg ginger rhizome). Stugeron and Scopoderm TTS were administered the evening prior to departure, with a second dose of Stugeron being given the morning of sea travel. The other medications were administered 2 hour prior to departure, with Touristil and Zintona being administered again 4 hour later. None of the study medications offered complete protection from seasickness, with all offering similar rates of efficacy. In each treatment group, 4.1 to 10.2% experienced vomiting and 16.4 to 23.5% experienced nausea and discomfort. There was no statistical difference between groups. No serious adverse reactions were reported (15). Though interesting, the study did not include a baseline measurement of nausea/vomiting sensitivity.

A 1999 randomized, double-blind drug comparison study found the efficacy of ginger extract (Zintona) and dimenhydrinate to be similar when given to 60 cruise ship passengers (age 10–77 yr) with a history of motion sickness (16). Side effects were significantly less in the ginger group (13.3%) than in those receiving dimenhydrinate (40%). Comorbid conditions were not ruled out and no power calculation was included in the report.

Another 1999 randomized, double-blind study compared the efficacy of a ginger extract (Zintona) and dimenhydrinate in the pediatric population (17). Twenty-eight children, aged four to eight years, with a history of motion sickness as determined by questionnaire were enrolled in the trial. Fifteen subjects received ginger and 13 received dimenhydrinate. Subjects (3–6 yr) in the ginger group received 250 mg of ginger extract half an hour before the trip and, if necessary, 250 mg every 4 hour; children aged 6 and above received 500 mg half an hour before the trip and, if necessary, 500 mg every 4 hour. Children randomized to receive dimenhydrinate took 12.5 to 25 mg half an hour before the trip and, if necessary, 25 mg every 4 hour. Physicians' rating of the therapeutic effectiveness

showed highly significant difference between the treatment groups ($P < 0.00001$). Results were good in 100% of treatment cases in the ginger group, while in the dimenhydrinate group, they were modest in 69.2% and good in only 30.8%. All subjects in the ginger group reported symptom reduction within 30 minutes of taking the extract, while 69.2% in the dimenhydrinate group reported a reduction in 60 minutes ($P < 0.00001$). No patient in the ginger group reported any side effects, while most (84.6%) of the dimenhydrinate patients suffered from side effects, including dryness of the mouth (69.23%) and vertigo (23.07%). The difference in the treatment group was highly significant ($P < 0.001$). It is unclear when reading the study whether all of the children traveled by the same mode(s) of transportation. Also, the randomization process did not appear to allow for well-matched groups with regard to severity of motion sickness.

While the studies all show a beneficial effect for ginger on motion sickness, all have methodological shortcomings.

Nausea and Vomiting of Pregnancy

Nausea is likely to affect more than 50% of pregnant women, and frequently disrupts family and work routines (18). The most extensively studied botanical for nausea and vomiting of pregnancy is dried ginger rhizome (*Z. officinale*). A systematic review of six double-blind, randomized controlled trials (RCT) assessing the safety and effectiveness of ginger in the treatment of pregnancy-induced nausea and vomiting concluded that ginger may be an effective treatment for nausea and vomiting in pregnancy (19). Four of the six RCT ($n = 246$) showed superiority of ginger over placebo; while the other two RCT ($n = 429$) indicated that ginger was as effective as the vitamin B6 in relieving the severity of nausea and vomiting episodes. Regarding safety, a prospective cohort observational study of 187 pregnant women and RCT (including follow-up periods) showed the absence of significant side effects or adverse effects on pregnancy outcomes. However, the authors noted that more observational studies, with a larger sample size, are needed to confirm the encouraging preliminary data on ginger safety.

Since the publication of the systematic review, three additional RCTs have been published. Two RCTs ($n = 196$) found that ginger (1000 mg/d and 650 mg t.i.d.) and vitamin B6 (40 mg/d and 25 mg t.i.d.) were both effective in reducing nausea and vomiting episodes (20,21). Chittumma et al. found that 650 mg t.i.d. of ginger was more effective than 25 mg t.i.d. vitamin B6 ($P < 0.05$), while the study by Ensiyeh et al. reported greater relief of nausea with ginger compared to vitamin B6. The other RCT compared 500 mg b.i.d. ginger powder to 50 mg b.i.d. dimenhydrinate over a period of 7 days (22). Dimenhydrinate was more effective for reducing vomiting episodes during the first 48 hours, though there was no difference from days 3 to 7. There was more drowsiness and sedation in the dimenhydrinate group.

In summary, clinical trials suggest that ginger may be considered a useful treatment option for women suffering from pregnancy-induced nausea and vomiting at doses ranging from 1000 to 1850 mg per day of powdered ginger.

Chemotherapy-induced Nausea and Vomiting

Chemotherapy-induced nausea and vomiting significantly reduces patients' quality of life, increases fatigue and anxiety, and increases costs of health care delivery. An abstract published in 1987 reported that of 41 patients with leukemia randomly assigned to receive either oral ginger or placebo after administration of intravenous compazine, there was a significant reduction in nausea in those who received ginger compared with those who received placebo (23). This report was followed by a small open study of 11 patients who were undergoing monthly photopheresis therapy (psoralen and the chemotherapy agent 8-MOP) and regularly complained of nausea as a result (24). Patients were given 1.6 g of powdered ginger 30 minutes prior to the administration of 8-MOP and then evaluated their nausea on a scale of 0 to 4. Total score for nausea decreased from 22.5 (individual average 2.045) prior to the trial to 8.0 (individual average 0.727) after administration of ginger. Three patients complained of heartburn. The study suffered from lack of blinding and placebo arm.

In 2003, a more rigorous randomized, prospective, crossover, double-blind study was carried out in 60 patients ($n = 50$) receiving cyclophosphamide in combination with other chemotherapeutic agents (10). Patients with at least two episodes of vomiting in the previous cycle were included and randomly assigned to receive one of three antiemetics: 1 g p.o. dried ginger powder given 20 minutes prior to chemotherapy and repeated 6 hours after chemotherapy; 20 mg IV metoclopramide 20 minutes prior to chemotherapy and 10 mg p. o. 6 hour after chemotherapy; or 4 mg IV ondansetron 20 minutes prior to chemotherapy and 4 mg p.o. 6 hours after chemotherapy. Lactulose capsules and normal saline IV were used, where appropriate, to maintain blinding. Patients were admitted to the hospital for 24 hours and observed for the incidence of nausea and vomiting, and adverse effects, if any, were recorded. Patients were crossed over to receive the other antiemetic treatments during the two successive cycles of chemotherapy. Complete control of nausea was achieved in 86% with ondansetron, 62% on ginger, and 58% with metoclopramide. Complete control of vomiting was achieved in 86% with ondansetron, 68% of patients on ginger, and 64% with metoclopramide. No adverse effects attributable to ginger were recorded. In summary, the antiemetic effect of ginger was comparable to that of metoclopramide, but ondansetron was found to be better than both.

A randomized, double-blind, placebo-controlled trial of 162 patients with chemotherapy-induced nausea and vomiting undergoing treatment at the University of Michigan failed to find any beneficial effect on nausea or vomiting when ginger was given after the start of chemotherapy with or without 5-HT₃ receptor antagonist and/or aprepitant. The study used two doses of ginger extract (1.0 g/d or 2.0 g/d) per day or a matching placebo. It should be noted that this was a 10:1 extract and each 250 mg capsule contained 15 mg (5%) total gingerols. A 10:1 extract typically means that 1-g extract is equivalent to 10 g crude herb equivalent. A relatively high dose compared to the doses of 1 to 2 g per day of powdered crude ginger used in other trials. This study did not pretreat with ginger but provided the first dose one hour after completing chemotherapy. Participants receiving the high dose

of ginger (2.0 g) reported having significantly more severe episodes of delayed nausea compared to both placebo and low-dose ginger (25).

A study of 644 patients undergoing chemotherapy randomly assigned them to receive either a placebo or one of three doses of ginger in capsule form (0.5 g, 1.0 g, or 1.5 g). The placebo or ginger was divided into two doses given each day for six days, starting three days before chemotherapy. Patients also received 5-HT₃ receptor antagonist plus dexamethasone (anti-nausea medications) on day 1 of chemotherapy. Information about nausea was collected during the first four days of the chemotherapy cycle. All three doses of ginger were more effective than the placebo at reducing nausea, though the two lower doses (0.5 g and 1.0 g) appeared to have the greatest effect and there was no effect on vomiting. These results suggest that the addition of ginger supplements to conventional antiemetic medications reduces chemotherapy-induced nausea (26).

Postoperative Nausea and Vomiting

Postoperative nausea and vomiting (PONV) is one of the most common complaints following anesthesia and surgery. The incidence of PONV is 20 to 30% during the first 24 hours after anesthesia. As a part of oral premedication, ginger is being studied due to its lack of known relevant side effects (e.g., sedation), high patient acceptance, and low cost (27). A meta-analysis of five randomized controlled trials ($n = 363$) found the relative risks of ginger for postoperative nausea and vomiting and postoperative vomiting were 0.69 (95%, confidence interval 0.54–0.89) and 0.61 (95%, confidence interval 0.45–0.84), respectively. Only one side effect—abdominal discomfort—was reported (28). Two additional placebo-controlled studies published after the meta-analysis were also positive for reduction of PONV when a minimum of 1 g ginger powder was given one hour prior to surgery (29,30).

Anti-inflammatory Activity

In vitro and animal models have shown that ginger inhibits both cyclo-oxygenase and lipo-oxygenase pathways (31). Intraperitoneal administration of crude hydroethanolic ginger reduced rat paw edema induced by carrageenan and inhibited serotonin-induced skin edema (32).

Osteoarthritis

Present-day therapy for osteoarthritis (OA) is principally directed at symptoms, since there is no well-established disease-modifying therapy. Treatments generally involve a combination of nonpharmacologic and pharmacologic measures, utilizing a combination of analgesia, and anti-inflammatory and intra-articular therapies (33). Srivastava and Mustafa (34) published two collections of anecdotal reports on the beneficial effects of ginger on rheumatological complaints more than a decade ago. Two clinical trials have been published since that time. Ginger extract (170 mg/day EV.EXT 33) was compared to placebo and ibuprofen (400 mg/day) in 67 patients ($n = 56$) with osteoarthritis of the hip or knee in a controlled, double-blind, double-dummy, crossover study with a wash-out period of one week followed by three treatment periods in a randomized sequence, each of three weeks'

duration. Acetaminophen was used as rescue medication throughout the study. The ranking of efficacy was ibuprofen > ginger extract > placebo for visual analog scale scores on pain and the Lequesne index, but no significant difference was seen when comparing ginger extract and placebo directly (35). The lack of positive effects may have been due to inadequate trial length and/or insufficient dose.

A randomized, double-blind, placebo-controlled study enrolled 261 ($n = 247$) patients with OA of the knee as diagnosed by the American College of Rheumatology classification criteria (36). The primary efficacy variable was the proportion of responders experiencing a reduction in "knee pain on standing," using an intent-to-treat analysis. A responder was defined by a reduction in pain of ≥ 15 mm on a visual analog scale. During the six-week treatment period, patients ingested one capsule twice daily of 255 mg ginger extract (EV.EXT 77, extracted from 2500 to 4000 mg of dried ginger rhizomes and 500 to 1500 mg of dried galanga rhizomes) or placebo. The percentage of responders experiencing a reduction in knee pain on standing was superior in the ginger extract group compared with the control group (63% vs. 50%; $P = 0.048$). Analysis of the secondary efficacy variables revealed a consistently greater response in the ginger extract group compared with the control group, when analyzing mean values: reduction in knee pain on standing (24.5 vs. 16.4 mm; $P = 0.005$) and reduction in knee pain after walking 50 ft (15.1 vs. 8.7 mm; $P = 0.016$). One group of adverse events showed a significant difference between treatment groups: GI adverse events were more common in the ginger extract group [116 events in 59 patients (45%)] compared with the placebo group [28 events in 21 patients (16%)]. None of the GI adverse events were considered serious by the investigators.

Both of these studies suggest the strong need for dose escalation studies that can determine the most efficacious dose that it is still safe and well tolerated. Studies of longer duration are also required before more definitive conclusions can be drawn about the safety, tolerance, and effectiveness of ginger for osteoarthritis.

Cardiovascular Effects

Ginger shows considerable anti-inflammatory, antioxidant, anti-platelet, hypotensive and hypolipidemic effect in in vitro and animal studies, all of which suggest that it may have a beneficial role in cardiovascular health (37). In vitro research has shown that constituents in ginger have an inhibitory effect upon cholesterol biosynthesis (38). Animal studies have demonstrated lipid-lowering activity via enhancement of the activity of hepatic cholesterol-7 α -hydroxylase, the rate-limiting enzyme in bile acid biosynthesis, thereby stimulating conversion of cholesterol to bile acids, an important mechanism for eliminating cholesterol from the body (39). A study in rabbits found that an orally administered ethanolic extract of ginger (200 mg/kg) reduced lipids after 10 weeks' feeding of a cholesterol-rich diet. The authors found that, at this dose, ginger produced results similar to those of gemfibrozil (40). In contrast to the in vitro and animal data, a study of patients with coronary artery disease found that three months ingestion

of 4 g/day dried ginger powder failed to lower blood lipids (41).

Ginger inhibits platelet aggregation in vitro, acting as a potent inhibitor of arachidonic acid, epinephrine, adenosine diphosphate, and collagen. A placebo-controlled study of eight healthy males found that ingestion of 2 g of ginger caused a dose-dependent reduction of thromboxane synthetase and prostaglandin synthetase; however, no differences were found in bleeding time, platelet count, or platelet function between the placebo and control groups (42). In patients with coronary artery disease (CAD), powdered ginger administered in a dose of 4 g/day for three months did not affect adenosine diphosphate- and epinephrine-induced platelet aggregation. However, a single dose of 10 g produced a significant reduction in platelet aggregation induced by the two agonists (41).

Gastrointestinal Effects

Ginger has long been valued in traditional medicine for a wide variety of GI complaints. Researchers are beginning to explore possible scientific explanations for these historical uses. In vitro research indicates that constituents present in ginger have antiulcer activity (43). Animal research demonstrates that ginger reduces the occurrence of gastric ulcers induced by nonsteroidal anti-inflammatory drugs (NSAIDs) and hypothermic-restraint stress (44). Chologogic activity has been documented in rats with the acetone extract of ginger (45).

Helicobacter pylori (HP) is the primary etiological agent associated with dyspepsia, peptic ulcer disease, and development of gastric cancer. Novel, inexpensive, and safe approaches to the eradication of HP are currently being sought. A methanol extract of the dried, powdered ginger rhizome, fractions of the extract, and the isolated constituents, 6-, 8-, and 10-gingerol and 6-shogaol, were tested against (19) strains of HP (46). The extract inhibited the growth of all (19) strains in vitro with a minimum inhibitory concentration range of 6.25 to 50 $\mu\text{g/mL}$. One fraction of the crude extract, containing the gingerols, was active and inhibited the growth of all HP strains with a minimum inhibitory concentration range of 0.78 to 12.5 $\mu\text{g/mL}$.

Ginger may be of benefit in patients with gastroparesis, which can present with signs and symptoms such as heartburn, gastroesophageal reflux, early satiety, abdominal bloating, and nausea and/or vomiting several hours after eating a meal. Twenty-four healthy volunteers were studied twice in a randomized double-blind manner (47). After an eight-hour fast, the volunteers ingested three ginger capsules (total 1200 mg) or placebo, followed after one hour by 500 mL of low-nutrient soup. Antral area, fundus area and diameter, and the frequency of antral contractions were measured using ultrasound at frequent intervals over 90 minutes, and the gastric half-emptying time was calculated from the change in antral area. Antral area decreased more rapidly ($P < 0.001$) and the gastric half-emptying time was less after ginger than placebo ingestion (13.1 ± 1.1 vs. 26.7 ± 3.1 min, $P < 0.01$), whereas the frequency of antral contractions was greater ($P < 0.005$). There was no significant difference in any GI symptoms.

DOSE

The dose for ginger varies considerably between products; especially with the growing numbers of highly concentrated ginger extracts being promoted for their anti-inflammatory effects. Reading and understanding the dose on product labels can be challenging for consumers and clinicians alike. For instance when the label states that each capsule contains "Ginger rhizome extract 250 mg," there is no way to know how much ginger is actually in the product as no strength is provided for the extract. If the product contains a 20:1 extract, each 250 mg capsule would actually provide 5000 mg of crude ginger. Imagine a pregnant woman being told to take 250 mg of ginger four times per day for nausea and vomiting of pregnancy, purchasing this type of product and actually getting 20,000 mg per day of ginger! This is a real problem in the marketplace. The following are a few doses (serving sizes) reported in the research and literature:

- Fresh or dried rhizome: 1 to 4 g daily (48). Note: dried is more effective for nausea and vomiting.
- Fluidextract: 1:1 (g/mL) 0.25 to 1.0 mL t.i.d.; tincture 1:5 (g/mL) 1.25 to 5.0 mL t.i.d. (48).

CONTRAINDICATIONS AND ADVERSE EFFECTS

Because of its cholagogic effect, some experts recommend that those with active gallstone disease avoid large doses of ginger. Patients treated with ginger have reported increased flatulence and heartburn compared to those on placebo.

HERB-DRUG INTERACTIONS

None are known. There have been anecdotal and speculative warnings about ginger and warfarin; however, there are no documented cases in the literature. Standardized ginger extract had no significant effects on coagulation parameters or on warfarin-induced changes in blood coagulation in rats (49). Though evidence is lacking for a direct interaction between warfarin and ginger (50), it is probably still wise for practitioners and patients alike to be cautious about the use of doses greater than 4 g/day of dried ginger in conjunction with antiplatelet/anticoagulant medications.

TOXICITY

There is little risk of toxicity when used as a spice. Acute toxicity tests in mice found no mortality or adverse effects when ginger extract was given at doses up to 2.5 g/kg (by lavage) over a seven-day period. Increasing the dose to 3.0 to 3.5 g/kg resulted in 10 to 30% mortality (51).

USE IN PREGNANCY

Two studies have been published examining the effect of ginger in pregnant rats. One found that ginger tea (20 or

50 g/L) administered from gestation days 6 to 15 and then sacrificed at day 20 significantly increased early embryonic loss and increased growth in surviving fetuses (52). No gross morphologic malformations were seen in the treated fetuses. Teratogenic studies on ginger extracts at doses of 100 to 1000 mg/kg failed to observe any toxic effects or early embryonic loss (53).

Researchers at the Hospital for Sick Children in Toronto, Canada, studied 187 pregnant women who used some form of ginger in the first trimester. They report that the risk of these mothers having a baby with a congenital malformation was no higher than that in a control group (7). Of the published human studies, there was 1 spontaneous abortion out of 32 in the ginger group (19), 1 spontaneous abortion of 27 in the crossover design study (20), and 3 spontaneous abortions of 60 in the ginger group (21), although one of these occurred in a woman who had not begun taking the treatment. Though the total number of women in these clinical trials is small, the rate of spontaneous abortion is not any greater than that seen in the general population.

CONCLUSIONS

The research to date for ginger is most compelling for its effects on the GI tract. There is little doubt that ginger has significant anti-emetic activity and research clearly shows that it can help ease the nausea and vomiting that occurs during pregnancy, or due to surgery or chemotherapy. The studies also suggest that lower doses of dried ginger (1–2 g) may be most efficacious for this purpose and that ginger must be given before and after chemotherapy for beneficial effects. As mentioned under the dosing section, researchers must be specific with regards to the type of extract used in the clinical trial as many readers will not understand that a 250 mg ginger extract (10:1) is equivalent to 2500 mg of crude ginger. And clinicians must be familiar with products in the marketplace in order to counsel patients about the safe and effective use of ginger for any medical condition.

The ability of ginger to enhance gastric motility and emptying in patients with heartburn and gastroparesis should be further explored. Many of our motility agents (e.g., metoclopramide) can have significant side effects, making natural remedies such as ginger an attractive alternative. In addition, ginger has gastroprotectant activity and is active against HP, an organism that is becoming more resistant to drug treatment and is a leading cause of gastric cancer around the world.

The potential role for ginger as an anti-inflammatory agent is intriguing based on basic science, though the limited human research has not been compelling. The studies also indicate that when patients consume high doses of 6 to 10 g of ginger each day, many will have gastric upset and irritation. Thus, better delivery systems and dosage forms will be necessary if this type of research is to continue. And if the National Institutes of Health is any indication of research interest, with more than eight clinical trials of ginger under way in 2010, there is probably much more we can learn from this popular and flavorful medicinal spice.

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Ginkgo

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INTRODUCTION

The tree *Ginkgo biloba* L. has a long history of use in traditional Chinese medicine. The ginkgo tree is classified in its own family and order, and holds a special position in evolutionary plant history because it provides a connection between the seedless vascular plants and seed plants. In recent years, the leaf extract of *G. biloba* has become one of the most widely used herbal remedies and is sold as a phyto-medicine in Europe and as a dietary supplement worldwide. Ginkgo extracts are used for the treatment of cerebral dysfunction and circulatory disorders, and have been studied in several animal experiments and clinical trials. There are a wide variety of chemical constituents in the extract, with the principal components being ginkgolides and bilobalide (terpene trilactones, TTLs) and flavonoids.

BACKGROUND

Ginkgo biloba L., or the maidenhair tree (Fig. 1), is the only surviving member of its family (Ginkgoaceae) and order (Ginkgoales), underscoring its unique phylogenetic status. Fossil records show that the *Ginkgo* genus was present some 180 million years ago. The Ginkgoaceae peaked 130 million years ago, with numerous widespread species, but gradually gave way to modern angiosperms. Today, only one species, *G. biloba*, survives, and it occurs naturally only in eastern parts of China. The morphology of the ginkgo tree itself appears to have changed very little over 100 million years, and for this reason it is often called a “living fossil.”

The ginkgo tree takes its name from *ginkyo* in Japanese and *yinhsing* in Chinese; both words translate to “silver apricot,” referring to the appearance of the ginkgo nuts. The term “ginkgo” was first used by the German physician and botanist Engelbert Kaempfer in 1712, but Linnaeus provided the terminology “*Ginkgo biloba*” in 1771. The ginkgo tree can grow up to 40 m high, with a stem diameter between 1 and 4 m, and can reach an age of more than 1000 years. Vertical growth generally slows down with the onset of sexual maturity at around 25 years. The appearance of the tree varies from slim and conical to full and rounded, with gray bark deeply furrowed on old trees. The ginkgo tree has characteristic green, leathery, fan-shaped leaves that turn golden yellow in autumn. In young specimens, the leaves are divided into two distinct lobes, and hence the notation *biloba* (from Latin *bi*, double; *loba*, lobes). The Ginkgo species is dioecious, having separate male and female trees.

Among seed plants with a reproductive system, the ginkgo is a primitive tree, and its reproductive organs resemble those of seedless vascular plants such as ferns. Ginkgo provides an important evolutionary connection between seedless vascular plants and seed plants, as discovered by Japanese botanist Sakugoro Hirase more than a 100 years ago. In the spring, before the leaves emerge, male *G. biloba* trees produce catkins rich in pollen, while female trees produce 2- to 3-mm long ovules. Each ovule secretes a small mucilaginous droplet that catches the airborne pollen and transports it inside the ovule, where multiflagellated spermatozoids are produced. A spermatozoid fertilizes the female egg cell, and the seed is shed from the tree approximately one month after fertilization. Fully mature ginkgo seeds, also known as ginkgo nuts, have a pungent smell due to the presence of butanoic and hexanoic acids in the fleshy sarcotesta surrounding the seed (Fig. 2) (1).



Figure 1 A *Ginkgo biloba* tree.



Figure 2 Leaves and nuts of *G. biloba*.

Cultivation and History of Use

The ginkgo tree has been cultivated in China for several thousand years and, according to some of the earliest written ginkgo tree references dating back to the Song dynasty of the early 11th century, the tree was appreciated for its beauty and for its edible nuts. The tree was introduced into Japan from China in the 12th century, and some 500 years later into Europe and North America. The use of *G. biloba* for medicinal purposes was first mentioned in 1505 A.D. in a book by Liu Wan-Tai. In the Chinese materia medica *Pen Tsao Ching* from 1578, *G. biloba* is described as a treatment for senility in aging members of the royal court. In these old records, it is mainly the use of nuts that is described. Raw ginkgo nuts without the fleshy sarcotesta are described in traditional Chinese medicine as a treatment for a variety of lung-related ailments, including asthma and bronchitis, as well as some kidney and bladder disorders. The use of *G. biloba* leaves has played only a minor role in traditional Chinese medicine, but in the modern Chinese Pharmacopoeia, the leaves are considered beneficial for the heart and lungs.

The ginkgo tree can persist in conditions of low light and nutrient scarcity and is highly resistant to bacteria, fungi, and viruses. Furthermore, it is resistant to air pollution; this has made *G. biloba* a popular roadside tree in urban areas of Japan, Europe, and northern America. In China, the ginkgo tree is cultivated partly to meet demands for ginkgo nuts, a delicacy in Chinese and Japanese cuisine alike. The kernel is obtained by boiling the nuts until the hard shell cracks open; this kernel is subsequently boiled with sugar or roasted. Unfortunately, raw ginkgo nuts contain the toxin 4-O-methylpyridoxine, which can result in serious food poisoning.

Ginkgo biloba Extract

For pharmaceutical purposes, an extract of *G. biloba* leaves was first introduced in Western countries in 1965 by the German company Dr. Willmar Schwabe under the trade name Tebonin. Later, Schwabe established a collaboration with the French company Beaufour-Ipsen, and to-

gether they developed a standardized *G. biloba* extract (GBE) termed EGb 761 (Extrait de *Ginkgo biloba* 761), which was sold under trade names such as Tanakan, Rökan, and Tebonin forte. Other *G. biloba* products have entered the market, and GBE is now among the best selling botanical preparations worldwide. Rising demand for GBE has spurred the increased harvesting of *G. biloba* leaves, and today, more than 50 million *G. biloba* trees are grown, especially in China, France, and the United States, producing approximately 8000 tons of dried leaves each year. The yellow or green leaves are harvested in mid-to-late summer and then dried and pulverized. Through various extraction procedures, the active constituents are concentrated and undesired constituents such as organic acids are discarded. The composition of the leaf extract varies considerably and is related to the age of the plant, growth conditions, and time of harvest. To ensure the quality of GBE and the concentration of flavonoids and terpene trilactones, the presumed active constituents have been standardized.

CHEMISTRY AND PREPARATION OF PRODUCT

Ginkgo leaves contain a wide variety of phytochemicals, including alkanes, lipids, sterols, benzenoids, carotenoids, phenylpropanoids, carbohydrates, flavonoids, and terpenoids, particularly terpene trilactones (2). The major constituents are flavonoids and polyphenolic compounds that are widely distributed in the plant kingdom and are found in all green plants. Flavonoids are pigments responsible for the colors yellow, orange, and red in autumn leaves and various flowers, and are also present in wine and tea. To date, more than 30 flavonoids have been found in *G. biloba*; the diversity arises from different glycoside substitutions of the flavonol aglycone. The flavonoids of GBE are almost exclusively flavonol-O-acylglycosides, including mono-, di-, or triglycosides of the flavonol aglycones quercetin and kaempferol primarily substituted at the 3-position (Fig. 3). Additionally, nonglycosidic biflavonoids, such as bilobetin, ginkgetin, and isoginkgetin (Fig. 3) and proanthocyanidins such as procyanidin and prodelphinidin have also been isolated from *G. biloba*.

The TTLs comprise five diterpenes named ginkgolide A, B, C, J, and M and the sesquiterpene bilobalide. These compounds are unique and found only in the ginkgo tree. The ginkgolide cage structure consists of six 5-membered rings, including three lactones, a tetrahydrofuran ring, and a spiro[4.4]nonane skeleton. The ginkgolides differ in the positions and numbers of hydroxyl groups on the spirononane framework. In bilobalide, rings A and F are absent and the tetrahydrofuran ring of the ginkgolides (ring D) is replaced by a lactone. The ratios of TTLs vary by season and between different parts of the tree.

The TTLs are unique constituents of the ginkgo tree and have attracted great interest because of their complex structures and reported biological activities (3). Ginkgolides were first isolated from the root bark of *G. biloba* by S. Furukawa in 1932, and their structures were elucidated in 1967. The structure of bilobalide was determined in 1972. The ginkgolides have inspired many studies, particularly as targets for complex total synthesis, as

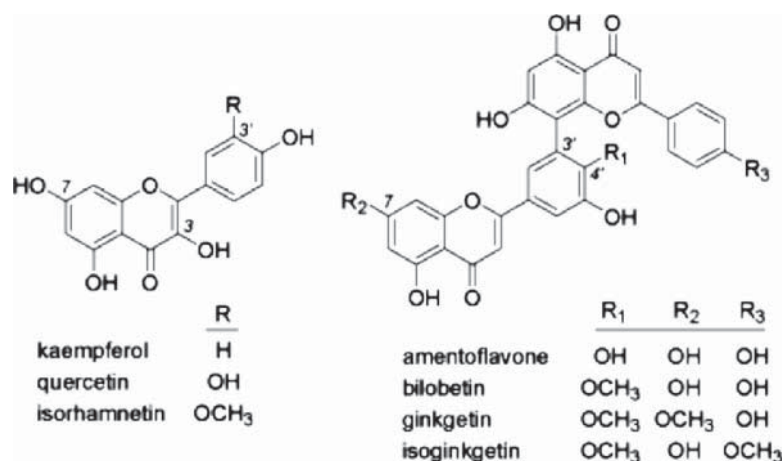


Figure 3 Structures of flavonoids.

templates for structure–activity relationship studies, and as terpenes with a surprising and novel biosynthetic pathway. Prior to these studies, it was thought that all terpenes were biosynthesized through the mevalonate pathway, but by examining the biosynthesis of ginkgolides, Arigoni and coworkers proved that terpenes can be synthesized through the deoxyxylulose phosphate or nonmevalonate pathway (4).

Formulation and Analysis

Standardized extracts (dry extracts from dried leaves, extracted with acetone and water) contain 22% to 27% flavone glycosides and 5% to 7% terpene lactones, of which approximately 2.8% to 3.4% are ginkgolides A, B, and C and 2.6% to 3.2% is bilobalide. Qualitative and quantitative determination of flavonoid glycosides is carried out after hydrolysis to the aglycones kaempferol, quercetin, and isorhamnetin. The qualitative presence or absence of biflavones is determined by high performance liquid chromatography. Qualitative and quantitative determination of TTLs (ginkgolides and bilobalide) is by high performance liquid chromatography or gas–liquid chromatography. Certain commercial products such as EGb 761 do not contain biflavones, and the level of ginkgolic acids should be below 5 mg/kg, because of their allergenic potential. Coated tablets and solutions for oral administration are prepared from these standardized, purified extracts.

PRECLINICAL STUDIES

A vast number of preclinical studies have investigated the *in vitro* and *in vivo* effects of GBE as well as the individual components of GBE, particularly the TTLs, ginkgolides and bilobalide. The major components of all GBEs are flavonoids and TTLs, and it is believed that these two classes of compounds are responsible for the biological effects of GBE. In most cases, GBE has been investigated in *in vitro* or *in vivo* assays, or the extracts have been

screened in DNA arrays. The other primary approach has been to investigate single chemical components, such as the flavonoids, ginkgolides, and bilobalide, in a wide variety of assays. Although many biological effects can be explained by the individual components, it has often been suggested that GBE acts by a synergistic mechanism. The effects of GBE can be grouped into three related categories: (i) effects related to antioxidant activity; (ii) effects on gene expression; and (iii) direct effect on protein function, with these effects sometimes overlapping or being related.

The antioxidant effects of GBE are well documented, particularly using *in vitro* studies. These effects are most likely because of the flavonoids, which are well-known free radical scavengers and antioxidants. Specifically, it has been shown that GBE can scavenge nitric oxide, protect against lipid peroxidation of low-density lipoproteins, and inhibit the formation of oxygen radicals. It is believed that numerous disease states are related to free radicals. Therefore, it has been speculated that the antioxidant effects of GBE could be used to treat diseases such as atherosclerosis and cancer, as well as a number of neurodegenerative diseases such as Alzheimer's and Parkinson's disease.

The effects of GBE on gene expression have been the subject of several investigations in recent years, and a general consensus has emerged that this effect is critically important when looking at the clinical effects of GBE (5). Gohil and coworkers used high-density oligonucleotide microarrays to study neuromodulatory effects in mice that had EGb 761-supplemented diets (6). Twelve thousand genes and expressed gene tags from the hippocampus and cerebral cortex of the mice were analyzed for changes in gene expression, and of these, 10 were found to change more than threefold as a result of EGb 761 administration. Several of these 10 genes may be relevant to neurodegenerative disorders.

In another approach, the expression of peripheral benzodiazepine (PB) receptors, proteins involved in cholesterol transport and many other biological events, was studied (7). Animal studies have shown that GBE treatment decreases cell proliferation in tumors with PB

receptor overexpression (8). Most clinical studies of GBE have looked at effects on various forms of neurodegenerative disease, particularly as potential treatment of Alzheimer's disease (AD). Together with the accumulation of intracellular neurofibrillary tangles, deposition of β -amyloid plaques is the primary indication for AD, and it is believed that an increase of β -amyloid plaques is central to the pathogenesis of AD. Therefore, the recent findings that GBE and individual ginkgolides (9) can inhibit β -amyloid aggregation (10), as well as prevent neuropathy and neuron dysfunction (11), form the basis for continued research into the effects of GBE in relation to AD. The two major components of GBE are flavonoids and TTLs, and in contrast to the wealth of studies that have been performed using GBEs, far fewer investigations have looked at the effect of the individual components of these extracts. However, flavonoids and TTLs are thought to be responsible for most of the pharmacological properties of GBEs. An important consideration when looking at the effects on the central nervous system (CNS) is the bioavailability, including penetration of the blood-brain barrier, of these components. It has been assumed that the bioavailability of flavonoids is low, whereas TTLs, in particular the ginkgolides, are nearly completely bioavailable. Recent studies indicate that ginkgolides can penetrate the blood-brain barrier, although only in limited amounts. Though such studies cannot predict which of the components of GBE are efficacious, bioavailability is obviously a critical parameter when evaluating the physiological effects of GBE.

Flavonoids possess many biological activities, and they act as antioxidants, free radical scavengers, enzyme inhibitors, and cation chelators. They also show anti-inflammatory, antiallergic, anti-ischemic, immunomodulatory, and antitumoral action (12). The pharmacological effects of flavonoids in GBE have mainly been attributed to their utility as antioxidants and free radical scavengers. Because the flavonoids present in GBE are almost entirely flavonol glycosides, it is expected that these compounds or their metabolites play a key role in these events; however, as mentioned above, their bioavailability might be a limiting factor.

The number of studies on the biological effects of ginkgolides increased dramatically in 1985, when it was reported that ginkgolides, particularly ginkgolide B (GB), are antagonists of the platelet-activating factor (PAF) receptor. The clinical application of GB (BN 52021) as a PAF receptor antagonist was investigated, but, as is true of all other antagonists of the PAF receptor, GB was never registered as a drug, primarily because of a failure to demonstrate efficacy. The clinical studies, however, showed that GB was well tolerated and showed very few, if any, side effects. A large number of ginkgolide derivatives that have been prepared and tested for their ability to antagonize the PAF receptor and several derivatives showed increased potency in comparison to the native ginkgolides. Together, these studies have led to a clearer understanding of the structural features required for PAF receptor antagonism (3).

Recently, it was found that ginkgolides are potent and selective antagonists of glycine (Gly) receptors. The Gly receptors are found primarily in the spinal cord and

brain stem, as well as in higher brain regions such as the hippocampus. They are, together with γ -aminobutyric acid (GABA_A) receptors, the main inhibitory receptors in the CNS. Electrophysiological studies showed that GB antagonizes Gly receptors in neocortical slices (13) and hippocampal cells (14), and suggested that GB binds to the central pore of the ion channel, acting as a noncompetitive antagonist. Molecular modeling studies showed a striking structural similarity between picrotoxinin, an antagonist of both GABA_A and Gly receptors, and ginkgolides (13). Thus, ginkgolides are highly useful pharmacological tools for studying the function and properties of Gly receptors. However, the physiological importance of this antagonism remains to be investigated.

Several studies have shown that ginkgolides, particularly ginkgolide A and ginkgolide B can modulate PB receptors. These receptors are located mainly in peripheral tissues and glial cells in the brain, and are distinct from the benzodiazepine site on GABA_A receptors. PB receptors are typically located on the outer membranes of mitochondria. The function of PB receptors is not entirely clear, but involvement in steroidogenesis, cell proliferation, and stress and anxiety disorders has been suggested. The primary action of GB is the inhibition of the expression of PB receptors (15). Several studies have indicated that ginkgolides also protect against various damaging CNS events, such as excitotoxicity, ischemia and other cerebrovascular and traumatic brain injury, as well as inflammation. The mechanisms behind these effects are not entirely clear and are probably multifaceted.

Bilobalide (BB) is the predominant TTL found in GBE. A wealth of pharmacological evidence indicates that BB might be a very important compound when looking at neuromodulatory properties of *G. biloba* constituents. Several studies have shown that BB affects the neuronal transmission mediated by the neurotransmitters glutamate, GABA and glycine, but the mechanism of action is still unclear. BB has been found to interfere with the release of glutamate under hypoxia/hypoglycemic conditions. Furthermore, it was demonstrated that BB is an antagonist of GABA_A receptors (16), but this effect seems only to account for a small part of the neuroprotective effect of BB (17).

Because antagonists of inhibitory receptors, particularly GABA_A and glycine receptors, are known convulsants, the result of BB acting on GABA_A receptors and ginkgolides on glycine receptors could pose a risk to patients ingesting GBE. In confirmation, a study of two epileptic patients showed an increased frequency of seizures with GBE administration. This increase was reversed when the patients stopped taking the extract (18). These results indicate that people with a low seizure threshold, such as epileptic patients, should be cautious when taking GBE.

Potential medicinal applications of BB have been described in patents, including use of BB for the protection of neurons from ischemia, as an anticonvulsant, and for treatment of tension and anxiety. BB inhibits brain phospholipase A₂ activity, leading to a neuroprotective effect, and several studies have shown that BB preserves mitochondrial respiration, especially under ischemic conditions.

CLINICAL STUDIES

A large number of clinical trials have been conducted using GBEs and, in most cases, these trials have examined effects related to dementia. Specifically, changes in memory, thinking, and personality in aging people were studied. In almost all of these investigations, the standardized extract EGb 761 was administered and, although various dosing regimens were employed, daily doses of 120 to 240 mg EGb 761 were most commonly used.

Generally, clinical studies have shown that GBE can lead to an improvement in the symptoms associated with cerebral insufficiency, such as memory loss, depression, and tinnitus. In Germany, GBE is registered as an herbal medicine to treat cerebral insufficiency. This is a diagnosis covering a range of conditions, as illustrated by the list of indications from the German Commission E: "disturbed performance in organic brain syndrome within the regimen of a therapeutic concept in cases of demential syndromes with the following principal symptoms: memory deficits, disturbances in concentration, depressive emotional condition, dizziness, tinnitus, and headache. The primary target groups are dementia syndromes, including primary degenerative dementia, vascular dementia, and mixed forms of both (19)." In two seminal clinical studies, a total of 549 AD patients were evaluated for effects of EGb 761 treatment (20,21). In both studies, EGb 761 significantly slowed the loss of cognitive function, which is a symptom of dementia, and regression on certain data points was delayed by 7.8 months, which is comparable to the currently available AD treatments—Aricept (donepezil, 9.5 mo) and Exelon (rivastigmine, 5.5 mo), both acetylcholinesterase inhibitors.

Kleijnen and Knipschild reviewed 40 GBE clinical studies, which examined the efficacy of GBE in cerebral insufficiency (22). In the studies, the standard dose was 120 mg/day for at least four to six weeks. Of the 40 trials, only 8 were considered to be of acceptable methodological quality. The problems with many of the studies included small patient numbers, inadequate description of randomization procedures, inadequate patient characterization, and insufficient data presentation. Essentially, all the eight acceptable trials reported positive results, and no serious side effects were reported. It was concluded that future studies could provide a detailed efficacy assessment of GBE treatment. A more recent review by Knipschild and colleagues summarized 55 additional clinical studies, which also looked at the effect of GBE on cerebral insufficiency. Knipschild reports that although there is good evidence for a GBE effect, this evidence was obtained from an excessively small patient population and further, larger trials are required (23).

A meta-analysis systematically reviewed more than 50 clinical studies on GBE for the treatment of dementia and cognitive malfunctions associated with AD. Only 4 of the 50 studies met the inclusion criteria for the evaluation; these 4 studies included more than 400 patients. It was concluded that administration of 120 to 240 mg of GBE for three to six months had a small but significant effect on objective measures of cognitive function in AD, without significant adverse effects in formal clinical trials (24).

In 2002 and 2003, two clinical studies cast doubt on the positive clinical effect of GBE seen in almost all previous investigations. Both these studies included a larger number of patients, were carefully designed, and were randomized, double-blinded, and placebo-controlled. Knipschild et al. completed a clinical trial with 214 patients, who received GBE for 24 weeks. The patients suffered from either dementia or age-associated memory impairment, and no GBE treatment-related improvement was seen (25). In another study with 203 people older than 60 years who were given GBE for six weeks, no beneficial effect from the GBE treatment was observed (26); the results of this investigation have been heavily debated. In an evaluation from the Cochrane Library, Birks and Evans have critically reviewed 33 clinical studies, which were all randomized and double blind (27). The duration of the studies varied from 3 to 52 weeks, although the majority were conducted for 12 weeks. The participants were all diagnosed with either dementia or age-associated memory impairment, although some of the earlier studies did not fully verify the diagnoses. In their conclusion, Birks and Evans state that GBE appears to be safe and with no side effects compared to placebo, and that there is promising evidence for improved cognition and function with GBE treatment. However, the authors also note the results of recent trials that did not show GBE-related improvement, and therefore suggest that further clinical trials are required.

From 2000 to 2008 the U.S. National Institutes of Health sponsored the Ginkgo Evaluation of Memory Study, which enrolled 3069 volunteers aged 75 and older across five academic medical centers. This randomized, double-blind, placebo-controlled clinical trial found no effects of GBE in the rate of AD and all-cause dementia (28). Because the study is regarded as sufficient in scale and observational duration, but contradicts convincing preclinical evidence, the focus of research is shifting to whether the current standard GBE schedule is sufficient in terms of both daily amount and dosing duration (29). In France, the pharmaceutical company Ipsen is sponsoring another clinical trial, the GuidAge study, which aims to examine prevention of AD in patients older than 70 years with memory impairment. The results of this 2854-patient study are expected in 2010 (30) and will be of major importance in the continued evaluation and determination of the effects of GBE in relation to dementia.

A primary function of GBE may be to improve blood flow and to inhibit platelet aggregation, the latter through inhibition of the PAF receptor by ginkgolides. Therefore, the vascular effects of GBE administration naturally invite examination, and a number of clinical trials have looked at effects of GBE treatment in relation to peripheral vascular conditions. A meta-analysis of clinical trials investigating the effect of GBE on intermittent claudication, an early symptom of peripheral arterial disease, was carried out by Pittler and Ernst (31). Of 12 clinical trials conducted, 8 were included in this analysis, and the authors concluded that GBE is superior to placebo in the symptomatic treatment of intermittent claudication. However, it was noted that the overall magnitude of the treatment effect was modest and its clinical relevance was uncertain.

The effect of GBE on healthy people has also been examined. Several clinical studies indicate improved cognitive functions. This effect is still controversial, as illustrated by a recent evaluation of the clinical trials studying GBE efficacy in healthy persons (32). Canter and Ernst found nine placebo-controlled double-blind trials, which were generally of acceptable methodological quality. None of these short-term (< 31 days) trials indicated consistent positive effects from GBE treatment. The authors conclude that the benefit from GBE on cognitive function is not proven and that there is a particular need for further long-term investigations with healthy subjects (32).

Several studies have looked at the adverse effects and toxicity of GBE. Generally very few, if any, serious side effects from GBE have been found. Studies of acute GBE toxicity in mice and rats showed that doses of up to 10 g/kg did not result in lethal effects and higher doses could not be administered. In chronic toxicity studies with rats, doses up to 500 mg/kg caused no significant changes that could be observed after sacrificing the animals. In vitro studies and studies in mice and rats showed no mutagenic, carcinogenic, or teratogenic effects. In humans, the toxicity of orally administered GBE is generally considered low, and no significant interactions with other medications have been reported. Hemorrhage has been reported in connection with the use of Ginkgo products but a clear causality between intake of GBE and bleeding could not be established.

CONCLUSION

In conclusion, there is mounting evidence from clinical studies to suggest that GBE may positively affect a range of cognitive and peripheral vascular conditions. Other recent studies have questioned the benefit of GBE on cognitive disorders, but ongoing clinical trials seek to settle this controversy in the future. There is no evidence for any adverse effects from GBE administration.

REGULATORY STATUS

In most European countries, GBE is registered as an herbal medicine, whereas it is available as a dietary supplement in the United States.

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Ginseng, American

Chong-Zhi Wang and Chun-Su Yuan

INTRODUCTION

Ginseng has been widely used for thousands of years as a traditional medicine in many oriental countries. During the past few decades, ginseng roots and their extracts have also become increasingly popular in the United States and Europe as dietary supplements and additives to foods and beverages. American ginseng (*Panax quinquefolius*) and Asian ginseng (*Panax ginseng*) are the two most recognized herbs around the world. Based on Traditional Chinese Medicine theory, Asian ginseng is considered to stimulate and invigorate yang, whereas American ginseng is considered to calm and nourish yin while promoting the secretion of bodily fluids (1,2).

American ginseng has gained impressive popularity with the American public in the past 20 to 30 years. It is now cultivated with a production of over 1000 tons of dried roots in the northern United States, Canada, and northeastern China. The biologically active constituents of this plant have been pursued extensively and it is accepted that the triterpene saponins called ginsenosides are the major active ingredients (3,4). Ginsenosides are also distributed in many plant parts of the herb, including the roots, leaves, flowers, and berries. American ginseng is reported to have a wide range of therapeutic and pharmacological applications, such as tonic, anti-aging, immunomodulating, anti-fatigue, antidepressant, anti-diabetic, and anti-tumor activities (5,6).

The medical importance of American ginseng has led to the development of a wide spectrum of analytical methods for the determination of the total saponin content, target compound analysis, and group-specific analysis or metabolite profiling. The pharmacokinetics and metabolism of different compounds from American ginseng have been studied and discussed in animals and human subjects (7).

American ginseng is generally considered safe when used orally. The Food and Drug Administration (FDA) has previously considered ginseng as "generally recognized as safe" or "GRAS." However, adverse effects after ginseng administration have been reported, and ginseng may also interfere with prescription drugs, and interact with food, and diseases or conditions. During the last 10 years, more than 1000 publications on American ginseng focused on biological activities, clinical investigations, chemistry and analysis, as well as pharmacokinetics and potential ginseng-drug interactions have been reported and highlighted.

BACKGROUND

As a member of the plant family Araliaceae, American ginseng (*Panax quinquefolius* L.) is distributed in the eastern temperate forest areas of North America from southern Quebec to Minnesota in the north to Oklahoma, the Ozark Plateau, and Georgia in the south. The part of the plant commonly used in remedies is the root. It was first introduced in the "New Compilation of Materia Medica" by Yi-Luo Wu in 1757 (8). In the western world, it was recorded in Quebec, Canada, by Father Lafitau in the early 18th century, and since then has generated a lot of interest (9). This perennial herb thrives in hardwood forests on the north- and east-facing slopes in predominantly porous, humus-rich soils that receive a lot of rain. American ginseng also grows on southwest-facing slopes, in soils where sand or clay is characteristic, and in forests with conifers and softwoods, but most wild populations thrive in upland, north- and east-facing woods where shade and loam soils are typical. Methods of cultivation, botanical characteristics, and authentication of this medicinal plant have been extensively described (10). There are three kinds of American ginsengs available on the market: cultivated (organic and inorganic), simulated wild, and wild. Like all ginsengs, those growing wild are the best, and the most expensive.

CHEMISTRY AND PREPARATION OF PRODUCTS

Chemistry

Up to 2009, approximately 150 constituents including saponins, polysaccharides, polyacetylenes, phytosterols, oils, acids, carbohydrates, flavonoids, nitrogen-containing compounds, and vitamins have been isolated and characterized from American ginseng (11). The major and most important bioactive components are triterpene saponins. Most of the saponins are dammarane glycosides (12).

More than 60 saponins have been isolated from different parts of this plant including roots, leaves, stems, flower buds, and berries. These different saponins, however, can be divided into two major groups; some such as ginsenosides Rb₁, mRb₁, Rb₂, mRb₂, Rb₃, Rc, mRc, Rd, mRd, Rg₃, 20(R)-Rg₃, Rh₂, and quinquenosides I, II, III, V, and R₁ belong to the protopanaxadiol group. Others like ginsenosides Re, Rg₁, Rg₂, 20R-Rg₂, Rg₈, Rh₁, and 20R-Rh₁ belong to the protopanaxatriol group (13–15). In addition, other saponins such as ginsenoside Ro (oleanane

type), pseudoginsenoside F₁₁ (ocotillol type) have also been isolated from American ginseng (5). During the past two years, new ginsenosides have been continuously isolated and elucidated, these include quinquenoside L₁₇ (16), quinquefoloside-Lc (17), quinquenosides L₁₀, L₁₄, and L₁₆ (18,19), and new acetylated ginsenosides (19). Zhu et al. (20) isolated a new ceramide from transgenic crown galls of American ginseng. While conducting a research on the flower buds, Nakamura et al. (21) isolated five new dammarane-type triterpene glycosides, floralquinquenosides A, B, C, D, and E, together with 18 known dammarane-type triterpene glycosides and 3 flavonoid glycosides.

Polysaccharides and glycoproteins are also important bioactive compositions in American ginseng. In bioassay-guided isolation, a water-soluble polysaccharide PPQ5-2, which shows lymphocyte stimulation and interleukin-induction activities, was isolated and characterized (22). Another polysaccharide, Cvt-e002, was isolated and observed to reduce the allergic immune response in vitro (23). Dried American ginseng root contains 24.9% to 28.9% of starch and 11.0% to 12.4% of proteins (11).

Preparation and Analysis

Different solvents and methods were utilized to prepare extracts from American ginseng roots. Refluxing in 100% methanol was found to have better extraction efficiency than water or 70% aqueous methanol extraction (24). Ligor et al. (25) compared accelerated solvent extraction, the ultrasound-assisted solvent extraction, and mechanical shaking-assisted solvent extraction, finding the highest extraction efficiency during shaking. Using supercritical fluid extraction, Wood et al. (26) observed that up to 90% of the total ginsenosides were extracted compared to conventional methanol Soxhlet. This led to the isolation and characterization of a novel 6''/6'''-mono-*O*-acetyl ginsenoside Rb₁.

For analysis of constituents in American ginseng, conventional spectroscopies such as Fourier transform infrared spectroscopy and Raman spectroscopy were seldom applied (27,28). High-performance liquid chromatography (HPLC) was the most often used technique for ginsenoside analysis because it is low-cost, readily available, and easy to use. The ultraviolet (UV)/diode array detector (DAD) or evaporative light scattering detector (ELSD) were the routinely used detectors. For the UV detector, the detection wavelength was normally set at 202 nm owing to very few chromophore groups of ginsenosides. The UV detection showed a good linearity in a 100 time ranges of concentration, and a limit of 10 ng of ginsenosides on the column (29). In contrast, the sensitivity of the ELSD was found to be five times lower than that obtained with the UV, with minimum detectable concentration of 50 ng of ginsenosides on the column (30). In view of the better sensitivity and easier handling, the UV is recommended for the routine analysis of American ginseng samples. Recently, HPLC coupled with mass spectrometry (MS) has become the widely used technique for both qualitative and quantitative analysis of ingredients in American ginseng. HPLC-MS combines the efficient separation capability of HPLC and great

power of structural characterization and high sensitivity and selectivity of MS detection. The glycosidic linkages, the core, and the attached sugar(s) of the ginsenosides can be determined (31). Using high performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (HPLC-APCI-MS), a total of 30 ginsenosides were identified and characterized, the differences of ginsenoside profile can be used to distinguish Asian and American ginseng (32,33).

Based on component analysis and comparison, individual and total ginsenoside contents vary substantially among *Panax* species and ages, among different origins, and among different plant parts. In general, the content in wild grown root is higher than that in cultivated ones. However, there is a high variation of dry weight and total ginsenoside contents of individual wild roots ranging from 1% to 15% (34), which is likely influenced by genotype and environmental factors. Ginsenosides Rb₁, Rb₃, Rc, Rd, Re, and Rg₁ are six major saponins in American ginseng, accounting for more than 70% of total ginsenoside contents (2). Overall, ginsenoside Rb₁ is the most abundant, followed by Re and Rg₁/Rd. It is interesting to note that concentrations of Rg₁ and Re are inversely related among and within populations (35). Among different plant parts of American ginseng, the leaves, berries, rootlets, and rhizomes contain higher ginsenoside contents, followed by the main roots and stems. The leaves contain dramatically high levels of ginsenoside Rb₃ and Rd, the berries have the highest concentration of ginsenoside Rb₃, up to 5.35% (36), while higher contents of Rb₁ and Re are present in the main roots, rootlet, and rhizomes. The total contents of ginsenosides in main roots, rootlet, and rhizomes increase with the age of the plant. In contrast, the ginsenoside contents in the leaves and stems decrease with a year of growth (37).

The chemical composition and content of the steamed American ginseng is quite different from the fresh one. During the steaming process for American ginseng roots, ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, Rb₃, and Rd decreased while ginsenosides Rh₁, Rg₂, 20R-Rg₂, Rg₃, and Rh₂ increased. Ginsenoside Rg₃, a recognized anti-cancer compound, increased significantly between 0.5 to 3 hours of steaming. Heat processing increased the antiproliferative effect of American ginseng significantly (38). A similar phenomenon is observed for American ginseng berries (36).

PRECLINICAL STUDIES

Effects on Cardiovascular System

Cardiovascular disease continues to be the leading cause of death in the United States. Recent studies have incriminated reactive oxygen species in the pathogenesis of both acute and chronic heart disease. Many botanicals possess anti-oxidant properties, and these herbal anti-oxidants may protect against cardiovascular diseases by contributing to the total anti-oxidant defense system of the human body. Earlier studies have suggested that extract from the stems and leaves of American ginseng improves the lipid profile of hyperlipidemic rats and has anti-oxidant properties in cultured rat cardiac myocytes (39). Total American ginseng saponins administered to rats having the

myocardium damaged by injury to the left anterior descending coronary artery were also shown to protect the myocardium with an anti-ischemic action probably related to a decrease in free fatty acid levels and an elevation of lactate dehydrogenase activity. The total saponins may also have produced a Ca^{2+} channel blocking effect (40). American ginseng root saponins also displayed the ability to significantly decrease platelet aggregation rates and to increase superoxide dismutase activity in hyperlipidemic rats (41).

The acute anti-oxidant and protective effect of American ginseng berry extract (AGBE) has been demonstrated in cultured cardiomyocytes (42). Later works suggest that pretreatment with AGBE upregulates peroxide detoxifying mechanisms, which could affect intracellular oxidant dynamics in cardiomyocytes (42,43). A detailed study has shown that ginsenoside Re, the major constituent in AGBE, functions as an anti-oxidant, protecting cardiomyocytes from oxidant injury induced by both exogenous and endogenous oxidants, and that its protective effects may be mostly attributed to scavenging H_2O_2 and hydroxyl radicals (44). In an acute myocardial infarction (AMI) rat model, the effect of American ginseng saponin can protect myocardium from ischemic injury in rats after AMI by way of promoting angiogenesis in the infarcted or ischemic area of myocardium and upregulating expressions of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in myocardial cells (45). The anti-oxidant components and properties of American ginseng and their potential role in treating cardiovascular illness have been reviewed, the anti-oxidant activities of active constituents, and the relationship between their chemical structures and biological functions was also discussed (46,47).

Antihyperglycemic Effects

Type II diabetes is a serious chronic metabolic disorder worldwide. This disease represents a syndrome with disordered metabolism of carbohydrate and fat. The hypoglycemic ability of ginseng root extracts has been known for a few decades (48). Based on previous reports, extracts from American ginseng root, leaf, and berry showed antihyperglycemic activities.

Early research showed that both American ginseng and Asian ginseng roots possess significant anti-diabetic effects in C57 BL/6J ob/ob and C57 BL/Ks db/db mice (49–51). Ginsenoside Rb₁, the major constituent in American ginseng root, was found to possess anti-diabetic and insulin-sensitizing activities (52). It was reported that ginsenoside Rb₁ stimulates glucose transport in insulin-sensitive cells by promoting translocations of glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) by partially activating the insulin signaling pathway (53). In a separate study, ginsenoside Rb₁ was observed to promote glucose-stimulated insulin secretion through protein kinase A (PKA), which augmented insulin receptor substrate 2 (IRS2) expression to enhance insulin/insulin-like growth factor 1 (IGF-1) signaling (54). In addition to ginsenosides, other compounds from American ginseng root also showed anti-diabetic effects. An activity-guided fractionation and isolation of the American ginseng root extract led to identification of three glycans, quinquefolans

A, B, and C, which displayed hypoglycemic effects in normal and alloxan-induced hyperglycemic mice (55).

It is believed that nutrients did not accumulate beyond the root of the American ginseng plant, and thus research into its parts shied away from testing the leaf and berry for medicinal effects. Not surprisingly, there was no previous information on the biological activity of American ginseng leaf and berry. Our work demonstrated significant antihyperglycemic activity with the American ginseng leaf and berry extracts. Following a 12-day treatment using ob/ob mouse model, which exhibits profound obesity and hyperglycemia, to test the pharmacological effects of the extracts, we observed that both 150 and 50 mg/kg groups of the American ginseng leaf extract significantly decreased fasting blood glucose and improved glucose disposal in ob/ob mice suggesting an improvement in insulin sensitivity (56). Pharmacological studies on the AGBE suggested that the berry extract significantly reduced fasting hyperglycemia and body weight following a 12-day treatment (49,57). Ginsenoside Re was identified as an active anti-diabetic constituent in AGBE (58).

It is reported that oxidative stress was linked to diabetes (59–61). As a botanical anti-oxidant, American ginseng may protect against diabetes by contributing to the total anti-oxidant defense system of the human body (62,63). However, a recent study demonstrated that the anti-diabetic effects of American ginseng may not be linked to anti-oxidant activity (62).

Effects on Central Nervous System

Ginsenosides in American ginseng are responsible for its effects on the central nervous system (CNS) and the peripheral nervous system (64). Ginsenosides regulate various types of ion channels, such as voltage-dependent and ligand-gated ion channels, in neuronal and heterologously expressed cells. Ginsenosides inhibit voltage-dependent Ca^{2+} , K^{+} , and Na^{+} channel activities in a stereospecific manner (65). Ginsenosides also inhibit ligand-gated ion channels such as *N*-methyl-D-aspartate, some subtypes of nicotinic acetylcholine, and 5-hydroxytryptamine type 3 receptors. Competition and site-directed mutagenesis experiments revealed that ginsenosides interact with ligand-binding sites or channel pore sites and inhibit open states of ion channels. The effect of ginsenoside Rg₁ or Rb₁ was also examined and they both enhance CNS activities, but the effect of the latter is weaker (66), sometimes even having an inhibitory effect on the CNS. American ginseng has a lower ratio of Rg₁ to Rb₁ content. Thus, American ginseng is “cool” or calming to the CNS. The protective effects of Rb₁, Rg₁, Rg₃, and Rh₂ on neurodegeneration are well studied (64,67). Ginsenoside Rb₁ obtained from American ginseng roots was also shown to be able to partially prevent the memory deficits caused by the cholinergic agent scopolamine in a rat model (68).

Anti-cancer Effects

Effects of American ginseng on cancer treatment and side-effect management were evaluated. These observations include in vitro and in vivo anti-cancer activity alone and in combination with chemotherapy, potential mechanisms of action of anti-cancer effect, chemopreventive effect

supported by epidemiologic data, and effects on preventing or alleviating chemotherapy-induced side effects.

In *in vitro* studies, American ginseng extracts were found to inhibit the growth of breast cancer cells (69,70). Recently, our group observed that after steaming treatment of American ginseng, its antiproliferative effects were improved significantly, possibly due to the altered ginsenoside profile (38,36). In another study, the anticolorectal cancer effects of AGBEs were evaluated. In comparison to unsteamed extract, steamed extract showed significantly stronger antiproliferative effects on cancer cells. Antiproliferative effects of representative constituents were also evaluated showing that ginsenoside Rg₃ had a positive effect. Steamed American ginseng extract inhibited the colorectal cancer growth both *in vitro* and *in vivo*, and this inhibition might be achieved through cell cycle arrest and induced apoptosis in the cells (71). In addition, the extract from American ginseng enhanced the antiproliferation effect of cisplatin on human breast cancer cells, suggesting that ginseng possesses its own anti-cancer activity (72).

Additional studies suggested that American ginseng and its purified ginsenosides may inhibit cancer cell proliferation by inducing gene and protein expression of the cell cycle regulatory protein p21, thus arresting tumor cell cycle progression (73), by inducing cancer cell apoptosis through activation of caspase-3 protease via a bcl-2-insensitive pathway and by sensitizing multidrug-resistant tumor cells to chemotherapy (74,75). To further characterize downstream genes targeted by steamed American ginseng extracts in a human colorectal cancer cell line, the gene expression profiling was assayed. This interesting research work showed that the most affected pathway was the Ephrin receptor pathway. The top upregulated and downregulated genes were identified. This important knowledge could further be used to develop ginseng derivatives as novel chemotherapeutic and/or chemopreventive agents for human cancer (76).

For cancer chemoprevention, epidemiologic evidence based on a case-control study on over 1000 subjects in Korea showed that Asian ginseng users had a decreased risk for many different cancers compared with nonusers (77,78). Data suggested that ginseng has a nonorgan-specific preventive effect against cancer (79).

The commonly used cancer chemotherapies are still limited by severe side effects and dose-limiting toxicity. The drug-related adverse events not only worsen patients' quality of life, but can also lead to their refusal to continue the potentially curative chemotherapy. Our group investigated the effects of several herbal extracts on reducing chemotherapeutic side effects and found that American ginseng can attenuate cisplatin-induced nausea and vomiting in a rat model (80) without affecting its anti-cancer properties in human cancer cells (72).

Effects on Immune System

It is generally accepted that ginseng's anti-cancer effects should be linked to its activity with the immune system. Ginsenosides from American ginseng enhance the formation of antibodies and immune functions in cancer patients and in microbe-infected experimental laboratory animals,

possibly by elevation of the cyclic adenosine monophosphate (cAMP) levels. Both antitumoral activity and stimulation of the immune function have been observed in early experimental animals and in human subjects (81).

Ginsenosides Rb₂, Rc, and Rg₁ were subsequently confirmed as stimulants of DNA synthesis in bone marrow cells possibly by the involvement of cyclic nucleotides (82). Therefore, preparations of American ginseng may be useful in the prevention and treatment of hematopoietic damage induced by chemical agents. An *in vivo* study showed that total saponins of American ginseng partially restored the activity of cyclophosphamide-depressed bone marrow stem cell proliferation and splenocyte proliferation in mice and enhanced production of interleukin-3 (IL-3) and IL-6-like substances from the splenocytes (45). It may also oppose the depressant effects of cyclophosphamide and other chemotherapeutic agents on the bone marrow stem cell proliferation by control of hemopoietic growth factor production in the splenocytes (83).

A polysaccharide-rich extract of American ginseng showed a number of biological effects, including the enhancement of lymphocyte transformation, induction of interferon- γ (IFN- γ) and IL-1 production, and stimulation of spleen cell production of an IL-3-like cytokine activity (84). Recently, in comparison to American ginseng saponin extract, the effect of this polysaccharide-rich extract on systemic and gut-associated immune function was evaluated. Feeding the polysaccharide-rich extract modifies systemic immune responses and appears to affect gut-associated immunity in a manner distinct from that of saponin-containing extracts of American ginseng (85).

CLINICAL STUDIES

Cardiovascular System

Several beneficial effects on the cardiovascular system have been attributed to the use of American ginseng, thus proving its popularity as an alternative in cardiovascular therapeutics (47). In a randomized, double-blinded, placebo-controlled trial, 16 hypertensive individuals received 3 g of American ginseng powder or placebo. After treatment, blood pressure was measured every 10 minutes for 160 minutes. There was no difference in the effects of American ginseng and the placebo on the overall (160 minutes) mean blood pressure change of the individuals. Since an early observational study suggested that Asian ginseng could elevate blood pressure, the administration of American ginseng caused concern. This observation suggested that American ginseng exerts a neutral acute effect on blood pressure in hypertensive individuals (86).

In another double-blinded, placebo-controlled, crossover trial in 52 hypertensive individuals to determine the effect of 12-week American ginseng intake on 24-hour blood pressure, after a 4-week placebo run-in, 52 participants were randomly assigned to 3 g/day of American ginseng or placebo for 12 weeks. American ginseng did not affect the blood pressure or serum cystatin C level. This study suggested that long-term American ginseng use had no effect on 24-hour blood pressure and renal function in hypertensive individuals (87).

The effects of American ginseng powder (1.6 g/day) on physically active male college students who received

exhaustive running exercise compared with placebo in a four-week randomized study have been reported. According to this study, although there was no noticeable improvement in the 80% $\text{VO}_{2\text{max}}$ of the experimental group over the placebo group, the production of plasma creatine kinase during the exercise significantly decreased for the American ginseng group than for the placebo group. Administration of American ginseng for four weeks prior to exhaustive aerobic treadmill running reduced the leakage of creatine kinase during exercise, but did not enhance aerobic work capacity. The reduction of plasma creatine kinase may be due to the fact that American ginseng is effective for the decrease of skeletal muscle cell membrane damage, induced by exercise during the high-intensity treadmill run (88).

Diabetes

Historical records on traditional medicinal systems reveal that ginseng root was used to treat a disease corresponding to diabetes (89). Description of ginseng's medicinal effects found in the ancient Chinese *Materia Medica* contained a reference to ginseng's ability to "quench thirst," amongst the other wide-ranging beneficial effects (90). This effect, as per Traditional Chinese Medicine, could be related to the anti-diabetic activity of ginseng. Research on the effects of treatment with ginseng root on blood sugar levels started in the early part of the last century (91). Ginseng root has since been used to treat diabetic patients (90). Results of clinical trials support the claim that ginseng root possesses anti-diabetic properties.

Seven clinical studies using American ginseng as a potential therapy for diabetes were found. A double blind placebo-controlled study in which ginseng root tablets (100 or 200 mg daily for eight weeks) were orally administered to 36 newly diagnosed type II diabetic patients showed a reduction in fasting blood glucose and glycosylated hemoglobin (92). On the other hand, some clinical trials from the same group were examining the herb's short-term effects. Vuksan reported antihyperglycemic activity when a single dose of 3 g American ginseng root was administered in both nondiabetic and type II diabetic individuals (93). Other short-term metabolic trials in healthy volunteers also found decreases in postprandial glucose (94–98). The available evidence for American ginseng in diabetes suggests a possible hypoglycemic effect; however, the trials are small and longer-term studies are needed (99).

Respiratory Infection

In a randomized, double-blind, placebo-controlled study, 89 (2000, 8 weeks) and 109 (2000–2001, 12 weeks) enrolled older adults, average age 81 and 83.5, respectively, were orally administered a proprietary American ginseng extract, 200 mg or placebo (twice daily) during the influenza season. An intent-to-treat analysis of pooled data corrected for drug exposure time showed that the incidence of laboratory-confirmed influenza illness was greater in placebo- (7 cases/101 subjects) than American ginseng-treated (1/97) groups ($P = 0.033$). Combined data for laboratory-confirmed influenza illness and respiratory syncytial virus illness were also greater in placebo- (9/101) than American ginseng-treated (1/97) groups ($P = 0.009$),

for an overall 89% relative risk reduction of acute respiratory illness in the American ginseng group. American ginseng was shown to be safe, well tolerated, and potentially effective for preventing acute respiratory illness due to influenza and respiratory syncytial virus (100).

In another double-blind placebo-controlled study, at the onset of the influenza season, 279 subjects (18–65 years of age) with a history of at least two colds in the previous year were instructed to take 400 mg daily of either the American ginseng poly-furanosyl-pyranosyl-saccharide-rich extract or a placebo for a period of four months. Data from this study suggested that the American ginseng extract reduced the mean number of colds per person, the proportion of subjects who experienced two or more colds, the severity of symptoms, and the number of days with cold symptoms (101).

Anti-fatigue

To investigate whether American ginseng might help cancer-related fatigue, in a randomized double-blind study, 290 cancer patients received American ginseng in doses of 750, 1000, or 2000 mg/day or placebo given in twice daily dosing over eight weeks. Overall, this study suggested that American ginseng, at a dose of 750 mg/day, did not provide any benefit over that seen with a placebo. However, the two highest doses of ginseng (1000 and 2000 mg/day) did appear to decrease fatigue more than did a placebo, as measured by various scales of fatigue, vitality, and well-being. There were no significant toxicities apparent in the active arms compared to the incidences seen in those taking the placebo. This was true despite the fact that many of these participants had advanced disease and were taking cytotoxic treatment for their disease. Data suggested that the higher doses studied may be helpful in cancer-related fatigue (102).

Prescription Drug Interactions

In a case report, the anti-coagulant effect of warfarin decreased after patients consumed ginseng (103). To evaluate the interactions between American ginseng and warfarin, a randomized, double-blind, placebo-controlled trial was conducted (104). In this four-week study, 20 subjects received warfarin for three days during weeks 1 and 4. Beginning in week 2, patients were assigned to receive either American ginseng or placebo. The peak international normalized ratio (INR) statistically significantly decreased after two weeks of ginseng administration compared with placebo. The INR area under the curve (AUC), peak plasma warfarin level, and warfarin AUC were also statistically significantly reduced in the ginseng group as compared with the placebo group. Although the study sample consisted of young, healthy volunteers in a research setting rather than patients taking therapeutic doses of warfarin, American ginseng was found to reduce warfarin's anti-coagulant effect. Data from this study suggested that when prescribing warfarin, physicians should ask patients about ginseng use (104).

Recently, interaction of American ginseng with antiretroviral chemotherapies was evaluated. In a clinical study, 24 healthy volunteers received indinavir 800 mg every 8 hours for 3 days and then indinavir and American ginseng 1 g every 8 hours or placebo for 14 days.

Indinavir pharmacokinetics and insulin sensitivity were assessed before and after American ginseng coadministration. There was no difference in the area under the plasma concentration time curve after the coadministration of American ginseng, compared to indinavir alone. Although insulin-stimulated glucose disposal per unit of insulin (M/I) decreased, M/I remained unchanged after coadministration of indinavir and American ginseng. This data suggest that American ginseng did not significantly affect indinavir pharmacokinetics (105).

In another recent study, 10 healthy volunteers received 300 mg of zidovudine (an antiretroviral agent) orally before and after two weeks of treatment with a ginsenoside-enriched American ginseng extract 200 mg twice daily. Pharmacokinetic profiles of zidovudine and oxidative stress marker concentrations were measured post-zidovudine dose. Two weeks of American ginseng did not alter zidovudine pharmacokinetics but reduced oxidative stress markers (106). It appears that American ginseng did not affect these two antiretroviral agents' pharmacokinetics.

PHARMACOKINETICS AND METABOLISM

The pharmacokinetic data are unavailable when American ginseng extract is administered. The metabolism and pharmacokinetics of single compounds from American ginseng have been studied using rats, dogs, and humans.

The average half-life time of ginsenoside Rd in plasma was detected as 19.29 hours, when 10 mg of ginsenoside Rd was administered intravenously to the volunteers. Seven metabolites including three oxygenated, two combined, and two hydrolyzed components were identified in rat urine samples by using liquid chromatography-mass spectrometry (LC-MS) (107). Ginsenoside Re was rapidly cleared from the body with a short half-life (0.2 ± 0.03 hours for male and 0.5 ± 0.08 hours for female mice after i.v.) and oral absorption was generally poor (108). The observed oral bioavailability of ginsenoside Rh₂ was 17.6% and 24.8% for male and female dogs, respectively (109). The absolute bioavailability of 20(S)-ginsenoside Rh₁ in rats was only 1.01% (110).

When taken orally, protopanaxadiol-type ginsenosides are mostly metabolized by intestinal bacteria to a protopanaxadiol monoglucoside, 20-O-beta-D-glucopyranosyl-20(S)-protopanaxadiol (M1). In humans, M1 is detected in plasma 7 hours after the intake of ginsenosides and in urine 12 hours after the intake, indicating that M1 is the final metabolite of this type of ginsenoside (111). Ginsenoside Rb₁ was not detectable in serum for 24 hours, indicating a major intestinal bacterial metabolism (112). In vitro and in vivo experiments showed that CYP450-catalyzed mono-oxygenation, intestinal bacteria-mediated deglycosylation, and gastric acid-mediated hydration reaction were the main metabolic pathways of this compound (110).

DOSAGE AND SAFETY

- No dosage suggestion (*U.S. Pharmacopoeia-NF 2008*)
- Up to 6 g, not for long-term use (*Chinese Pharmacopoeia, 2005 Edition*)

As references, Asian ginseng dosage: 1 to 2 g of root per day for up to three months, and a repeated course is feasible (113). Based on *PDR for Herbal Medicines*, the average daily dosage of Asian ginseng is 1 to 2 g root, and may be taken three to four times a day over three to four weeks (114).

In China, doses have been given up to 50 g, and some large doses have resulted in insomnia, depression, and nervousness. Regarding the so-called ginseng abuse syndrome (115), most reported adverse effects were increased blood pressure, nausea, headache, insomnia, nervousness, and diarrhea. To avoid this syndrome, recommended doses to be therapeutically effective were as low as 0.5 g (115). As Siegel stated, this dose can be used in a wide variety of commercial ginseng preparations, including Asian ginseng or American ginseng (115).

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Ginseng, Asian

Lee Jia and Fabio Soldati

INTRODUCTION

Recent revolutions in life science and technology including genomics, proteomics, nanotechnology, and highly sensitive chemical analysis methods have enriched our knowledge of *Panax ginseng* (*P. ginseng*; Fig. 1) and improved our understandings of this traditional herb medicine. The newly isolated and characterized chemical entities from *P. ginseng* added many new members to ginseng species. Preclinical in vitro and in vivo tests on the active ingredients of this species discovered some previously unknown biological functions of those ingredients. This review covers etymology and history of *P. ginseng*, the ways to cultivate and process it, and the related cookery. The review also lists ginseng's active ingredients (i.e.,

mainly ginsenosides) and standardized analytical methods used for quality control of various ginseng products as well as relevant government regulations. The active and inactive chemical entities obtained from *P. ginseng* can be classified into five categories that are saponins, polysaccharides, polyynes, flavonoids, and volatile oils. The recent preclinical findings of pharmacological effects of *P. ginseng* are narrated herein with critical discussions. These new findings include effects of ginseng on upregulating biological production of nitric oxide, which result in its aphrodisiac effect and cardiovascular effect; its anti-hyperglycemic effect that may benefit type II diabetics as evidenced by in vitro and in vivo studies using β -cells and obese diabetic mouse models; its neuropharmacological effect and its roles in cancer treatment. We also summarized pharmacokinetics and metabolism profiles of active ginseng ingredients, and analyzed its safety test results obtained from ginseng extract or ginsenosides. Various human clinical trials to test ginseng effectiveness on diabetics, psychophysiological performance, immunomodulation, and others are also discussed followed by information on ginseng overdose and drug interactions.

ETYMOLOGY AND HISTORY

The English word "ginseng" derives from the Chinese term "rénshēn" (simplified: 人参; traditional: 人蔘), literally "man root" (referring to the root's characteristic forked shape, resembling the human body and the legs of a man). The current Japanese word for "ginseng" is 人参, or "ninjin" meaning carrot, or "chosen ninjin," adopting the name of ninjin from the last dynasty of Korea (Choson). The Korean name of ginseng is 高麗人蔘, or "goryo insam."

Ginseng refers to the root of several species in the plant genus *Panax* (C.A. MEYER Araliaceae). The botanical name "*Panax*" was given by the Russian botanist Carl Anton von Meyer (1795–1855) in 1843 in the Bulletin of Physics and Mathematics published by the Academy of Petersburg Institute(1). In Greek, "pan" means all and "axos" means cure. *Panax* was applied to this genus after Carl Linnaeus was aware of its wide use in Chinese medicine. Carl Linnaeus (1707–1778, Swedish) had introduced over 9000 binomial names for both plants and animals. The earliest record of ginseng in Europe goes back to 1711 when a Jesuit, Father Jartoux, who worked in Chinese missions, sent a letter to the general procurator in Paris, describing this plant, which he had never seen before, as having immense therapeutic properties. The ginseng plant then remained practically unknown to the scientific Western world until the early 1960s when researchers

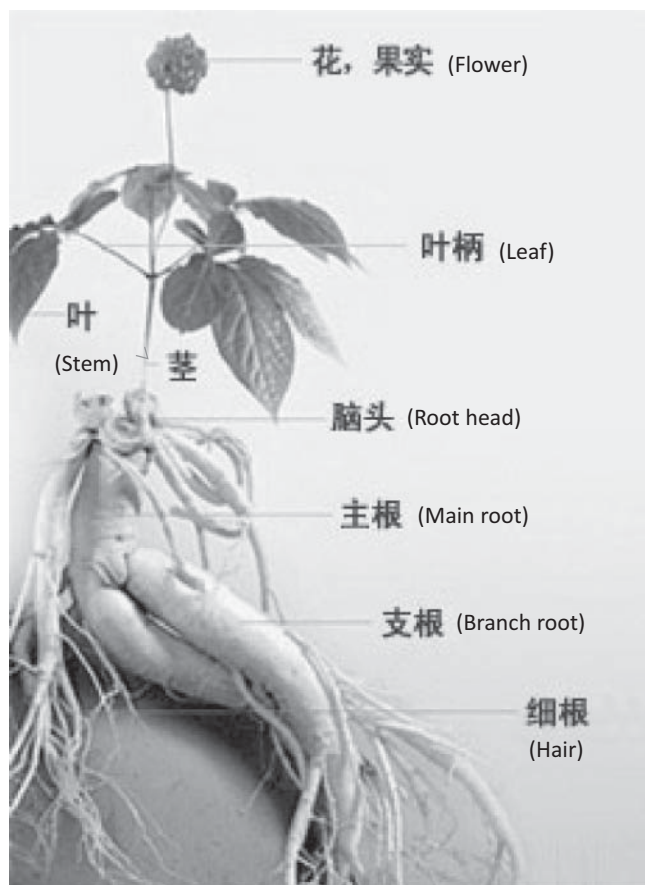


Figure 1 A typical *Panax ginseng*.

started to investigate its pharmacological and therapeutic properties. One of the major difficulties was the lack of demonstration of its healing power following protocols accepted by Western medicine (2).

Among them, *Panax ginseng* (*Panax ginseng* C.A. Meyer) is the most widely used ginseng and is indigenous to the Far East countries, most notably China and Korea (2). Cultivation of *P. ginseng* began at around 11 BC and has a medical history of more than 2000 years (3).

CULTIVATION AND PROCESSING

Ginseng grows in the Northern Hemisphere, typically in cooler climates. They are slow-growing perennial plants with fleshy roots (4,5). The plant grows 6 to 18 in. tall with 1 to 3 umbels of 15 to 30 flowers and greenish-yellow corollas. The fruit is a pea-sized, globular to reniform, scarlet, smooth, and glossy drupe, which contains two seeds (6). The plant usually bears three leaves, each with three to five leaflets 2 to 5 in. long. In China, Korea, Japan, and Russia, the cultivated ginseng roots are harvested when the plant is 3- to 6-year-old. The treasured aromatic root resembles a small parsnip that forks as it matures. It should be noted that ginseng is not derived from the rhizomes (the thick stem that lies flat along the ground with roots and leaves growing from it).

There are two different ways of processing ginseng after harvest: air drying and steaming the roots; the former results in white ginseng and the latter produces red ginseng. In Asia, the roots (which in nature are white) are sometimes treated with steam at approximately 100°C for differing times in order to protect them from microbiological pathogens. This high-temperature treatment results in the reddening of the epidermis of the roots producing the so-called "red ginseng." Not only are the sugars in the epidermis of the root caramelized by the heating, but the ginsenosides are also partially chemically modified and/or destroyed (7). In addition, the distribution of the ginsenosides is different in the various parts of the plant, both in total content and in the relative ratio of the different ginsenosides.

Ginseng cultivated in Korea is classified into three types, depending on how it is processed: fresh ginseng (less than 4 years old), white ginseng (4–6 years old and dried after peeling), and red ginseng (harvested when 6 years old, steamed and dried) (8). Red ginseng is not skinned before it is steamed or otherwise heated and subsequently dried. In the course of the steaming process, ginseng starch is gelatinized, causing an increase in saponin content. Traditionally red ginseng has been used to restore and enhance normal well-being, and is often referred to as an adaptogen.

COOKERY

The fresh root, after peeled, can be directly chewed, or soaked in various wines for a period of time before drinking and chewing. Ginseng is most often available in dried form, either in whole or sliced form. Ginseng leaf, although not as highly prized, is also used; as with the root it is most often available in dried form. The correct and

efficient cooking processes for the dried ginseng are as follows: washing the dried roots with running water; immersing the dried roots in boiled water and maintaining the roots in hot water for at least three hours or overnight; taking the soaked and hence soft roots out of water and slicing the roots with a knife as thin and small as possible; and finally stewing the sliced roots slowly and gently in water in a closed vessel before drinking all extracted liquid.

Ginseng is usually sliced and simmered in hot water to make a decoction. Ginseng ingredient may also be found in some popular energy drinks, tea varieties, or functional foods. Ginseng root is often sliced and steamed with chicken meat as a soup in China (including Taiwan) and Korea. Usually ginseng is used at subclinical doses for a short period and as such, it does not produce measurable medicinal effects. Daily consumption of ginseng root varies individually from 2 to 20 g depending on whether hypertension or high heart rate occurs at a given dose.

GINSENOSES: CHARACTERISTIC INGREDIENTS

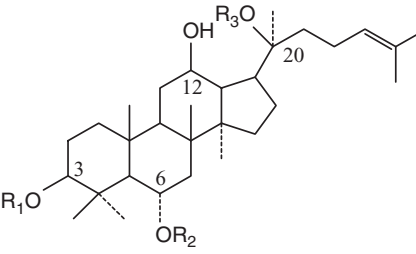
Ginseng is characterized by the presence of ginsenosides. Ginsenosides are triterpenes saponins considered to be the main bioactive principles of ginseng (Fig. 1). The content of ginsenosides and their metabolites varies depending on the *Panax* species, the plant age, the part of the plant, the preservation method, the season of harvest, and the extraction method (9,10). The ginsenoside content variability can also be in part ascribed to differing growth conditions, such as kind of soil, weather conditions, geographical location, and different production procedures. Table 1 lists various concentrations of ginsenoside and its metabolites that develop in complexity with age of ginseng (11). Soldati and Tanaka (12) also documented a relationship between the age of the plant and the content of ginsenosides: the major development of the roots occurs between the fourth and fifth year of growth, during which time the root doubles its weight, and the optimum yield of ginsenosides occurs at the fifth year.

Total ginsenosides content in *P. ginseng* varied from 0.2% to 2% for main roots and from 4% to 9% for root hair. Although the main source of ginsenosides is from ginseng root, the leaf, stem, and berry parts of ginseng also contain significant quantities of ginsenosides (13).

STANDARDIZED EXTRACTION AND QUALITY CONTROL

The U.S. Pharmacopoeia gives a detailed description of the quality of a standardized Asian ginseng extract: "Powdered Asian Ginseng Extract is prepared from Asian Ginseng by maceration, percolation, or both processes performed at room temperature with suitable solvents such as alcohol, methanol, water, or mixtures of these solvents, and by concentrating the fluid extract at temperatures below 50°. The ratio of the starting crude plant material to Powdered Extract is between 3:1 and 7:1. It contains not less than 3.0 per cent of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd combined, calculated on the anhydrous basis. It may contain other added substances." Moreover, the

Table 1 Structures of 7 Ginsenosides Commonly Used for Quality Control of Ginseng Products

				ST AN DA RD IZ ED EX TR AC TI ON
R1	OR2	R3		
Rb1	Glucose-2→1-glucose	2H	Glucose-6→1-glucose	
Rb2	Glucose-2→1-glucose	2H	Glucose-6→1-arabnose(pyr)	
Rc	Glucose-2→1-glucose	2H	Glucose-6→1-arabnose(fur)	
Rd	Glucose-2→1-glucose	2H	Glucose	
Re	H	O-glucose-2→1-rhamnose	Glucose	
Rf	H	O-glucose-2→1-glucose	H	
Rg1	H	O-glucose	Glucose	

USP gives detailed information on the methods for identification [thin layer chromatography (TLC)] and content determination [high-performance liquid chromatography (HPLC)] of the ginsenosides as well as limits for microbial contamination, water, pesticide residuals, heavy metals, organic volatile impurities, and alcohol content (14).

HPLC methods developed for the quantification of ginsenosides contained in the roots and in the finished products are also included in the U.S. Pharmacopoeia and European Pharmacopoeia for the definition of *P. ginseng* roots and *P. ginseng* extracts. Ginseng species were well described in the Chinese Pharmacopoeia when the second edition of the Pharmacopoeia was published in 1963, and the information is periodically updated to the current seventh edition, including the methods used for the quantification of ginsenosides.

According to the U.S. Pharmacopoeia published monographs, *P. ginseng* roots are defined to contain not less than 0.2% Rg₁ and 0.1% Rb₁ and *P. ginseng* extract, contains not less than 3.0% ginsenosides, calculated as the sum of ginsenosides Rb₁, Rb₂, Rc, Re, Rg₁, and Rd and determined by HPLC-UV. European Pharmacopoeia published a monograph on *P. ginseng* roots in which the content of ginsenosides Rb₁ and Rg₁, determined by HPLC-UV, is not less than 0.4%; moreover, the peak due to the ginsenoside Rf is present in *P. ginseng* roots (in case of a substitution with *P. quinquefolium* no peak due to ginsenoside Rf is present) (15). Concerning the literature data, besides the natural variation due to the heterogeneity of the plant material, the main reason of this variability of ginsenosides content is attributable to the choice of the compounds to be quantified in ginseng. Some authors quantified neutral ginsenosides Rb₁, Rb₂, Rc, Re, Rg₁, and Rd stating that they make up 90% of total saponin content (16). However, other authors showed that the content

of acidic saponins malonyl-ginsenoside Rb₁, Rb₂, Rc, and Rd represents between 35% and 60% of the total content of ginsenosides in both *P. ginseng* and *P. quinquefolius* (17). Since malonyl-ginsenosides are likely to release ginsenosides upon consumption of ginseng products, the content of sole neutral saponins may not reflect the potency of the product. Furthermore, the relative contents of acidic and neutral ginsenosides could be used for the determination of the age and the processing of ginseng samples (Table 1). In fact the malonyl-ginsenosides Rb₁, Rb₂, Rc, and Rd are quite unstable, difficult to be isolated and degraded to the corresponding ginsenosides Rb₁, Rb₂, Rc, and Rd during steaming (Red ginseng) or extraction at temperatures above 70°C (158°F). This is the reason why Pharmacopoeias do not include such compounds as reference standards in the Radix ginseng monographs. Some companies for marketing reasons increase “artificially” the genuine content of the ginsenosides by heating the roots or extracting them at high temperatures.

In order to evaluate the quality of the products on the U.S. market, the American Botanical Council started the Ginseng Evaluation Program (GEP) in 1993 (18). The GEP is an analytical evaluation that measures product content and consistency, and compares these to the label claims. The GEP developed and validated testing methodologies for consumer products, and analyzed more than 500 *P. ginseng* (*P. quinquefolius* and *Eleutherococcus senticosus*) products using HPLC to profile and assay ginsenosides (and/or eleutherosides), for the full text of the methodologies used by the GEP, please refer to www.herbalgram.org. The identities of the reference ginsenosides (and eleutherosides) were determined by spectroscopic means (nuclear magnetic resonance, liquid chromatography/mass spectrometry, LC/MS), and their purity was established by HPLC analysis (19). Of the

numerous ginsenosides that have been identified from ginseng, Rb₁, Rb₂, Rc, Rd, Re, and Rg₁ have been chosen for reference standards for ginseng products (20). However, the GEP evaluated quality of ginseng products by testing for the presence and amount of seven major ginsenosides Rb₁, Rb₂, Rc, Rd, Re, Rf, and Rg₁ (19) (Table 1). Each product was evaluated for lot-to-lot consistency as demonstrated by the following three factors: 1% of lots with an Rb₁/Rg₁ ginsenoside value within an acceptable range for *P. ginseng*; 2% of lots that met claim for total ginsenoside content; and 3% relative standard deviation (%RSD) of the lot-to-lot total ginsenoside content.

GINSENG PRODUCT REGULATIONS

In China, there are more than 900 ginseng-related products registered to the Chinese State Food and Drug Administration (SFDA, www.sfda.gov.cn) as a kind of Chinese medicinal formula that contains different amounts of ginseng and its ingredients. These products are formulated as extract, compounded extract, or tincture of ginseng root, capsules containing extract of ginseng root or leaves, and ginsenosides. In addition, there were 46 types of food products containing ginseng and its extracts under the SFDA inspection.

In United States, the Center for Food Safety and Applied Nutrition of the U.S. Food and Drug Administration (FDA) monitors ginseng quality and safety. According to federal regulations, products making claims fall into two classes. Class I refers to manipulated products (e.g., standardized ginseng extract) or products containing added ingredients (e.g., softgels containing standardized ginseng extract). These products must contain at least 100% of the claim throughout the shelf life of the product. Thus, an Asian ginseng product standardized to 4% ginsenosides must contain at least 4% ginsenosides throughout the shelf life of the product. Class II refers to naturally occurring, nonmanipulated ingredients (e.g., Asian ginseng root powder). These products must contain at least 80% of the claimed amount of the specified ingredient over the shelf life of the product [Code of Federal Regulations 21 CFR101]. If an Asian ginseng root powder product claims 4% ginsenosides, then that product must contain at least 80% of 4% (i.e., 3.2%) for the shelf life of the product.

GENERAL CLASSIFICATION OF GINSENG INGREDIENTS

The detailed chemical structures and related bioactivities of more than 100 chemical entities of ginseng can be found in the recent report (21). So far, approximately 200 substances have been isolated and characterized from *P. ginseng*, both from primary and secondary metabolism of the plant. These include 30 ginsenosides, 40 ether-soluble compounds, 8 nitrogen-containing compounds, 32 volatile compounds, 13 carbohydrates/polysaccharides, 9 glycanes, 56 lipids and fatty acids, and 9 trace elements. The ginsenosides are characteristic substances of ginseng (11). In general, active or inactive chemical entities obtained from ginseng species can be classified into five categories

ranked below in the order of their bioactivities: saponins, polysaccharides, polyynes, flavonoids, and volatile oils.

1. Saponins (or sapogenin glycosides). Saponin is a type of glycoside that is widely distributed in plants. Each saponin consists of a sugar and a sapogenin, the latter constitutes the aglucon moiety. The sapogenin may be a steroid or a triterpene. The sugar moiety may be a glucose, maltose, fructose, galactose, pentose, or methylpentose. Ginseng's saponins are generally called ginsenosides (Rx), which are considered as the main active principals of ginseng and often used as a marker for the quality control of ginseng drugs and commercial products. The basic structure of ginsenosides is similar. They consist of a gonane steroid nucleus with 17 carbon atoms arranged in four rings. The characteristic biological responses for each ginsenoside are attributed to the differences in the type, position, and number of sugar moieties attached by the glycosidic bond at C-3, C-6, and C-20 (3,11,22). Ginsenosides are amphipathic in nature. The hydroxyl (OH) group of ginsenosides allows both interactions between the polar head of the membrane phospholipids and the β -OH group of cholesterol, while the hydrophobic steroid backbone can interact with the hydrophobic side chains of fatty acids and cholesterol. Indeed, these physiochemical interactions are greatly determined by the numbers and sites of polar hydroxyl groups on each ginsenoside. Up to now more than 100 ginsenosides have been isolated from *Panax* species and most of them exhibit four types of aglycone moieties:

- Protopanaxadiol [or aglycone (20S)-protopanaxadiol]: dammarane-type ginsenosides including ginsenosides Ra₁, Ra₂, Ra₃, Rb₁, Rb₂, Rb₃; notoginsenoside R₄, Rs₁, Rs₂, Rs₃, Rs₄; and malonyl-ginsenoside Rb₁, Rb₂, Rc, and Rd. The metabolism pathway of the protopanaxadiol has been extensively investigated resulting in identification and characterization of several active metabolites.
- Protopanaxatriol [or aglycone (20S)-protopanaxatriol]: also dammarane-type ginsenosides including ginsenosides Re, Rf, Rg₁, and notoginsenoside R₁. The main structural difference between protopanaxatriol and protopanaxadiol is that the latter holds only H element at C6.
- Oleanolic acid (or aglycone oleanolic acid): including ginsenosides Ro that is an oleanane triterpenoid, chikusetsusaponin-V Rb₁, Rb₂, Rc, Rd, Re, and Rg₁.
- Ocotillol type.

2. Polysaccharides: These are water soluble and include panaxane A to U. The acidic polysaccharides (MW 10,000–150,000 Da) have immunomodulating and antiproliferative effects. They contain various sugar moieties, uronic acid, and less than 5% protein by weight. Recent studies have identified an acidic polysaccharide, referred to as "Ginsan," with noted immunostimulatory activity (23,24).
3. Polyynes: The polyynes are a group of organic compounds with alternating single and triple bonds. The term polyyne simply implies the presence of several alkynes. In ginseng, these include falacrinol

(panaxynol), falcarintriol (panaxytriol), acetic acid or linolenic acid.

4. Flavonoids
5. Volatile oils: Qiu et al. (25) recently used comprehensive two-dimensional gas chromatography/time-of-flight MS or flame ionization detector to characterize and quantify the chemical composition of volatile oil in the radices of *P. ginseng* harvested from Changbai Mountain in Jilin province, China. These ginseng samples were 3, 5, and 8 years old, respectively. They tentatively identified total 36 terpenoids in the ginseng volatile oil based on the mass library search and retention index of each oil entity. The study found that the following components of the volatile oil significantly increased with the age of the ginseng: α -cadinol, α -bisabolol, thujopsene, and *n*-hexadecanoic acid.

Four malonyl derivatives of ginsenosides Rb₁, Rb₂, Rc, and Rd were well described by Fuzzati (26). The malonyl derivatives and ginsenoside Ro (an oleanane-type triterpenoid) are also called "acidic" ginsenosides while the other are usually named "neutral" ginsenosides. Due to the fact that ginseng is a very popular phyto-medicine used all over the world, a huge quantity of work has been carried out during the last 30 years in order to develop analytical methods for the identification, quantification, and quality control of ginsenosides in raw plant materials, extracts, and marketed products. One of the main goals of these researches was to differentiate the ginsenosides pattern between the different *Panax* species in order to avoid adulteration or misidentification. Moreover, studies of changes in ginsenosides composition due to different traditional processing of *P. ginseng* roots such as white and red ginseng have been undertaken.

PRECLINICAL STUDIES

Ginseng is mainly used, as it has been for hundreds of years, to increase resistance to physical, chemical, and biological stress and boost general vitality. In addition, we have begun to recognize its newly found activities, including antihyperglycemic effect that may benefit type II diabetics, its modulation to immune system that may serve as an immunotherapeutic agent for combination with chemotherapy.

Glucose Metabolism and Diabetes Mellitus

Ginseng is one of the most studied anti-diabetic herb (27). Ryu et al. (28) investigated the anti-oxidant activity of Korean red ginseng and its effect on erectile function in noninsulin-dependent diabetes mellitus (NIDDM) rats induced by intraperitoneal injection of 90 mg/kg of streptozotocin on day 2 after birth. According to the diabetic period, the rats were classified as either short-term (22 weeks) or long-term (38 weeks) diabetics. The rats were fed 30 mg/kg of Korean red ginseng three times per week for one month. For those rats treated with Korean red ginseng, their erectile function and levels of glutathione (used as an index of the free radical-scavenging activity) (29) and malondialdehyde (an end product of lipid peroxidation used as an index of oxidative stress) were comparable to their age-matched normal controls. In con-

trast, the untreated diabetic rats showed impaired erectile function and increased levels of glutathione and malondialdehyde. Compound K, a major intestinal metabolite of ginsenosides derived from *P. ginseng*, exhibited antihyperglycemic effect through its insulin-secreting action, similar to that of insulin secretagogue sulfonylureas. Treatment of diabetic db/db (db: dependent diabetes) mice (type II diabetic model) for eight weeks with compound K (10 mg/kg), or combined with metformin resulted in significant improvements in plasma glucose and insulin levels (30).

A very interesting study showed antihyperglycemic and antiobese effects of *P. ginseng* berry extract and its major constituent, ginsenoside Re, in obese diabetic C57BL/6J ob/ob mice and their lean littermates (31). The animals received daily intraperitoneal injections of *P. ginseng* berry extract for 12 days. On day 12, 150 mg/kg extract-treated ob/ob mice became normoglycemic and had significantly improved glucose tolerance. The overall glucose excursion during the two-hour intraperitoneal glucose tolerance test decreased by 46% ($P < 0.01$) compared with vehicle-treated ob/ob mice. The improvement in blood glucose levels in the extract-treated ob/ob mice was associated with a significant reduction in serum insulin levels in fed and fasting mice. A hyperinsulinemic-euglycemic clamp study revealed a significant increase in the rate of insulin-stimulated glucose disposal in treated ob/ob mice. In addition, the extract-treated ob/ob mice lost a significant amount of weight associated with a significant reduction in food intake and a very significant increase in energy expenditure and body temperature. Treatment with the extract also significantly reduced plasma cholesterol levels in ob/ob mice. Additional studies demonstrated that ginsenoside Re plays a significant role in antihyperglycemic action. This anti-diabetic effect of ginsenoside Re was not associated with body weight changes, suggesting that other constituents in the extract have distinct pharmacological mechanisms on energy metabolism. Ginseng decreases acute postprandial glycemia (32).

Cardiovascular Effect

Ginseng has been shown to produce a number of actions on the cardiovascular system. Intravenous administration of ginseng to anesthetized dogs resulted in reduction, followed by an increase in blood pressure, and transient vasodilatation (33). Recent studies found close relationship between ginseng's effects and the NO production pathway, including regulation of cGMP and cAMP levels (34).

Korean red ginseng can improve the vascular endothelial dysfunction in patients with hypertension possibly through increasing NO (35). In addition to endothelium-derived NO release, Li et al. (36) reported that ginsenoside-induced vasorelaxation involves Ca²⁺ activated K⁺ channels in vascular smooth muscle cells. It has also been reported that crude saponin fractions from Korean red ginseng enhanced cerebral blood flow in rats (37), and ginsenosides reduced plasma cholesterol levels and the formation of atheroma in the aorta of rabbits fed on a high cholesterol diet (38). This antiatherosclerotic

action of ginseng components is apparently due to the correction in the balance between prostacyclin and thromboxane, inhibition of 5-hydroxytryptamine (5-HT) release, and adrenaline and thrombin-induced aggregation of platelets, regulation of cGMP and cAMP levels, and prolongation of the time interval between conversion of fibrinogen to fibrin. Also, ginsenosides have been shown to be relatively potent platelet-activating factor antagonists (39).

In parallel with these findings, Nakajima et al. (40) concluded that red ginseng was found to promote the proliferation of vascular endothelial cells, to inhibit the production of endothelin (that is known to constrict blood vessels and result in raising blood pressure), and to increase the production of interleukin-1 beta (IL-1 β), which suppresses the formation of thrombin in blood coagulation. The role of ginseng in angiogenesis has also been reported. For instance, Kim et al. (41) demonstrated that water extract of Korean red ginseng stimulated in vitro and in vivo angiogenesis via activation of phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), phosphatidylinositol 3-kinase (Akt), and endothelial nitric oxide synthase (eNOS) resulting in an increase in NO production (41). Ginsenoside Rg₁ promoted functional neovascularization into a polymer scaffold in vivo and tubulogenesis by endothelial cells in vitro (42). Therefore, ginsenoside Rg₁ might be useful in wound healing as it can induce therapeutic angiogenesis.

Neuropharmacology

Recently, it has been shown that ginseng and its components, ginsenosides, have a wide range of actions in the central nervous system (43). These effects include protective effects of ginsenosides against homocysteine-induced excitotoxicity, increased cell survival, extension of neurite growth, and rescuing of neurons from death in consequence of different insults either in vivo or in vitro. Ginseng roots appeared to be able to facilitate survival and neurite extension of cultured cortical neurons (44), and ginsenosides Rb₁ and Rg₃ protected neurons from glutamate-induced neurotoxicity. Following forebrain ischemia in gerbils, Wen et al. (45) demonstrated that central infusion of ginsenoside Rb₁ rescued the hippocampal CA1 neurons against lethal damage of cellular hypoxia.

Using a spinal neuron model, ginsenosides Rb₁ and Rg₁ proved to be potentially effective therapeutic agents for spinal cord injuries as they protected spinal neurons from excitotoxicity induced by glutamate and kainic acid and oxidative stress induced by hydrogen peroxide (46). The most recent studies further confirmed that Rg₁ and Rb₁ could enhance glutamate exocytosis and release in rat nerve terminals through affecting vesicle mobilization and activation of protein kinase A and/or C, respectively, resulting in an increase in synaptic vesicle availability (47).

Although the processes and mechanisms underlying the neuroprotective effects of ginseng upon dopaminergic neurons remain to be elucidated, several reports demonstrated the inhibition of ginseng on MPP⁺-(1-methyl-4-phenylpyridinium) uptake in dopaminergic neurons, the suppression of oxidative stress induced by auto-oxidation of dopamine, the attenuation of MPP⁺-induced apoptosis, and the potentiation of nerve growth factor (NGF). It has

been shown that certain ginsenosides inhibit dopamine uptake into rat synaptosomes, and consequently, provide protection against MPP⁺ through blockade of its uptake by dopaminergic neurons (48). Ginsenoside Rg₁ was shown to interrupt dopamine-induced elevation of reactive oxygen species (ROS) or NO generation in pheochromocytoma cells (PC12). *P. ginseng* attenuated MPP⁺-induced apoptosis as it decreased the intensity of MPP⁺-induced DNA laddering in PC12 cells and ginsenoside Rg₁ had protective effects against MPTP-induced apoptosis in the mouse substantia nigra. These anti-apoptotic effects of ginseng may be attributed to the enhanced expression of Bcl-2 and Bcl-xl, reduced expression of bax and nitric oxide synthase (NOS), and inhibited activation of caspase-3. Ginseng may also reverse the neurotoxic effects of MPP⁺ through elevation of NGF mRNA expression (48). In accordance, ginsenosides Rb₁ and Rg₁ elevated NGF mRNA expression in rat brain and potentiated NGF-induced neurite outgrowth in cell culture. Furthermore, it has been reported that ginsenosides Rb₁, Rg₁, Rc, and Re inhibited tyrosine hydroxylase activity and exhibited antidopaminergic action since they reduced the availability of dopamine at presynaptic dopamine receptors (49).

Long-term oral consumption of ginsenosides prevents memory loss in aged SAMP8 mice (a model of accelerated senescence that was established through phenotypic selection from a common genetic pool of AKR/J strain of mice) by decreasing oxidative stress and upregulating the plasticity-related proteins in hippocampus (50).

Immunology

The effect of oral administration of a standardized ginseng extract to mice for four consecutive days (10 mg/day) on immune response was investigated. The extract enhanced antibody plaque-forming cell response and circulating antibody titer against sheep erythrocytes. This finding was confirmed by oral administration of an extract with defined ginsenoside content to mice at doses of 10, 50, or 250 mg/kg body weight daily for five to six days, which resulted in enhanced immune responses in a battery of six ex vivo tests including primary and secondary immune responses against sheep red cells, natural killing activity, mitogen-induced proliferation, interferon production, and T-cell-mediated cytotoxicity (51). A standardized extract from ginseng roots and several fractions of the extract were found to possess anti-complement and mitogenic activities in mice spleen cell cultures, with the strongest anti-complement activity being observed in the crude polysaccharide fraction. The polysaccharide with the major anti-complement activity consisted of arabinose, galactose, and glucose, and small amounts of galacturonic acid, glucuronic acid, and rhamnose. Its molecular weight was estimated to be 3.68 – 105 kDa (52). An acidic polysaccharide fraction containing galactose, arabinose, and uronic acids showed inhibition of *Helicobacter pylori*-induced hemagglutination with a minimum inhibitory concentration of 250 mg/mL. Digestion of the fraction with pectinase resulted in a lower molecular weight oligosaccharide fraction, which was noninhibitory at a concentration of 4 mg/mL (53). A high output of inducible nitric oxide synthase (iNOS) was shown in mice

administered intraperitoneally with the acidic polysaccharide from ginseng. Newly synthesized iNOS protein was also observed in peritoneal macrophages cultured with interferon- λ and the acidic polysaccharide. Spleen cells from acidic polysaccharide-treated mice did not proliferate in response to concanavalin A, but responsiveness was restored by the cotreatment of NG-monomethyl-L-arginine (NMMA) with concanavalin A. The treatment of mice with aminoguanidine, a specific iNOS inhibitor, alleviated the acidic polysaccharide-induced suppression of antibody response to sheep red blood cells. Present results suggest that the immunomodulating activities of the acidic polysaccharide were mediated by the production of NO (54).

It was demonstrated that *P. ginseng* extract and purified ginsenosides exert an adjuvant effect on the immune responses against porcine parvovirus, Erysipelothrix rhusiopathie, and against Staphylococcus aureus in dairy cattle (55).

Innate immunity is the front line host defense that acts within minutes of infection to counter invasion by microorganisms. Members of the toll-like receptor (TLR) family recognize conserved pathogen-associated molecular patterns of virus, bacteria, fungi, and parasites. A *P. ginseng* extract administered orally to mice was able to modulate proinflammatory cytokine production throughout the increase of macrophage toll-like receptor 4 expression during physical stress (56).

Ginseng in Cancer Treatment

Although there is no conclusive evidence that ginseng itself can cure cancer, it makes sense that use of ginseng for cancer therapy should focus on synergistic combinations or palliative treatment. During active cancer therapy, ginseng should generally be evaluated in combination with chemotherapy and radiation. In this role, it acts as a biological response modifier and an adaptogen to synergistically enhance efficacy of the conventional therapy (57). It can improve immune system activity of patients and their appetite, and functions as a supplementary agent of chemotherapy. For instance, Xing et al. (58) treated 35 rectal cancer patients with retention enema containing 85% ginsenoside for four to six hours per day for six to eight consecutive days before surgical operation. The control group ($n = 15$) received retention enema containing saline in the same way. They reported that after ginsenoside treatment symptoms such as frequent defecation, hematochezia, and tenesmus were palliated in most patients (25 out of 35), and abdominal pain was relieved in 7 patients with incomplete intestinal obstruction. Electron microscopic examination showed apoptosis in 23 treated patients. In comparison, the above-mentioned changes were not observed in the control group. Preclinical studies have showed some immune-stimulating activity of ginseng and ginsenosides (59).

Recent in vitro experimental studies have showed anti-cancer effects of various ginsenosides that will be narrated below. However, we have to point out that these in vitro studies, although inexpensive to carry out, should only serve as an adequate screening mechanism, or help us understand the mechanisms of actions of ginsenosides. In addition, the concentrations of ginsenosides applied in

some in vitro experiments are as high as 100 μ M level in order to produce some biological effects. This fact suggests that the observed biological effects of ginsenosides are not specific to a particular pathway. Therefore, we propose that when a difference arises between in vitro and in vivo findings, the in vivo results should always take precedence over in vitro studies.

Rg₃ is one of the most effective cytostasis ginsenosides separated from ginseng. Rg₃ inhibits human prostate cancer cells and other androgen-dependent cells from proliferating (60). The mechanisms of actions of Rg₃ include (i) decreasing genetic expression of 5 α -reductase, (ii) inhibiting cell cycle evolution genes such as proliferating cell nuclear antigen gene and cell cycle protease D1 gene that would stop cells from proliferating, (iii) increasing cyclinase suppressor genes such as p21 and p27 so as to make cells arrest at G1 stage, (iv) downregulating Bcl-2 (the anti-induction apoptosis gene), and (v) activating caspase-3 (the induction apoptosis gene) to induce cell death. Rg₃ inhibited tumor cell proliferation and induced cell apoptosis in mice with induced liver cancer (61). In addition, Rg₃ can affect the differential expression of cell signaling genes and other related genes in human lung adenocarcinoma cell line A549, and induce apoptosis in the A549 tumor cells and HUVEC 304 cell lines. It was showed that Rg₃ and Rg₅ had significant inhibition on benzo(a)pyrene-induced adenocarcinoma (62) and dimethylbenz(a)anthracene (DMBA)-induced lung tumor in mice. The ginsenosides had strong inhibitory effects on the development of rat mammary adenocarcinoma induced by methyl-N-nitrosourea and N-ethyl-N-nitrosourea administration, as well as on DMBA-induced uterine and vaginal tumors (63). Using athymic mice transplanted with ovarian SKOV-3 cancer cells, Xu et al. (64) showed that intraperitoneal injection of ginsenoside Rg₃ alone, or Rg₃ combined with cyclophosphamide to the mice for 10 days improved life quality and survival of the mice, and reduced tumor weight in the mice in comparison to the control untreated mice. Chen et al. (65) reported that Rg₃ produced apoptosis in human bladder transitional cell carcinoma cell line EJ at IC₅₀ 125.5 μ g/mL after 48 hours of incubation. When treated with 150 μ g/mL of Rg₃ for 24 hours and 48 hours, the cells showed significant DNA ladders and apoptotic morphological characteristics including condensed chromatin, nuclear fragmentation, apoptotic bodies, and bright fluorescent granules as well as a higher caspase-3 expression. When the cells were treated with 75 μ g/mL of Rg₃ for 24 hours and 48 hours, or 150 μ g/mL of Rg₃ for 48 hours, the percentage of cells in S phase and G2/M phase was increased, whereas the percentage of cells in G0–G1 was decreased.

Ginsenoside Rh₂ induced apoptosis in various tumor cells by different pathways. Rh₂ interfered with B-cell lymphocyte/leukemia-2 (Bcl-2) family proteins-related apoptosis and activated protein kinase caspase-3 to cause cell apoptosis. Rh₂ induced apoptosis of rat C6Bu-1 glioma cells and human SK-N-BE(2) neuroblastoma cells through protein kinase C pathway. Ginsenoside Rh₂ also induced apoptosis in human malignant melanoma, which was partially dependent on caspase-8 and caspase-3 (66). Ginsenoside Rh₂ induced apoptosis and inhibited cell growth in C6 glioma cells, human lung adenocarcinoma A549 cells, and various ovarian cancer cell lines (67). Mediating G1

growth arrest and apoptosis in human lung adenocarcinoma A549 cells appeared to be the molecular mechanisms of ginsenoside Rh₂. Rh₂ inhibited human hepatoma Bel-7404 cell lines via arresting cell cycle, upregulating Bax protein expression, and downregulating mutated p53 protein expression. Rh₂ inhibited the growth of MCF-7 cells by inducing p21 protein expression and reducing cyclin D levels. As a result, cyclin/Cdk complex kinase activity, pRb phosphorylation, and E2F release could be inhibited.

Another ginsenoside, that is, compound K (or IH901), was shown to induce apoptosis in human hepatoblastoma HepG2 cells (68) and KMS-11 cells through a cytochrome C-mediated activation of caspase-3 and caspase-8 proteases and inhibition of the fibroblast growth factor receptor 3 (FGFR3) expression. Incubation of leukemic cells HL260 with compound K produced apoptosis in the cells in a concentration- and time-dependent manner with morphologic changes in chromatic agglutination, atrophy, and nuclear fragmentation (69). Compound K suppressed melanoma cell proliferation in B16-BL6 mice by activating the protein kinase caspase-3 and releasing cytochrome C in mitochondria into cytoplasm. Western blot test revealed that compound K could elevate p27Kip1 expression, degrade expression of c-Myc and cyclin D1, and induce cell LLC apoptosis through activating caspase-3 protein kinase at the same time. Recently, IH-901 has been reported to induce both G1 arrest and apoptosis, and the apoptosis could be inhibited by COX-2 induction (70).

PHARMACOKINETICS AND METABOLISM

Pharmacokinetics of *P. ginseng* cannot be directly performed using the conventional analytical methods because *P. ginseng* root contains many ingredients, and the amounts of each ingredient vary with different conditions. All these make it more complex and even impossible to directly conduct pharmacokinetics of ginseng itself. The practical way, however, to conduct ginseng's pharmacokinetics is to measure individual ginsenoside's absorption, distribution, metabolism, and elimination in animals and human beings.

Rg₁ and Rb₁ were shown to be metabolized at the gastrointestinal level. In the acidic medium of the stomach, they are immediately decomposed into different ginsenoside metabolites whose chemical structures have been partially determined. Pharmacokinetic studies conducted in rats revealed only 23% absorption of ginsenosides (Rb₁) after a period of 2.5 hours (71). Very small recoveries of these ginsenosides were made in the liver (e.g., 0.25% dose) and heart (<0.1% dose), while the majority of the material was recovered in the small intestine. The bioavailability of bioactive ginseng constituents appears very limited, as evidenced by the low absorption rates for orally administered Rg₁ (0.1% dose) and Rg₂ (1.9% dose), and only little of the original ginsenoside material was recovered in the feces (<1% dose) (72). In our view, the limited amounts of ginsenosides recovered from tissues, urine, and feces are not surprising because the total content of ginsenosides (saponins) in a ginseng is usually less than 5% of its weight, as we mentioned earlier. Moreover, ginsenosides have poor oral bioavailability as demonstrated earlier mainly because of their extensive presystemic metabolism

and poor membrane permeability. A study performed with mini-pigs showed, after intravenous administration, that the derivatives of protopanaxatriol, such as Rg₁, have a one-compartment pharmacokinetic profile, and a half-life of approximately 30 minutes. On the other hand, for those of protopanaxadiol, such as Rb₁, the half-life is much longer (approx. 16 hours), and their pharmacokinetics are described by a two-compartment model (73).

Metabolism pathway of ginsenosides in the gastrointestinal tract of humans could be elucidated following the oral administration of a standardized *P. ginseng* extract. Within the framework of a pilot study, human plasma and urine samples of two subjects were screened for ginsenosides and their possible degradation products. In general, the urine data coincided well with the plasma data, and in both volunteers, the same hydrolysis products, which are not originally present in the extract ingested, were identified. It was shown that two hydrolysis products of the protopanaxatriol ginsenosides, namely G-Rh₁ and G-F₁, may reach the systemic circulation. In addition, compound K, the main intestinal bacterial metabolite of the protopanaxadiol ginsenosides, was detected in plasma and urine. In contrast to previous reports, G-Rb₁ was identified in the plasma and urine of one subject (74).

In conclusion, the ginsenosides are degraded by the acidic medium of the stomach as well as by the intestinal enzymes and intestinal bacteria, therefore the ginsenosides are considered as prodrugs, and G-Rh₁ and G-F₁ are probably the metabolites responsible for the ginsenoside action in humans (74,75).

PRECLINICAL SAFETY EVALUATION

The results of several toxicity studies in animals with a *P. ginseng* extract have been reviewed (76) and demonstrate that a standardized *P. ginseng* extract has a very large therapeutic index. These studies included acute, subacute, chronic toxicity, and teratogenic activity performed in mice, rats, rabbits, pigs, and dogs, and mutagenicity tests (Ames, DNA-repair, mouse-bone micronucleus, chromosomal aberration in human lymphocytes), as well as the safety assessment on the cardiovascular and hormone system.

In a single dose toxicity study, the LD₅₀ after oral administration is >5 g/kg in rats, >2 g/kg in mini-pigs, and >1 g/kg in mice, and after i.p. administration, it is >1 g/kg in rats and mice. No noticeable changes of cardiovascular parameters such as electrocardiogram (ECG), pulse, blood pressure, cardiac output, and stroke volume were observed after single dose oral administration of 0.25, 0.5, and 2.0 g/kg in mini-pigs.

In repeated dose toxicity study, no hematological or histological abnormalities were observed in rats after 20 days of daily oral administration of 4.0 g/kg. Treatment-related hematological or histopathological effects were not noticed in beagle dogs after oral administration of 1.5, 5.0, and 15 mg/kg for 90 days.

In reproduction toxicity study, no decrease of growth rate or reproduction and no treatment-related hematological or histopathological findings were seen in rats for 33 weeks in a two-generation study with daily oral administration of 1.5, 5.0, and 15 mg/kg.

In terms of embryo, fetal, and perinatal toxicity concerns, no abnormalities of fetal development have been detected in rats after daily oral administration of 40 mg ginseng extract/kg on days 1 to 15 after mating, or in rabbits after daily oral administration of 20 mg/kg on days 7 to 15 after mating. In an in vitro study using whole rat embryo culture model, ginsenoside Rb₁ induced teratogenicity (77). The significance of this study is uncertain due to the concentration of Rb₁ used, and to the fact that it is known that ginsenosides that are not metabolized by the acidic medium and intestinal flora exert hemolytic activities (as is generally observed with saponins).

No genotoxicity was observed in the hepatocyte DNA repair test using concentrations of 0.1 to 10 mg/mL of ginseng extract with or without ginsenosides or using 1 to 50 µg/mL of ginsenoside Rg₁. Neither has mutagenicity been observed in *Salmonella typhimurium* and Chinese Hamster V79 cells.

CLINICAL STUDIES

Learning and Memory Effects

In humans, ginseng or ginseng extract showed significant effects on neurological and psychiatric symptoms in aged humans and psychomotor functions in healthy subjects (78). This positive effect of ginseng on cognition performance is due to the direct action of ginseng on the hippocampus. Consistent with the study, Wen et al. (45) demonstrated that red ginseng, ginseng powder, and ginsenoside Rb₁ administration for seven days prior to ischemia rescued the hippocampal CA1 pyramidal neurons and subsequently ameliorated learning deficits in gerbils. The influence of ginsenoside Rg₁ on the proliferating ability of neuronal progenitor cells may serve as an important mechanism underlying its nootropic and antiaging effects particularly on learning and memory (79).

On the other hand, Persson et al. (80) recruited 3500 community-dwelling volunteers to investigate whether the use of ginseng or ginkgo biloba for a long period of time (up to two years) has positive effects on performance on learning and memory. They found that the use of neither ginkgo biloba ($n = 40$) nor ginseng ($n = 86$) was associated with enhanced memory performance in any of the eight memory tests examined, relative to control groups either using or not using nutritional supplements. The study did not provide any quantifiable beneficial effects of ginseng on memory performance. This result coincides with the finding of Sorensen and Sonne (81) who reported that ginseng intake did not enhance memory functions. The investigators admitted, however, that "the study lacked direct control of dosage and specificity of formulas used such as measure of daily or weekly intake and the specific brands that were used."

The effects of 400 mg/day of a ginseng extract on a variety of cognitive functions were compared with placebo in a double-blind randomized study in which 112 healthy volunteers older than 40 years (55 on ginseng, 57 on placebo) were treated for eight to nine weeks. The ginseng group showed a tendency to have faster simple reactions and significantly better abstract thinking than the controls. However, there was no significant difference between the two groups in concentration, memory, or subjective experience (81).

A study investigated whether acute administration of standardized ginseng extract had any consistent effect on mood and four aspects of cognitive performance (quality of memory, speed of memory, quality of attention, and speed of attention) that can be derived by factor analysis of the Cognitive Drug Research computerized assessment battery. The study followed a placebo-controlled, double-blind, balanced crossover design. Twenty healthy young adult volunteers received 200, 400, and 600 mg of the extract, and a matching placebo, in counterbalanced order, with a seven-day washout period between treatments. Following a baseline cognitive assessment, further test sessions took place 1, 2.5, 4, and 6 hours after the day's treatment. The most striking result was a significant improvement in "quality of memory" and the associated "secondary memory" factor at all time points following 400 mg of ginseng. Both the 200 and 600 mg doses were associated with a significant decrement of the "speed of attention" factor at later testing times only. Subjective ratings of alertness were also reduced 6 hours following the two lowest doses (82).

Physiological Performance

In a double-blind crossover study, 12 student nurses working night shifts (three to four consecutive nights followed by three days of rest) were given 1.2 g of ginseng roots or placebo for the first three consecutive nights and tested on the morning after the third night. Crossover medication was given after an interval of at least two weeks. A third series of tests was carried out during normal daytime working, after no medication and following a good night's sleep (GNS). The subjects assessed their mood, physical well-being, and degree of lethargy by means of linear self-rating scales. Two psychophysiological performance tests and hematological tests were also carried out.

The detrimental effects of night shifts were clearly seen. A constant trend in favor of ginseng compared with placebo was noted. Ginseng ratings were favorable for mood criteria, but not for physical well-being symptoms. Ginseng restored blood glucose levels raised by night shift stress. A small but consistent anti-fatigue activity of ginseng was concluded (83).

Various tests of psychomotor performance were carried out in a cohort of 16 healthy male volunteers given a standardized ginseng extract (100 mg ginseng extract twice a day for 12 weeks) and in a similar group given placebo under double-blind conditions. A favorable effect of ginseng relative to baseline performance was observed in attention (cancellation test), processing (mental arithmetic, logical deduction), integrated sensory-motor function (choice reaction time), and auditory reaction time. However, end performance of the ginseng cohort was only statistically superior ($P < 0.05$) to the placebo group in mental arithmetic. No difference between ginseng and placebo was found in tests of pure motor function (tapping test), recognition (digit symbol substitution), and visual reaction time (78).

In a double-blind, placebo-controlled, crossover study, 43 top triathletes received either placebo or 200 mg of a standardized ginseng extract per day for periods of 10 weeks, respectively. Significant differences ($P < 0.05$) in various endurance parameters were only seen after the

second treatment phase. It was concluded that ginseng improves endurance (resistance against end of season stress), but not optimum performance (84).

Twenty top class male athletes received 200 mg standardized ginseng extract per day for nine weeks. In the bicycle ergometer exercise test lasting eight minutes, the post-treatment values were higher for maximal oxygen absorption and lower for blood lactate level and heart rate during exercise compared with pretreatment values. The differences were significant ($P < 0.001$) (85).

A double-blind study involved 30 athletes who received daily either placebo ($n = 10$), 200 mg ginseng extract standardized to 7% ginsenosides ($n = 10$), or 400 mg vitamin E and 200 mg ginseng extract standardized to 4% ginsenosides ($n = 10$) for nine weeks. The same bicycle ergometer test was used and statistically significant variations in heart rate ($P < 0.05$), blood lactate ($P < 0.01$), and maximal oxygen absorption ($P < 0.01$) after exercise between either of the two ginseng preparations and placebo were found. Differences between the two ginseng preparations were not statistically significant. The levels of testosterone and luteinizing hormone in plasma, and free cortisol in urine, were unchanged after all treatment periods (86).

A further double-blind placebo-controlled study with 28 top class male athletes examined the persistence of the effects of nine-week's treatment (placebo or 200 mg ginseng extract with 4% ginsenosides) beyond the treatment period. Ginseng resulted in a significant improvement of maximal oxygen uptake during exercise ($P < 0.01$), heart rate at maximal exercise ($P < 0.001$), forced expiratory volume ($P < 0.01$), forced vital lung capacity ($P < 0.05$), and visual reaction time ($P < 0.01$) compared with placebo. These positive effects lasted for at least three weeks after treatment, and it was concluded that the effects of ginseng are based on clinically relevant metabolic changes that persist for a certain period after treatment (87).

In a double-blind placebo-controlled study with 50 ambulatory patients suffering from asthenia, depressive syndrome, or neurovegetative disorders, the effects of eight weeks' treatment with 200 mg/day of a standardized ginseng extract on performance in two psychometric tests and on results from a comprehensive psychological questionnaire (Sandoz Clinical Assessment Geriatric) were studied. Significant improvement ($P < 0.05$ and $P < 0.01$) was seen in most of the parameters (88).

In a randomized double-blind study, 31 healthy male volunteers received 200 or 400 mg ginseng extract per day for eight weeks. Ginseng had no effect on oxygen consumption, respiratory exchange ratio, minute ventilation, blood lactic acid concentration, heart rate, and perceived exertion (89).

In another randomized double-blind study, 19 healthy female volunteers received daily 200 mg ginseng extract or placebo for eight weeks. It had no effect on maximal work performance and resting, exercise, recovery oxygen uptake, respiratory exchange ratio, minute ventilation, heart rate, and blood lactic acid levels (90).

In a double-blind, placebo-controlled, crossover study in eight healthy volunteers (mean age 25 years) who regularly practiced physical activities, 30 days of daily oral treatment with 400 mg of a standardized ginseng ex-

tract did not improve performance at supramaximal exercise (125% of the maximum aerobic power on bicycle ergometer), nor did it influence blood lactate or blood testosterone (91).

In a study on blood oxygenation status of eight males and two females middle-aged subjects (average 50-year-old), a significant ($P < 0.05$) increase of resting arterial PO_2 was found after four weeks' oral treatment with 200 mg standardized ginseng root extract per day. The resting arterial PO_2 was increased by 4.5 mmHg. In synergy with oxygen treatment, the increase was 10.1 mmHg. Venous PO_2 was decreased (4.3 mmHg) (92).

The effects of a standardized ginseng extract on psychological mood states and the perceptual response to submaximal and maximal exercise stress were examined in a study with 19 young adult females who received either 200 mg/day of a standardized ginseng root extract ($n = 10$) or placebo ($n = 9$). The results did not support claims of the efficacy of ginseng to alter psychological function characteristics at rest and during exercise stress (93).

The effects of a standardized ginseng extract (300 mg/day) on healthy, untrained male students and on healthy male students who received regular bicycle ergometer training were compared with placebo in an eight-week, randomized, double-blind study ($n = 41$). Ginseng administration at the prescribed dose exhibited training-like effects on VO_2 max as well as anaerobic power and leg muscle strength. But no synergistic effect on these fitness variables occurred when both ginseng administration and exercise training were combined (94).

The effect of acute administration of standardized ginseng extract was investigated on mood and four aspects of cognitive performance mentioned previously derived from factor analysis of the cognitive drug research computerized test battery. Following a double-blind, placebo-controlled, balanced, crossover design, 30 healthy young adult volunteers received 400 mg of ginseng, and a matching inert placebo, in a counterbalanced order, with a seven-day washout period between treatments. Following baseline evaluation of cognitive performance and mood measures, participants' cognitive performance and mood were assessed again 90 minutes after drug ingestion. In line with previous research, a fractionation of the effect of ginseng administration was observed. Ginseng significantly improved speed of attention, indicating a beneficial effect on participants' ability to allocate attention processes to a particular task. However, no significant effect was observed on any other aspect of cognitive performance. In addition, participants' self-reported mood measures did not differ significantly across treatments. It is interesting to note that previous research demonstrated no improvement on attention processes, but significant improvements on quality of memory following administration of 400 mg of ginseng when participants were tested 1, 2.5, 4, and 6 hours postingestion (82). It may be the case that ginseng may offer performance at varying time points (95).

Immunomodulation

The effects of ginseng root extract (200 mg orally/day) on immune parameters were studied in an eight-week

three leg trial involving 60 healthy volunteers of both sexes aged between 18 and 50 years. Study medication was either a standardized ginseng extract or a nonstandardized aqueous ginseng extract or placebo. The statistically significant differences from baseline that have been observed are listed below. The standardized extract led to an increase in the following: chemotaxis of circulating polymorphonuclear leukocytes ($P < 0.05$ at week 4 and $P < 0.001$ at week 8), phagocytosis index and phagocytosis fraction ($P < 0.001$ at weeks 4 and 8), total lymphocytes (T3) ($P < 0.05$ at week 4 and $P < 0.001$ at week 8), T-helper (T4) subset ($P < 0.05$ at week 4 and $P < 0.001$ at week 8), helper/suppressor (T4/T8) ratio ($P < 0.05$ at weeks 4 and 8), induction of blastogenesis in circulating lymphocytes ($P < 0.05$ at weeks 4 and 8 after induction by concanavalin A and pokeweed mitogen, $P < 0.001$ at weeks 4 and 8 after induction by lipopolysaccharide), and natural killer cell activity ($P < 0.05$ at week 4 and $P < 0.001$ at week 8). With the aqueous extract, a rise was observed in the following: chemotaxis of circulating polymorphonuclear leukocytes ($P < 0.05$ at weeks 4 and 8), phagocytosis index and phagocytosis fraction ($P < 0.05$ at week 8), total (T3) lymphocytes ($P < 0.05$ at week 4 and $P < 0.001$ at week 8), T-helper (T4) subset ($P < 0.05$ at week 8), induction of blastogenesis in circulating lymphocytes ($P < 0.05$ at week 8 after induction by concanavalin A and pokeweed mitogen), and natural killer cell activity ($P < 0.05$ at week 8). With the placebo, only an enhancement in natural killer cell activity was statistically significant ($P < 0.05$) after 8 weeks. It was concluded that ginseng extracts act as an immunostimulant in humans, and that the standardized extract was more active than the aqueous one (96).

Healthy volunteers ($n = 227$) were enrolled in a multicenter, randomized, double-blind, placebo-controlled clinical trial to investigate potential effects of a standardized ginseng extract on resistance against influenza and the common cold. Study duration was 12 weeks and the study medication was either 200 mg standardized ginseng extract ($n = 114$) or placebo ($n = 113$) per day. All participants received an anti-influenza polyvalent vaccine at week 4. Results from examinations at weeks 4, 8, and 12 showed highly significant differences ($P < 0.0001$) between ginseng extract and placebo with regard to the frequency of influenza or colds between weeks 4 and 12 (15 cases in the verum group vs. 42 cases in the placebo group). Antibody titers at week 8 were also much higher after verum (272 units vs. 171 units after placebo) as well as natural killer cell activity that was almost twice as high in the verum group compared with the placebo group (97).

A controlled single-blind study was performed to investigate the effects of standardized ginseng root extract (200 mg/day) in 40 patients suffering from chronic bronchitis. It was shown that the extract significantly ($P < 0.001$) improved alveolar macrophage activity compared with baseline (98).

The effects of a standardized ginseng root extract (200 mg orally per day for three months) were studied in a pilot trial involving 15 patients with severe chronic respiratory diseases. Respiratory parameters, such as vital capacity, expiratory volume and flow, ventilation volume, as well as walking distance, were examined. The results led to the conclusion that the extract

improves pulmonary function and oxygenation capacity, which seems to be the reason for improved walking capacity (99).

A study in two equal groups of 10 young healthy males was undertaken to investigate the effects of eight weeks' administration of a standardized ginseng extract (300 mg/day) in comparison with the effects of placebo. It was concluded that ginseng caused no significant changes in peripheral blood leukocytes and lymphocyte subsets (100).

INTERACTIONS AND SIDE EFFECTS

Data from clinical trials suggest that the incidence of adverse events with *P. ginseng* preparations is similar to that with placebo. The most commonly experienced adverse effects are headache, sleep, and gastrointestinal disorders. The possibility of more serious side effects is indicated in isolated case reports and data from spontaneous reporting schemes. However, causality is often difficult to determine from the evidence provided. Combination products containing ginseng as one of several constituents have been associated with serious adverse events and even fatalities. Interpretation of these cases is difficult, as ingredients other than *P. ginseng* may have caused the problems. Collectively, these data suggest that *P. ginseng* monopreparations are rarely associated with adverse events or drug interactions. The ones that are documented are usually mild and transient. Combined preparations are more often associated with such events, but causal attribution is usually not possible (101).

However, probable drug-drug interactions, sometimes serious, have been reported between *P. ginseng* and warfarin, phenelzine, and alcohol with much attention toward ginseng and warfarin interaction (102–107). In 1997, Janetzky and Morreale reported a case that triggered scientific debates on whether there is a drug-drug interaction between ginseng and warfarin (103). The case report described that a patient with a mechanical heart valve and five-year warfarin anticoagulation therapy had a decline in his international normalized ratio (INR) of the prothrombin time to a subtherapeutic value (i.e., 1.5) after taking a ginseng product Ginsana for two weeks. The manufacturer Pharmaton claims that each capsule of Ginsana contains 100 mg of standardized levels of the eight most potent ginsenosides. After discontinuance of Ginsana, the patient's INR returned to the warfarin-controlled range from 3.0 to 4.0. In 2003, Rosado (104) reported a case of thrombosis on a mechanical bileaflet aortic valve prosthesis caused by inadequate anticoagulation levels (INR 1.4) that occurred after the patient took a period of a commercial ginseng product. He had been optimally maintained on anticoagulation with warfarin for three months. His INR declined to 1.4 after he began using the ginseng product obtained abroad.

These two case reports triggered three investigations (105–107) that all concluded, based on their independent studies, that ginseng has no significant impact on pharmacokinetics and pharmacodynamics (anticoagulation and INR) of warfarin: (i) in Zhu's study (105), rats were orally dosed with warfarin (0.2 mg/kg/day) and ginseng decoction (2 g/kg, twice daily; the aqueous

extract of ginseng root powder was evaporated to dryness) concomitantly for five days. The control group was given warfarin only. Coadministration of ginseng did not significantly change warfarin's pharmacokinetic parameters and prothrombin time. (ii) In Lee's study (106), 25 ischemic stroke patients were randomly divided into two groups: the ginseng group received both *P. ginseng* (the aqueous ginseng extract evaporated to dryness) and warfarin for two weeks, and the control group received only warfarin for two weeks. The warfarin dose was restricted to 2 mg in the first week and 5 mg in the second week. The ginseng aqueous extract (0.5 g) was given three times daily. This study suggests that coadministration of *P. ginseng* and warfarin to the ischemic stroke patients does not influence the INR and prothrombin time of warfarin. (iii) In a double-blind, randomized, two-period crossover study (107), 1 g of Korean red ginseng extract and warfarin (no dose information was reported) were given to 25 patients with cardiac valve replacement and stable INR resulted from warfarin therapy. One group initially received both warfarin and the ginseng extract for six weeks followed by a three-week washout period, and then warfarin and a placebo. Another group received the treatments in the opposite order. This study showed a slight decline in the INR values in ginseng plus warfarin treatment in comparison with the placebo plus warfarin treatment although the differences are not statistically significant.

Zhu's study provides evidence that coadministration of oral ginseng appears not to affect oral absorption of warfarin, its bioavailability, and distribution profile (105). However, there is an 8- to 12-hour delay in the action of warfarin, and an one- to three-day delay between the peak plasma concentration of warfarin and its maximum hypoprothrombinemic effect. Warfarin's anticoagulant effect results from a balance between partially inhibited synthesis and unaltered degradation of the four vitamin K-dependent clotting factors. The hypoprothrombinemic effect is dependent on their degradation rate in the circulation. These half-lives are 6, 24, 40, and 60 hours for factors VII, IX, X, and II, respectively (108). Therefore, a proper study design is to give tested subjects warfarin alone for a period long enough to allow the drug to reach its maximum effect before adding ginseng to the regimen. The results of this study design may be more convincing to tell us whether or not a drug-drug interaction between ginseng and warfarin exists.

A study in humans has shown that *P. ginseng* extract after oral administration for 14 days does not induce the cytochrome P450 3A (CYP3A) activity (109). A *P. ginseng* extract at dosages of 300 to 100 mg/kg orally administered to rats in single or multiple doses did not affect the induction of CYP2B1, CYP3A23, and CYP1A2 gene expression (110).

In a single-dose study and a steady state dose study in rats, the pharmacokinetic of warfarin (peak plasma concentration, time to peak, elimination half-life, oral clearance) as well as the pharmacodynamics (area under prothrombin time vs. time curve, maximum prothrombin time) was not altered by the per oral administration of 2 g/kg *P. ginseng* roots (105).

A three-way crossover randomized study was performed to check the pharmacokinetics and pharmacodynamics interaction of *P. ginseng* with warfarin in 12 healthy

male subjects. *P. ginseng* roots 0.5 g daily pretreatment during seven days and a single dose of 25 mg warfarin and ginseng administration was continued after the warfarin dose. Platelet aggregation, INR of prothrombin time, warfarin enantiomer protein binding, warfarin enantiomer concentrations in plasma, and S-7-hydroxywarfarin concentration in urine were measured. Coadministration of warfarin with *P. ginseng* in humans did not affect the pharmacokinetics or pharmacodynamics of either S-warfarin or R-warfarin (111).

PREGNANCY AND LACTATION

In animals, no effect on fetal development has been observed. No human data are available.

In accordance with general medical practice, ginseng should not be used during pregnancy or lactation without medical advice (112).

OVERDOSE

In 1979, Siegel first coined the term "ginseng abuse syndrome" (113) to describe several side effects of ginseng caused by inappropriate uses of ginseng and its products. Although Siegel did not define the syndrome clearly, the syndrome now can be described, after decades of clinical observation, as symptoms such as heart palpitations, high blood pressure, insomnia, restlessness, skin rash, edema, and diarrhea. It is true that a small portion of people are sensitive to ginseng and its products.

One case of ginseng-associated cerebral arteritis has been reported (114) in which a routine ginseng user who ingested approximately 200 mL of ginseng extract developed severe headache, nausea and vomiting, and chest tightness eight hours after drinking the ginseng extract. The extract was made by stewing 60 slices of dry ginseng root (~25 g) with 400 mL of rice wine (22% alcohol). The patient had a sore throat and took 500 mg acetaminophen two hours before she drank the ginseng extract. The headache was severe and was temporarily relieved by acetaminophen. She was referred to a local hospital six days later and diagnosed as cerebral arteritis. The symptoms gradually disappeared over the next 10 days.

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Glucosamine

Karla L. Miller and Daniel O. Clegg

INTRODUCTION

Glucosamine (2-amino-2-deoxy-D-glucose) is a naturally occurring substance derived from the exoskeletons of arthropods. Glucosamine-6-phosphate is a precursor in the biosynthesis of the glycosaminoglycans (GAGs) found in cartilage. Premature loss of cartilage is part of the clinical syndrome recognized as osteoarthritis (OA). The hypothetical role that dietary glucosamine may play in the treatment of OA is to delay, halt, or even reverse this degenerative process. There have been a number of interesting clinical experiments suggesting these effects. However, carefully designed, objective trials are needed to confirm them. If glucosamine is shown to have disease-modifying effects on OA, more basic studies will be necessary to determine the mechanism of action. Additionally, if it is effective in the treatment of the syndrome development of a rational plan to regulate its manufacture and distribution is imperative, so that the patient can be assured of a reliable and pure product.

CHEMISTRY AND PHYSIOLOGY

D-Glucosamine (2-amino-2-deoxy-D-glucose) is a naturally available amino sugar (hexosamine) with a molecular weight of 179.17. The chemical structure is shown in Figure 1.

When taken up by living cells, glucosamine reacts with adenosine triphosphate (ATP) to form glucosamine-6-phosphate, the natural precursor of GAGs that contain *N*-acetylglucosamine (keratan sulfate and hyaluronan) and those that have *N*-acetylgalactosamine (heparan sulfate and chondroitin sulfate). These GAGs are polysaccharides composed of hexosamines and monosaccharides (e.g., galactose and glucuronic acid) arranged as a linear chain of repeating disaccharide units (such as the

glucuronic acid and *N*-acetylgalactosamine-6-sulfate of chondroitin sulfate). With the exception of hyaluronan, GAGs do not exist alone in nature but are attached to specific "core" proteins, and the composite structures are called proteoglycans (protein glycosaminoglycans). Both hyaluronan and many different kinds of proteoglycans (such as aggrecan, versican, and syndecan) are abundant throughout the body where they perform diverse functions (1).

The most abundant proteoglycan of adult human articular cartilage is aggrecan, and it is composed of a protein core substituted with about 100 chondroitin sulfate and about 50 keratan sulfate chains. Because of the high fixed charge density (about 4000 sulfate groups per molecule), and its retention by the collagen network of the tissue, aggrecan generates an osmotic gradient, which retains water within the tissue, thereby providing the articular cartilage with high compressive resistance. This property of the cartilage, together with its capacity to generate a mucin-like molecule called lubricin on its surface (2), is critical to the smooth, essentially frictionless motion observed in normally functioning joints. In patients with early OA, there is a loss of aggrecan from the cartilage, and this compromises the integrity and function of the tissue, which, if left uncontrolled, leads to advanced joint disease, pain, and disability. Because dietary glucosamine could theoretically increase the production of glucosamine-6-phosphate (if it reaches the joint space and is taken up by cartilage cells) and therefore tissue proteoglycans (including cartilage aggrecan), there has been much interest in the possibility that it might represent a means of preventing cartilage loss in OA. However, the pharmacokinetics of dietary glucosamine taken by the general public for this purpose has not been established. In particular, the concentration of that reaches the articular cartilage is unknown, and whether such a level would be sufficient to alter the intracellular concentration of glucosamine-6-phosphate (and aggrecan production) in human cartilage is yet to be determined.

Generally, glucosamine is produced from glucose inside the cell through the hexosamine biosynthetic pathway. Under normal physiological conditions, glucosamine levels in the extracellular fluids are below detection, but if provided in the diet, it is rapidly taken up into cells by glucose transporters (3,4) and is phosphorylated to produce glucosamine-6-phosphate, which enters the hexosamine biosynthetic pathway as such (see Fig. 2 for details).

There have been numerous recent studies directed toward examining how a change in glucosamine concentration (in or around the painful joint) might result in

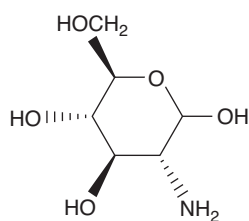


Figure 1 Chemical structure of glucosamine.

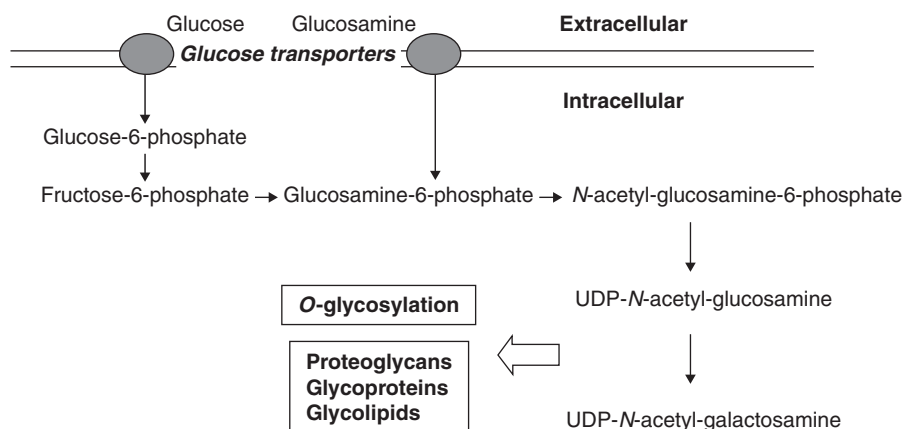


Figure 2 Glucosamine production from glucose by the hexosamine pathway and glucosamine uptake by glucose transporters.

therapeutic benefit. For example, experiments on the effect of glucosamine on chondrocyte or cartilage metabolism have shown how it can inhibit interleukin-1 (IL-1)-induced and aggrecanase-mediated cartilage degradation (5,6), a glucosamine effect that appears to be due to a blockade of the NF κ B signaling pathway (7,8). There are also data demonstrating that glucosamine suppresses the activation of T-lymphoblasts and dendritic cells in vitro as well as allogeneic mixed leukocyte reactivity. Further, glucosamine administration prolonged allogeneic cardiac allograft survival in vivo (9).

All of these studies (5–9) have used high concentrations of glucosamine (0.5–10 mM), either administered IV over a short time in in vivo studies or added to cells and tissue cultures in in vitro studies. However, since the pharmacokinetics of human dietary glucosamine has not been described, the importance of these observations at high concentrations remains undetermined.

PHARMACOLOGY AND PHARMACOKINETICS

Information on the absorption and serum pharmacokinetics for dietary glucosamine is very limited, and in some cases, the available data are contradictory. For example, in one series of studies (10–12), ¹⁴C-glucosamine was given orally to rats, dogs, and humans, and in all cases, the radiolabel was described as “efficiently” absorbed, reaching a plasma peak after about four hours. A high percentage of the radiolabel (about 35%) was excreted in the urine, and a similar amount was lost in expired air. On the other hand, the laboratory that conducted this experiment was unable to detect chemical amounts of glucosamine in human serum after a single oral dose at 100 mg/kg (five times the clinical dose) using a chromatographic assay with a limit of detection of about 14 μ M (13). This suggests that the bioavailable glucosamine in human serum after the normal recommended dosage (20 mg/kg) is well below 10 μ M.

USE OF GLUCOSAMINE IN OSTEOARTHRITIS

Glucosamine has acceptance as a symptomatic slow acting drug for osteoarthritis (SYSADOA) in Europe (14). However, its use in the United States has been controversial. It is marketed in the United States as a dietary supplement, which results in availability without prescription. The public’s access to glucosamine is regulated under the 1994 Dietary Supplement Health and Education Act (DSHEA), which was enacted for less-rigorous regulation of the manufacture, packaging, and claims requirements for complementary and alternative medicine (CAM) agents compared with traditional drugs. This less-regulated environment can result in the arbitrary promotion or advocacy of CAM products and in unsubstantiated scientific claims or empiric utilization. There has been a great deal of interest and information in the lay press. In their books, Theodosakis et al. (15,16) advocated the use of glucosamine as part of a defined therapeutic approach to OA which in addition to the supplements glucosamine and chondroitin, recommends regular exercise, healthy diet, and weight control, “traditional medications” as indicated, and a positive attitude.

OA has been described as “the coming epidemic of arthritis” (17). It is the most common form of arthritis, affecting 12.1% of U.S. adult population or 20.7 million people. It is expected to increase in prevalence substantially in upcoming years due in large part to the aging of the U.S. population (18). Patients with OA frequently seek medical care for improvement in their symptoms. The Arthritis Foundation estimates that there are over 7 million physician visits annually for OA. A recent study addressing primary care utilization found that OA patient visits accounted for more than one-half of general medical visits that involved rheumatologic complaints (19). Currently, recommended medical therapy includes patient education in joint protection, weight reduction, physical therapy, and analgesia, most often with acetaminophen (14,20–22). Often times, these recommendations fail to meet the patient’s expectations, and miscreate a frustrating gap

between hopes and reasonably attainable results. In this setting, patients are turning to complementary and/or alternative therapies in an effort to obtain an added measure of improvement. Likewise, physicians are frustrated by the lack of evidence-based information to establish a foundation for the rational use of these therapies. Often, studies attempting to demonstrate the efficacy of CAM have been hampered by serious flaws. Publication bias in the medical literature, even of trials that have been poorly developed and performed, is toward reports of positive results. Consequently, due to a lack of scientifically credible information, both patients and health care practitioners are often unable to develop rational therapeutic strategies that include CAM.

In an effort to encourage rigorously designed scientific trials that address CAM efficacy, the National Institutes of Health (NIH) established the Office of Alternative Medicine and, subsequently, the National Center for Complementary and Alternative Medicine (NCCAM). The stated mission of NCCAM is to “support rigorous research on complementary and alternative medicine, to train researchers in CAM, and to disseminate information to the public and professionals on which CAM modalities work, which do not, and why” (23). In this complex medical/political milieu, the use of glucosamine in the treatment of OA has become very popular over the past several years.

GLUCOSAMINE PREPARATIONS

Glucosamine is prepared commercially by acid hydrolysis of chitin [poly- β -(1 \rightarrow 4)-*N*-acetyl-D-glucosamine], which is a major component of the shells of Crustacea such as crabs and shrimps. Because it is derived from shellfish hydrolyzates, persons with shellfish allergies should probably avoid exposure, or use with caution. Along with cellulose, chitin is the most widely prevalent natural biopolymer. In shellfish, chitin is clustered with proteins and calcium carbonate. The purification of chitin and its subsequent hydrolysis yields glucosamine. As a weak organic base, glucosamine can be transformed into either a hydrochloride or a sulfate salt form. Commercially available forms of glucosamine include: (i) glucosamine sulfate, (ii) cocrystals and coprecipitates of glucosamine sulfate with potassium or sodium chloride, (iii) glucosamine hydrochloride, and (iv) physical mixtures of glucosamine hydrochloride and potassium or sodium sulfate. Glucosamine is available in highly purified final forms. Details of the various preparations are summarized below.

Glucosamine Sulfate

The “pure” sulfate salt of glucosamine is intensely hygroscopic, and due to the resultant hydration and subsequently low pH, there is potential for oxidation of the amino group (Fig. 3). Because of these properties, this formulation must be preserved with a desiccant under extremely controlled conditions and would be prohibitively expensive. For these reasons, commercial manufacture of “pure glucosamine sulfate,” and development of a clinical application of this formulation is not feasible.

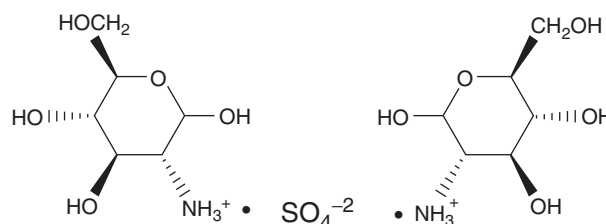


Figure 3 Chemical structure of glucosamine sulfate.

Glucosamine Bonded with Sulfate (Not as a Salt)

There are other substances available that are sometimes termed “glucosamine sulfate.” These compounds are not the sulfate salts of glucosamine. Rather, they are composed of glucosamine with sulfate groups covalently bonded to the hexosamine at different sites. Examples are D-glucosamine 2,3-disulfate, D-glucosamine 2,6-disulfate, D-glucosamine 3,6-disulfate, and D-glucosamine-6-sulfate. Their structures are available through the International Union of Pure and Applied Chemistry (IUPAC). These molecules are not a component of the so-called “stabilized glucosamine sulfate” (which is a salt form of glucosamine discussed later), nor are they available in oral dosage forms. One example is illustrated in Figure 4.

Cocrystals and Coprecipitates of Glucosamine

Because glucosamine hydrochloride was readily available but could not be patented, efforts were directed toward the use of glucosamine sulfate for commercial purposes. However, due to the issues described earlier, commercial development for mass distribution could not be accomplished. To overcome these obstacles, a process was developed and patented that yielded glucosamine sulfate in a cocrystallized matrix with sodium chloride (Fig. 5). This method “stabilized” the glucosamine sulfate, in that addition of sodium chloride led to a reduction in the

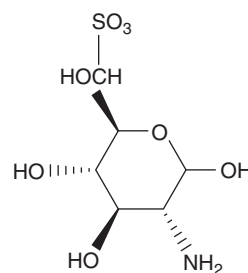


Figure 4 Glucosamine-6-sulfate (not a salt form; sulfate covalently bonded to structure).

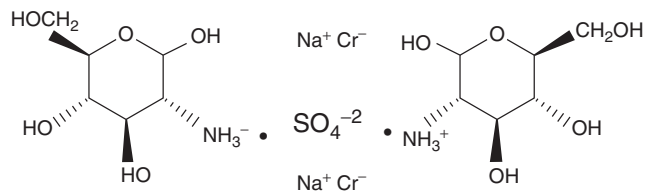


Figure 5 Chemical structure of glucosamine sulfate/sodium chloride coprecipitate.

hygroscopic properties of the compound and made it possible to produce oral dosage forms. This product has since been used in commercially sponsored clinical trials of glucosamine in OA.

Subsequent to the award of patent protection for the production of this "stabilized glucosamine sulfate," there has been commercial promotion alleging that this preparation is therapeutically superior to others, though no clinical studies have been conducted to prove it. In this regard, it is important to recognize that since the biological acid in the stomach is HCl, all dietary glucosamine (independent of the salt form ingested) likely enters the small intestine for absorption as glucosamine HCl. It is also interesting to note that all of the published pharmacokinetic studies on glucosamine in humans have been conducted using ^{14}C radiolabeled glucosamine hydrochloride mixed with unlabeled "stabilized glucosamine sulfate" (11–13).

At least one other glucosamine stabilization method has been patented. Similar to the process described earlier, this technique utilizes lyophilization (freeze drying) to coprecipitate glucosamine sulfate with potassium chloride. This method has also been patented, and the resultant product is commercially available in the United States. In both instances, glucosamine hydrochloride (see below) is the glucosamine substrate that is either cocrystallized or coprecipitated to produce the final "stabilized glucosamine sulfate" salt.

Glucosamine Hydrochloride

Glucosamine hydrochloride is a much more stable salt form of glucosamine than glucosamine sulfate and is produced from chitin in an acid extraction using hydrochloric acid (Fig. 6). It is available as an extremely (>99%) pure

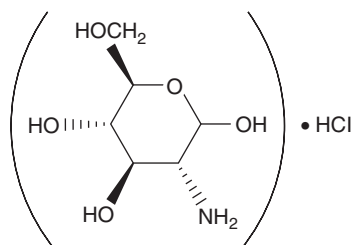


Figure 6 Chemical structure of glucosamine hydrochloride.

compound that is very stable and has a long shelf life. The material can be certified by the Food and Drug Administration (FDA) as compliant with current Good Manufacturing Practices (cGMP) and can be produced to strict pharmacologic standards.

Physical Mixtures

Because of the patent issues described earlier, some products marketed as "glucosamine sulfate" are simply a physical combination of glucosamine hydrochloride and a sulfate salt such as potassium sulfate. The rationale for using this combination is the possibility that both glucosamine and ionic sulfate may be therapeutic, or that ionic sulfate may promote glucosamine absorption in the gut.

However, it must be recognized that when the sulfate dissociates from the glucosamine in the gastrointestinal (GI) tract, highly charged anion(s) are generated that do not readily cross GI tract membranes and potentially result in an osmotic diarrhea. In large doses, this is the basis of the mechanism of action for cathartic laxatives that contain sulfate salts. Normal levels of sulfate in the blood (about 0.3 mM) are critical for many cellular functions, including the synthesis of GAGs. However, the pharmacokinetics of dietary sulfate and its potential effect on cartilage metabolism are unknown, though some authors have suggested a role for sulfates in OA (24,25).

Comparison of Salt Forms

As detailed in the subsections earlier, there are practical therapeutic considerations inherent in the physical characteristics of the various glucosamine preparations. These are summarized in Table 1. The actual quantity of glucosamine found in the preparations varies due to the size of the associated salt form. Thus, as can be seen, in the usual daily dose of 1500 mg/day, the actual level of glucosamine can range from 895 to 1245 mg/day.

Most often, salt forms of pure substances are prepared to improve solubility characteristics. Hydrochloride salts are among the most commonly used forms of salts of weak organic bases, because chloride is readily available, is found naturally in the human body, and produces salts with good stability characteristics. An additional advantage is that on a molecule-per-molecule basis, these salts are smaller than those made with ions such as citrate, lactate, and sulfate (e.g., the molecular weight of HCl is 36, whereas that of H_2SO_4 is 101). This is important when considering whether a dose is referenced to the amount of active parent drug or to the quantity of the salt form as seen in Table 1. Finally, therapeutic drug monitoring in patients receiving a salt form of a drug is conducted using blood concentration of the parent drug, not the salt. In the instance of glucosamine, the salt form dissociates when it dissolves in the GI tract. Hence, in performing pharmacokinetics of glucosamine HCl (or H_2SO_4), only the glucosamine moiety is measured.

In the case of glucosamine hydrochloride, glucosamine sulfate, and any of its stabilized forms, the dissolution of these molecules will also involve dissociation of the salt. There has been no published evidence, nor have we observed any difference in the rate of dissolution of any of the glucosamine-containing preparations.

Table 1 Quantities of Glucosamine Present in Different Preparations^a

Comparative attributes	Glucosamine HCl	Glucosamine SO ₄ -2 NaCl	Glucosamine SO ₄ -2 KCl
Purity (as the salt form) (%)	99 +	79.5 (20.5% NaCl)	75 (25% KCl)
Weight percentage as glucosamine	83.1	62.7	59.5
Dose (mg) to yield 1500 mg of glucosamine	1805	2392	2521
Glucosamine content (mg) per 1500 mg of substance	1246.5	940.5	892.5

^aDoes not include comparison of physical mixtures of glucosamine hydrochloride and potassium sulfate because these mixtures can be prepared in varying concentrations.

CLINICAL TRIALS USING GLUCOSAMINE IN OSTEOARTHRITIS

Many of the controlled clinical trials with glucosamine in OA patients have been of marginal quality due to insufficient sample size, lack of statistical rigor, potential for sponsor bias, inadequate concealment, and lack of intention-to-treat principles. A systematic review on glucosamine in OA was published in 2005 and included 20 randomized controlled trials (RCTs) for analysis (26). Results from this analysis showed a significant improvement in OA pain and function. Overall, a mean difference of 28% between glucosamine and placebo was noted, and a mean difference in function of 21%. These differences were substantially reduced when compared with the previously published meta-analysis reviewing glucosamine use in OA, which was published in 1999. These differences were largely explained by the inclusion of eight new studies and focusing the analysis to the most well-designed trials. Additionally, if only studies with adequate blinding were included in the efficacy analysis, glucosamine offered no significant improvement for pain or function (27).

Increasing interest in the use of glucosamine for treatment of OA has provided the impetus for larger, multicenter clinical trials to investigate its efficacy and safety more carefully. The Glucosamine/Chondroitin Arthritis Intervention Trial (GAIT) evaluated the use of glucosamine, chondroitin, and both in combination compared with placebo in patients with symptomatic OA of the knee (27). This study enrolled 1583 patients with knee pain secondary to OA and randomized them to glucosamine HCl 1500 mg daily, sodium chondroitin sulfate 1200 mg daily, glucosamine and chondroitin sulfate, celecoxib 200 mg daily, or placebo. These patients were followed over 24 weeks with the goal of attaining a 20% decrease in knee pain as assessed by the Western Ontario MacMaster Osteoarthritis Questionnaire (WOMAC). The results of this study indicated that glucosamine did not significantly reduce pain due to knee OA in the overall group of patients. However, an analysis of the patients with moderate to severe pain showed a significant improvement in knee pain with the combination of glucosamine and chondroitin sulfate. A trend toward significance with glucosamine alone was noted in this subgroup of patients, but these results must be interpreted with caution in light of the small number of patients in the subgroup (27). In general, clinical trials that have larger sample sizes and those without industry support tended to have smaller effect sizes.

Three other glucosamine clinical trials merit specific comment. These studies present data from patients who

received long-term glucosamine therapy with the primary objective being to evaluate progressive loss of joint space in the knee and thus assess the potential for disease modification using serially obtained knee X-rays as the outcome measure. Other outcomes were also addressed aimed at evaluating improvement in joint pain over the duration of the trial. Two of the three studies were industry supported.

The first study (28) evaluated 212 patients followed for three years on 1500 mg glucosamine per day versus placebo. It assessed change in medial compartment joint space width as determined on standing, weight-bearing anteroposterior knee radiographs as the primary outcome. Symptomatic outcomes were assessed using the WOMAC instrument. The authors reported that the patients taking glucosamine experienced no loss in joint space, while those on placebo continued to show progressive cartilage loss. Glucosamine-treated subjects also experienced improved symptoms in total WOMAC index based on intent-to-treat statistical principles.

In the second study evaluated (29), 202 patients received 1500 mg glucosamine per day or placebo. Once again, radiographic medial joint space narrowing as described in the study earlier was the primary outcome measure. Symptomatic evaluation was measured using both the WOMAC and Lequesne instruments. The researchers found that patients taking glucosamine showed no progression of medial joint space narrowing, while the placebo-treated subjects experienced progressive joint space narrowing. The study also reported a completer's analysis that demonstrated significant improvement in symptoms based on both the above-mentioned indices.

The third study was an ancillary report from GAIT wherein 572 patients with knee OA were followed for two years (30). These patients had been randomized to receive glucosamine 500 mg three times daily, chondroitin sulfate 400 mg three times daily, the combination of both supplements, celecoxib 200 mg daily, or placebo as part of the GAIT study and were followed over 24 months with the primary outcome measure of mean change in joint space width (JSW). This study found no statistically significant difference in the loss of JSW in any of the treatment groups compared with placebo, but was limited by the smaller sample size and smaller than expected loss in JSW.

At least two major concerns have been raised regarding the validity of the selected radiographic outcome measure in the first two studies. The first is that because of anatomic positioning in the extended AP view of the knee, the JSW does not actually measure articular cartilage only, but others as well such as the meniscus and status of the collateral ligaments and therefore may not indicate true JSW. Secondly, positioning of the joint for

radiography in terms of extension may be influenced by the amount of joint pain at the time the film was taken (31). Thus, patients with less painful knees may have had less guarding and therefore more extension that could give the appearance of wider JSW. The third study addressed these concerns by utilizing the semiflexed metatarsophalangeal (MTP) view protocol described by Buckland-Wright et al. for use in trials assessing efficacy of structure-modifying drugs in knee OA (32).

A seemingly overlooked, yet remarkable, finding in the first two trials was the improvement that was seen in joint pain over the years of study follow-up. Sustained lessening in pain of the degree and duration suggested by these trials has never been reported before for any agent in the management of OA. This is certainly puzzling information regarding glucosamine efficacy in a controlled setting.

Study data regarding the use of glucosamine in hip OA is limited. Two of the studies published in the last few years found no significant differences in symptom reduction or joint space loss between glucosamine sulfate and placebo (33,34).

It remains unclear whether true clinical differences between glucosamine sulfate and glucosamine HCl exist, or whether these apparently discordant efficacy results are due to inadequate blinding and industry bias (35,36). A large clinical trial investigating glucosamine sulfate efficacy versus placebo or analgesic medications would help to further elucidate this issue.

SAFETY

The safety profile of glucosamine in the published studies described earlier is uniformly favorable and comparable to placebo. A few minor adverse events have been reported, including GI complaints, such as heartburn, diarrhea, constipation, epigastric pain, and nausea (37). One concern regarding the use of glucosamine is its potential to cause or worsen diabetes. In animal models, increased glucosamine levels in cells have been associated with insulin resistance (a major factor in the genesis of type II diabetes mellitus) and alterations in insulin production (38–40). Whether the doses commonly used in humans are sufficient to cause significant alterations in glucose homeostasis is not clear at this time. A study by Scroggie et al. (41), however, found that glucosamine treatment of known diabetics did not change either their diabetes management or their diabetes control as assessed by levels of hemoglobin A1c.

There has been some concern about potential interactions between glucosamine and glucosamine chondroitin sulfate combination with warfarin. A recent case report with additional literature review was published (42). This review described 20 cases suggesting interactions of glucosamine with or without chondroitin sulfate that resulted in supratherapeutic international normalized ratio (INR) and/or increased bleeding complications. Importantly, other factors known to alter hemostasis, and other important clinical/environmental factors could not be assessed in the majority of the MedWatch reported cases. To date, there are no clinical series that document glucosamine and warfarin interactions. Nevertheless, pa-

tients taking warfarin should be advised of this potential interaction and monitored for INR changes.

RECOMMENDATIONS

Glucosamine is a natural aminosaccharide present in the exoskeletons of arthropods and is obtained from chitin by acid decomposition. It does not exist as a natural biosynthetic product in cells. Instead, it is generated as glucosamine-6-phosphate by the reaction of fructose-6-phosphate and glutamine (see Fig. 2 pathway for detail). Glucosamine-6-phosphate precedes in the biosynthesis of the GAG component of proteoglycans such as cartilage aggrecan. Loss of cartilage aggrecan due to excessive proteolysis is part of the clinical syndrome identified as OA. Dietary glucosamine may slacken, stall, or even counter this degenerative process. While there have been smaller clinical studies suggesting these effects, larger, more meticulous experiments have not been able to validate them consistently. The best current evidence suggests that the effect of glucosamine on OA pain, function, and radiographic change is minimal at best. Some patients may decide to continue taking glucosamine due to a perceived benefit that may represent placebo response, varied individual metabolism, or even differences in preparation. Reported results from recent trials that suggest lack of efficacy do not appear to have altered consumer behavior regarding use of oral supplements (43). Studies to further clarify differences in glucosamine preparations would be helpful, but efficacy results may have limited impact on public behavior toward OA (44). In light of this possibility, public funding for such studies may be limited. Safety data from the more recent, larger trials further support the benign side-effect profile of glucosamine, and patients who want to continue taking it may be reassured that it is not considered dangerous.

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Glutamine

Steven F. Abcouwer

INTRODUCTION

Glutamine is the most abundant amino acid in the body, due to its relatively high concentration in the blood and comparatively large stores of free glutamine in muscle tissue. It is found in all proteins, and thus any protein source provides glutamine in the diet. The free form is also found in meat, milk, fruits, and vegetables. Glutamine has become a popular nutritional supplement due to claims that its consumption can boost immune function and increase muscle mass and volume. However, while glutamine is nontoxic and probably harmless, the benefits of regularly supplementing the diet with this amino acid have not been proven. Glutamine is not an essential amino acid, since it can be readily produced in all tissues, with muscle being the primary source of glutamine in the blood. Although there is no dietary reference intake (for under normal conditions, glutamine is not a required dietary constituent), during critical illness, severe trauma, intestinal disease, starvation, total parenteral nutrition (intravenous feeding), wasting (excessive loss of lean body mass), and extreme endurance exercise, the body's consumption of glutamine can exceed dietary intake plus tissue production of this amino acid. Hence, glutamine is referred to as a "conditionally essential" amino acid. Under catabolic conditions, lack of glutamine may contribute to immunodepression and limit reparative processes. Glutamine also may help to prevent intestinal atrophy during total parenteral nutrition. Thus, in specific instances glutamine may be quite beneficial as an adjunct to other nutritional support regimens. However, convincing large scale, blinded clinical trials proving the benefits of glutamine supplementation are still lacking.

NAME AND GENERAL DESCRIPTION

Glutamine (L-glutamine, Gln, Q, CAS Registry number 56-85-9) is a nonessential, neutral, polar amino acid, one of the 20 common amino acids found in proteins. Its molecular weight is 146.15, and its molecular formula is $C_5H_{10}N_2O_3$. Unlike most amino acids, glutamine contains two nitrogen molecules: one is part of the " α -amino" group, and the other is part of an amide or "amido" group of the amino acid side chain (Fig. 1). The addition of the amide group differentiates glutamine from the closely related amino acid glutamate (L-glutamic acid, Glu, E). More than any other amino acid, glutamine is key to nitrogen shuttling, metabolism, and energy production. Glutamine is highly abundant in the bloodstream. The interconversion of glutamine and glutamate by addition and removal

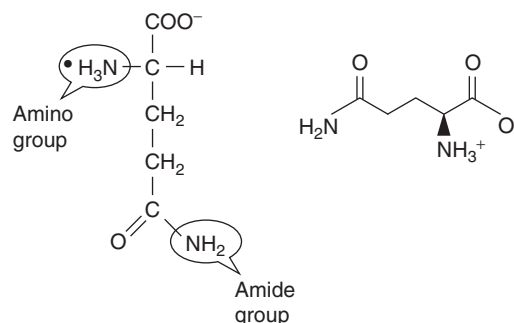


Figure 1 The structure of glutamine. Two representations of glutamine: On the left is the structural formula, with the amino nitrogen and amide nitrogen side groups indicated. On the right is a conformational formula. In solution at neutral pH, both the amino and carboxylic acid groups of glutamine are charged.

of this amido group makes glutamine a convenient interorgan nitrogen shuttle and a nitrogen donor for many critical synthetic biochemical reactions. As glutamate, the α -amino nitrogen is used for additional synthetic reactions, including the formation of amino acids from keto acids, thus converting glutamate to the keto acid α -ketoglutarate (2-oxoglutarate). As α -ketoglutarate, glutamine carbons enter the tricarboxylic acid cycle (TCA cycle). In this way, glutamine can serve as an important oxidative fuel source, providing reducing equivalents [e.g., NAD(P)H, FADH₂] and, ultimately, cellular energy. Entry of glutamine carbons into the TCA cycle serves the equally important function of replenishing TCA cycle substrates (anaplerosis), thus maintaining the cells capacity to oxidize glucose and fatty acids.

BIOCHEMISTRY AND FUNCTIONS

Biological Synthesis and Utilization

Glutamine is formed directly from cellular glutamate by the addition of ammonia in an adenosine triphosphate (ATP)-requiring reaction catalyzed by the enzyme glutamine synthetase (GS, E.C. 6.3.1.2) also referred to as glutamate-ammonia ligase (GLUL). This enzyme is found in the cell cytoplasm. Its function is solely to form glutamine at the expense of cellular glutamate and energy. Glutamine may be formed because it is needed for synthetic reactions, ammonia detoxification, or for export to other tissues. In turn, glutamine is readily converted to glutamate by several amidotransferase enzymes and

glutaminase enzymes (GA or GLS, E.C. 3.5.1.2). The former enzymes transfer the amide nitrogen of glutamine to other molecules in the course of biosynthesis. In this way, glutamine is necessary for the production of other amino acids, purine and pyrimidine bases, amino sugars, and several coenzymes (1). The glutaminase reaction produces glutamate and ammonia. Two related glutaminase genes exist: *Gls1* is expressed in kidney and other tissues, and the protein encoded by the gene is referred to as the kidney-type, or K-type isoform (KGA). *Gls2* is expressed predominantly, but not exclusively, in the liver and is referred to as liver-type or L-type glutaminase (LGA) (2,3). *Gls1* and *Gls2* each give rise to more than one isoform of the proteins due to alternative splicing of the mRNA formed from these genes (4,5). The functional significances of these GA isoforms are not fully known. However, the expression of one KGA isoform known as GAC is controlled by acidity, which has important implications for the control of chronic metabolic acidosis by the kidney (6).

Metabolic Functions

Being one of the 20 amino acids is found in proteins, free glutamine is needed for protein synthesis. However, glutamine and molecules derived from glutamine serve numerous vital roles in other metabolic processes. Glutamine serves as a primary source of cellular glutamate. The normal plasma concentration of glutamine is relatively high, whereas the plasma concentration of glutamate is quite low (7,8). Conversely, in most tissues (with the notable exception of muscle), the intracellular concentration of glutamine is much lower than that of glutamate. Cells exhibit large capacities for the import of extracellular glutamine (9). Once inside the cell, glutamine is readily converted into glutamate by the action of amidotransferase and glutaminase enzymes. Glutamate is a substantial intracellular anion, playing a vital role in maintenance of cell osmolarity and therefore cell volume (10). Thus, extracellular glutamine can act as a key mediator of cell swelling (11). Glutamate formed from glutamine is indispensable for many cellular processes in addition to protein synthesis (for review, see Refs. 12,13). For example, glutamate serves as a precursor for proline synthesis. It also supports the synthesis of the tripeptide molecule glutathione, the cell's major store of reducing equivalents (14). Glutamate does this directly by serving as a substrate for glutathione synthesis, and indirectly by providing a means for the cell to import cysteine, another substrate for glutathione synthesis (15). Transamination reactions convert glutamate to α -ketoglutarate (2-oxoglutarate), as they utilize the amino nitrogen of glutamate to convert keto acids to amino acids. In this way, glutamate is central to the cell's amino acid economy. α -Ketoglutarate is also formed as glutamate is oxidatively deaminated by the enzyme glutamate dehydrogenase (GDH), with the concurrent reduction of NAD(P)^+ to NAD(P)H . As α -ketoglutarate, the carbon backbone of glutamate enters the TCA cycle. In this way, glutamine is utilized for anaplerosis, the replenishment of TCA cycle intermediates. In this capacity, glutamine supports the oxidative catabolism of glucose and fatty acids. Once in the TCA cycle, glutamine-derived carbons may themselves be oxidized to CO_2 making

glutamine an important source of cellular energy (16). Partial traversing of α -ketoglutarate around the cycle forms malate, and conversion of malate to pyruvate by the *malic enzyme* completes a process referred to as "glutaminolysis" (in analogy to glycolysis). Glutaminolysis not only results in conversion of glutamine to pyruvate, but also results in production of reducing equivalents in the forms of nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), and flavin adenine dinucleotide (FADH₂). These reducing equivalents are utilized for ATP synthesis by oxidative phosphorylation, synthetic reactions, and cellular protection against oxidative stress (Fig. 2) (17). Pyruvate formed from glutamine may be converted to CO_2 and acetyl-CoA by *pyruvate dehydrogenase*. As these acetyl groups enter the TCA cycle, glutamine-derived carbons can potentially undergo complete oxidation to CO_2 .

PHYSIOLOGY

Cellular Functions

In 1955, Harry Eagle pioneered the growth of mammalian cells in culture. In the course of developing culture media for these cells, he tested the requirements for numerous salts, vitamins, minerals, carbohydrates, and amino acids (18). The studies found that glutamine was necessary to support the growth and viability of cells in culture, and at concentrations greater than that of any other amino acid (19). Eagle and colleagues subsequently determined that both protein synthesis and nucleic acid synthesis were dependent on glutamine (20). Now it is known that nearly all mammalian cell cultures benefit from the addition of glutamine to their media. Thus, cell culture media is almost always supplemented with concentrations of glutamine (or a glutamine-containing dipeptide) that are an order of magnitude greater than those of other amino acids. However, during all this time, the exact nature of this dependence on glutamine has not been fully clarified. Perhaps this is because the metabolic functions of glutamine and glutamate are so varied. Indeed, a supply of glutamine is needed to support numerous cellular processes.

Support of Cell Proliferation

The need for glutamine is particularly acute for proliferative cells. In the adult, cell proliferation is most active in the intestine, immune system, and during wound healing. Cells within the intestinal epithelium constantly divide to cope with cell loss and renewal. Replacement of damaged epithelial cells through a process of crypt cell proliferation and differentiation along the crypt-villous axis seems to be supported by glutamine (21). In culture, intestinal epithelial cells are avid glutamine consumers, and their growth is glutamine dependent (22). Cell growth and replacement also characterize the immune system. In response to immune challenge, immune cells of both T and B lineage undergo clonal expansion followed by programmed cell death (apoptosis) when the infection has abated. Immune cells of all types exhibit marked glutamine dependence for both activation and proliferation (23). Glutamine also inhibits cell death caused by several cellular stresses, and thus has been referred to as an "apoptosis suppressor" (17). Highly proliferative cancer cells tend to require and

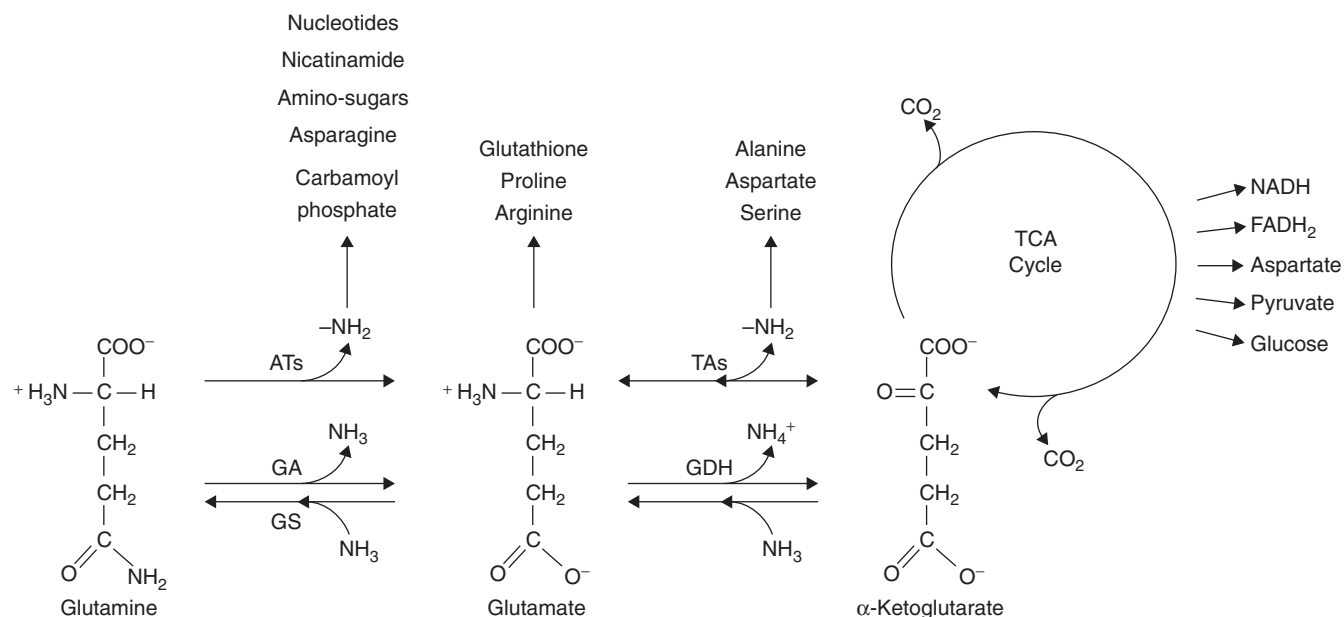


Figure 2 Metabolic functions of glutamine. Abbreviations: GA, glutaminase enzymes; ATs, amidotransferase enzymes; GS, glutamine synthetase; GDH, glutamate dehydrogenase; TAs, transaminase enzymes; TCA cycle, tricarboxylic acid cycle.

consume large amounts of glutamine (12,24). Recently, a novel finding provided great insight into the mechanism by which glutamine consumption is increased in transformed tumor cells. Gao and colleagues described a mechanism by which activation of the *c-Myc* oncogene greatly increases the expression of GLS1 (KGA) in cancer cells (25). MYC accomplishes this indirectly by downregulating the expression of two micro-RNAs (miR-23a and miR-23b) that function to limit expression of KGA protein. The expression of glutamine and glucose transporters plus several other activities that support both energy production and biosynthetic metabolism is increased by MYC (For review see Ref. 26). Thus, an oncogene central to stimulation of normal and cancerous growth does so in part by increasing uptake and metabolism of glutamine in order to produce the energy and anabolic substrates necessary for cell growth and division.

SYSTEMIC METABOLISM

Glutamine Cycling in Brain and Liver

Glutamine synthetase occurs in all tissues and is especially abundant in brain and liver. In the brain, GS activity is vital for the conversion of glutamate (one of the most important neurotransmitter molecules) to glutamine by astrocytes (27). This serves to prevent the accumulation of glutamate, which is toxic at high levels, and to detoxify ammonia. Glutamine is then transferred from the astrocytes to neurons, which convert it back to glutamate to be released at synapses in response to stimuli. In addition, since the neurotransmitter γ -aminobutyric acid (GABA) is derived from glutamate and can be eventually converted to glutamine in astrocytes, a glutamate/GABA-glutamine

cycle is central to neural function and prevention of neurotoxicity (for review, see Ref. 28).

In the liver, another type of glutamine–glutamate cycle operates due to zonation of glutamine synthesis and catabolism (29). Blood entering the liver from the gut via the portal vein carries waste nitrogen that must be disposed of by conversion to urea. This nitrogen is primarily carried by the amino acids alanine and glutamine. Periportal hepatocytes, in zone 1 of the hepatic acinus, contain high GA activity. These cells extract glutamine from incoming blood and convert it to glutamate, producing ammonia that then enters the urea cycle. In addition, the α -amino nitrogen of glutamine is converted to ammonia by oxidative deamination of glutamate. In a transaminase reaction, the α -amino group of glutamate is transferred to oxaloacetate to form aspartate that can enter the urea cycle. Thus, as the blood moves down the liver sinusoids, it is stripped of glutamine. GS activity in the liver is concentrated in perivenous hepatocytes, in zone 3 of the liver acinus. In the perivenous section of the sinusoid, excess ammonia is scavenged and incorporated into glutamine by GS; ammonia is removed and the concentration of glutamine in the outgoing venous blood is adjusted (30). In this way, the liver both packages nitrogen into urea for disposal by the kidney and exerts control over plasma glutamine level.

Glutamine Production by Muscle and Lung

The muscle, lung, and adipose tissue are major sources of glutamine in circulation, and these tissues increase glutamine production during catabolic states. The expression of the GS gene in these tissues is increased in response to stress hormones, principally glucocorticoids (31). The human GS gene has not been characterized. However,

the rat GS gene includes two regions containing glucocorticoid response elements (GRE), which are responsible for increased transcription in response to glucocorticoid hormones (32). This phenomenon of increased gene expression is particularly evident in muscle and lung tissues (33). Thus, in response to stress hormones, muscle and lung tissues produce increased amounts of GS mRNA that is translated into GS protein, facilitating the production of increased amounts of glutamine for release into the bloodstream.

In addition, the ultimate accumulation of GS protein is regulated by a unique feedback mechanism that responds to the need for glutamine synthesis. Namely, the degradation rate of the GS protein is increased by glutamine (34). Thus, when glutamine is abundant, GS protein is rapidly degraded. When intracellular glutamine is depleted, GS protein degradation decreases and the GS level increases. In this way, the amount of GS protein is indexed to the need for glutamine. This seems to occur through regulation of GS protein proteosomal degradation rate by glutamine. In times of stress, glucocorticoid hormones increase GS transcription, and the resulting elevated level of GS mRNA leads to increased GS protein production. Nevertheless, GS protein will not appreciably accumulate unless there is a need for increased glutamine production, as signaled by intracellular glutamine depletion (34). Importantly, stress hormones also signal changes that lead to increased glutamine export from cells (35). Thus, as these hormones signal for increased GS expression, the intracellular glutamine stores become depleted. This leads to a synergistic mechanism by which GS protein is produced at an accelerated rate and degraded at a reduced rate. This results in a robust increase in GS activity, until such time that the production of glutamine is sufficient to match the rate of its export.

Control of Acidosis

Glutamine utilization by the kidney is essential for controlling the amount of acid in the blood. This is accomplished through ammonia formation by the kidney-type GA and GDH, using glutamine as the source (36). Once GA forms glutamate and ammonia, GDH catalyzes the oxidative deamination of glutamate to form α -ketoglutarate and ammonia. The ammonia formed from these two reactions binds hydrogen ions to form ammonium ions that are eliminated in the urine along with acid anions. During shock, starvation, uncontrolled type I diabetes, and severe diarrhea, metabolic acidosis can occur due to the increased production of ketoacids (e.g., acetoacetate and β -hydroxybutyrate) or the loss of bicarbonate ions. To dispose of these extra acid anions, the kidney greatly increases its utilization of glutamine, thereby producing ammonia. This is accomplished by increasing the expression of GA and GDH within the kidney tubules. The expression of KGA (the GAC isoform) and GDH in response to acidosis is controlled by a unique mechanism that involves stabilization of these mRNA via multiple RNA-binding proteins within cytoplasmic stress granules that form in response to acidic pH (37). The consequence of greater utilization of glutamine by the kidney during acidosis is

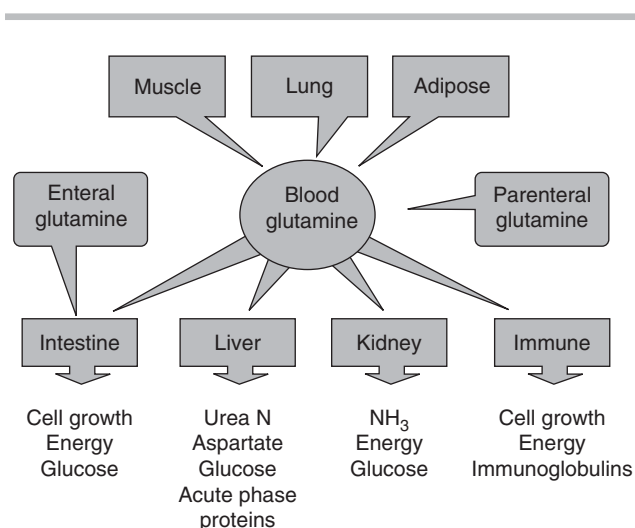


Figure 3 Systemic glutamine metabolism.

that the demand for systemic glutamine synthesis is correspondingly increased (Fig. 3).

ROLE IN CATABOLIC DISEASE

Catabolic States

Catabolic states describe any metabolic situation in which fat and lean body masses are utilized faster than they are restored. However, the term catabolic state is usually used to describe a pathological state where fat and lean body mass (primarily muscle) is utilized in response to hormonal signals and/or increased metabolic demands that are not being met by nutrient uptake. Severe trauma, burn, infection, starvation, or chronic diseases such as HIV/AIDS and cancer cachexia all cause catabolism to increase and metabolism to decrease so that the result is net loss of body mass. Increased protein degradation during these states produces greater amounts of waste nitrogen and thus requires increased nitrogen shuttling by glutamine (38). These states are also associated with ketosis causing metabolic acidosis. Glutamine demand by the kidney is increased to counter this acidosis. In addition, immune activation and expansion may also cause an increased glutamine demand as immune cell consumption increases. If infection occurs, the liver increases its consumption of glutamine and other amino acids to support acute phase protein synthesis. If sustained, catabolic states can lead to severe depletion of lean body mass, thereby diminishing the ability of muscle tissue to produce glutamine and satisfy this increased demand, and ultimately leading to impaired immunity, poor wound healing, and loss of intestinal barrier function (39,40).

Interorgan Transport

During catabolic states, stress hormones trigger an increased rate of muscle protein degradation, while decreasing muscle protein synthesis (35). Thus, free amino

acids are produced. The carbon skeletons of branched-chain amino acids can be utilized for oxidative energy production by the muscle, thus sparing glucose for use by other tissues (41). The muscle also releases amino acids produced from protein degradation, which are then utilized in other tissues, especially those in the intestine, kidney, liver, and immune system. In the muscle, the amino groups from oxidation of branched-chain amino acids ultimately become waste nitrogen in the form of ammonia. In order to dispose of this nitrogen, muscle tissue utilizes glutamine and alanine as nitrogen carriers (42). Thus, lean body mass is converted into energy, and a mixture of amino acids is released that is dominated by glutamine and alanine (43). The lung also increases production of glutamine during catabolic states (44). Adipose tissue has also been implicated as a producer of glutamine (45). However, the source of nitrogen to support the production of glutamine by these two tissues has not been established. Glutamine may be produced from glutamate and ammonia extracted from blood (46). It is also possible that branched-chain amino acids produced in the liver could be extracted by these tissues and used to support glutamine synthesis (42).

Glutamine is an ideal interorgan nitrogen shuttle because it can be readily formed from intracellular glutamate, and because each molecule carries two ammonia equivalents. Alanine is also an ideal nitrogen shuttle because it can be readily formed from pyruvate by a single transamination reaction, and as it can then serve as a ready source of energy and glucose when it is converted back to pyruvate. Thus, both glutamine and alanine carry nitrogen in nontoxic forms, serve as energy sources for visceral tissues, and serve as gluconeogenic precursors for the liver, kidney, and intestine (46,47). Glutamine is used by the kidney to help control metabolic acidosis. Therefore, most of the nitrogen carried by these two amino acids will ultimately be converted to urea by the liver, but a portion of the nitrogen carried by glutamine will be disposed of as ammonia in the kidney.

NUTRITIONAL SUPPLEMENTATION

Total Parenteral Nutrition (TPN)

In severe catabolic states or situations where oral nourishment cannot be tolerated, intravenous feeding, referred to as TPN, is used. Until recently, TPN formulations did not include glutamine. This was in part due to the fact that glutamine is unstable in solution; it slowly decomposes to form ammonia and pyrrolidonecarboxylic acid (48). Lack of enteral feeding during TPN leads to intestinal atrophy (49). Because glutamine is such an important substrate for the intestine, it was reasoned that its inclusion in TPN solutions would alleviate intestinal atrophy. Numerous studies using animal models have confirmed this assumption (for review, see Ref. 50). In addition, many human trials have demonstrated significant benefits in inclusion of glutamine in TPN solutions (for review, see Ref. 51). Including glutamine-containing dipeptides that are cleaved in the circulation to produce free glutamine solved the problem of glutamine instability (52). However, the inclusion of up to 25 g/day glutamine in TPN solution has very little effect on glutamine concentrations in blood and muscle (53).

In contrast to TPN, the benefits of enteral glutamine supplementation are still being debated (54). In fact, several efforts to reverse wasting and cachexia by glutamine feeding have not been successful (55,56). Meta-analyses of clinical trials of glutamine nutritional support concluded that it was associated with an insignificant reduction in mortality and infections in critically ill and surgery patients (57) and failed to discern any significant consistent improvements in patients undergoing bone marrow transplantation and cancer therapy (58).

Support of Intestinal Renewal and Function

Glutamine's ability to support intestinal renewal and function has made it a prime candidate for nutritional support of patients with intestinal diseases, including short-bowel syndrome and inflammatory bowel disease, such as Crohn's disease (for review, see Ref. 50). However, most studies showing the benefits of enteral glutamine feeding on intestinal repair and function have been conducted using rats and pigs. Although glutamine is now being routinely incorporated into treatments that include various growth factors and nutrients, clinical data confirming these beneficial effects in humans are lacking (59,60). Glutamine feeding is also believed to improve intestinal mucositis caused by chemotherapy or radiation treatment (61,62). Results of studies with animals have suggested that it can accomplish this. Although clinical studies show that glutamine supplementation is safe and may have benefits for patients given chemotherapy or radiation, there are not sufficient controlled clinical data on humans to reach a final conclusion regarding glutamine nutritional support for critically ill patients in general (63–65). Many of the positive conclusions have been based upon small, poorly controlled and unblinded studies. Given the bias to publish studies showing a positive effect, it is likely that the conclusions of small studies will not be recapitulated in larger trials. For example, a phase III, randomized, double-blind clinical study conducted by the North Central Cancer Treatment Group found that glutamine feeding had no effect on acute diarrhea in patients receiving pelvic radiation therapy (66).

Very Low-Birth Weight Infants

Another group of patients who may benefit from glutamine feeding is very low-birth-weight infants. These babies are born with underdeveloped alimentary systems that make them unable to tolerate oral feedings and render them prone to necrotizing colitis and susceptible to sepsis due to poor barrier function of the intestinal epithelium. Several early studies found that glutamine feeding of premature infants can decrease morbidity and reduce hospital costs (for review, see Ref. 67). However, subsequent clinical trials have not demonstrated appreciable benefits of glutamine in this patient group. A trial of glutamine in TPN for very low-birth-weight infants found that glutamine reduced the time until these babies could tolerate full enteral feeding (13 vs. 21 days) (68). However, glutamine did not reduce the incidence of sepsis or age at discharge. A clinical trial of glutamine feeding for extremely low-birth-weight babies found that glutamine fed infants did not exhibit greater tolerance of enteral feeding, suffer less necrotizing enterocolitis, or exhibit greater weight

gain (69). A recent meta-analysis found that glutamine supplementation did not significantly affect neonatal morbidities including invasive infection, necrotizing enterocolitis, time to achieve full enteral nutrition, or duration of hospital stay of preterm infants (70).

Sickle Cell Disease

It has been suggested that glutamine feeding may alleviate the anemia associated with sickle cell disease. In 1975, a study found that incubation of sickle cells in high concentrations of homoserine, asparagine, and glutamine reduced the sickling of the red blood cells (71). A subsequent study discounted the effects of these amino acids when it was found that they did not restore the deformability of sickle cells and did not raise the minimum gelling concentration of deoxyhemoglobin S, in spite of noticeable morphological effects on the cells (72). On the other hand, more recent studies found that oral glutamine does improve the redox state of sickle cells and decrease their adhesion to endothelial cell layers (73,74). A small study (27 participants) of children and adolescents with sickle cell disease suggested that dietary glutamine supplements decreased resting energy expenditure and improved nutritional parameters (body mass index, body fat percentage, and muscle strength) of these patients (75).

Athletic Performance Enhancement

Glutamine has recently become a popular dietary supplement marketed to athletes. Its use to boost athletic performance and promote muscle gain is based on three observations: (i) Glutamine concentration in the plasma is decreased following extreme endurance exercise, such as marathon running (76), (ii) muscle breakdown leads to release of large amounts of glutamine from the muscle (43), and (iii) glutamine, in its role as the primary source of glutamate, is vital for the maintenance of cell volume (10). It is rational to believe that consumption of glutamine would prevent depletion of plasma glutamine, even boosting its concentration in the blood. However, studies in animals and humans have shown that enteral consumption of large amounts of glutamine causes only slight and transient increases in blood glutamine levels (7). This is partly due to extensive first pass extraction and oxidation of enterally delivered glutamine by the gut. (77). If the muscle were no longer required to supply glutamine, then increasing glutamine supply might be expected to deter muscle protein breakdown. Although this is seemingly logical, there is no scientific evidence that glutamine supplementation, either by enteral or parenteral routes, can decrease muscle glutamine release or increase muscle glutamine import. Both muscle protein breakdown and glutamine release are hormonally controlled (35). Thus, the balance between anabolic and catabolic hormones is more likely to influence net buildup or loss of lean body mass. Furthermore, increasing plasma glutamine concentration would increase muscle cell glutamate concentration, osmolarity, and cell volume only if muscle glutamine uptake is appreciably increased for sustained periods. Although there is experimental evidence to suggest that this can occur in rats (78), there is no evidence to suggest that oral glutamine consumption can increase muscle glutamine uptake or decrease muscle glutamine release in humans. Though only

short-term studies have been performed so far, they do not support the hypothesis that oral glutamine supplementation improves athletic performance (for recent review, see Ref. 79).

Immune System Enhancement

Because immune cells are dependent upon glutamine, this amino acid has been touted as an "immune booster." Commercial energy drinks and juice vendors offer immune booster additions that include glutamine. Several animal studies have found beneficial effects of glutamine feeding on measures of immune function, especially mucosal immune function (80). Because glutamine feeding has minor effects on plasma glutamine concentration, this effect is probably not due to delivery to immune cells in the circulatory system. Oral glutamine may directly affect the proliferation and development of gut-associated lymphoid tissue (GALT). These immune cells mature and expand their numbers while residing in the intestine. They are later associated with mucosal membranes and, therefore, are vital for protection against infection through these barriers (81). In the intestine, these cells are exposed to ingested glutamine, which may stimulate their growth and development (80). Enteral glutamine decreased mortality and infectious morbidity in burn patients, perhaps by reducing intestinal permeability and bacterial translocation (82). Although several small studies suggest that inclusion of glutamine in nutritional formulas may benefit critically ill patients (83), the true utility of glutamine supplementation as an immune modulator and the mechanism by which oral glutamine consumption may support immune function have yet to be determined (84).

CONCLUSIONS

In conclusion, glutamine is a nitrogen donor in metabolic reactions and the main interorgan nitrogen shuttle. As a source of glutamate, it is essential for maintenance of cellular volume, amino acid economy, glutathione, reducing equivalents, and energy production. In catabolic states, muscle protein breakdown and conversion of unknown substrates by the lung produce glutamine that is released by these tissues. The kidney, intestine, immune system, and healing tissues utilize this glutamine. The amino acid also serves as a major precursor for glucose formation. Nitrogen carried by glutamine is disposed of as urea produced in the liver and as ammonia produced primarily in the kidney. If a catabolic state persists, loss of lean body mass diminishes the ability of muscle to produce glutamine and maintain interorgan glutamine flux. In these instances, enteral or parenteral glutamine intake may be beneficial. Thus, glutamine is a conditionally essential amino acid, and several limited studies have suggested that metabolic support of catabolic patients with glutamine may improve their condition and speed up their recovery. Glutamine, together with other nutrients, may also benefit those with intestinal deficiencies or sickle cell disease. Dietary supplementation is claimed to increase athletic performance, muscle mass buildup, and improve immune function. However, controlled clinical studies have not yet substantiated any of these claims. Further, several recent clinical studies have suggested that the

significant benefits of glutamine feeding observed in animals are not recapitulated in humans. Regardless, the National Institutes of Health website for clinical trials, ClinicalTrials.gov, currently lists 40 open clinical trials of glutamine supplementation.

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FURTHER READINGS

1. Many of the most recent and comprehensive reviews of glutamine metabolism, function, and nutrition were cited in the text and thus appear in the reference list above. The author apologizes for not including citations to many seminal primary publications that are relevant to the subject matter.
2. For an update on U.S. government sponsored clinical trials of glutamine, see <http://www.clinicaltrials.gov/ct2/results?term=glutamine>.

Goldenseal

Dennis J. McKenna and Gregory A. Plotnikoff

INTRODUCTION

Goldenseal (*Hydrastis canadensis* L.) is a plant native to North America and is used in its herbal traditions. A principal active ingredient, the alkaloid berberine, is shared with several medicinal plants used in traditional Asian medicines. Traditional uses include soothing irritated skin and mucous membranes, easing dyspepsia, and reducing debility. Preclinical studies suggest clinically relevant activity for cancer, cardiac diseases, gastrointestinal, and infectious diseases among others. There are no published clinical trials of goldenseal, and most of the available preclinical and clinical data are on the alkaloids berberine and β -hydrastine. Accordingly, much of the information summarized in this entry applies to berberine, and only indirectly to goldenseal, under the assumption that extracts of the plant containing berberine or β -hydrastine will display activities similar to those of the alkaloids. A few clinical trials of berberine support use for cardiac arrhythmias, congestive heart failure, diarrhea, and protozoal infection. Berberine has poor oral absorption, but recent human pharmacokinetic studies of goldenseal have demonstrated significant *in vivo* inhibition of important P450 isozymes. Injected, inhaled, or skin-absorbed berberine may displace albumin-bound bilirubin and pharmaceuticals. Goldenseal certainly effects cytochrome P450 metabolism via enzymes CYP2D6 and CYP3A4, and in a study of 21 commercial ethanolic herbal extracts potentially inhibitory to the cytochrome P450 system, goldenseal displayed the most pronounced activity, at a concentration of 0.03% of the full strength preparation (1). Thus, there is a significant potential for goldenseal extracts to elicit herb/drug or herb/herb interactions in patients concomitantly taking some of the most commonly used and most important life-saving pharmaceutical medications or other herbal supplements. However, reported adverse reactions to goldenseal or berberine are rare (2).

CLASSIFICATION AND NOMENCLATURE

- Scientific name: *Hydrastis canadensis* L.
- Family: Ranunculaceae
- Common names: Goldenseal, yellow root, turmeric root, eye root, Indian dye, yellow puccoon, ground raspberry.

H. canadensis (Fig. 1) is a perennial herbaceous plant found in rich, shady woods and moist meadows in eastern



Figure 1 Goldenseal (*Hydrastis canadensis* L.): whole plant, flower, and rhizome.

North America, especially in Ohio, northern Kentucky, Indiana, and Virginia, whereas in Canada, it is restricted to southwestern Ontario (3). The name “goldenseal” comes from the yellow scars left on the rhizome by the stem that bursts forth every spring; these scars look like the imprint of an old-fashioned letter seal. *Hydrastis* is a Greek word meaning “to accomplish with water” (3,4).

Goldenseal grows to about 30 cm in height with a simple, hairy stem, usually bearing a single-lobed basal leaf and two-lobed cauline leaves near the top. The flower is terminal, solitary, and erect, with small greenish-white sepals and no petals, and blooms in May and June. The fruit is an oblong, compound, orange-red berry containing two black seeds in each carpel. The medicinal rhizome is horizontal, irregularly knotted, bears numerous long slender roots, and is bright yellow with an acrid smell (4,5).

Populations of goldenseal in the wild have been greatly diminished in recent years due to overcollection

and habitat loss, which has placed this plant on the endangered species list. Due to concerns about overharvesting and increasing market demand, it is now commercially cultivated across the country, especially in the Blue Ridge Mountains (4,5). Recently, other species of plants purported to be *H. canadensis* have been sold as the bulk dried herb on the U.S. wholesale market. This substitution is due to the high market price goldenseal now commands, and the shortage of cultivated supply. Care must be taken in ascertaining accurate identification of the dried material (6,7).

HISTORY AND TRADITIONAL USES

Goldenseal has been used both as a dye and a medicine in North America. The root of goldenseal supplied Native Americans with a brilliant yellow dye for coloring their clothing and weapons, as well as for painting their skin (4). Goldenseal's ability to soothe irritated mucous membranes led to its topical and oral use for numerous uncomfortable conditions. Native Americans taught the first European settlers to use goldenseal root to treat skin diseases, ulcers, gonorrhea, and arrow wounds. The Iroquois employed goldenseal for heart troubles, fevers, and tuberculosis (8). The Cherokee utilized it for cancer and general debility (9). Both tribes used the plant for dyspepsia, appetite improvement, and inflammatory dermatoses. Folk use expanded later to include treatments of sore eyes, hepatitis, and menstrual difficulties.

Goldenseal became known as one of the most powerful North American medicinal plants, and was included in the *U.S. Pharmacopoeia* from 1831 to 1936, and then in the *National Formulary* until 1960 (4,5). It is now commonly used in the United States as a treatment for canker sores, and sore mouths and throats (7). Many herbal practitioners advise that topical use, such as gargling with a solution of goldenseal for sore throat, is more effective than similar amounts taken orally in capsules (10). Today, many North American herbal practitioners consider it to be indispensable for its many purported medicinal effects: digestive, antibiotic, immunostimulatory, antispasmodic, sedative, hypotensive, uterotonic, choleric, carminative, antifungal, and antimicrobial (11). Goldenseal is frequently used by practitioners of functional medicine for treatment of dysbiosis, potentially pathogenic intestinal flora imbalances including yeast overgrowth. No clinical trials have been published to date.

Goldenseal's medicinal effects are primarily attributed to the alkaloid berberine, on which there are the most preclinical data (12). Berberine is also found in barberry or Oregon grape root (*Mahonia aquifolium* Nutt.), another traditional Native American herbal medicine used for similar symptoms (12). Likewise, in traditional Asian medicines, berberine-containing species are used for similar indications as in North America. For example, in traditional Chinese medicine, three species of *Coptis* are used for problems affecting the cardiovascular and gastrointestinal systems (13). They are widely used in China today for treatment of congestive heart failure (14). Kampo, the Japanese herbal medicine tradition, incorporates the berberine-containing Chinese cork

tree (*Phellodendron amurense* Rupr.) as a cooling agent for diseases of summer season including irritated skin and membranes (12). Ayurveda, a traditional Indian system of herbal medicine, utilizes *Berberis aristata* for intestinal infections. Vietnamese traditional herbal medicine uses *B. aristata* Griff. for dyspepsia, dysentery, eye inflammation, and toothache (15).

Some studies have commenced on the other abundant alkaloid, β -hydrastine (6). This is a central nervous system (CNS) stimulant and has direct myocardial and intestinal smooth muscle depressant effects (12,16). The *British Herbal Compendium* (16) states that the activity of goldenseal is mainly due to β -hydrastine, which is vasoconstrictive, and active on the nervous, reproductive, respiratory, and cardiac systems. Both berberine and β -hydrastine are choleric, spasmolytic, sedative, and antibacterial; canadine is a stimulant to uterine muscle (16).

CHEMISTRY

Alkaloids

The primary active constituents of goldenseal are the alkaloids β -hydrastine (1.5–4%) and berberine (0.5–6%). The plant contains lesser amounts of the alkaloids canadine (tetrahydroberberine), berberastine, hydrastindine, isohydrastindine, (S)-corypalmine, (S)-isocorypalmine, and 1- α -hydrastine (Fig. 2) (12).

Other Constituents

Other constituents include meconin, chlorogenic acid, lipids, resin, starch, sugars, and a small amount of volatile oil (12).

Formulation and Analysis

High-performance liquid chromatography (HPLC) analysis of commercial goldenseal products demonstrates wide

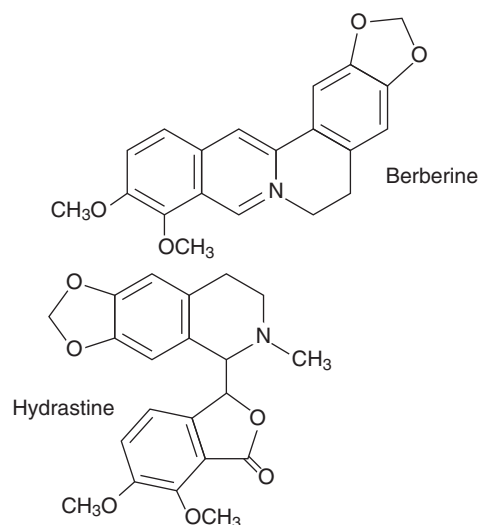


Figure 2 Structures of berberine and β -hydrastine, the major alkaloids of *Hydrastis canadensis* (goldenseal).

variation in berberine, β -hydrastine, alkaloid ratio, and total alkaloid content. Berberine content ranged from 0.82% to 5.86%, while that of β -hydrastine between 0% and 2.93% (17). HPLC analysis guidelines have been published (18). Since the first edition of this monograph in 2004, several new analytical methods have been published for goldenseal and its associated alkaloids, including quantitative methods for determining berberine and hydrastine in human serum (19–22).

PRECLINICAL STUDIES ON GOLDENSEAL AND BERBERINE

As noted earlier, there have been no clinical and few preclinical investigations of goldenseal itself, but a rather large number of studies have investigated the activities of its major alkaloid, berberine. In this entry, we have reviewed what little preclinical data have been published on goldenseal itself, and have summarized the preclinical and clinical data on berberine.

Cardiovascular and Circulatory Functions (In Vitro, Organ Isolates)

Yao et al. (23) reported that berberine could relax 5-hydroxytryptamine-induced muscle contractions. Palmery et al. (24) have shown that an extract of goldenseal exhibits an inhibitory action on adrenaline-induced contractions in rat thoracic aorta in vitro. The constituents responsible were identified as the alkaloids canadine, berberine, and canadine. Including the inactive alkaloid β -hydrastine, the alkaloid mixture showed an IC_{50} of 2.86×10^{-7} M. Therefore, Palmery et al. (24) concluded that the mixture of active alkaloids, producing a greater adrenolytic action than any one alone, acted synergistically. Moreover, acting in a dose-dependent manner, the total extract of the roots and rhizomes was able to inhibit contractions induced by higher doses of adrenaline, whereas the individual alkaloids did not. The authors concluded that these alkaloids appear to account for the vasoconstrictive activity of goldenseal that has led to its popular use.

Goldenseal may also exhibit an anti-cholesterol effect. Abidi et al. (25) reported that goldenseal extracts displayed greater activity than berberine and the related alkaloid, hydrastine, as an upregulator of liver low-density lipoprotein receptors (LDLR) in HepG2 cells and in reducing plasma cholesterol and low-density lipoprotein cholesterol in hyperlipidemic hamsters. They attributed the activity to canadine and two other constituents of goldenseal. Unlike berberine, canadine was resistant to MDR1-mediated efflux from liver cells, and this finding explained why canadine was more active than berberine as an upregulator of LDLR.

Immune Functions (In Vivo, Male Rats)

Goldenseal may provide both an accelerated IgM antibody response as well as an enhanced cytokine response. Rehman et al. (26) examined the effects of continuous treatment with a goldenseal root extract on antigen-specific immunity in male rats over a six-week treatment period (6.6 g

in glycerin solvent/L drinking water) compared with glycerin-only treated rats. No significant difference was found in the consumption levels of treated and nontreated controls. Rehman et al. recorded changes in immunoglobulins G and M (IgG and IgM), finding no significant difference in IgG levels during the first three weeks and a trend toward lower levels in the last three weeks of treatment compared with the controls that reached significance on day 42 only. The IgM levels became significantly higher in the goldenseal group on day 4 and continued to remain so on days 11 and 15. The effect amounted to an accelerated antibody response, which permitted a more rapid increase in levels of IgM, or an enhancement of “the acute primary IgM response.” The authors commented that further studies of immunomodulatory medicinal plants should take the matter of time dependence into consideration to pinpoint times of maximal effects. Another group of investigators (27) compared the effects of goldenseal and Astragalus root extracts on proinflammatory cytokines in cultured macrophages. Neither herb significantly stimulated macrophages directly. However, when the macrophages were stimulated with lipopolysaccharide, subsequent exposure to extracts from both plants resulted in reduction of tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6), IL-10, and IL-12 in a dose-dependent manner. The results indicated that both plants can modulate macrophage response during stimulation, and the authors speculate that this reduction in proinflammatory response may provide a basis for their traditional uses.

Antimicrobial and Antifungal Activity (In Vitro)

Goldenseal appears to have a greater antifungal than antibacterial activity. Scazzocchio et al. (28) evaluated a “total” standardized extract of goldenseal for relative killing time in a low-density inoculum. The undiluted extract showed the most activity, and there was correspondingly weaker activity at two lower dilutions. The standardized extract and four derivative alkaloids were least effective against *Candida albicans* and *Escherichia coli*. Compared with the alkaloids tested, however, the undiluted standardized extract showed the most potent activity, killing the fungus at 15 seconds versus 1 to 2 hours for the alkaloids (canadine, canadine, berberine, and β -hydrastine). Undiluted, berberine was the most potent alkaloid against *C. albicans* (killing time, one hour at 3.0 mg/mL), equivalent to the standardized extract at a 50% dilution. Canadine killed *C. albicans* at over two hours. The results suggested that isoquinoline alkaloids with an open C ring, such as canadine, appear to show greater antimicrobial activity. In most of the micro-organisms tested, canadine also showed more potency than berberine.

Goldenseal is a very weak antibiotic against common bacteria such as the gram-negative *Pseudomonas aeruginosa* and the gram-positive *Staphylococcus aureus* and *Streptococcus pyogenes*. For the latter, a weak pathogen, the mean inhibitory concentration of goldenseal was also quite weak at 4000 times higher than that of penicillin (29). Goldenseal’s value and potential value lie elsewhere. For example, it demonstrates relevant activity against the oral pathogens *Streptococcus mutans* and *Fusobacterium nucleatum* (30).

Respiratory and Pulmonary Functions (In Vitro, Organ Isolates)

Goldenseal may effect smooth muscle relaxation. A relaxing effect was shown from an ethanolic extract of goldenseal roots in carbachol-precontracted guinea pig trachea (31). Complete relaxation of carbachol-precontracted isolated guinea pig trachea was obtained from a total extract of the roots in a cumulative dose of 5 mg/mL. Further studies have shown that the constituents responsible are the alkaloids canadoline, canadine, berberine, and hydrastine (EC₅₀ 2.4, 11.9, 34.2, 72.8 µg/mL, respectively). Although as yet unclear, the activity appears to involve interactions of the alkaloids with adenosine and adrenergic receptors (32).

PRECLINICAL STUDIES ON BERBERINE

Cancer

Berberine continues to attract attention for its potential applications in treatment and prevention of cancers. Since 2004, over 95 papers have appeared on berberine and related alkaloids in cancer therapy. Two thorough reviews published in 2009 (33,34) cite the antineoplastic and antiproliferative mechanisms for berberine ranging from cell cycle arrest, to induction of apoptosis, to anti-inflammatory activities, to inhibition of angiogenesis and metastasis. Anti-cancer activities appear among the most promising future uses of goldenseal.

Growth Inhibition and Antiproliferative Activity

Berberine interventions have demonstrated positive results in multiple cancer cell lines. First, in six types of esophageal cancer cell lines cocultured with berberine-containing *Coptidis* rhizome extract, in vitro antiproliferative activity existed at relatively low concentrations of the herbal extract (ID₅₀ 0.25–3 µg/mL) (35).

Second, in prostate cancer cell lines, berberine in vitro and in vivo, inhibited cell cancer growth in a concentration (0–50 mM) and time-(0–48 hours) dependent manner without any growth inhibition in normal human prostate epithelial PWR-1E cells. This effect, both in vitro and in vivo, was strongest in p53 expressing LNCaP cells as measured by cell arrest in G0/G1 phase, apoptotic cell death, and the expression of apoptotic cell death proteins Bax and caspase-3 (36).

Third, in breast cancer cell lines, as compared with untreated control cells, berberine efficiently inhibited growth in anoikis-resistant MCF-7 and MDA-MB-231 cells by inducing cell cycle arrest at G0/G1. Most remarkably, this effect was greater than that seen with the classic chemotherapy agent doxorubicine (37).

Fourth, in leukemia cell lines, Khan and colleagues recently demonstrated that berberine is a potent antineoplastic compound that acts via antiproliferative and proapoptotic mechanisms independent of genotoxicity. At just 1.2 µg/mL, berberine significantly inhibited HL-60 cell proliferation in S phase. They documented that this was preceded by a strong activation of Chk2, phosphorylation and degradation of Cdc25 A, and the subsequent inactivation of Cdc2 [cyclin-dependent kinase (CDK)1]. They also noted that berberine both inhibited the expression of the proto-oncogen cyclin D1 and induced

acetylation of α -tubulin correlated with the induction of apoptosis (38).

Finally, consecutive intraperitoneal (i.p.) dosing of berberine has inhibited ascites tumor proliferation in Swiss albino mice and resulted in a 32% increase in life span compared with controls (39).

Chemopreventive activity

Ongoing research in chemoprevention appears warranted. In vitro, berberine inhibited carcinogenicity of arylamine and its main metabolizing enzyme, *N*-acetyltransferase, in colon tumor cells (40) and leukemia cells (41).

Berberine displayed dose-dependent in vivo activity against carcinogenesis induced by 20-methylcholanthrene or *N*-nitrosodiethylamine (NDEA) in mice and rats (42).

Berberine also suppressed tumor induction by proinflammatory tumor promoters teleocidin and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (43).

Chemotherapy Adjunct Activity

Berberine displayed a synergistic effect with radiation treatment and cyclophosphamide in Swiss albino mice implanted with Dalton's lymphoma ascites tumor cells (42).

Cytotoxicity

In vitro cytotoxic activity of berberine has been demonstrated in a wide variety of tumor cells including uterine, ovary, and larynx carcinomas (43), gliomas (44), leukemias (45), and hepatomas (46).

Cardiovascular and Circulatory Functions

Antiarrhythmic Effects

Berberine was shown by Huang et al. (47) to inhibit experimental ventricular arrhythmias induced by aconitine, ouabain, and barium chloride in rats by 62%. A new berberine derivative, CPU86017, has been evaluated in animal models as an experimental antiarrhythmic agent, and has displayed improved pharmacological profile, solubility, and bioavailability compared with the parent compound (48). The compound displayed an ED₅₀ of 0.22 mg/kg in suppressing ischemia-reperfusion arrhythmia in rats, compared with lidocaine ED₅₀ of ~2.23 mg/kg. Additionally, CPU86017 had anti-oxidant properties that are likely to contribute to its antiarrhythmic effect.

Cardiotonicity, Cardioprotection

Berberine significantly reduced creatine phosphokinase release during the reoxygenation period, and ultrastructural damage was reduced (49). A new study by Korean investigators reevaluated the cardioprotective properties of tetrahydropalmatine, a berberine-related alkaloid, in a rat model of ischemia-reperfusion injury (50). Palmatine significantly reduced infarct size, improved myocardial function, and delayed the posttreatment decline in anti-oxidant enzymes such as SOD and catalase. It also limited the increase in plasma levels of malonyl dialdehyde (MDA), lactate dehydrogenase (LDH) and creatinine kinase (CK), and inhibited Cyclooxygenase-2 (COX-2) inducible nitric oxide synthase (iNOS) expression in I/R myocardium and increased heme-oxygenase. The

authors conclude that palmatine protects from ischemia-reperfusion injury by reducing oxidative stress and modulating the inflammatory process following injury.

Congestive Heart Failure

Berberine and its derivatives have positive inotropic, negative chronotropic, antiarrhythmic, and vasodilator properties, each of which can be beneficial in congestive heart failure (51).

For rats with verapamil-induced cardiac failure, pretreatment with berberine resulted in significantly less severe cardiac failure compared with untreated controls (52). In a rat model of cardiac hypertrophy, berberine administered for eight weeks, beginning four weeks after aortic banding, resulted in significant reductions in whole heart, left ventricular weight, and left ventricular size compared with control aorta-banded rats (53).

Hypertension

Berberine is reported to have an antihypertensive effect at low concentrations ($<1 \times 10^{-6}$ M). Denuded aorta and methylene blue pretreated aorta did not demonstrate aortic relaxation at these concentrations. Methylene blue is a direct nitric oxide synthesis inhibitor and a direct inhibitor of guanylyl cyclase. These data indicate that aortic relaxation observed in response to low concentrations of berberine in isolated rat aorta was solely endothelium dependent at these concentrations. Those higher than 1×10^{-6} M induced aortic relaxation regardless of the presence of intact endothelium or methylene blue pretreatment (54). A recent study offered insight regarding the mechanisms of action of berberine using cultured endothelial cells and blood vessels isolated from rat aorta (55). Berberine enhanced phosphorylation of endothelial nitric acid synthase and stimulated an increase in nitric oxide production. It attenuated glucose-induced generation of reactive oxygen species, cellular apoptosis, NF κ B activation, and expression of adhesion molecules. In isolated mouse aortic rings, berberine induced endothelin-dependent vasodilatation and alleviated high glucose-mediated endothelial dysfunction. All of these effects were mediated by the adenosine monophosphate protein kinases (AMPK) signaling cascade, and all were abolished by pharmacological inhibition of AMPK.

Digestive, Hepatic, and Gastrointestinal Functions

Diarrhea

Berberine sulfate appears to inhibit the intestinal secretory response induced by *Vibrio cholerae* and *E. coli* by 70% in vivo. However, the drug was effective when given either before or after enterotoxin binding (56). In the human colon (in vitro), berberine was shown to inhibit ion transport. Based on studies using a human model of intestinal ion transport, the antisecretory activity of berberine appears to be due to a direct action on epithelial cells, possibly through the blockade of potassium channels (57).

Hepatic Functions

Berberine has shown significant pre- and posttreatment hepatoprotective effects in rat models of acetaminophen-induced hepatotoxicity, as measured by reduction of serum alkaline phosphatases and serum transaminases. Although the alkaloid does display some ameliorative

effects as a pretreatment in CCl₄-induced models of hepatotoxicity, it did not have comparable posttreatment activity. In both instances, berberine was more effective in the animals exposed to acetaminophen than those exposed to CCl₄ (58).

Endocrine and Hormonal Functions

Adrenal Functions

Berberine has exhibited α -adrenergic antagonist activities in isolated animal organs (23,59).

Immunology: Immunopotentialiation

Berberine alkaloids have displayed potent macrophage-activating activity, in turn inducing cytostatic activity against tumor cells. In mice, and in vitro against human brain tumors, berberine produced an average of 91% tumor inhibition against six malignant brain tumor cell lines (60).

Infectious Diseases

Fungal Infections

The overgrowth of *Candida* on mucous membranes may respond well to the use of goldenseal extract. Berberine sulfate has demonstrated antifungal activity against *C. albicans*, *C. tropicalis*, *Trichophyton mentagrophytes*, *Microsporum gypseum*, *Cryptococcus neoformans*, and *Sporotrichum schenckii* (61,62). Two more recent studies have demonstrated a potent synergistic effect of berberine/fluconazole combinations against 40 fluconazole-resistant strains of *C. albicans* (63) and a similarly marked synergy with Amphotericin B in a mouse model of disseminated candidiasis (64).

Microbial Infections

Berberine is active against important gastrointestinal pathogens including *Helicobacter pylori* and *V. cholerae*. For the first, a concentration-dependent inhibitory activity was shown from berberine (0.08–160 μ M) against 21 strains of *H. pylori* obtained from human peptic ulcer patients (65). Further study on 15 strains of *H. pylori* demonstrated that a crude methanol extract of both *H. canadensis* and extracted berberine showed an MIC₅₀ of just 12.5 μ g/mL (66).

For *V. cholerae*, berberine sulfate has exhibited in vitro bactericidal activity with more rapid antibacterial activity in vitro than tetracycline and chloramphenicol (61). On *S. aureus*, at very high concentrations of 35 and 50 mg/mL, achievable only in the intestines, berberine was bacteriostatic (61).

Certain microbial agents can block the adherence of micro-organisms to host cells at doses much lower than those needed to kill cells or inhibit cell growth. Strategies that interrupt the adhesive functions of bacteria before host tissue invasion occurs may be an effective prophylactic approach against bacterial infectious diseases. Berberine caused an eightfold increase in release of lipoteichoic acid, the major ligand responsible for adherence of *Streptococci* to epithelial cells, fibronectin, and hexadecane (67).

Berberine is antimicrobial, but as a cation it is extruded from bacterial cytosol by multidrug resistance (MDR) ion pumps and as a result, is relatively ineffective as an antibacterial agent. In one promising approach,

berberine was conjugated with INF 55, and inhibitor of major facilitation MDRs, resulting in a highly effective antimicrobial agent that readily accumulated in bacteria. The formulation was effective in a worm model of enterococcal infection, curing the worms of the pathogen (68).

Parasitic infections

Berberine demonstrated antiplasmodial (IC_{50}) values less than 1 M for multidrug-resistant *Plasmodium falciparum* (69). When derived from *H. canadensis*, it is active against multiple drug-resistant *Mycobacterium tuberculosis* (70). It has also demonstrated effectiveness against *Entamoeba histolytica*, *Trichomonas*, *Giardia*, *Leishmania*, and *Echinococcus granulosus* (61,71–74).

In vitro studies and strong anecdotal evidence indicate that berberine sulfate, berberine hydrochloride, or goldenseal is effective in inhibiting the growth of protozoan parasites such as *E. histolytica*, *Giardia lamblia*, *Trichomonas vaginalis*, and *Leishmania donovani* (61,73). The alkaloid does not have the mutagenic side effects of metronidazole, the current agent of choice for treating these conditions; yet it has in some cases appeared equally effective (73).

The protozoan parasite *L. donovani* and its treatment with berberine chloride were studied in hamsters. Berberine was effective in reducing by 90% the number of parasitic amastigotes formed by the protozoan parasite *L. donovani* in the liver and spleen of hamsters, and was much better tolerated at levels of 50 and 100 mg/kg/day than the medication pentamidine (72). Based on the ability of β -hydrastrine to dissolve *E. granulosus* cysts in mice, Ye et al. (74) showed that it may be a promising agent for treating hydatidosis.

Integumentary, Muscular, and Skeletal Functions

Connective Tissue Functions

Berberine-type alkaloids inhibit the activity of elastase, a serine proteinase that degrades elastin, an important structural component of blood vessels, lung, skin, and other tissues (75). The authors suggest that these alkaloids might therefore be effective in treating certain inflammatory diseases, such as pulmonary emphysema, chronic bronchitis, arthritis, and rheumatoid arthritis.

Osteoporosis

Berberine demonstrated inhibition of parathyroid-induced bone resorption in ovariectomized rats (76). When administered orally (10 mg/kg/day for 22 weeks) to male and female senescence-accelerated mice, it resulted in significant increases in bone mineral density compared with controls (77). In osteoblasts, berberine enhanced the expression of osteogenic marker genes including osteocalcin and osteopontin, and stimulated the transcriptional activity of the key osteogenic transcription factor Runx2. Berberine also activated p38 mitogen-activated protein kinase (MAPK) and increased cyclooxygenase expression, both key factors in osteoblasts differentiation. These results indicated that berberine promotes osteoblasts differentiation through activation of Runx2 by MAPK. Berberine and/or goldenseal herb may therefore be a therapeutic agent for the treatment of osteoporosis (78).

Receptor and Neurotransmitter-Mediated Functions

Berberine has shown concentration-dependent noncompetitive monoamine oxidase (MAO) inhibitory activity in vitro in mouse brain mitochondria (79). Berberine has demonstrated antidepressant activity in various animal models of depression such as the immobility test for despair and the forced swim test. Administered over 15 days, berberine significantly elevated levels of serotonin and dopamine, but not norepinephrine. Berberine was synergistic with the Sigma-1 receptor agonist pentazocine, but its effect was blocked by antagonists. The investigators interpreted the results as an indication that berberine exerted its antidepressant effects by modulating brain biogenic amines and that the effect was partially mediated by Sigma 1 receptors and the nitric oxide pathway (80).

Respiratory and Pulmonary Functions

An ethanolic extract of goldenseal roots produced a relaxing effect in carbachol-precontracted guinea pig trachea (31). Despite remaining unclear, the effect seems to involve interactions of the alkaloids with adenosine and adrenergic receptors (32).

CLINICAL STUDIES

Cardiovascular and Circulatory Disorders

Berberine has several potential actions relevant to the care of cardiac patients including antiarrhythmic, vasodilating, and anti-cholesterol functions. In a study of 100 patients with ventricular tachyarrhythmias, berberine treatment (dose not specified in summary) resulted in significant reductions in the number of beats per hour (from 452 ± 421.8 to 271 ± 352.7). There were no side effects except for mild gastroenterologic symptoms in some patients (52). In China, berberine is a class III antiarrhythmic agent, with oral berberine (tablets, 1.2 g/day) reportedly of value in the treatment of ventricular premature complexes (VPCs) in patients with congestive heart failure (81). Subjects were administered oral berberine 1.2–2.0 g/day in a randomized controlled trial of 156 cases with New York Heart Association (NYHA) class II–IV heart failure. Those treated with berberine demonstrated significant improvement in the six-minute walking distance test ($P = 0.001$) and left ventricular ejection fraction ($P < 0.02$). VPCs decreased by 84% ($P < 0.001$) and nonsustained ventricular tachycardia decreased by 96% ($P < 0.001$) in the treatment groups with nonsignificant differences in those receiving placebo. Total mortality in the treatment group was 8.8% and 16.4% in the placebo group ($P < 0.02$) (82).

Wang et al. (83) evaluated the ability of berberine to improve endothelial function by reducing endothelial microparticles (EMP) in 14 healthy subjects. Berberine therapy significantly reduced the number of CD31(+)/CD42(–) microparticles while increasing flow-mediated vasodilation. Parallel in vitro studies on human umbilical vein endothelial cells showed that EMPs diminished eNOS protein expression, and this EMP-mediated detrimental effect was significantly inhibited by berberine. Berberine significantly upregulated endothelial function, and the authors speculated that EMP reduction may represent a novel therapeutic target for ameliorating endothelial dysfunctions.

Berberine is known as a lipid-modulating agent, and two clinical studies have examined its efficacy in reducing serum triglyceride and cholesterol levels. In an open-label study, Cicero et al. (84) compared berberine alone with a combination formula composed of berberine, policosanol, red yeast extract (containing monocolin), folic acid, and astaxanthin in two groups of 20 moderately hyperlipidemic subjects over four weeks of treatment. Both formulations significantly reduced total cholesterol (16% and 20%, respectively) LDL (20% and 25%, respectively), ApoB by 15% and 29%, and triglycerides by 22% and 26%. There were no adverse reactions, indications of impairment of liver transaminase or creatinine phosphokinase. Another combination formula containing berberine + simvastatin in 63 hypercholesterolemic patients reduced serum LDL cholesterol by 31.8%, a significant difference compared with either agent alone (85).

Metabolic Disorders

Berberine has been evaluated as a hypoglycemic agent in clinical trials. In one controlled comparison trial with the hypoglycemic agent metformin, berberine was evaluated in 36 patients newly diagnosed with type II diabetes over three months (86). Berberine significantly reduced hemoglobin A1c, fasting blood glucose, postprandial blood glucose, and serum triglycerides. In a follow-up study in 48 patients with poorly controlled type II diabetes, berberine significantly reduced all of these parameters and also decreased fasting plasma insulin by 28% and insulin resistance by 44%. The authors concluded that berberine acted similarly to metformin and could be used effectively for the control of type II diabetes.

Another group of investigators (87) evaluated berberine versus placebo in a randomized study of 166 patients with type II diabetes and dyslipidemia. Following three-month's treatment at 1.0 g/day, berberine significantly lowered plasma cholesterol, fasting and post load plasma glucose, plasma triglycerides, and low-density lipoprotein. Plasma glucose disposal rate was increased by berberine, but changes were not significant compared with placebo. No side effects were observed other than transient constipation in some patients. The authors concluded that berberine was a safe and effective treatment for controlling glucose levels in type II diabetes.

Cancer

The effects of berberine on radiation-induced lung injury (RILI) was assessed in 90 patients with nonsmall cell lung cancer that were treated with radiotherapy (88). The trial group received radiation therapy plus berberine for six weeks, while the control group received only radiation. Soluble intermolecular adhesion factor 1 (sICAM-1), transforming growth factor β -1 (TGF- β -1), RILI, and pulmonary function were measured. RILI was significantly lower in the berberine-treated group at six weeks and six months compared with controls (45.2% vs. 72.1% and 35.7% vs. 65.1%, respectively, both $P < 0.05$). Plasma levels of both sICAM-1 and TGF- β -1 were significantly lower at week 3 and 6, and significant improvements in various measures of pulmonary function were observed at week 6 and month 6. In this study, berberine was effective in

reducing RILI and in maintaining pulmonary functions, but the exact mechanism was not elucidated.

Receptor and Neurotransmitter-Mediated Functions

A berberine analog may prove to be helpful in the treatment of heroin addiction. Chinese investigators (89) assessed the effect of tetrahydropalmatine (THP), a berberine analog, on reducing craving and increasing abstinence in 120 heroin users. Patients were treated for four weeks in hospital, followed by four weeks of observation, with follow-up three months after discharge. Outcome measures were the severity of the protracted abstinence withdrawal syndrome (PAWS) and the abstinence rate. Patients who completed the treatment and remained in the program following treatment had a significantly higher abstinence rate compared with the control group (47.8% vs. 15.2%, respectively). THP treatment also significantly ameliorated PAWS symptoms, including craving, somatic syndrome, mood states, and insomnia. These results supported the use of THP in treating heroin addiction.

Digestive, Hepatic, and Gastrointestinal Disorders

Diarrhea

In a randomized controlled experiment, the efficacy of berberine sulfate in the treatment of diarrhea due to enterotoxigenic *E. coli* (ETEC) and *V. cholerae* was evaluated in 165 patients. The berberine sulfate group showed significantly reduced stool volumes for three consecutive eight-hour periods following treatment. The results suggest that berberine can be an effective and safe antisecretory drug for ETEC diarrhea, but that it has only slight activity against cholera and is not additive with tetracycline (90).

In a study by Khin-Maung et al. (91), 400 adults presenting with acute watery diarrhea were entered into a randomized, placebo-controlled, double-blind clinical trial of berberine, tetracycline, and a tetracycline plus berberine combination to study the antisecretory and vibriostatic effects of berberine. Of 185 patients with cholera, those given tetracycline or tetracycline plus berberine showed considerably reduced volume and frequency of diarrheal stools, duration of diarrhea, and volume of required intravenous and oral rehydration fluid.

In a trial of 65 children younger than five years of age affected by acute diarrhea, a superior response was observed in those receiving berberine tannate (25 mg every six hour) compared with those receiving standard antibiotic therapy. Berberine tannate was effective against diarrhea caused by *E. coli*, *Shigella*, *Salmonella*, *Klebsiella*, and *Faecalis aerogenes* (56).

In 200 adult patients with acute diarrhea, standard antibiotic treatment in conjunction with berberine hydrochloride [150 mg orally (p. o.)/day] resulted in a faster recovery than in those given antibiotic therapy alone. In addition, in 30 subjects treated with berberine hydrochloride alone, diarrhea was arrested in all with no side effects or toxicity (92).

Cirrhosis

In patients with alcohol-related liver cirrhosis, berberine prevented the elevation of serum tyramine following oral tyrosine load by inhibiting bacterial tyrosine

decarboxylase in the large intestine. The accumulation of tyramine causes lowering of peripheral resistance, resulting in high cardiac output, reduction in renal function, and cerebral dysfunction (93).

Parasitic Disease

Pediatric patients 5 months to 14 years of age infected with giardiasis were administered berberine (10 mg/kg/day), while a control group received a standard anti-giardial drug (metronidazole). After 10 days, 90% of the berberine group showed negative stools versus 95% in the metronidazole group; at 1 month, 83% of the berberine group remained negative compared with 90% of the metronidazole group (86).

DOSAGE AND TOXICITY

- Dried root as decoction: 0.5–1.0 g, 3× daily.
- Tincture (1:10, 60% ethanol): 2–4 mL, 3× daily.
- Fluid extract (1:1, 60% ethanol): 0.3–1.0 mL, 3× daily.

All the earlier formulations and dosages are derived from the recommendations given in the *British Herbal Compendium* (16,94). Specific indications are not mentioned, and these dosages therefore represent a range of recommended ones.

Werbach and Murray (60) advised the use of 250 to 500 mg, 3× daily, of a standardized extract containing 5% total alkaloids for indications such as alcoholic liver diseases, infections, and diarrhea. All of the applications cited in this publication, however, are for berberine, and not for goldenseal per se.

Contraindications

According to Bergner (10), some practitioners recommend that goldenseal not be administered to a child younger than two years of age. Berberine-containing herbs are contraindicated for the treatment of newborn infants because of the capacity to displace bilirubin from albumin (95). It is also not advisable for children at risk for glucose-6-phosphate-dehydrogenase deficiency. This follows the observation that shortly after administration of berberine-containing herbs, they developed hemolytic anemia and jaundice. After a subsequent ban of berberine-containing herbs by the Government of Singapore in 1979, incidences of jaundice dropped, whereas they remained at a high level among infants in southern China and Hong Kong. Using serum from neonates, *in vitro* tests of herbal teas rich in berberine showed that bilirubin protein binding was decreased and that the effect was at least partly due to berberine (2). Brinker (96) cautions against the local use of goldenseal to treat purulent ear discharge because of a possible underlying rupture in the ear drum. He also lists it as a bitter herb that could ostensibly aggravate gastrointestinal irritations.

Drug Interactions

In herbal medicine, goldenseal is widely believed to enhance the activity of other botanicals; however, no studies are available to support this contention. There is a theoretical risk that berberine can increase the activity of pharmaceuticals. Yao et al. (97) found that berberine acted like

yohimbine and prazosin in showing competitive blocking activity against α_1 - and α_2 -adrenoreceptors. Therefore, goldenseal may have an additive effect with those agents. Because berberine can displace albumin-bound bilirubin, goldenseal theoretically could displace highly protein-bound pharmaceuticals. This could precipitate significant toxicity.

Most concerning is goldenseal's inhibition of P450 isozymes. When normalized to alkaloid content, goldenseal, both *in vitro* and *in vivo*, can strongly inhibit CYP2C8, CYP2D6, and CYP3A4/5 (98–100). This is significant because a very large percentage of prescription drugs are metabolized via either the CYP2D6 or CYP3A4 systems. Of all herbal medicines, goldenseal has one of the highest inhibitory activities against human cytochrome P450 (CYP) isoforms (1,5,98,100). In human volunteers, goldenseal has significantly affected CYP2D6 and CYP3A pharmacokinetics. Comparisons of pre- and post-supplementation of the CYP2D6 substrate debrisoquine revealed significant inhibition (approximately 50%) of CYP2D6 activity for goldenseal, but not for other herbal extracts (98). Comparisons of pre- and post-supplementation midazolam pharmacokinetic parameters revealed significant inhibition of CYP3A by goldenseal [AUC(0-infinity), 107.9 ± 43.3 versus 175.3 ± 74.8 ng \times h/mL; Cl/F/kg, 1.26 ± 0.59 versus 0.81 ± 0.45 l/h/kg; T(1/2), 2.01 ± 0.42 versus 3.15 ± 1.12 h; C_{max} , 50.6 ± 26.9 versus 71.2 ± 50.5 ng/mL] (99). This new *in vivo* data confirms previous observations. Among 21 commercial ethanolic herbal extract products sold in Canada, *in vitro* inhibitory activity on CYP3A4 was found from about 66%. Significant inhibition of CYP3A4 was shown at concentrations less than 10% of their full strength source preparations. An extract of goldenseal (*H. canadensis*, 0.03% full strength) was the most potent inhibitor (1). These findings have clinical significance with patients being at risk for undertreatment or toxicity. *In vitro* studies using various colon, gastric, and oral cancer cell lines pretreated with berberine (32 mM) 24 hours before treatment with the anti-cancer drug taxol (paclitaxel) found that the anti-cancer activity of the agent was compromised (80). Others have made similar observations in murine and human hepatoma cell lines in which berberine compromised the retention of chemotherapy agents (tamoxifen and verapamil) in tumor cells (101). Goldenseal's P450 inhibition can significantly affect cyclosporine metabolism. In a placebo-controlled group of renal transplant patients receiving CsA, 52 patients were also given 200 mg berberine 3×/day for 12 weeks, while a control group of 52 received CsA alone. The trough blood concentrations and concentration/dose of CsA in the berberine treated group were elevated by 88.9% and 98.4% relative to baseline, respectively, versus 64.5% and 69.4%, respectively, in the untreated group. Final blood concentrations and concentration/dose of CsA were elevated by 29.3% and 27.8% respectively, compared with untreated patients. No significant effects on renal or liver functions were observed after coadministration of berberine. In six renal transplant patients, pharmacokinetic profiles were assessed following CsA (3 mg/kg, 2×/day) before and after oral administration of 200 mg berberine 3×/day for 12 days. Following this treatment, the mean area under the curve (AUC) for CsA was significantly increased by 34%, the mean time to

reach peak plasma concentration and the mean half-life were increased by 1.7 hours and 2.7 hours, respectively, and average percentage increase in steady-state plasma concentration (C_{ss}) and minimum blood concentration (C_{min}) were increased by 34.5% and 88.3%, respectively. The authors concluded that berberine can markedly enhance the bioavailability of CsA, probably by the inhibition of CYP3A4, and could be used to reduce the effective dose of CsA (102).

Certain pharmaceuticals can elevate serum levels of berberine. Under normal conditions, oral administration demonstrates very poor bioavailability (<5%) (103). This suggests that the risk of toxicity is low. However, in a rat recirculating perfusion model, concomitant use of *P*-glycoprotein inhibitors, such as cyclosporin A and verapamil, significantly enhanced berberine absorption (104). This 6× rise in absorption increases the risk of berberine toxicity.

Pregnancy and Lactation

Herbs that contain berberine are not recommended for use during pregnancy. Berberine has been shown to cause uterine contractions in experimental animals (2,11,16). Developmental toxicity evaluation in mice demonstrated no effect on maternal body weight or gravida uterine weight. Maternal liver weight was increased in the absence of histopathologic changes at 12,500 ppm. Prenatal mortality, live litter size, and fetal sex ratio were unaffected. At 50,000 ppm, slight increases in the incidence of cleft palate and exencephaly were documented. Also noted was an 8% reduction in fetal body weight. The developmental toxicity of NOAEL was 12,500 ppm, while the LOAEL was 50,000 ppm. These correspond to 75 to 300 times greater than the estimated human intake (105).

The Botanical Safety Handbook (106) does not recommend its use during lactation. No pharmacokinetic studies of goldenseal have been found to confirm to what extent alkaloids cross in breast milk. Use by breastfeeding mothers of babies with jaundice or a deficiency of glucose-6-phosphate-dehydrogenase should be carefully scrutinized (see sect. "Contraindications"). In vitro tests of serum from neonates fed berberine-containing herbal teas showed that bilirubin protein binding was decreased and that the effect was at least partly due to berberine (2). The dose of plant constituents received in direct feeding with herbal teas is many times larger than that possible through breast milk.

Toxicity

No oral toxic dose has been established for goldenseal. The oral LD_{50} in mice of berberine is variously reported from 3.29 mg/10 g to 1000 mg/kg body weight, suggesting that toxicity is extremely low (6). The German Commission E gives the LD_{50} of berberine in mice as 24.3 mg/kg i.p. (107). Berberine in adults is well tolerated up to a dose of 500 mg. Above that, side effects have been reported: eye and skin irritation, nephritis and kidney irritation, nose bleed, lethargy, and dyspnea (108). In humans, a massive overdose of berberine would be expected to result in CNS-depressant effects (108), including central paralysis (109).

Special Precautions

Goldenseal and berberine-containing plants are usually considered nontoxic and are generally so at the recommended dosages. However, higher dosages may interfere with vitamin B metabolism (4). Tinctures may cause irritation of the mucous membranes (96). Bergner (10) reports that large doses over a long duration can overstimulate and eventually exhaust the mucous membranes. In support of this, he cites the traditional Chinese medical point of view that bitter herbs taken inappropriately can injure the spleen.

Duke (4) warns that topical overdose can cause skin or membrane ulceration, and for that reason advises against its traditional use as a douche. Foster (5) counters that this fear is from Millspaugh's 1887 homeopathic text. Modern studies have been unable to confirm this.

REGULATION

Because of threatened loss over much of its natural range, since June of 1997, goldenseal has been regulated as an endangered species under Appendix II of the Convention on International Trade in Endangered Species of wild fauna and flora (CITES). In the United States, export now requires a federal permit from the U. S. Fish and Wildlife Service (USFW) that certifies harvesting is not detrimental to the survival of the species. Interstate commerce is not regulated. However, individual states may regulate commercial activity in goldenseal.

Sales of goldenseal as a dietary supplement requires notification of intent but does not require approval from the U. S. Food and Drug Administration (FDA) (USC, 1994). In Canada, products containing goldenseal are considered Natural Health Products (NHPs) and are required to undergo a premarket assessment for safety, efficacy, and quality by the Natural Health Products Directorate. Licensure from the Directorate must be obtained prior to sales in Canada.

CONCLUSIONS

Goldenseal has a long history of application in Western phytotherapy and herbalism, as well as in Native American ethnomedicine. Its popularity as a dietary supplement continues to the present, and this has led in part to overharvesting of wild populations such that the species has been placed on the endangered list. Abundant preclinical and clinical evidence on the activity of its major alkaloid, berberine, has shown that the plant potentially has numerous therapeutic indications, especially in microbial, fungal, and parasitic infections, in diarrhea and other gastrointestinal disorders, and in cardiac disorders such as arrhythmias and mild congestive heart failure. Promising data exists for its potential role in treating cancer. Berberine occurs in other plant species, and both the alkaloid itself and extracts of berberine-containing plants have been investigated clinically. The greatest risk with goldenseal use is its effect on P450 CYP2D6 and CYP3A4/5. Oddly, there have been few published clinical studies on goldenseal itself, despite its popularity and position as a mainstay of Western herbalism. Thus, the possible therapeutic

applications of goldenseal are speculations extrapolated from the data on the activities of the alkaloids and other species containing these alkaloids.

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Grape Seed Extract

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INTRODUCTION

Grape seed extract (GSE) refers to mixtures of catechin monomers, procyanidin oligomers, and procyanidin polymers extracted from *Vitis vinifera* seeds. These bioactive components are reducing agents and hydrogen-donating antioxidants. Their intrinsic properties are due to their polyphenolic nature. They exert diverse biologic effects in vitro at pharmacologic concentrations and seem to critically influence and modulate cell function, cell activation, cell signal transduction, endothelium-dependent vasodilation, vascular reactivity, inflammatory cascades, immune function, cellular and humoral immunity, cell survival, carcinogenesis, etc. in experimental situations. Recent clinical research has clarified benefits in areas such as blood pressure, inflammation, and other aspects of the metabolic syndrome and also suggested new areas of protection, such as in the case of Alzheimer disease.

CHEMISTRY

GSEs have varying amounts of monomers, oligomers, higher oligomers, and polymers of catechin, a flavan-3-ol molecule (Fig. 1).

The relative percentage of polyphenols in the grape seeds versus their content in the whole grape depends on the grape variety and generally appears to be a little above 4.0% of the total weight of the seed. The pulp contains about 10% of the total extractable phenolic substance in the berry. The seeds and the skin constitute two-thirds and one-third, respectively, of the remainder (1). The components that compose the phenolic fraction in the grape seed are essentially all flavonoids, designated as monomeric flavan-3-ols identified as (+)-catechin (Fig. 1), (–)-epicatechin, and (–)-epicatechin-3-gallate esterified with a gallic acid residue. The flavan-3-ol repeated molecules show extensive distribution in grape seeds, and considerable chain extensions occur to produce polymers (procyanidins) (Fig. 2). Fuleki and Da Silva assessed the composition of catechin and procyanidins in 17 different red and white cultivars (2). They isolated and identified 11 different monomers, dimers, and trimers by reverse-phase high-performance liquid chromatography (HPLC). In earlier studies reported in 1994, Prieuer et al., employing gel permeation chromatography and normal-phase HPLC, demonstrated that the degree of polymerization of the procyanidins could reach 16 (3). A certain amount of higher polymer content may arise from oxidative polymerization of oligomers after extraction.

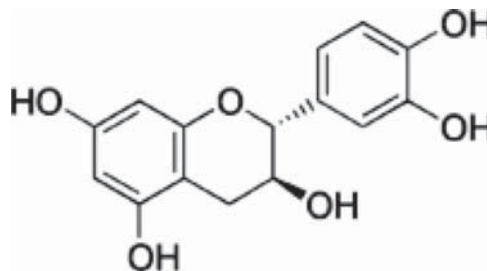


Figure 1 (+) catechin.

Known by a number of names—leucoanthocyanidins, oligomeric proanthocyanidins (OPCs), procyanidin, procyanidolic oligomers (PCOs), pycnogenols, and others—GSE first gained widespread prominence as a nutritional supplement due to the work of Schwitter and Masquelier (4). Wine is a source of GSE procyanidins, but extraction of the active substances into wine requires extended exposure of the juice to the crushed skins and seeds, something typical only of red wines. In contrast, GSEs are made from many varieties, including white (i.e., green) grapes. Commonly claimed concentrations in GSEs range from 60% to 95% polyphenols.

PRECLINICAL STUDIES

Pharmacokinetics

Relatively few studies address the absorption, distribution, metabolism, and elimination of procyanidins. The information available on the factors that control the disposition of flavonoids in general is limited, and there are few reliable methods for identifying possible metabolites in tissues. Recent experiments concerning the metabolism of flavanol monomers, such as epicatechin and catechin, confirm findings first made some 30 years ago. Flavanol monomers seem to be extensively metabolized to O-methylated forms and/or conjugated to glucuronides and sulfates during absorption into circulation, whereas the higher procyanidin oligomers can undergo cleavage to mixtures of monomers and dimers in the stomach that may act to enhance their absorption in the small intestine. Higher oligomers may have very limited absorption. The major bioactive forms of monomers and procyanidins in vivo are likely to be metabolites and/or conjugates of epicatechin, such as 3'-O-methyl epicatechin (5). This being

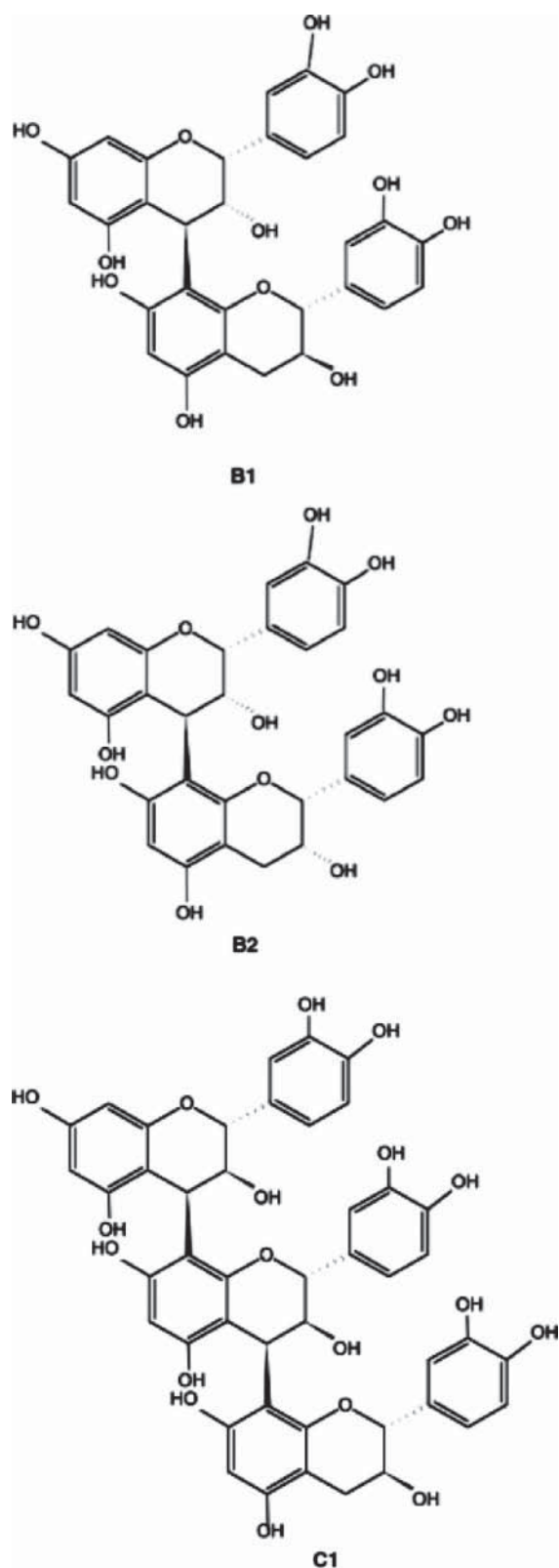


Figure 2 Showing procyanidins B1, B2, and C1.

said, newer techniques, such as an off-line solid-phase extraction and ultra-performance liquid chromatography–tandem mass spectrometry method, are being developed. In one report that describes application to the analysis in rat plasma obtained 2 hours after ingestion of GSE, monomers (catechin and epicatechin), dimers, and trimers in their native forms were detected and quantified in plasma samples with concentrations ranging from 0.85 to 8.55 μM . Several metabolites, including catechin and epicatechin glucuronide, catechin and epicatechin methyl glucuronide, and catechin and epicatechin methyl sulfate, were also identified (6).

Lappara et al. showed the rapid appearance of small, but still significant, radioactivity in the blood of rats after oral administration of a grape preparation containing radioactive carbon-labeled procyanidin dimers (7). Harmand and Blanquet documented similar results and reported that the amount of radioactivity excreted into the feces increased with the dose (8). Yamakoshi et al. detected procyanidins in the plasma of GSE-fed rabbits 1 hour after administration of the extracts (9). In a subsequent study, these authors reported the occurrence of metabolites of GSE procyanidins in rat plasma 15 minutes after administration, with three peaks identified by HPLC as gallic acid, (+)-catechin, and (–)-epicatechin (10). Lapidot et al. reported that red wine anthocyanidins were detected in the urine of volunteers, a very surprising finding because they are stable only at very low pH (11). The authors deduced that proanthocyanidins entering the body from the administered red wine in this instance were degraded to anthocyanidins in the urine (under its acidic conditions) upon storage.

Several studies have reported the absorption of procyanidins. OPCs from grape seed in an early animal study were found to have an affinity for the collagen structures that support the arteries, capillaries, and veins. Administration of procyanidin B2 [epicatechin-(4 β -8)-epicatechin] to rats resulted in it being excreted in urine, a portion of which was degraded to (–)-epicatechin and to the metabolized conjugated and/or methylated (–)-epicatechin internally (12). In a human trial, administration of 2.0 g of GSE led to the presence of procyanidin B1 [epicatechin-(4 β -8)-catechin] 2 hours later in serum (13). At least one large animal study supports the position that GSE is more active when given in conjunction with grape skin extract (14).

PHARMACOLOGY

The antioxidant and free radical scavenging actions of GSE procyanidins can explain only some of the biologic effects of GSE. Other factors include the inhibition of collagen-degrading enzymes, the upregulation of endothelial nitric oxide synthase, the downregulation of inducible nitric oxide synthase (iNOS) in circulating blood cells, the modulation of cell activation, cellular protein phosphorylation, cellular signaling, interference with matrix proteases, and others. Moreover, caution is in order when interpreting these statements. Many animal studies have involved dietary administrations of 100–300 mg/kg or more of body weight, much more than can normally be achieved from consumption.

Anticarcinogenic and Anticancer Effects

Animal experiments indicate that GSE interferes with the initiation and promotion of carcinogenesis. The extract elicits antiproliferative, apoptosis-promoting, and direct cytotoxic actions against prostate and other cancer cell lines in vitro (15). In an animal model, the dietary administration of GSE prevented photocarcinogenesis in SKH-1 hairless mice. Grape seed proanthocyanidins significantly decreased tissue fat level (24–27%, $P < 0.05$) without changing the total body mass of the animals compared with controls as a result of increased lipolysis or decreased synthesis of fat. The conclusion was that there was a reduction in UVB-induced oxidative damage and tissue fat content (16). Antitumor-promoting actions have been similarly shown with the topical application of GSE in an animal model (17). Procyanidins from various sources have elicited antitumor-promoter effects in in vivo carcinogenesis studies, often with dimers and trimers showing seemingly greater activity (18). In one study, feeding female rats diets containing 0.1–1.0% GSE procyanidins led to 72–88% inhibition of azoxymethane-induced aberrant crypt foci formation and a 20–56% prevention of ornithine decarboxylase activity in the distal third of the colon with no effect on the activity of liver cytochrome P450 2E1 (19).

Alzheimer Disease

It has been shown that polyphenols extracted from grape seeds are able to inhibit amyloid beta (A β) aggregation, reduce A β production, and protect against A β neurotoxicity in vitro. The therapeutic effects of a polyphenol-rich GSE were investigated in a mouse model of Alzheimer disease. APP(Swe)/PS1dE9 transgenic mice were fed with normal AIN-93G diet (control diet), AIN-93G diet with 0.07% curcumin, or diet with 2% GSE beginning at 3 months of age for 9 months. Total phenolic content of GSE was 592.5 mg/g dry weight, including gallic acid (49 mg/g), catechin (41 mg/g), epicatechin (66 mg/g), and proanthocyanidins (436.6 mg catechin equivalents/g). The A β levels in the brain and serum of the mice fed with GSE were reduced by 33% and 44%, respectively, compared with the Alzheimer mice fed with the control diet. Amyloid plaques and microgliosis in the brain of Alzheimer mice fed with GSE were also reduced by 49% and 70%, respectively (20). Interestingly, animal experiments have demonstrated that repeated dosing with a GSE previously found to attenuate cognitive deterioration in a mouse model of Alzheimer disease increased the plasma area under the curve 0–8 hours of gallic acid, catechin, and epicatechin. Catechin and epicatechin were undetectable in brain tissues after a single acute dose of GSE but reached levels of 290.7 ± 45.9 and 576.7 ± 227.7 pg/g in brain tissues from rats administered GSE for 10 days, a finding that the researchers view as having implications for Alzheimer disease (21).

Metabolic, Nutritional, Renal, and Cardioprotective Effects

Antioxidant protection against low-density lipoprotein (LDL) modification may not be the primary mechanism by which dietary agents protect against coronary artery disease. Benefits could accrue by way of protection against

endothelial dysfunction, changes in cholesterol transport, and so forth. Plasma protein binding studies show that (+)-catechin and procyanidins from GSE mainly bind to a protein of about 80 kDa in rats and 35 kDa in humans. The sequencing indicates that these proteins are apo A-1 in humans and transferrin in rats. The fact that red wine procyanidins bind to both proteins suggests that they may have a role in reverse cholesterol transport and in preventing the oxidizing action of iron (22). In one rat study, administration of GSE procyanidins caused a significant increment of volatile fatty acids pool and beneficial effects on lipid disposition (23). GSE procyanidins appear to exert an antihypercholesterolemic effect not only by enhancing reverse cholesterol transport but also by reducing intestinal cholesterol absorption and increasing bile acid excretion in rats (24). GSE added to the diet exerted a positive effect on serum lipids in rabbits fed with high-fat diet (25). At the 0th, 6th, and 12th week of the experiment, there was a lower serum TC, TG, LDL-C, and a higher HDL-C in experimental groups in comparison with those in the control group.

Studies with animal and human models have reported that GSE exerts wide-ranging protection against nonlipid factors implicated in cardiovascular disease. Supplementation of the extract in studies has improved postischemic left ventricular function; reduced myocardial infarct size, ventricular fibrillation, and tachycardia; decreased the amount of reactive oxygen species as detected by electron spin resonance spectroscopy; and lessened malondialdehyde formation in the heart perfusate (26). In an animal model, there was inhibition of cardiomyocyte apoptosis under ischemia/reperfusion conditions (27). A 1984 GSE study showed protection against elevations in blood cholesterol and damage to the aorta on a high-cholesterol diet in rabbits (28). A more recent trial in rabbits (0.1% and 1% in the diet) using immunohistochemical analysis revealed a decrease in the number of oxidized LDL-positive macrophage-derived foam cells in atherosclerotic lesions in the aorta of the animals fed a proanthocyanidin-rich extract (9).

Cardioprotective effects would also be expected from hypotensive actions of GSE. One mechanism explored is the inhibition of angiotensin-I-converting enzyme (ACE) activity. OPCs from *V. vinifera* L. (two fractions), *Cupressus sempervirens* L. (three fractions), and the monomers (+)-catechin and (–)-epicatechin have been tested for their effects on ACE activity in vitro. The oligomers appeared to be the most active; fraction A of *Vitis* (the most inhibitory test substance) had an IC₅₀ of 0.08 mg/mL. Monomers had little activity. Under in vivo conditions, the vasopressive response to angiotensin I was inhibited by approximately 20–40% in the rabbit after administration of OPCs (5 mg/kg IV). Angiotensin II was also inhibited, which indicated another action, perhaps due to the formation of new compounds from reactions between angiotensin I and II and the oligomers (29). Many sources of procyanidins seem to be active in lowering blood pressure.

Protection of renal functions has been demonstrated with procyanidins. Administration of GSE procyanidins resulted in the reversal of experimental myoglobinuric acute renal failure induced by glycerol in rats (30).

Hair Growth Effects

Topical application studies of GSE have determined that procyanidin dimers and trimers exhibit higher growth-promoting activity than do the monomers. Procyanidin B2, an epicatechin dimer, exhibits the maximum growth-promoting activity for hair epithelial cells (300% of control). A clinical trial using a topical application of procyanidin B2 produced significant results (31). Procyanidin C1, an epicatechin trimer, is second in hair growth promotion (approximately 220% of control). Other fractions display much less efficacy (32). Procyanidin B3 also has potential according to an *in vitro* study (33).

Hepatoprotective, Tissue Protective, and Premenstrual Syndrome Effects

Considerable interest has developed in the hepatoprotective effects of GSE. In one rodent study, OPCs decreased the activities of cytochrome P450 1A1 and of other inducible P450 isozymes while significantly increasing those of the phase II enzymes generally involved in the detoxification of reactive metabolite intermediates. This demonstrates that GSE can modulate the generation of reactive intermediates mediated by distinct molecular forms of P450, thus revealing their potential in chemoprevention (34). A wide-ranging study reported that prior oral exposure of mice for a period of 7–10 days afforded near-complete protection of GSE against the action of four agents that induced hepatotoxicity, pulmonary toxicity, cardiotoxicity, nephrotoxicity, spleen toxicity, and neurotoxicity while significantly reducing DNA damage in various tissues triggered by these agents (35).

Vascular, Postoperative Edema, and Skin and Wound Healing Effects

Procyanidins and flavonoids may be beneficial to connective tissue for several reasons, including the decrement of inflammation and associated tissue degradation, the improvement of local circulation, as well as the promotion of a strong collagen matrix. Certain proteins, such as elastins, are important in the interaction of cells and the vascular wall. Procyanidin oligomers appear to be potent inhibitors of elastases, and this has particular implications for vascular protection. They stabilize collagen *in vitro* and *in vivo*. The shorter chains of dimers and trimers are more effective in inhibiting lipid peroxidation and in their radical scavenging activity, whereas the longer pentamers and hexamers are more effective in inhibiting elastase activity. The dimers to pentamers, in particular, act on the vascular wall, especially on mesenchymal cells, as well as on the extracellular matrix, where they bind to fibrous proteins and prevent the degradation of elastin and collagen (36).

Tixier et al. showed that procyanidins bind to thin elastin fibers when injected intradermally into young rabbits resulting in the resistance of the fibers to hydrolytic attack by porcine pancreatic elastase (37). Catechin-related molecules possess an inherent ability to interact with glycoproteins and cross-link with the ϵ -amino groups of lysyl residues in polypeptides from biomolecules such as collagen (38). Endothelial cells from vein explants from patients with edema placed in a medium containing procyanidins incorporated significantly less glucosamine than those in the control and secreted more into the medium. The great-

est impact was on the biosynthesis of glycoprotein and sulfated glycosaminoglycan, which may explain the beneficial effect of procyanidins on vein disorders (39). Similarly, corneas incubated in the presence of collagenase were quickly attacked, and their degradation was practically complete after 24 hours. With a low concentration of procyanidins from grape seeds (0.066 mg/mL), proteolysis was only slightly inhibited. A higher concentration (1 mg/mL) completely prevented collagenolysis, and there was complete preservation of the corneas against proteolytic attack (40).

One animal study showed that OPCs from ingested GSE have a particular affinity for the cell membranes and that it lodges in the lamina densa of the basal membrane, where it seems to facilitate the formation of collagen microfibrils (41).

Topical application of GSE showed positive effects on wound healing in one study. There was accelerated wound contraction and closure. GSE treatment was associated with a more well-defined hyperproliferative epithelial region, higher cell density, enhanced deposition of connective tissue, and improved histological architecture (42). Oral administration has proved effective in lightening the UV-induced pigmentation of guinea pig skin (43). One study selected GSE procyanidins as natural cross-linking agents, which suggests that GSE may be of significant value in the field of tissue engineering (44).

Visual Effects

Animal trials have indicated protection against cataract formation. Yamakoshi et al. recently reported that GSE prevented and postponed development of cataract formation in rats fed a standard diet containing 0.213% GSE [0.082% procyanidins in the diet (wt/wt)] for 27 days and suggested the antioxidant role of larger molecular procyanidins to explain this anticataract effect (9). Subsequent work in isolated canine lens epithelial cells identified a second potential mechanism for the inhibition of cataractogenesis: the attenuation of oxidative stress-induced cell signaling pathways (MAPK and PI3 K) that lead to induction of downstream proinflammatory cytokines and cataract progression (45).

Obesity Management and Positive Effects on Energy Metabolism

GSE *in vitro* shows inhibitory activity on the fat-metabolizing enzymes pancreatic lipase and lipoprotein lipase, thus suggesting that it might be useful as a treatment to limit dietary fat absorption and the accumulation of fat in adipose tissue. The observed reduction in intracellular lipolytic activity of cultured 3T3-L1 adipocytes indicates that such extracts may, in turn, reduce the levels of circulating free fatty acids, which are linked to insulin resistance in obese patients (46). A more complex picture emerges from another *in vitro* test in which differentiated 3T3-L1 cells were treated with catechin, epicatechin, or procyanidin extracts with varying degrees of polymerization at 150 μ M for different periods of time (0.5–24 hours). There was an increase in the release of glycerol from stored fat into the medium in 3T3-L1 cells treated with procyanidin extract that reached a plateau after 15-hour exposure. Procyanidins from grape and wine, by implication, affect

lipid metabolism, but their monomers (catechin and epicatechin) do not. This effect is more pronounced when the degree of polymerization is higher. The administration of grape extract has been shown to increase oxygen consumption in isolated guinea pig heart, suggestive of an increased oxidation of energy sources (47). GSE may also decrease the availability and assembly of lipid particles at the gene transcription level (48). In rats fed a high-fat diet, 25 mg/kg of GSE downregulated mRNAs for VLDL assembly (MTP) and triglyceride synthesis (DGAT2), as well as SREBP1, a transcription factor that regulates key lipid metabolism genes (particularly the genes of fatty acid synthesis and the LDL-receptor gene). GSE, therefore, may increase fat hydrolysis and oxidation while inhibiting further lipid synthesis or storage.

In vivo work has yielded inconsistent results with regard to weight loss. In one animal trial, supplying 250 mg of GSE procyanidins (equivalent to the procyanidins of 0.5 L of wine/day in humans) in the diet of rats for 12 weeks significantly decreased weight gain with no influence on daily food intake (23). However, two more recent oral toxicity animal trials using much higher intakes of up to 2.5% of diet did not report weight loss (49). In a third trial, a significant increase in food consumption without increase in weight gain was observed in male and female rats provided GSE diets compared with control rats, especially in male rats, consuming 2.0% GSE (50).

CLINICAL STUDIES

Metabolic, Nutritional, Renal, and Cardioprotective Effects

Clinically, a randomized, double-blind, crossover study undertaken in 24 healthy male heavy smokers indicated a protective effect from GSE (150 mg/day), administered for 4 weeks with a significant reduction in thiobarbituric acid-reactive substance (TBARS) (51). The mechanisms underlying cardioprotection against oxidative stress are becoming clearer. In one study, 10 healthy volunteers received a daily dose of 110 mg of GSE procyanidins for 30 days. Fasting venous blood samples were taken before and at the end of the supplementation period and after 7 days of washout. There was no modification in the total antioxidant activity and the plasma concentrations of α -tocopherol. However, the levels of α -tocopherol in red blood cell membranes increased significantly from 1.8 ± 0.1 to 2.8 ± 0.2 mg/g. Similarly, oxidized DNA in the lymphocytes was reduced, and red blood cell membrane fatty acid composition was shifted to a higher level of polyunsaturated fatty acids. It was suggested that dietary procyanidins exert their antioxidant protection in vivo by sparing lipid soluble vitamin E and reducing DNA oxidative damage (52). Recent findings suggest that the cardioprotective effects of GSE may be of clinical significance, indeed, and may have a therapeutic role in decreasing cardiovascular risk. In metabolic syndrome subjects randomized into 3 groups—(i) placebo, (ii) 150 mg GSE per day, and (iii) 300 mg GSE per day for 4 weeks—systolic and diastolic blood pressures were reduced significantly by both dosages, with the 300 mg dosage also reducing serum oxidized LDL cholesterol level (53). Results reported have varied with the nature of the GSE and the study pop-

ulation. A double-blind, randomized, placebo-controlled trial in diabetics using a different GSE at 600 mg/day for 4 weeks found significant improvement in markers for inflammation and glycemia as well as in a marker of oxidative stress, yet did not find a significant change in endothelial function (54).

Premenstrual Syndrome Effects

Premenstrual syndrome is often linked to problems with the clearance of sex hormones and other compounds by the liver, leading to circulatory and other issues. In one uncontrolled clinical study, GSE was judged to offer significant benefits with regard to mammary symptoms, abdominal swelling, pelvic pains, weight variations, and venous problems of the legs (55).

Vascular, Postoperative Edema, and Skin and Wound Healing Effects

In a double-blind, placebo-controlled clinical trial examining postoperative edema, GSE was administered at the rate of 300 mg/day for 5 days prior to facelift and then postoperatively days 2 through 6 in 32 female patients. It was judged to be significantly superior to placebo in reducing postoperative swelling (56).

Classically, GSE has had an application in Europe in the treatment of capillary fragility and weakness, also known as peripheral venous insufficiency. In a small placebo-controlled study with 28 active subjects (150 mg of OPCs per day) and 25 controls, capillary resistance rose from 14.6 ± 0.98 to 18 ± 3.35 cm Hg ($P < 0.0005$) in the treated group, while no significant variation was observed in the placebo group (15.5 ± 1.3 vs. 14.7 ± 1.3 cm Hg initially) (57). In a more recent open-label study involving 4729 patients, GSE was administered at the rate of 150 mg twice per day. Evaluations were carried out at 45 and 90 days of treatment based on factors such as nocturnal cramps, sensation of warmth in the legs, cyanosis, and edema. Heaviness in the legs was reduced in 57% of the cases at the first evaluation and in 89.4% of the cases at the second evaluation. Other symptoms improved in 66% of the cases by day 45 and in greater than 79% of cases by the 90-day evaluation (58).

Visual Effects

Clinical work seems to support claims for visual benefits. Two human intervention studies give evidence of significant improvements in visual acuity, response to glare, and in other areas. In one double-blind study, 100 patients were supplemented with 200 mg of GSE/day for 5 weeks, whereas controls received no treatment. Significant improvements occurred in visual performances after glare as well as in visual adaptation to low luminance (59). In another clinical study, 40 myopic patients received either GSE (150 mg/day) or placebo for 30 days. Of 14 patients in the OPC group with low light-emitting diode visual evoked potentials, 12 demonstrated significant improvement compared with 0/17 in the placebo ($P < 0.0001$). Significant electroretinographic improvements were noted in eight patients (40%) in the OPC group and zero patients in the placebo group ($P < 0.0001$) (60).

Obesity Management and Positive Effects on Energy Metabolism

One trial in humans lasting only 3 days found that giving GSE 30–60 minutes prior to lunch and dinner reduced energy intake in a small subgroup of the trial population that consumed substantially more calories than did the majority of the study's subjects. But it yielded no significant impact compared with placebo in the subjects as a whole (61).

Usage and Dosage

In view of the paucity of data concerning the composition of procyanidins in foodstuffs, the dietary intake of GSE-related components and their metabolic disposition, it is not possible to indicate a dose as a nutritional supplement. Based on French red wine consumption of 180 mL/day, the current daily intake of phenolics was estimated at 400.2 mg/day/person and catechins (monomers, dimers B1, B2, B3, B4) at 83.2 mg/day/person, including 40% of monomers for the French population (62). Based on European studies and practices, therapeutic dosages are typically 150–300 mg/day.

Safety

No significant health hazards have been reported with the use of GSE as directed. The extract was examined for acute and subchronic oral toxicity using Fischer 344 rats and for mutagenic potential by the reverse mutation test using *Salmonella typhimurium*, the chromosomal aberration test using CHL cells, and the micronucleus test using ddY mice (63). No evidence of acute oral toxicity at dosages of 2 and 4 g/kg and mutagenicity was found. Administration of GSE as a dietary admixture at levels of 0.02%, 0.2%, and 2% (wt/wt) to the rats for 90 days did not induce noticeable signs of toxicity. The no-observed-adverse-effect level (NOAEL) of GSE in the subchronic toxicity study was 2% in the diet (equal to 1410 mg/kg of body weight/day in males and 1501 mg/kg of body weight/day in females). A subchronic 3-month trial found NOAEL at intakes of approximately 1.78 g/kg of body weight/day of GSE or grape skin extract in male rats and 2.15 g/kg of body weight/day in female rats (49). Similarly, researchers elsewhere concluded that administration of the GSE IH636 to male and female Sprague Dawley rats in the feed at levels of 0.5%, 1.0%, or 2.0% for 90 days did not induce any significant toxicological effects (50).

MECHANISMS OF ACTION

Antioxidant

In vitro tests of GSE show it to be a more powerful antioxidant than either vitamin C or E, as assessed by the inhibition of lipid peroxidation and the scavenging of radical species (64). The antioxidant activity of GSE components in the lipid phase decreases with degree of polymerization, whereas that in the aqueous phase increases from monomer to trimer and then decreases from trimer to tetramer. Galloylation of catechin and dimeric procyanidins decreases lipid-phase antioxidant activity while increasing that of the aqueous phase (65).

Numerous studies demonstrate that GSE inhibits free radical generation mediated by xanthine oxidase (possibly by inhibiting the enzyme itself), metal catalysis (66), and arachidonic acid oxygenation (67). Procyanidins strongly inhibit superoxide generation and prevent oxidative discharge from activated neutrophils at the site of their adhesion. This action may be due to their inhibition of neutrophil NADPH oxidase and protein kinase C. Similarly, they protect cells from peroxynitrite damage (68). One recent study indicates that the high condensation rate and the gallate ester moiety in GSE procyanidins might play an important role in nitric oxide (NO) scavenging activity. The suggested mechanism for NO scavenging activity of GSE procyanidins is the direct reaction of NO with phenolic compounds to generate phenoxy radicals (69).

In vitro, GSE inhibits the oxidation of polyunsaturated fatty acid moieties in LDL particles by intercepting free radical propagation of LDL oxidation. Of the different antioxidants tested, including grape skin extracts, GSE is most effective in protecting LDL in the peroxynitrite generator initiated system. Procyanidin dimers and trimers isolated from GSE inhibited the oxidation of LDL and prevented early aortic atherosclerosis in hypercholesterolemic golden Syrian hamsters (70). Some in vitro studies have ascribed an indirect protective role for red wine phenolic compounds. These substances may protect plasma LDL against oxidative degradation by sparing the endogenous α -tocopherol of LDL (71). Clinically, several studies report the effects of GSE supplementation on the reduction of LDL oxidation and the increase in plasma antioxidant capacity (72–74). This increase in antioxidant capacity was accompanied by an increase in plasma antioxidant vitamins (ascorbic acid, retinol, or carotenoids; differences in plasma levels of vitamin E exhibited conflicting results between the studies). Taken with a meal, GSE minimizes the postprandial oxidative stress in the blood by decreasing the oxidants, increasing the antioxidant levels in plasma, and enhancing the resistance to oxidative modification of LDL (75).

Smoking is a model of oxidative stress. GSE can protect the mitochondrial membrane from the oxidative damage caused by gas-phase cigarette smoke (76). In healthy male heavy smokers, GSE supplementation (150 mg/day) significantly reduced the concentration of TBARS, a marker of oxidative stress (53).

GSE polyphenols may increase antioxidant capacity through indirect means by stimulating endogenous antioxidant enzyme (AOE) systems. This stimulation may occur at the level of gene transcription, as seen in the GSE-mediated increased transcription of glutathione-related enzymes (glutathione *S*-transferase, glutathione peroxidase, and glutathione reductase) (77), or by the direct interaction of GSE polyphenols and AOE as has been observed for copper/zinc superoxide dismutase (Cu/Zn-SOD) (78). In vitro analysis of the GSE/Cu/Zn-SOD interaction is consistent with a direct binding event and significantly increased SOD activity; in silico docking experiments identified several potential candidate GSE compounds (all substituted epicatechins or epicatechin-containing procyanidins) that can act as allosteric effectors to a binding site near the enzyme active site.

Anti-Inflammatory Effects

GSE significantly suppressed the expression of the gene for iNOS in lipopolysaccharide-activated peripheral blood mononuclear cells (PBMC) in a dose-dependent manner, as determined by iNOS mRNA expression in the cells, an action indicating that GSE may mediate an anti-inflammatory cellular process (79). However, another mechanism may be just as significant. A primary factor in the onset of inflammation is the increased permeability of tissues due to the action of collagenase. Animal experiments show that grape seed oligomers prevent the increased permeability of cerebral capillaries, aorta, and cardiac muscle capillaries induced by collagenase injections (80). Other studies have demonstrated the inhibition of hyaluronidase activation in isolated rat mast mesentery cells (81). As discussed above, procyanidins bind with biopolymers, such as proteins and carbohydrates; they can bind to collagen and prevent its degradation by elastases. In an animal model of inflammation, a naturally occurring procyanidin decreased to a statistically significant extent the magnitude of rat-paw edema induced with serotonin, carrageenan, or prostaglandin E₁. In the case of carrageenan-induced edema, this procyanidin was about twice as effective as phenylbutazone (82). GSE (10–40 mg/kg IP) inhibited carrageenan-induced paw edema in rats and croton oil-induced ear swelling in mice in a dose-dependent manner. At 10 mg/kg, it reduced the malondialdehyde content in inflamed paws; inhibited N-acetyl glucosamine (β-NAG) and NOS activity; and lowered the content of NO, interleukin-1β, tumor necrosis factor α (TNF-α), and prostaglandin E₂ in exudates from edema paws of rats induced by carrageenan (83). These experiments are sufficient to show that GSE qualitatively inhibits a variety of inducers of inflammation, but given that there is no univocal linear relation between injection of substances into the body cavity and oral ingestion, they are insufficient to establish the degree of quantitative impact.

Immunostimulatory, Antiviral, and Anticancer

Procyanidins in vitro exert marked antiviral and antitumor effects by inducing production of the Th1-derived cytokine interferon gamma (IFN-γ) by PBMC from healthy donors. GSE significantly induces the transcription of IFN-γ mRNA as demonstrated by reverse transcriptase-polymerase chain reaction, but has no effect on that of the proinflammatory Th2-derived cytokine, interleukin-6. In addition to the enhancement in IFN-γ expression, there is a concomitant increase in the number of cells with intracytoplasmic IFN-γ, as well as in the synthesis and secretion of IFN-γ. This demonstrates that the potentially beneficial immunostimulatory effects of GSE may be mediated through the induction of IFN-γ (84). In vitro and ex vivo effects seem to be dependent on the molecular size. One study compared the effects of monomers, dimers, and a trimer on NO production, TNF-α secretion, and oxidant-responsive transcription factor, nuclear factor κB (NF-κB). Monomers and dimers depressed NO production, TNF-α secretion, and NF-κB-dependent gene expression induced by IFN-γ, whereas the trimeric procyanidin C2 enhanced these parameters (85). GSE also downregulates the expres-

sion of coentry receptors required by HIV and perhaps other viruses for entry into cells (86).

Vasodilation

Dysfunctional vasodilation is found in a number of pathologic conditions. Procyanidins display endothelium-dependent vasorelaxation (EDR) activity in vitro. This EDR-enhancing activity involves the release of NO, mediated by endothelial NOS, and subsequent increase in cyclic guanosine monophosphate levels in the vascular smooth muscle cells. The activity of isolated procyanidins tends to increase with the degree of polymerization, epicatechin content, and galloylation (87,88). Cishek et al. clearly established that GSE elicits dose-dependent EDR, whereas monomers derived from grape seeds are inactive (89). Further studies deduced that acute exposure to GSEs elicits an EDR, which was mediated by the release of NO, but did not involve the muscarinic receptors.

Oral administration of GSE (60 mg/kg/day) to New Zealand white rabbits for 7 weeks showed a positive effect on endothelial function by increasing EDR (90). There was impairment of EDR in cholesterol-fed rabbits compared with that animals fed standard chow. However, in those fed GSEs together with cholesterol, this impairment was attenuated. This finding is significant in that endothelial dysfunction is an integral component of atherosclerosis. Harrison has demonstrated that such dysfunction precedes angiographically recognizable plaques in the coronary arteries (91). Endothelial dysfunction (i.e., loss of EDR) also exists in hypertensives, diabetics, smokers, postmenopausal women, and individuals with hyperlipidemia. These conditions are recognized conventional cardiovascular risk factors (92). Lipid disturbances may not be the common causal factor. da Luz et al. have demonstrated lipid-independent protection by red wine and nonalcoholic wine components (polyphenols) against atherosclerosis development (93). These substances prevented plaque formation in hypercholesterolemic rabbits despite significant increases in LDL. Antiplatelet effect, impairment of the expression of endothelial cell adhesion molecules, and/or EDR (and associated NO stimulation) by polyphenols are likely explanations. Studies have indicated that GSE attenuates the expression of adhesion molecules in human endothelial cells in culture and in human subjects with systemic sclerosis (94).

Additional Mechanisms

Yet unknown mechanisms of action may be revealed through proteomic analyses of GSE effects on a variety of disease states. Two dimensional difference gel electrophoresis, coupled with mass spectroscopy techniques, has allowed a broad-spectrum analysis of protein regulation by GSE in different animal disease models, such as diabetic retinopathy (95) and nephropathy (96), as well as in healthy animals fed GSE (97,98). Experimentally induced diabetic rats exhibit a marked downregulation of several proteins that are involved in mitigation of oxidative stress, protection from glycosylation damage, protein degradation, and amino acid metabolism; rats fed GSE for 24 weeks (250 mg/kg) back-regulated these proteins to their normal, nondiabetic levels. These data reveal

several potential protein targets for GSE study. Regulation of several classes of proteins is evident when GSE is administered to healthy animals as well. GSE stimulates the production of several proteins involved in protein folding (Hsp60, Hsc70/71) and energy production (creatine kinase) in healthy rat brain tissue. Interestingly, these same proteins have been shown to be downregulated in Alzheimer disease and mouse models of dementia. These effects may explain some of the utility exhibited by GSE for neuroprotection in animal models of Alzheimer disease and dementia.

REGULATORY ISSUES

In the United States, grape seed and grape skin extracts are generally recognized as safe. European regulations vary from country to country, but GSE is commonly allowed in pharmacopeias as a nonprescription herbal drug for the treatment of venous disorders.

CONCLUSIONS

GSE components exhibit a wide range of biological effects as modifiers of inflammation and modulators of various enzyme systems. Potential benefits demonstrated in experimental studies include endothelium-dependent relaxation, impact on inflammatory mediator (cytokine) release by cells and on the oxidation of LDL cholesterol, and effects on platelet aggregation and nitric oxide metabolism. Data from human intervention trials are relatively scant. Furthermore, little is known about the absorption, bioavailability, and bioactivity of GSE-associated flavanol oligomers and polymers because of difficulties associated with their reliable quantification in physiological fluids. The small number of adequately controlled human studies generally indicates that sufficient absorption takes place to accomplish transitory changes in the antioxidative capacity of plasma in humans and to afford cellular protection, even though the identification of the metabolites is elusive. However, none of these studies has adequately considered long-term effects. No clear picture has emerged regarding the appearance of catechin monomers or procyanidin dimers in the plasma of human subjects following GSE ingestion. Metabolites arising through bacterial biotransformation by ring fission may have their own unique pharmacologic effects deemed to be beneficial for health. More well-controlled in vivo and clinical studies are needed to extend the application of these compounds outside the realm of scientific research per se.

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Green Tea Polyphenols

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INTRODUCTION

Tea (*Camellia sinensis*, Theaceae) is second only to water in worldwide popularity as a beverage. The three major types of tea—green, oolong, and black—differ in terms of the manufacture and chemical composition. There are numerous studies in humans, animal models, and cell lines to suggest potential health benefits from the consumption of tea, including prevention of cancer and heart diseases. Many of the health benefits have been attributed to the polyphenolic components: Epigallocatechin-3-gallate (EGCG) and the related catechins have been the most widely studied in terms of disease prevention and treatment. The present entry summarizes the data concerning the preventive effects of the green tea polyphenols on heart diseases, neurodegenerative disorders, obesity and diabetes, and cancer. Greater attention is given to cancer prevention, as this area has been most widely studied. Importance is also accorded to the bioavailability and biotransformation of the catechins. Such factors are likely to influence the potential health benefits of tea consumption, and a complete understanding of them will aid in better assessing the beverage's disease-preventive activity.

BACKGROUND

Tea is one of the most widely consumed beverages in the world; it has been used for medicinal purposes in China and Japan for thousands of years. More than 300 different varieties of tea are produced from the leaves of *C. sinensis* by various manufacturing processes. In general, tea is divided into three types: green (nonfermented), oolong (semifermented), and black (fermented). Green tea and oolong tea are more popular in China, Japan, Korea, and some African countries, whereas black tea is preferred in India and the Western countries. Experimental and epidemiological studies have linked the consumption of tea to reduced risk of cardiovascular diseases and cancer (1,2). These effects have been attributed to its polyphenol compounds. Catechins are the most abundant polyphenols in green tea. A typical cup of brewed green tea contains, by dry weight, 30–40% catechins including EGCG, epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC) (Fig. 1). EGCG is the most abundant catechin in green, oolong, and black teas. Green and oolong teas typically contain 30–130 mg of EGCG per cup (237 mL), whereas black tea may contain up to 70 mg of EGCG per cup (3). The main pigments in black tea are theaflavins (Fig. 1) and thearubigins, which are formed by the oxidation and polymerization of catechins during fermentation.

The resulting brewed black tea contains 3–10% catechins, 2–6% theaflavins, and more than 20% thearubigins (4).

In the present entry, we discuss the disease-preventive activities of green tea and tea polyphenols, potential mechanisms for these activities, and the current knowledge of the bioavailability and biotransformation of these compounds.

CARDIOVASCULAR DISEASE

Both animal and human studies have suggested a cardio-protective effect for tea (1). Tea polyphenols have been shown to modulate biomarkers of atherosclerosis, hypertension, and vascular function. For example, Bursill et al. have reported that dietary supplementation of high cholesterol-fed New Zealand White Rabbits with 0.5–2% green tea catechins dose-dependently reduced total plasma cholesterol and plasma low-density lipoprotein (LDL) (5). In another study of cholesterol-fed New Zealand Rabbits, 0.3% green tea as the sole source of drinking fluid for 21 weeks was shown to reduce the formation of aortic atherosclerotic lesions compared with water-treated controls (6). Black tea supplementation had no effect. Green tea supplementation (0.6% wt/vol) for 2 weeks was shown to reduce angiotensin-II-induced hypertension and cardiac hypertrophy in rats (7). Similar results were observed in diabetic rats. Treatment of a type 2 diabetes rat model, the Otsuka Long-Evans Tokushima fatty rat, with 30 mg/kg/day tea catechins for 12 weeks reduced systolic blood pressure and enhanced the vasodilatory effects of sodium nitroprusside (8). These effects appear to correlate with decreased NADH oxidase expression and activity.

Studies in humans have yielded mixed results. A meta-analysis of randomized controlled trials of cardiovascular disease risk showed only weak effects of green tea and black tea interventions (9). The overall results of four studies of green tea showed a decrease in LDL (−0.233 mM). The meta-analysis found that black tea intervention resulted in a slight increase in both systolic and diastolic blood pressure following acute treatment. Widlansky et al. have shown that both acute and chronic supplementation with black tea (450–900 mL) increased plasma levels of tea catechins but had no effect on plasma antioxidant capacity or C-reactive protein (10). In contrast, a 12-week, double-blind study of obese Japanese subjects has shown that supplementation with green tea reduced systolic blood pressure and reduced plasma LDL levels (11). Further intervention studies are needed to more

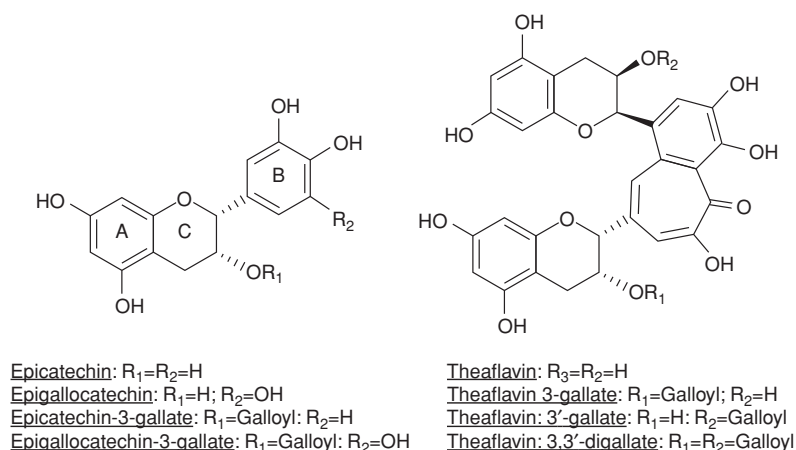


Figure 1 Structures of the major tea polyphenols.

clearly establish the efficacy of tea in the prevention of cardiovascular disease.

NEURODEGENERATIVE DISORDERS

Green tea and its components, especially EGCG, have been shown in some experiments to inhibit the development of Parkinson disease (PD). A recent publication reviewed all observational studies, including 11 case-control and 1 cohort studies that evaluated the association between PD risk and tea consumption (12). These observation studies indicate that tea consumption can protect against PD, and this protective effect is clearer in Chinese populations (12). Laboratory trials with mice further support a potential protective effect for green tea. Choi et al. reported that oral administration of green tea (1 g dry green tea in 360 mL water) and EGCG (25 mg/kg, IG) attenuated the development of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced PD in mice (13). EGCG prevented the loss of tyrosine hydroxylase positive cells in the substantia nigra and decreased the expression of neuronal nitric oxide synthetase (13). Potential mechanisms for the antiparkinsonian effects of EGCG include inhibition of catechol-*O*-methyltransferase (COMT) and 6-hydroxydopamine-induced neuronal cell death (14,15).

OBESITY AND DIABETES

Obesity and diabetes have become widespread health problems and contribute to increased risk of other diseases, including heart diseases and cancer. Green tea and catechin-enriched oolong tea have been shown in several studies to improve biomarkers related to obesity and diabetes including body mass index, waist-to-hip ratio, and fasting blood glucose (11,16,17). These effects have been observed in both diabetic and nondiabetic subjects.

Several studies have reported obesity- and diabetes-preventive effects of tea polyphenols in animal models. For example, Bose et al. have observed that treatment of high-fat fed C57bl6/J mice with 0.32% dietary EGCG reduced body weight gain (41% decrease), percentage of

body fat (45% decrease), fasting blood glucose (25% decrease), and insulin resistance (76% decrease) (18). Green tea extract has also been shown to reduce biomarkers of obesity and diabetes in B6.V-Lepob/J (ob/ob) leptin-deficient mice (19). Treatment with 0.5% green tea extract for 12 weeks reduced total white adipose and retroperitoneal fat pad weight, fasting blood glucose levels, and plasma triglycerides. Although the underlying mechanisms for these obesity- and diabetes-preventive effects remain unclear, both in vitro and in vivo studies have shown that tea and tea polyphenols can modulate lipid absorption from the gut, inhibit de novo lipogenesis, enhance fat oxidation, and improve glucose utilization (20). Further studies are required to determine which of these mechanisms are of primary importance.

CANCER

Animal Studies

Green tea has shown cancer chemopreventive activity against ultraviolet-light, chemical-induced, and genetic models of carcinogenesis. The organ sites include the lung, skin, oral cavity, esophagus, stomach, liver, pancreas, bladder, small intestine, colon, and prostate (2). Both tea polyphenols and caffeine have been shown to be important cancer-preventive components of tea (2,21,22). In a recent study, both 0.5% polyphenol E, a standard green tea polyphenol preparation containing 65% EGCG, and 0.044% caffeine could significantly reduce the progression of lung adenomas to adenocarcinomas in the 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in mice (22). A new approach for cancer prevention using tea polyphenols is the combination with other agents. Lu et al. recently demonstrated the synergistic inhibitory action of a combination of polyphenon E (PPE) and the lipid-lowering agent atorvastatin against NNK-induced lung carcinogenesis in A/J mice (23).

Epidemiological Studies

Although data from animal models of carcinogenesis suggest that tea and its components may be efficacious

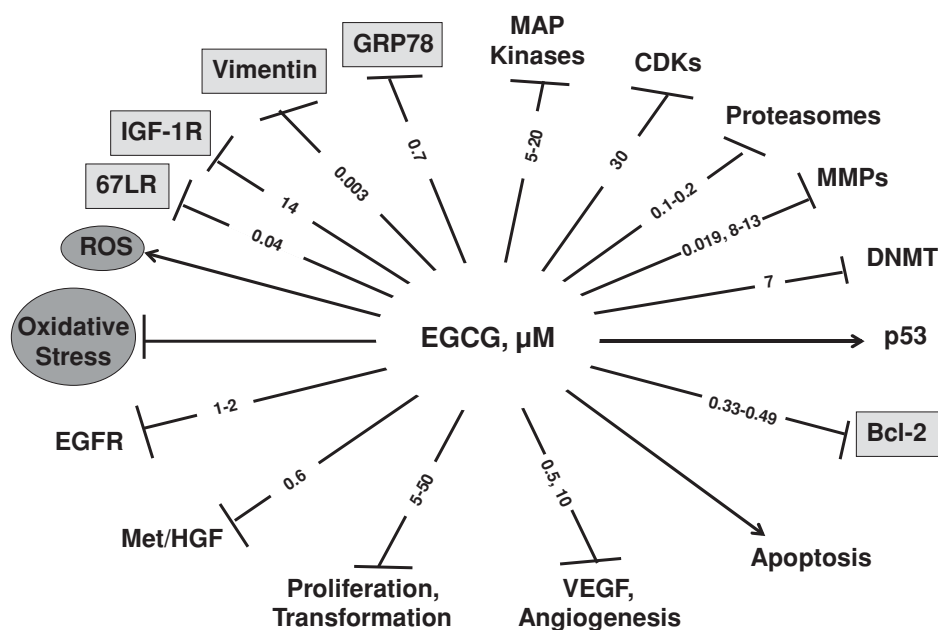


Figure 2 Possible targets for the cancer-preventive activity of (–)-epigallocatechin-3-gallate (EGCG) (2). Abbreviations: HGF, hepatocyte growth factor; IGF-1R, insulin-like growth factor 1 receptor; MMP, matrix metalloproteinase; ROS, reactive oxygen species.

cancer-preventive agents, they are epidemiologically inconclusive (reviewed in Ref. 2). Of the 127 case-control studies and 90 cohort studies on the relationship between tea consumption and human cancer risk published up to December 2008 (PubMed database), only 51 case-control studies and 19 cohort studies showed an inverse association between tea consumption and cancer risk, whereas other studies showed no such association (2). The overall impression is that the cancer-preventive effects are observed more frequently in studies on green tea than those on black tea and in case-control studies rather than in prospective cohort studies. Such inconsistent results may be due to a number of confounding factors, including diet, smoking status, age, and alcohol consumption, difficulties in quantifying tea consumption, and interindividual differences in cancer susceptibility and in the biotransformation of tea constituents.

Mechanisms of Cancer Prevention

Numerous potential mechanisms have been proposed for the cancer-preventive activity of tea and its constituents based on studies with cancer cell lines (Fig. 2). In vitro, tea polyphenols, especially EGCG, can serve as an antioxidant or a pro-oxidant and can also bind to target molecules and trigger cascades of signaling or metabolic pathways that lead to the inhibition of carcinogenesis (2). However, the concentrations of EGCG used in most of the cell culture experiments (20–100 μM) are higher than the plasma and tissue concentrations observed in humans and rodent in cancer-prevention experiments (usually $<0.5 \mu\text{M}$) (24). Whether the mechanisms obtained from cell lines with high EGCG concentrations can be extent to cancer prevention in vivo remains to be determined.

Antioxidant/Pro-Oxidant Activity

EGCG, as well as other tea polyphenols, has been shown to have strong antioxidant activity in vitro by scavenging reactive oxygen and nitrogen species and chelating redox-active transition metal ions. However, their antioxidative effects in vivo can be demonstrated only in certain studies. For example, it has been reported that EGCG-treated old rats had decreased levels of lipid peroxidation and increased levels of antioxidants and antioxidant enzymes, and no effects were observed in young rats, suggesting that the antioxidative effects of EGCG are apparent only in the presence of excessive oxidative stress (25,26). In addition, the antioxidative activity of tea polyphenols could decrease oxidative DNA damage, which has been shown in human and animal models (27,28).

In contrast to the potential antioxidative activity of tea polyphenols, recent experiments have suggested that the cell-killing activity of these compounds, at least in vitro, may be related to their pro-oxidant activity. Many studies have shown that under cell culture conditions, EGCG is subject to oxidation, resulting in the formation of reactive oxygen species (ROS) (29–33). The generated ROS may also activate the nuclear factor erythroid 2-related factor 2 and antioxidant-responsive element pathway to activate antioxidant and detoxifying enzymes (34). It remains to be determined whether this mechanism is relevant in vivo.

Binding to Molecular Targets

The eight phenolic groups of EGCG can serve as hydrogen bond donors to many biomolecules. EGCG has been shown to bind to salivary proline-rich proteins, fibronectin, fibrinogen and histidine-rich glycoproteins,

67-kDa laminin receptor, and Bcl-2 proteins (reviewed in Ref. 2). More recently, Dong et al. identified vimentin, insulin-like growth factor 1 receptor (IGF-1R), FYN, glucose-regulated protein 78 kDa, and ZAP70 as high-affinity EGCG-binding proteins (reviewed in Ref. 2). All of these proteins were demonstrated to be important for the inhibitory activity of EGCG in cell lines, but higher EGCG concentrations than the K_d values were needed. The difference in effective concentrations is probably due to the nonspecific binding of EGCG to other proteins, which compete with the target protein. The concentrations of EGCG used in the earlier binding studies are lower or similar to those observed in vivo, indicating that the discovery of the high-affinity EGCG-binding proteins is promising. The general applicability of these mechanisms for cancer prevention remains to be investigated.

Inhibition of Enzyme Activities

EGCG has also been shown to inhibit many enzyme activities that may contribute to the prevention of carcinogenesis (reviewed in Ref. 2). These include the inhibition of topoisomerase I activity in several human colon carcinoma cell lines (by 3–17 μ M EGCG); chymotryptic activity of the 20S proteasome in leukemic, breast cancer, and prostate cancer cell lines (1–10 μ M EGCG); matrix metalloproteinases 2 and 9 (9–13 μ M EGCG); the phosphorylation of JNK, JUN, MEK1, MEK2, ERK1, ERK2, and ELK1 in JB6 epidermal cell lines (5–20 μ M EGCG); DNA methyltransferase ($K_i = 7 \mu$ M EGCG) in KYSE 510 human esophageal cancer cell lines; dihydrofolate reductase (0.1–1.0 μ M EGCG); glucose-6-phosphate dehydrogenase; and glyceraldehyde-3-phosphate dehydrogenase. These enzyme inhibition results are biochemically interesting, but it is unclear whether these activities can be demonstrated in vivo at a nontoxic dose of EGCG.

Inhibition of Receptor-Dependent Signaling Pathways and Angiogenesis

Members of the epidermal growth factor receptor (EGFR) family are frequently overexpressed in human cancers and are associated with poor prognosis. Many studies have demonstrated the inhibitory effects of EGCG on the EGFR-signaling pathways (reviewed in Ref. 2). Deregulation of the hepatocyte growth factor (HGF)–HGFR pathway occurs in several types of human cancers and can lead to increased tumorigenesis and metastasis. It has been reported that 0.6 μ M EGCG could completely block the HGF-induced phosphorylation of HGFR and AKT1, and 5 μ M EGCG could significantly decrease cell invasion in MDA-MB-231 cells (2,35). EGCG has also been reported to decrease the levels of several growth factors, such as vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2), IGF, and EGF, which serve as chemical stimuli to initiate angiogenesis (2).

It is evident that different pathways may be involved in the protective effects that tea polyphenols exhibit. The relative importance of each of these potential mechanisms in vivo depends on whether effective tissue concentrations of the tea polyphenols can be achieved. In most cases, this remains to be determined.

BIOTRANSFORMATION AND BIOAVAILABILITY OF TEA POLYPHENOLS

The catechins have been demonstrated to have low bioavailability and to undergo considerable biotransformation (Fig. 3) (36,37). The low bioavailability of the tea polyphenols is likely due to their relatively high molecular weight and the large number of hydrogen-bond-donating hydroxyl groups (38). In addition, the efflux of these compounds by multidrug resistance-associated proteins (MRPs)-2 may play a vital role in limiting the bioavailability of tea catechins. Recently, Hong et al. found that EGCG and its methyl metabolites were substrates for MRP1 and MRP2 but not for P-glycoprotein (39). Vaidyanathan and Walle reported that ECG was also the substrates of MRP1 and MRP2 and that P-glycoprotein was partially responsible for ECG efflux (40). Correlating mechanistic data in vitro with effects in vivo should be done with careful consideration of the poor bioavailability of the tea polyphenols.

Enzymology of Biotransformation

Glucuronidation, sulfation, methylation, and ring-fission metabolism represent the major metabolic pathways for tea catechins (Fig. 3) (37). EGCG-4''-O-glucuronide is the major metabolite formed in human, mouse, and rat microsomes (41). Mouse small intestinal microsomes have the greatest catalytic efficiency (V_{max}/K_m) for glucuronidation followed in decreasing order by mouse liver, human liver, rat liver, and rat small intestine. Lu has shown that EGCG is time- and concentration-dependently sulfated by human, mouse, and rat liver cytosol (42). Methylated catechins including 3'- and 4'-O-methyl-EC, 4'-O-methyl EGC and 4''-O-methyl EGC and EGCG, and 4'- and 4''-di-O-methyl EGCG have been observed in vivo and in incubations with rat liver homogenates (43–45). Rat liver cytosol shows higher COMT activity toward EGCG and EGC than do human or mouse liver cytosol.

Following tea ingestion by human, EGCG is present mainly in the free form in the plasma (46). 4'-O-methyl EGC (mostly in the glucuronidated or sulfated form) is a major metabolite of EGC, reaching its peak level within the first 2 hours in human plasma at a concentration of four to six times higher than those of EGC [mainly as the glucuronidated form (57–71%) or sulfated form (23–36%) and with only a small amount present as the free form (3–13%)] (36,47). And the sulfated form of EC is more abundant (66%) than the glucuronidated form (33%) (47,48). Our recent results from data-dependent MS/MS analysis of mouse urine samples after administration of EGCG by IG or IP show that phenolic groups of methylated EGCG (or glucuronidated or sulfated EGCG) can be further methylated, glucuronidated, and/or sulfated to form multiple conjugated metabolites (Sang et al. 2010). We also identified a novel EGCG metabolite, 7-O- β -D-glucopyranosyl-EGCG-4''-O- β -D-glucopyranoside, indicating that glucosidation represents a novel pathway in the metabolism of EGCG in mice (49). At toxic doses of EGCG (200 mg/kg, IP), 2'-cysteinyl and 2''-cysteinyl EGCG were detected in the urine samples of mice (50).

In addition to these conjugation reactions, the tea catechins undergo metabolism in the gut to form

EGCG (16 μg) over 24 hours (45). Chow et al. compared the pharmacokinetic parameters of pure EGCG (200–800 mg, PO) and Polyphenon E (decaffeinated green tea catechin preparation containing 65% EGCG) (46). The authors found that the C_{max} of EGCG ranged from 73.7 to 438 ng/mL depending on the dose and was not significantly different when EGCG was given as a pure compound or in the mixture. Area under the curve (AUC) (22.5–161.4 min/ $\mu\text{g/mL}$), $t_{1/2}$ (118–113.5 minutes), and T_{max} (127–249 minutes) were also similar between pure EGCG and Polyphenon E. Interestingly, plasma levels of EGCG increased significantly when the dose increased from 400 to 600 mg. This may be due to a saturable first-pass elimination mechanism. In their recent study, they found that there was a greater than 3.5-fold increase in the average maximum plasma concentration of free EGCG and EGC when Polyphenon E was taken in the fasting condition than when taken with food (55). Taking Polyphenon E in the fasting condition did not have a significant effect on the plasma levels of total (free and conjugated) EGC but resulted in lower plasma levels of total EC. They also indicated that Polyphenon E up to a dose that contains 800 mg EGCG was well tolerated when taken under the fasting condition.

Although the tea polyphenols have a rather low systemic bioavailability, we have demonstrated that after holding green tea solution (7 mg/mL green tea solids in water) in the mouth without swallowing followed by extensive rinsing, the salivary concentrations of EGCG and EGC are 153 and 327 μM , respectively (56). These concentrations are 400–1000 times greater than those observed in plasma following ingestion of tea. Such locally high levels may support the use of green tea in the prevention of oral cancer and caries. More recently, Lee et al. have reported that holding green tea leaves for 2–5 minutes in the mouth resulted in high oral concentrations of catechins (131–2 μM) even after vigorously rinsing the mouth, suggesting that tea leaves are a low-cost product for sustained delivery of tea polyphenols in the oral cavity (57).

The pharmacokinetic parameters of tea catechins have been thoroughly determined in the rat by both IV and IG routes of administration. The kinetics of IV EGCG, EGC, and EC fit to a two-compartment model with elimination half-lives of 212, 45, and 41 minutes, respectively. The absolute bioavailability of EGCG, EGC, and EC following IG administration of decaffeinated green tea are 0.1%, 14%, and 31%, respectively (58). In another study, the EGCG levels in the tissues and blood correspond to 0.0003–0.45% of the ingested dose, further demonstrating the poor bioavailability of EGCG in the rat (59). Studies with bile duct-cannulated rats have shown that after oral administration of 100 mg EGCG, 3.28% of the dose is recovered in the bile as EGCG (2.65%) and methylated metabolites (0.63%) (44). With the exception of 4''-O-methyl EGCG and 4',4''-di-O-methyl EGCG, which were present as the sulfated form, the other metabolites and EGCG are present largely (>58%) as the glucuronidated form.

Treatment of rats with a green tea polyphenol preparation (0.6% wt/vol) in the drinking fluid has been shown to result in increasing plasma levels over a 14-day period with levels of EGC and EC being higher than those of

EGCG (60). Plasma levels then decrease over the subsequent 14 days suggesting an adaptive effect. EGCG levels have been found to be highest in the rat esophagus, intestine, and colon, which have direct contact with tea catechins, whereas EGCG levels are lower in the bladder, kidney, lung, and prostate, which depend on systemic bioavailable EGCG. When the same polyphenol preparation is given to mice, the EGCG levels in the plasma, lung, and liver are much higher than in rats (60). These levels appear to peak on day 4 and then decrease to less than 20% of the peak values in days 8–10.

In mice, the absolute bioavailability of EGCG has been found to be higher than in rats (26.5% vs. 0.1%) (61). This may be one of the reasons that inhibitory effects of green tea and EGCG on tumorigenesis in mice, but not in rats, have been consistently observed in different laboratories. Concentrations of EGCG in the small intestine and colon are 45 and 7.9 nmol/g following IG administration of 75 mg/kg EGCG (61). The levels in other tissues are less than 0.1 nmol/g. Following IV administration of EGCG, levels are highest in the liver (3.6 nmol/g), lung (2.7 nmol/g), and small intestine (2.4 nmol/g). Greater than 50% of plasma EGCG is present as the glucuronide, whereas EGCG is present mainly as the free form in the tissues (61).

The dose-dependent study of EGCG plasma and tissue levels in mice (50–2000 mg/kg, IG) indicated that there was a linear dose relationship in the plasma, prostate, and liver but not in the small intestine and colon, which showed a plateau between 500 and 2000 mg/kg, IG (62). These results may suggest that absorption of EGCG from the small intestine is largely via passive diffusion; however, at high concentrations, the small intestine and colonic tissues become saturated.

POTENTIAL HEPATOTOXICITY OF GREEN TEA POLYPHENOLS

There have been 34 case studies linking consumption of green tea-based supplements to hepatotoxicity (reviewed in Ref. 63). In most cases, elevations in serum transaminase levels, as well as increased serum bilirubin, were observed. Histological examination revealed inflammatory, cholestatic, or necrotic liver damage depending on the subject. In a subset of cases, additional liver damage following rechallenge with the same preparation was observed, suggesting a causal relationship between hepatotoxicity and green tea. Laboratory studies of green tea-derived preparations in rodents and dogs have generally supported the potential toxicity of those preparations at high doses (64,65). Oral administration of a green tea extract containing 91.8% EGCG for 13 weeks to Beagle dogs resulted in dose-dependent toxicity and death (65). Toxicity included vomiting, diarrhea, proximal tubule necrosis, and elevated serum bilirubin. Oral administration of this extract to rats resulted in lethality in 80% of animals treated (65). Histological analysis revealed hemorrhagic lesions in the stomach and intestine. Intraperitoneal administration of EGCG to CD-1 mice resulted in dose-dependent increases in alanine aminotransferase and lethality beginning at 0.33 mmol/kg (64). These findings suggest that caution should be exercised in the

use of green tea-based dietary supplements and that further studies are needed to determine the upper limit of safety for bolus dosing with tea polyphenols as well as the underlying mechanisms of toxicity.

CONCLUSIONS

Despite the demonstration of cancer prevention by tea in many animal studies, epidemiological trials have yielded mixed results concerning its effectiveness as a cancer chemopreventive agent in humans. This may be due to several factors: (i) the dose of the chemopreventive agent is generally higher in animal studies than is typically consumed by humans; (ii) the model of carcinogenesis, especially certain chemical carcinogens, may not be relevant to human carcinogenesis; (iii) interindividual variation in metabolism of tea constituents as well as other confounding factors may mask the effects of tea consumption on cancer. A clearer understanding of the bioavailability of tea polyphenols may resolve some of these confounding factors. These same limitations apply to the current knowledge of the beneficial effects of tea against other chronic diseases. Definitive conclusions on the effectiveness of tea as a preventive agent for chronic human disease will require well-designed intervention and prospective epidemiological studies.

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Hawthorn

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INTRODUCTION

Hawthorn is one of the most popular herbal medicines for the heart worldwide. In Europe, particularly in Germany, Austria, and Switzerland, hawthorn preparations are eligible for marketing authorization as drugs for the treatment of mild forms of heart insufficiency. In the United States, hawthorn products are regulated as dietary supplements. Numerous preparations are available, including teas, homeopathic preparations, and tinctures as well as simple and standardized extracts. Different starting materials are used. Extracts are prepared from leaves, flowers, or fruits. A substantial number of hawthorn products in the United States consist of comminuted hawthorn leaves, flowers, or fruits.

Over the last decades, the pharmacodynamic action of standardized extracts from leaves and flowers as well as different fractions thereof has been extensively evaluated in *in vitro* studies and animal experiments. Similarly, the clinical efficacy of hawthorn extracts has been assessed in more than a dozen double-blind, placebo-controlled clinical trials. The results of these studies are reviewed in this entry.

GENERAL DESCRIPTION

Plant Description

Hawthorn species (*Crataegus* L.; family Rosaceae) grow as shrubs or trees with hardwood and generally thorny twigs throughout the temperate zones of the world. Leaves are more or less lobed, with margins typically slightly serrated. Flowers are arranged in clusters and are mostly white and sometimes red. Small false fruits (berries) are formed and are red, black, or yellow and mealy.

Starting Material

The starting material consists of collected wild plant parts.

Leaves and Flowers

Hawthorn leaves and flowers consist of the whole or cut, dried, flower-bearing branches of *C. monogyna* Jacq. (Lindm.), *C. laevigata* (Poir.) DC. (*C. oxyacanthoides* Thuill.), or their hybrids or, more rarely, other European *Crataegus* species, including *C. pentagyna* Waldst. et Kit ex Willd., *C. nigra* Waldst. et Kit, and *C. azarolus* L. The preparation should contain not less than 1.5% flavonoids, calculated as hyperoside ($C_{21}H_{20}O_{12}$; M_r 464.4), with reference to the dried substance (European Pharmacopoeia, EP).

The United States Pharmacopoeia (USP) only recognizes the first two species. According to the USP, the prepa-

ration should contain not less than 0.6% C-glycosylated flavones, expressed as vitexin ($C_{21}H_{20}O_{12}$), and not less than 0.45% O-glycosylated flavones, expressed as hyperoside ($C_{21}H_{20}O_{12}$), calculated on a dry basis (United States Pharmacopoeia–National Formulary, USP-NF).

Fruits (Berries)

Hawthorn berries consist of the dried false fruits of *C. monogyna* Jacq. (Lindm.) or *C. laevigata* (Poir.) DC., or their hybrids, or a mixture of these (EP). They should contain not less than 1.0% procyanidins, calculated as cyanidin chloride ($C_{12}H_{11}ClO_6$; M_r 322.7) with reference to the dried product (EP).

Constituents of the Starting Material

Leaves and Flowers

The major constituents are flavonoids (up to 2%) such as vitexin-2''-O- α -L-rhamnoside, hyperoside, rutin, and vitexin as well as procyanidins formed by the condensation of catechin and/or epicatechin with varying degrees of polymerization (Fig. 1). The most important are oligomeric procyanidins (OPCs) containing 2–8 monomeric units, for example, the dimeric procyanidin B₂ (Fig. 2). The content of OPCs is approximately 3% (1). Further constituents are triterpenoid acids (approximately 0.6%), for example, ursolic, oleanolic, and crataegolic acid and phenol carboxylic acids such as chlorogenic and caffeic acid, as well as various amines (2).

Fruits (Berries)

The fruits contain relatively low levels of flavonoids. The procyanidins contained in the fruits reportedly have a higher degree of polymerization than those in the leaves and flowers. Total procyanidins amount up to 3% of which about 1.9% is OPCs. Triterpenoid acids are also present in the fruit (approximately 0.45%) (3).

Preparations

Hawthorn extracts from leaves, flowers, and fruits are characterized by different quantitative flavonoid patterns (4).

Leaves and Flowers

Extracts are produced from the herbal product by a suitable procedure using either water or a hydroalcoholic solvent equivalent in strength to a minimum of 45% ethanol. Aqueous extracts contain a minimum of 2.5% flavonoids and hydroalcoholic extracts contain a minimum of 6.0% flavonoids expressed as hyperoside (dried extracts) (EP).

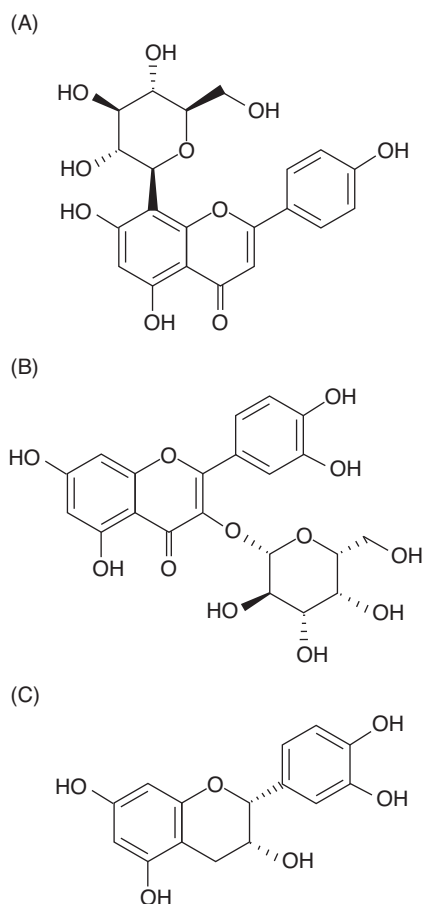


Figure 1 (A) Vitexin [β-D-glucopyranosyl-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one]; (B) hyperoside [2-(3,4-dihydroxyphenyl)-3-(β-D-galactopyranosyloxy)-5,7-dihydroxy-4H-1-benzopyran-4-one]; (C) L-epicatechin [(2R-cis)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol].

Standardized dry extracts are adjusted to 18.75% OPCs (WS[®] 1442; extraction solvent 45% ethanol) or 2.2% flavonoids (LI 132; extraction solvent 70% methanol), with a ratio of starting material to genuine extract (DER) of 4–7:1. The daily recommended dose is currently set at 160–900 mg in two or three divided doses (1,5).

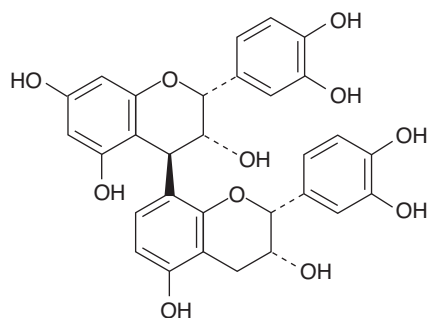


Figure 2 Procyanidin B₂.

Fruits (Berries)

Extracts are produced either from dried fruits, in compliance with the European Pharmacopoeia using alcohol (25–60%, vol/vol), or from fresh fruits. To date, no official monograph is available advocating the use of preparations from hawthorn fruits. Water extracts, water–alcohol extracts, wine infusions, and fresh juice from hawthorn fruits have been utilized traditionally to strengthen and invigorate heart and circulatory function (6).

ACTION AND PHARMACOLOGY

Pharmacological investigations with *Crataegus* preparations have been reported in a great number of publications. Unfortunately, many of these studies have been performed with insufficiently characterized extracts: Information on plant species, plant parts, and solvent and production conditions, for example, is not provided. Moreover, the applied pharmacological models and experimental details are frequently ill defined. Thus, formation of a clear judgment on the pharmacological activities of many of these products is not possible.

Almost all clinical studies reported until now have been performed with two different extracts, prepared from leaves and flowers of selected *Crataegus* species with either 70% methanol (LI 132) or 45% ethanol (WS 1442). The present entry will mainly review the pharmacological actions of these well-defined preparations.

Positive Inotropic Action

At concentrations between 30 and 180 μg/mL, LI 132 was found to raise the contraction amplitude of isolated cardiomyocytes of rats by up to 53% (7) and to improve oxygen utilization in comparison to β-adrenergic agonists or the cardiac glycoside ouabain. It is supposed that the inotropic action of LI 132 may be due to enhanced intracellular Ca²⁺ sensitivity. An increase of contraction amplitude was also observed in electrically stimulated canine papillary muscles (8).

In isolated, electrically stimulated left ventricular muscle strips of human failing myocardium, WS 1442 significantly augmented force of contraction by about 30% (50 μg/mL) and improved the frequency-dependent force generation (9). In normal human myocardial tissue, WS 1442 raised the Ca²⁺ gradient as well as the force generation and displaced bound ³H-ouabain from cell membranes. As the extract did not influence the activity of adenylate cyclase, the pharmacological mechanism of WS 1442 is suggested to be similar to the cAMP-independent positive inotropic action of cardiac glycosides. However, this conclusion is weakened by the fact that an extract fraction enriched for water-soluble, low-molecular-weight constituents displaced ³H-ouabain but did not elicit any inotropic effect.

Likewise, a significant dose-dependent effect of WS 1442 on the shortening of isolated and electrically stimulated myocytes isolated from right atria and left ventricles (LVs) of failing human hearts has been reported (10).

Using an isolated guinea pig heart preparation, two independent research groups (11,12) observed a maximal

increase of contraction force between about 10% and 20% at concentrations of 10 $\mu\text{g/mL}$ LI 132.

Increase of Coronary Flow and Vasorelaxing Effects

An increase of coronary flow has repeatedly been reported after perfusion of isolated hearts with medium-containing, ill-defined hawthorn extracts. These earlier observations were confirmed by a comprehensive study investigating the influence of LI 132 on different functional parameters in isolated guinea pig hearts. At a concentration of 3 $\mu\text{g/mL}$, LI 132 maximally enhanced coronary flow by 64%. A similar effect was brought about by amrinone and milrinone, while epinephrine had only a marginal effect, and digoxin concentration-dependently reduced coronary perfusion (11). Addition of the nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine (L-NNA) and the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) completely abolished the increase in coronary flow induced by WS 1442 in the isolated rat heart, while addition of indomethacin (a cyclooxygenase inhibitor) or aminoguanidine (a selective inhibitor of inducible NOS) had no effect on the coronary flow. Furthermore, in the presence and absence of L-NNA, WS 1442 significantly enhanced the relaxant effect of the NO donor nitroprusside. Thus, it has been concluded that *Crataegus* extracts increase the endothelial release of NO and may also inhibit NO metabolism possibly due to its antioxidative properties (13).

In a recent investigation, an endothelium-dependent vasorelaxing effect of WS 1442 was confirmed using rat aorta and human mammary artery. This effect was mainly mediated by the OPC fraction. It was found that WS 1442 induced an enhanced NO liberation from human coronary artery endothelial cells following activation of endothelial NO synthase (eNOS) by phosphorylation at serine 1177, while no eNOS translocation or phosphorylation at serine 114 or threonine 495 was observed (14).

Further studies on the molecular mechanism(s) involved in the vasorelaxing action of WS 1442 were performed by Anselm and coworkers (15). Vascular reactivity was assessed in porcine coronary artery rings, whereas reactive oxygen species (ROS) formation in artery sections was judged by microscopy, and phosphorylation of Akt and eNOS in endothelial cells was determined by Western blot analysis. The effect of hawthorn on endothelium-dependent relaxation was reduced by L-NNA and by charybdotoxin plus apamin (two inhibitors of endothelium-derived hyperpolarizing factor-mediated responses). Relaxation to WS 1442 was also inhibited by intracellular ROS scavengers and inhibitors of Src and PI3-kinase but not by an estrogen-receptor antagonist. Thus, the authors conclude that WS 1442 stimulates the endothelial formation of ROS in artery sections and subsequently induces endothelium-dependent, NO-mediated relaxations of coronary artery rings through the redox-sensitive Src/PI3-kinase/Akt-dependent phosphorylation of eNOS. However, based on these investigations, endothelium-derived hyperpolarizing factor, besides release of NO, also appears to contribute to the vasorelaxing activity of WS 1442.

Endothelium-dependent, NO-mediated relaxation has also been observed by an extract from hawthorn fruits

in rat mesenteric arteries (16) and a procyanidin-enriched fraction in the rat aorta (17). Therefore, relaxation of the noradrenalin-precontracted rat aorta has been proposed as a bioassay to investigate the pharmacological equivalence of different hawthorn extracts (18).

In unanesthetized dogs, the effect of oral (PO) treatment with WS 1442 on local blood flow in the myocardium of the LV was measured by means of chronically implanted heat-conduction probes. WS 1442 led to a dose-dependent temporary rise in blood flow, and repeated application caused a sustained increase of basal blood flow (19).

In a pilot study, the effect of LI 132 on the microcirculation in the mesenteric vessels of rats was compared with those of β -acetyldigoxin by intravital microscopy. Compared with digitalis, the *Crataegus* extract improved the erythrocyte flow rate in all investigated vessel types and reduced both leukocyte adhesion to the endothelium and leukocyte diapedesis (20).

Antiarrhythmic Effects

Evidence for an antiarrhythmic potential of LI 132 was provided by Poepping et al. (7), who observed a prolongation of the refractory period in isolated rat cardiac myocytes. Similarly, in isolated guinea pig hearts, an increase in left ventricular pressure and coronary flow was obtained, while at the same time the duration of the refractory period was prolonged (11). This combination of effects was unique among inotropic drugs, as epinephrine, amrinone, milrinone, and digoxin shortened the effective refractory period in a concentration-dependent manner.

Using cultured unpaced neonatal murine cardiomyocyte, several hawthorn preparations were found to have negative chronotropic effects (21). As compared with conventional cardiac drugs (i.e., epinephrine, milrinone, ouabain, or propranolol), hawthorn extracts had a unique activity profile. They appeared to be antiarrhythmic and capable of inducing rhythmicity in quiescent cardiomyocytes. Hawthorn extracts did not cause β -adrenergic-receptor blockade at concentrations that caused negative chronotropic effects. Commercial hawthorn preparations, extracts prepared from dried leaves and those made from dried berries, had similar chronotropic activities. When crude extracts are separated using size-exclusion chromatography, several fractions retain multiple-cardiac activities and revealed that multiple dissimilar cardioactive components may exist within the extract, making the identification of individual active constituents more challenging.

In guinea pig papillary muscles, LI 132 was observed to significantly increase action potential duration and time required for recovery of the maximum upstroke velocity of the action potential. These effects indicate class III and class I antiarrhythmic effects, respectively (12). Using patch-clamp techniques, the researchers obtained evidence that the prolongation of the action potential duration in isolated guinea pig ventricular myocytes is due to a weak blockade of both delayed and inward rectifier potassium currents (12). These investigators also attempted to get information on the mechanism responsible for the positive inotropic action of LI 132. As no influence on the L-type calcium current was detected, an inhibition of

phosphodiesterase or a β -sympathomimetic action, which had previously been proposed to account for the cardiogenic action of hawthorn extracts, can be excluded.

In vivo, antiarrhythmic effects of *Crataegus* extract WS 1442 were investigated in a rat model of ischemia/reperfusion-induced arrhythmia. Oral treatment for 7 days (100 mg/kg/day) effectively protected animals from reperfusion-induced arrhythmias, mortality, and hypotensive crisis following 7 minutes of occlusion of the left coronary artery. Treatment with the extract, however, did not modify the elevated plasma creatine kinase concentrations during reperfusion (22).

The influence of intake of a diet containing 2% LI 132 for 3 months on the incidence of reperfusion arrhythmias was also studied ex vivo in isolated rat hearts after global no-flow ischemia. Depending on the duration of ischemia, the average prevalence of malignant arrhythmias was significantly reduced by up to 83% in hearts of treated animals (23). However, in a recent publication, no protection against reperfusion-induced arrhythmias in isolated hearts was reported after 8 weeks' treatment (0.5 g/kg/day) of Wistar rats with LI 132 (24).

Cardioprotective Effects

Besides protecting against arrhythmias, hawthorn extracts have also been shown to prevent the leakage of intracellular enzymes upon ischemic injury. For this investigation, male Wistar rats were fed for 3 months with a diet containing 2% LI 132. As an index of myocardial cell damage, the concentration of lactate dehydrogenase (LDH) was determined in the perfusate of the isolated heart. LDH activity increased slightly during occlusion of the left coronary artery and was elevated dramatically after reperfusion. However, in treated animals, LDH release was suppressed significantly (control: 3795 ± 512 mU/min; LI 132: 1777 ± 452 mU/min) (25).

Fractionation of WS 1442 established that its cardioprotective effect is almost exclusively due to its standardized content of 18.75% OPC. A subfraction of WS 1442 enriched for OPCs was found to exert potent antioxidative action and to inhibit the enzymatic activity of neutrophil elastase (26). Since restoration of blood flow into a previously ischemic tissue is associated with the formation of oxygen free radicals as well as the accumulation and activation of leukocytes, it has been suggested that these activities may contribute to protection against reperfusion injury.

As ischemia lasting for more than 20–30 minutes causes irreversible tissue damage and cell death, Veveris et al. (2004) (27) evaluated whether treatment of rats with WS 1442 also improves cardiac function and prevents myocardial infarction during prolonged ischemia and reperfusion lasting for 240 and 15 minutes, respectively. Oral administration of WS 1442 (10 or 100 mg/kg/day) for 7 days before ligation of the left coronary artery dose-dependently suppressed the decrease of the pressure rate product. Treatment also attenuated the elevation of the ST segment in the ECG, diminished the incidence of ventricular fibrillations, and reduced the mortality rate. Furthermore, the area of myocardial infarction within the ischemic zone was significantly smaller in treated rats when compared with controls. It is suggested that these

pharmacological effects are accounted for by the combined antioxidative, leukocyte elastase-inhibiting, and endothelial NO synthesis-enhancing properties of WS 1442.

A protection against ischemia/reperfusion-induced brain damage was also described after administration of *Crataegus* flavonoids in a Mongolian gerbil stroke model (28). Likewise, an alcoholic extract of *Crataegus oxyantha* was shown to preserve mitochondrial function during isoproterenol-induced myocardial infarction in rats (29).

Cardiac hypertrophy (CH) is an adaptive enlargement of the myocardium in response to diverse pathophysiological stimuli such as hypertension, valvular disease, or myocardial infarction. Although this process is generally a beneficial response that temporarily augments cardiac output, sustained hypertrophy often becomes maladaptive and is a leading cause for the development of heart insufficiency. Activation of the protein phosphatase calcineurin (PP2B) is discussed as a major intracellular signaling pathway that contributes to the growth of cardiomyocytes. Using an in vitro test system, it was observed that WS 1442 inhibits the enzymatic activity of calcineurin. Thus, the effect of WS 1442 on the development of CH in animal models of hypertension was investigated. Hypertension and subsequent CH were induced in rats by aortic constriction (AC) or administration of deoxycorticosterone acetate (DOCA) in combination with NaCl/KCl-substituted drinking water, respectively. Animals were treated orally for a period of 14 (AC) or 28 days (DOCA-salt) with vehicle (0.2% agar suspension) or WS 1442 (100 and 300 mg/kg/day). In both experimental models, a marked increase in blood pressure (BP) and enlargement of the heart and the LV were observed. Treatment with WS 1442 dose-dependently lowered the pathologically increased BP but had no effect on the BP in normal control animals. In parallel with the reduction in the BP, development of CH was inhibited. This study demonstrates that oral treatment of rats with WS 1442 prevents development of CH induced by primary or secondary hypertension and thus supports its therapeutic use in the treatment of mild forms of heart failure (30).

The effects of WS 1442 treatment on remodeling and function of the LV was observed after 1 month of pressure overload-induced CH in male Sprague–Dawley rats (31). Animals were subjected to sham operation or AC for 4 weeks and treated orally with WS 1442 (1.3, 13, and 130 mg/kg/day) for 3 weeks after surgery. AC increased the LV/body weight ratio by 34% in vehicle- and WS 1442-treated rats, but WS 1442 markedly reduced LV chamber volumes and augmented relative wall thickness. In addition, WS 1442 attenuated the AC-induced reduction of velocity of circumferential shortening. The authors conclude that WS 1442 treatment modifies left ventricular remodeling and counteracts myocardial dysfunction in early pressure overload-induced CH.

In a subsequent study, the effect of WS 1442 (1.3, 13, or 130 mg/kg, PO) on left ventricular remodeling and function in pressure overload-induced heart failure was observed over a period of up to 5 months (32). AC increased the LV/body weight ratio by 53% in vehicle-treated rats, and administration of WS 1442 did not significantly affect this ratio. LV volumes and dimensions at systole and diastole significantly increased 5 months after AC compared with baseline in rats given vehicle (>20%

increase) but not in those given WS 1442 at 130 mg/kg (<10% increase). The decrease of velocity of circumferential shortening was prevented in rats treated with the medium or high dose of the extract. In the AC-vehicle group, the induced increases in messenger RNA expression for atrial natriuretic factor (approximately 1000%) and fibronectin (approximately 80%) were significantly attenuated in animals given the high dose of WS 1442 by approximately 80% and 50%, respectively. Thus, treatment with WS 1442 exhibited modest beneficial effects on cardiac remodeling and function during long-term, pressure overload-induced heart failure in rats.

The mechanism(s) by which hawthorn extract treats heart failure is not yet known but may include, theoretically, immunological effects. Therefore, the effect of WS 1442 (1.3, 13, or 130 mg/kg, PO) on the immunomodulatory response in a pressure overload model of heart failure was investigated. Six months after surgical procedure, animals were sacrificed and plasma samples obtained for the measurement of the following immunomodulatory markers: interleukin (IL)-1, IL-2, IL-6, IL-10, and leptin. Hawthorn extract had no effect on the immunomodulatory markers measured in this study, although there appeared to be a trend suggesting suppression of IL-2 plasma concentrations. Thus, in this animal model of heart failure, WS 1442 had no significant effect on the immunomodulatory response, and other than immunological activities may better define hawthorn's effect in treating heart failure (33).

Other Pharmacological Activities

Koch and Chatterjee (34) investigated the effects of WS 1442 in a rat model of endotoxin shock, in which the observed cardiovascular pathologies are suggested to be mediated by an excessive formation of oxygen-derived free radicals and enhanced production of NO by inducible NO synthase. Oral treatment (100 mg/kg) 1 hour before injection of endotoxin significantly inhibited the endotoxin-induced deterioration of cardiac output and prevented an increase in peripheral resistance. As WS 1442 had no effect on heart rate and mean arterial BP, the increased cardiac output in treated animals appears to be due to improved ventricular diastolic filling and/or enhanced myocardial contractility. The beneficial effects of WS 1442 in endotoxin shock may also be related to its positive effects on endothelial NO synthesis and/or its antioxidative properties.

Platelet-derived growth factor (PDGF) has been reported to play an important role in the pathogenesis of atherosclerosis as well as restenosis after angioplasty. Because polyphenols have been reported to inhibit tyrosine phosphorylation of the PDGF receptor beta (PDGFR- β) and *Crataegus* extracts are rich in these constituents, the effect of WS 1442 on the PDGF signal transduction pathway and neointimal formation was investigated in a rat balloon angioplasty model. WS 1442 concentration-dependently inhibited phosphorylation of the human PDGFR- β (IC₅₀: 1.4 μ g/mL). In PDGF-stimulated NIH3T3 fibroblasts, auto phosphorylation of PDGFR- β and DNA synthesis were suppressed half-maximally at concentrations of 31 and 3.1 μ g/mL, respectively. Oral treatment (300 mg/kg) of rats from day 2 before to day 13 after carotid artery balloon angioplasty significantly reduced neointimal

formation and increased the luminal area in parallel. These results provide evidence that WS 1442 may also have therapeutic potential for the prevention of restenosis in humans (35).

Hypocholesterolemic activity of different hawthorn extracts has been repeatedly reported. In one study, rats fed with an atherogenic diet were treated for 6 weeks with a tincture prepared from the fruits of *C. oxyacantha*. In the treated animals, the total as well as the low-density, very low-density, and high-density lipoprotein (LDL, VLDL, and HDL) cholesterol concentrations were significantly reduced. Detailed investigations revealed that application of the tincture enhanced cholesterol uptake into the liver by an elevated expression of LDL receptors. However, accumulation of cholesterol in the liver was prevented by suppression of cholesterol biosynthesis as well as increased cholesterol degradation (36). The same authors reported that under identical experimental conditions, the tincture reduced lipid peroxidation and precluded atherosclerotic changes in the aorta of treated rats. In addition, decrease of the glutathione and α -tocopherol content of the liver, aorta, and heart was inhibited (37). The total serum cholesterol and triacylglycerol concentrations were also decreased in hamsters that were fed for 4 weeks with a hypercholesterolemic diet containing an ethanolic extract from the fruits of *C. pinnatifida* (0.5%). Treatment with the extract led to greater excretion of both neutral and acidic sterols. Enzymatic tests indicated that the hypocholesterolemic activity may be mediated by upregulation of hepatic cholesterol-7 α -hydroxylase and downregulation of intestinal acyl-CoA:cholesterol acyltransferase (ACAT) (38).

In another study, it was found that hawthorn extracts inhibited ACAT activity in Caco-2 cells. The inhibitory activity was positively associated with the content of triterpenic acids (i.e., oleanolic acid and ursolic acid) in the extracts. Cholesterol-lowering action of hawthorn and its potential additive effect in combination with plant sterol esters were further studied in hamsters. The effects of these ingredients were conversely associated with their capacity to increasing fecal neutral sterol excretion. Oleanolic acid and ursolic acid were responsible for the cholesterol-lowering effect of hawthorn by inhibiting intestinal ACAT activity. In addition, hawthorn and both of these bioactive compounds (oleanolic acid and ursolic acid) enhanced the cholesterol-lowering effect of plant sterols (39).

The influence of WS 1442 on the cellular secretion of apolipoprotein B-100 (ApoB) and on the transcription of LDL-R in vitro using human HepG2-cells was investigated. Both parameters represent important elements in the regulation of serum cholesterol levels, as two-third of human serum cholesterol is transported in the form of LDL particles that contain ApoB as structure protein. ApoB is not only essential for the buildup of LDL particles but also represents a ligand for LDL-R in nearly all body cells that bind ApoB with high affinity and eliminate serum LDL through a receptor-mediated endocytosis. The incubation of HepG2-cells in the presence of WS 1442 (10–100 μ g/mL) led to a concentration-dependent inhibition of ApoB secretion (IC₅₀: 23 μ g/mL) and an increase in the transcription of LDL-R up to 5.6-fold compared with baseline. Applying the fractions of the total WS 1442 extract

revealed that the observed effect on LDL-R expression was exclusively mediated by an OPC-rich fraction, while other constituents of WS 1442 contribute to the inhibition of ApoB secretion (40).

Other pharmacological effects reported for different hawthorn extracts include stimulation of superoxide dismutase activity in erythrocytes of treated mice (41), inhibition of thromboxane A₂ synthesis (42), stimulation of prostaglandin I₂ production (42), and inhibition of angiotensin-converting enzyme (ACE) (43). Furthermore, hypoglycemic effects (44), inhibition of human isolated neutrophil functions (45), as well as protection of human blood lymphocytes against genotoxicity induced by gamma irradiation (46) have been described.

Pharmacokinetics

The absorption and distribution of ¹⁴C-labeled catechins, trimeric and higher polymeric procyanidins, and OPC total fraction have been determined in mice after oral administration. Total radioactivity was measured in blood and different organs without determination of individual metabolites. As early as 1 hour after oral administration, absorption of radioactivity could be detected for all labeled substances. The absorption rate for the OPC total fraction was about 31% and those for individual substances ranged from 16% to 40%. The accumulation of radioactivity was higher after repeated oral administration than after a single dose (47).

A liquid chromatography method coupled with tandem mass spectrometry for determination and in vivo pharmacokinetic studies of vitexin rhamnoside, a constituent of hawthorn extracts, has been reported. The method was applied to assess pharmacokinetics and bioavailability of vitexin rhamnoside after intravenous and oral administration to rats. The oral bioavailability of vitexin rhamnoside was only 3.57%, which indicated that vitexin rhamnoside has poor absorption or underwent extensive first-pass metabolism (48).

TOXICOLOGY

Doses of up to 3000 mg/kg of WS 1442 were given to rats and mice by the oral route without any sign of toxicity. Following intraperitoneal injection, LD₅₀ values of 1170 and 750 mg/kg were calculated in mice and rats, respectively. No abnormalities in the general state of health as well as clinical, chemical, hematological, gross morphological, and histological findings were observed after oral treatment of rats and dogs at doses of 30, 90, or 300 mg/kg/day for 26 weeks. Similarly, in a battery of genotoxicity assays, no evidence of mutagenic or clastogenic action was obtained (49). While reports on carcinogenicity studies with *Crataegus* extracts are not available, animal as well as clinical and postmarketing surveillance studies do not indicate any carcinogenic potential.

Oral application of WS 1442 at doses up to 1600 mg/kg body weight to rats and rabbits did not induce teratogenic effects. Furthermore, this extract affected neither the peri- and postnatal development nor the fertility of treated male and female rats and their F1 descendants (Schlegelmilch, oral communication, 1996).

To determine the safety of hawthorn to the developing fetus, pregnant rats were dosed daily by gavage using 56 times the human dose of hawthorn on either gestation days 1–8 or 8–15. On gestation day 20, fetuses were weighed and examined for signs of external, internal, or skeletal malformations. Rat fetuses were also explanted on gestation day 10.5 and cultured with hawthorn extract for 26 hours. No adverse effect on embryonic development in vivo or in vitro was observed (50).

INDICATIONS AND USAGE

Hawthorn extracts from leaves and flowers are recommended as an oral treatment option for chronic heart failure (CHF) (51), for example, declining cardiac performance corresponding to functional stage II of the New York Heart Association (NYHA) (1,5,6,52). Stage II NYHA heart failure is characterized by freedom from symptoms at rest and a slight limitation of physical activity; ordinary physical activity results in fatigue, palpitations, dyspnea, or anginal pain. The majority of clinical studies have been performed with standardized hydroalcoholic extracts of hawthorn leaves and flowers (extract designations: WS 1442, LI 132).

Table 1 provides an overview of 17 clinical trials on the efficacy of *Crataegus* extracts with a total of 4880 patients, of whom 1791 were suffering from CHF NYHA II, 2801 from CHF NYHA II/II, and 209 from CHF NYHA III. One study was performed with 79 diabetes type 2 patients. Duration of treatment lasted between 4 weeks and 24 months. In CHF NYHA II and NYHA II/III studies performed with the standardized extract of leaves and flowers (WS 1442, LI 132), the daily doses ranged from 160 to 900 mg extract. In the CHF NYHA III study, daily doses of 900 and 1800 mg WS 1442 were used.

In mainly placebo-controlled, double-blind studies, a statistically significant reduction in subjective discomfort and an improvement in cardiac performance due to an increase in left ventricular ejection fraction (LVEF), more efficient cardiac work (reduction in pressure-rate product), and an increase in physical stress tolerance (increase in exercise tolerance, elevation of anaerobic threshold) have been demonstrated in CHF NYHA II patients treated with hawthorn extracts (51,52).

In a placebo-controlled, double-blind study of 40 patients with CHF NYHA II resulting from coronary heart disease (CHD), an increase in LVEF of approximately 1.5%, measured during bicycle ergometric loading, was found with WS 1442 (daily dose: 480 mg PO; 4 weeks), whereas the LVEF in the placebo group dropped by around 0.2% ($P = 0.0002$). At rest, the LVEF increased by about 2.5% with WS 1442 and decreased by about 0.3% with placebo ($P = 0.0001$) (58). In another placebo-controlled, double-blind study of 136 patients with CHF NYHA II, WS 1442 (daily dose: 160 mg; 8 weeks) led to a reduction in the pressure-rate products (difference 50 W load vs. resting) of approximately 6.2, whereas no effect was observed with placebo ($P = 0.018$) (64).

In a double-blind, comparative study of 132 patients with CHF NYHA II, LI 132 (daily dose: 900 mg; 7 weeks) was shown to be as effective as the angiotensin-I-converting enzyme inhibitor captopril (daily dose:

Table 1 Clinical Studies with Hawthorn Extracts

Author(s) (Ref.); type of study	No. of patients/indication	Daily oral dose and preparation (duration of therapy)	Efficacy
<i>Standardized hydroalcoholic extracts from leaves and flowers^a</i>			
Zick et al. (53); double-blind, placebo-controlled study (as add-on to standard therapy defined as ACE inhibitor, AT-receptor antagonist, β -blocker, or diuretics)	120 / NYHA II and III with LVEF \leq 40%	900 WS 1442 (6 mo)	<i>Change in 6-min walk distance:</i> WS 1442: 14 ± 62 m Placebo: 5 ± 32 m; $P = 0.61$ <i>Change of LVEF in %:</i> WS 1442: change from 37 ± 15 to 37 ± 16 Placebo: change from 35 ± 14 to 33 ± 13 , $P = 0.04$
Holubarsch et al. (54); double-blind, placebo-controlled study (as add-on therapy to preexisting treatment of CHF)	2681/NYHA II and III with LVEF \leq 35%	900 WS 1442 (24 mo)	<i>Time to first cardiac event:</i> WS 1442: average time 620 days, event rates: 27.9% Placebo: average time 606 days, event rates 28.9%, $P = 0.476$ <i>Reduction of cardiac mortality at month 24:</i> WS 1442: 9.7%, $P = 0.269$ <i>Reduction of cardiac mortality at month 18</i> WS 1442: 20.3%, $P = 0.046$ <i>Reduction of cardiac mortality at month 6:</i> WS 1442: 40.7%, $P = 0.009$ <i>Reduction of sudden cardiac death at month 24 in the subgroup with LVEF \geq 25%:</i> WS: 39.7%, $P = 0.025$
Walker et al. (55); double-blind, placebo-controlled study	79/Type 2 diabetes	1200 mg, 16 wk	<i>Diastolic blood pressure:</i> LI 132 showed greater reduction [baseline: 85.6 mm Hg, 95% confidence interval (CI) = 83.3–87.8; outcome: 83.0 mm Hg, 95% CI = 80.5–85.7) than the placebo group (baseline: 84.5 mm Hg, 95% CI = 82–87; outcome: 85.0 mm Hg, 95% CI = 82.2–87.8) ($P = 0.035$) <i>Systolic blood pressure:</i> No group difference; reduction from baseline (3.6 and 0.8 mm Hg for LI 132 and placebo, respectively; $P = 0.329$)
Koller et al. (56); open, two-armed, cohort study	711/NYHA II due to CHD	900 mg WS 1442 (cohort 1), 6 mo additional to conventional treatment Conventional treatment with chemical substances (cohort 2), 6 mo	<i>Quality of life:</i> Significant improvement in the WS 1442 cohort in comparison with the cohort 2 <i>Direct medical costs:</i> WS 1442: €469 \pm €655 Cohort 2: €1086 \pm €4848 <i>Hospitalization costs due to heart failure:</i> WS 1442: €49 \pm €615 Cohort 2: €703 \pm €4821, $P = 0.043$
Tauchert (57); double-blind, placebo-controlled study (add-on therapy to preexisting diuretic treatment)	209/NYHA III	<ul style="list-style-type: none"> 1800 mg WS 1442 + 50 mg triamterene/25 mg hydrochlorothiazide 900 mg WS 1442 + 50 mg triamterene/25 mg hydrochlorothiazide Placebo + 50 mg triamterene/25 mg hydrochlorothiazide (16 wk) 	<i>Maximal tolerated workload:</i> WS 1442 1800 mg: increase ($P = 0.013$ vs. placebo; $P = 0.01$ vs. 900 mg) <i>Symptoms of heart failure and complaints reported by patients:</i> WS 1442 1800 mg: decrease in typical symptoms of heart failure ($P = 0.004$ vs. placebo) and in complaints ($P = 0.03$ vs. placebo) WS 1442 900 mg: decrease in typical symptoms of heart failure ($P = 0.004$ vs. placebo) and in complaints ($P = 0.03$ vs. placebo)
Eichstaedt et al. (58); double-blind, placebo-controlled study	40/NYHA II	480 mg WS 1442 (4 wk)	<i>Left ventricular ejection fraction</i> During exercise WS 1442: + 1.5%; placebo: - 0.2% ($P = 0.0002$) At rest: WS 1442: + 2.5%; placebo: - 0.3% ($P = 0.0001$)
Zapfe G (59); double-blind, placebo-controlled study	40/NYHA II	240 mg WS 1442 (12 wk)	<i>Exercise tolerance</i> WS 1442: + 10.8%; placebo: - 16.9% ($P = 0.06$) <i>Pressure-rate product</i> WS 1442: - 26.8%; placebo: - 2.7%
Boedigheimer K, Chase D (60); double-blind, placebo-controlled study	85/NYHA II	300 mg LI 132 (4 wk)	<i>Exercise tolerance</i> LI 132: + 13 W; placebo: + 3 W ($P = 0.143$) <i>Pressure-rate product, symptom score:</i> No statistically significant difference between therapy groups
Schmidt U, et al. (61); double-blind, placebo-controlled study	78/NYHA II	600 mg LI 132 (8 wk)	<i>Exercise tolerance</i> LI 132: + 28 W; placebo: + 5 W ($P < 0.001$) <i>Pressure-rate product</i> Significant group difference in favor of LI 132 ($P < 0.05$) <i>Symptom score</i> Significant group difference in favor of LI 132 ($P < 0.001$)

Table 1 Clinical Studies with Hawthorn Extracts (Continued)

Author(s) (Ref.); type of study	No. of patients/indication	Daily oral dose and preparation (duration of therapy)	Efficacy
Foerster A, et al. (62); double-blind, placebo-controlled study	72/NYHA II	900 mg LI 132 (8 wk)	<i>Ergospirometry</i> LI 132: Improved O ₂ uptake ($P < 0.05$), and the anaerobic threshold was reached later ($P < 0.05$) <i>Symptom score</i> LI 132: improvement in 86% of patients; placebo: improvement in 47% of patients ($P < 0.01$)
Tauchert et al. (63); double-blind study, comparison of hawthorn and captopril	132/NYHA II	900 mg LI 132; 37.5 mg captopril (7 wk)	<i>Exercise tolerance</i> LI 132: Increase from 83 to 97 W; captopril: increase from 83 to 99 W. No difference between therapy groups <i>Pressure-rate product</i> Downwards trend in both therapy groups <i>Symptom score</i> Reduction in frequency and severity in both therapy groups by approximately 50%
Weigl et al. (64); double-blind, placebo-controlled study	136/NYHA II	160 mg WS 1442 (8 wk)	<i>Pressure-rate product</i> WS 1442: – 6.2; placebo: + 0.1 ($P = 0.018$) <i>Main symptoms</i> WS 1442: improvement in 59% of patients; placebo: improvement in 44% of patients ($P = 0.05$)
Leuchtgens H (65); double-blind, placebo-controlled study	30/NYHA II	160 mg WS 1442 (8 wk)	<i>Pressure-rate product</i> WS 1442: – 11.6; placebo: – 4.9 ($P < 0.05$) <i>Complaints</i> WS 1442: – 16.5; placebo: – 4 ($P < 0.05$)
Weigl A, Noh HS (66); open	7/NYHA III	240 mg WS 1442 (4 wk)	<i>Left ventricular ejection fraction</i> Increase from 29.8% to 40.4%
Eichstaedt H, et al. (67); open	20/NYHA II	480 mg WS 1442 (4 wk)	<i>Left ventricular ejection fraction</i> During exercise: + 5.05% At rest: + 3.32% <i>Exercise tolerance</i> : + 10% <i>Subjective condition</i> Improvement in 65% of patients according to the patients themselves and in 75% according to the doctor
<i>Extracts from fresh berries</i>			
Degenring FH, et al. (68); double-blind, placebo-controlled	143/NYHA II	3 × 30 drops ethanolic (49% vol/vol) extract of fresh berries (drug-extract ratio 1:3.2) (8 wk)	<i>Exercise tolerance</i> Difference between the treatment groups: 8.3 W in favor of the extract ($P = 0.045$) <i>Pressure-rate product, cardiac symptoms</i> No statistically significant difference between the treatment groups
Rietbrock N, et al. (69); double-blind, placebo-controlled	88/NYHA II	3 × 25 drops ethanolic (60% vol/vol) extract of fresh berries (drug-extract ratio 1:1.3–1.5) (12 wk)	<i>Total exercise time</i> Extract: increase of 38.9 sec vs. placebo <i>Minnesota Questionnaire total score</i> : Extract: decrease from 44.1 to 30.6 (31%); placebo: decrease from 42.4 to 34.6 (18%) <i>Dyspnea–Fatigue Index total score</i> : Extract: increase from 8.37 to 9.41 (12%); placebo: increase from 8.26 to 8.92 (8%) <i>Dyspnea (visual analog scale)</i> Extract: decrease from 56.6 to 50.5 mm (11%); placebo: decrease from 57.3 to 54.8 mm (4%)

^aWS 1442: standardized to contain 18.75% oligomeric procyanidins; LI 132: standardized to contain 2.2% flavonoids.

37.5 mg; 7 weeks). The work tolerance determined during bicycle exercise increased in the *Crataegus* group from 83 to 97 W, whereas in the captopril group, it increased from 83 to 99 W; in both groups, the frequency and severity of the symptoms decreased by about 50% (63).

A recent placebo-controlled, double-blind study of 209 patients with CHF NYHA III investigated the efficacy of WS 1442 (daily dose: 1800 or 900 mg; 16 weeks) as an add-on therapy to the basic treatment with a diuretic (daily dose: 50 mg triamterene/25 mg hydrochlorothiazide). After therapy with 1800 mg WS 1442, the maximal tolerated workload during bicycle exercise showed a statistically significant increase in comparison with both placebo ($P = 0.013$) and 900 mg WS 1442 ($P = 0.01$). Typical heart failure symptoms as rated by the patients were reduced to a greater extent by WS 1442 than by placebo (1800 mg: $P = 0.004$; 900 mg: $P = 0.04$) (57).

In a 6-month prospective, open, two-armed, multicenter cohort study of 711 patients with CHF NYHA II due to CHD, the influence of the treatment with WS 1442 as add-on treatment or monotherapy (cohort 1) on quality of life and medical costs in comparison with conventional treatment with only chemical substances (cohort 2) was investigated. The results of 158 matched pairs revealed that the treatment with WS 1442 in cohort 1 significantly improved the quality of life and decreased the severity of disease-related symptoms in comparison with cohort 2. The calculated mean hospitalization costs due to heart failure were significantly lower in cohort 1 than in cohort 2 (WS 1442: €49, cohort 2: €703; $P = 0.043$). The mean direct medical costs (ambulant treatment, prescribed drugs, hospitalization) were €469 and €1086 for cohort 1 treated with WS 1442 and for cohort 2, respectively (56).

In a controlled trial, the effect of LI 132 on hypertension in patients with type 2 diabetes taking prescribed drugs was performed in general practices in Reading, United Kingdom (55). Patients ($n = 79$) were randomized to daily 1200 mg hawthorn extract ($n = 39$) or placebo ($n = 40$) for 16 weeks. At baseline and outcome, a well-being questionnaire was completed and BP and fasting blood samples taken. There was a significant group difference in mean diastolic BP reductions ($P = 0.035$): The hawthorn group showed greater reductions [baseline: 85.6 mm Hg, 95% confidence interval (CI) = 83.3–87.8; outcome: 83.0 mm Hg, 95% CI = 80.5–85.7] than the placebo group (baseline: 84.5 mm Hg, 95% CI = 82–87; outcome: 85.0 mm Hg, 95% CI = 82.2–87.8). Systolic BP reduction from baseline (3.6 and 0.8 mm Hg for hawthorn and placebo groups, respectively; $P = 0.329$) was similar in both groups. No herb–drug interaction was found, and minor health complaints were reduced from baseline in both groups. This is the first randomized controlled trial to demonstrate a hypotensive effect of hawthorn in patients with diabetes taking medication.

In a randomized, double-blind, placebo-controlled, multicenter clinical study (SPICE trial), the efficacy and safety of *Crataegus* extract WS 1442 as an add-on treatment were investigated in adults suffering from congestive heart failure (NYHA II or III) with impaired LVEF ($\leq 35\%$). In this study, 2681 patients were included and randomized to additional treatment with WS 1442 (daily dose: 900 mg) or placebo for 24 months. The primary endpoint was time to first cardiac event (death of cardiac cause such as sudden cardiac death, death due to progressive heart failure, fatal myocardial infarction as well as nonfatal myocardial infarction, hospitalization due to progression of heart failure). In the subgroup with LVEF $\geq 25\%$, WS 1442 significantly reduced sudden cardiac death (39.7% at month 24, $P = 0.025$), whereas the trend for the combined endpoint did not reach statistical significance. Most patients in this study were already treated with three or more concomitant drugs according to current treatment guidelines (especially ACE inhibitors, AT-II antagonists, β -blockers, diuretics, spironolactone, digitalis) and may not have gained an additional benefit from WS 1442 taken on top of optimal pharmacological therapy due to a severely reduced overall health status. Nevertheless, cardiac mortality was significantly reduced in the total population after 6 ($P = 0.009$) and 18 months ($P = 0.046$) (54).

A randomized, double-blind, placebo-controlled trial in 120 ambulatory patients with NYHA class II–III CHF was conducted in 2004 (53). All patients received standard medical therapy, defined as ACE inhibitors or AT-receptor antagonists, β -blockers, and diuretics, as tolerated, and were randomized to receive additionally either WS 1442 (450 mg twice daily) or placebo for 6 months. There were no significant differences between groups in change in 6-minute walk distance at 6 months ($P = 0.61$), whereas for the LVEF, a significant difference ($P = 0.04$) in favor of WS 1442 was found. It should be noted that the drug used in this study (*Crataegutt*[®] novo, 450 mg) is approved in a number of European countries for the treatment of impaired performance of the heart corresponding to NYHA II. Moreover, despite of the entry criteria of an LVEF $\leq 40\%$, 38 patients were included in this study with

an LVEF of more than 40% at baseline. Furthermore, given the modest sample size of the study, the possibility of a type II error must be considered. In addition, this trial had not been powered to investigate an effect on hospitalization or mortality.

A Cochrane review on the use of hawthorn extracts for treating CHF was published in 2008 (51). The benefits and harms as reported in double-blind, randomized clinical trials of hawthorn extract compared with placebo for treating patients with CHF were evaluated. To be included, studies were required to state that they were randomized, double blind, and placebo controlled and used hawthorn leaf and flower extract monopreparations. Fourteen trials met all inclusion criteria and were included in this review. In most of the studies, hawthorn was used as an adjunct to conventional treatment. Ten trials including 855 patients with CHF (NYHA classes I–III) provided data that were suitable for meta-analysis. For the physiologic outcome of maximal workload, treatment with hawthorn extract was more beneficial than placebo [weighted mean difference (WMD) (Watt): 5.35, 95% CI: 0.71–10.00, $P < 0.02$, $n = 380$]. Exercise tolerance was significantly increased by hawthorn extract [WMD (Watt \times minute): 122.76, 95% CI: 32.74–212.78, $n = 98$]. The pressure–heart rate product, an index of cardiac oxygen consumption, also showed a beneficial decrease with hawthorn treatment [WMD (mm Hg/min): -19.22 , 95% CI: -30.46 to -7.98 , $n = 264$]. Symptoms such as shortness of breath and fatigue improved significantly with hawthorn treatment as compared with placebo (WMD: -5.47 , 95% CI: -8.68 to -2.26 , $n = 239$). No data on relevant mortality and morbidity such as cardiac events were reported at that time apart from one trial, which reported deaths (three in active, one in control) without providing further details. Reported adverse events were infrequent, mild, and transient; they included nausea, dizziness, and cardiac and gastrointestinal complaints. The authors conclude that these results suggest that there is a significant benefit in symptom control and physiologic outcomes from hawthorn extract as an adjunctive treatment for CHF.

CONTRAINDICATIONS

Contraindications for the use of hawthorn extracts have not been reported (1,5,6).

PRECAUTIONS AND ADVERSE REACTIONS

Precautions

A physician must be consulted in cases where symptoms continue unchanged for longer than 6 weeks or when fluid accumulates in the legs. Medical intervention is absolutely necessary when pain occurs in the region of the heart, spreading out to the arms, upper abdomen, or the area around the neck, or in cases of dyspnea (1,5,6).

Adverse Effects

The safety data of all available human studies on hawthorn monopreparations were evaluated in a very comprehensive review in 2006 (70). Systematic searches were conducted on MEDLINE, EMBASE, AMED, The

Cochrane Library, the U.K. National Research Register, and the U.S. ClinicalTrials.gov (up to January 2005). Data were requested from the spontaneous reporting scheme of the WHO, and hand searches were also conducted in a sample of relevant medical journals, conference proceedings, reference lists of identified articles, and own files of the authors. Eight manufacturers of hawthorn-containing preparations were contacted and asked to supply any information on adverse events or drug interactions. Twenty-nine clinical studies were identified, of which 24 met the inclusion criteria. A total of 7311 patients were enrolled, and data from 5577 patients were available for analysis. The daily dose and duration of treatment with hawthorn monopreparations ranged from 160 to 1800 mg and from 3 to 24 weeks, respectively. The extracts most used in the clinical trials were WS 1442 and LI 132. Overall, 166 adverse events were reported. Most of these events were, in general, mild to moderate; in eight cases, the adverse events were considered severe (palpitation, $n = 1$; gastrointestinal complaints, $n = 3$; vertigo, $n = 2$; chest pain, $n = 1$; migraine, $n = 1$). All these eight cases derived from a single observational study with 3664 patients monitored by 940 medical practitioners to evaluate the efficacy and tolerability of high-dose LI 132 extract. During this study, 72 adverse events were reported in 48 patients. The causal relationship with hawthorn treatment could not be excluded in 26 cases.

The SPICE trial included 2681 patients with CHF NYHA II or III and LVEF $\leq 35\%$ and revealed an excellent safety and tolerability of WS 1442. In this trial, 1338 patients were randomized to WS 1442 (daily dose: 900 mg) or placebo (1343 patients) as an add-on therapy to preexisting treatment of the CHF. WS 1442 was safe to use in patients receiving optimal medication for heart failure. Frequency, type, and severity of adverse events were comparable in both groups (54).

In conclusion, none of the adverse events that could definitely be related to the use of hawthorn were considered serious, and their incidence did not seem to be correlated with the dosage (70). In fact, in the study by Tauchert (57), the occurrence of adverse events was even lower for the WS 1442 1800 mg/day group than for the WS 1442 900 mg/day group. The data indicated that hawthorn extracts are exceptionally well tolerated.

Drug Interactions

In a recent systematic review based on data from 24 controlled clinical studies with more than 5500 patients, Daniele et al. (70) did not find any evidence for drug interactions. Similarly, the absence of drug interactions was also confirmed in the SPICE study, which investigate the efficacy and safety of an add-on treatment with *Crataegus* extract WS 1442 in 2681 patients (WS 1442: 1338; placebo: 1343) with congestive heart failure (54). No reports on drug–drug or herb–drug interactions with *Crataegus* extracts could be found during a literature search.

A randomized, crossover trial with eight healthy volunteers was performed to evaluate the effect of WS 1442 on pharmacokinetic parameters of digoxin. Subjects were randomized into one of two groups: digoxin 0.25 mg/day alone for 10 days or digoxin 0.25 mg/day with 900 mg WS 1442/day for 21 days. There were no statistically significant differences in any measured pharmacoki-

Table 2 Regulatory Status of Hawthorn

Australia	Listed in Australian Register of Therapeutic Goods
Austria	Authorized as prescription (Rx) or over-the-counter (OTC) drug
Belgium	Authorized as OTC drug
Canada	Available without restriction; not suitable for self-medication of cardiac diseases
Denmark	Authorized as herbal medicinal product
France	Authorized as traditional herbal medicine
Germany	Authorized as OTC drug
Poland	Authorized as OTC drug
Switzerland	Authorized as OTC drug
United Kingdom	Not included in general sales list
United States	Regulated as dietary supplement

netic parameters. This suggests that WS 1442 and digoxin, in the doses and dosage forms studied, may be coadministered safely (72).

OVERDOSAGE

Symptoms of overdosage have not been reported.

CONCLUSIONS

Extracts from different parts of *Crataegus* species are used commonly throughout the world as herbal remedies for the treatment of mild forms of heart insufficiency. The majority of pharmacological, toxicological, and clinical studies have been performed with standardized hydroalcoholic extracts from leaves and flowers (WS 1442, LI 132). These extracts have been demonstrated to possess cardiogenic as well as cardioprotective activities. Clinical trials, many of them conducted in accordance with the highest standards of good clinical practice, have confirmed that these extracts can be used as an alternative in early CHF and as an adjunct in the therapy of later stages of CHF. The evidence for the efficacy and safety of other hawthorn preparations needs to be evaluated on a case-by-case basis. Promising results from pharmacological studies on vasoprotective effects of hawthorn extracts warrant further clinical trials in the prophylaxis and treatment of other cardiovascular diseases.

COMPENDIAL/REGULATORY STATUS

The regulatory and compendial status of hawthorn are summarized in Tables 2 and 3.

Table 3 Compendial Status of Hawthorn

European Pharmacopoeia	Hawthorn leaf and flower, 2003 Hawthorn leaf and flower dry extract, 2003 Hawthorn fruits, 2003
British Pharmacopoeia	Hawthorn fruits, 1998
U.S. Pharmacopoeia–National Formulary	Hawthorn leaf with flower, 2003 Powdered hawthorn leaf with flower, 2003
American Herbal Pharmacopoeia	Hawthorn leaf with flower, 1999
ESCOP monograph	Hawthorn leaf and flower, 1999
WHO monograph	Folium cum Flore Crataegi, 2001

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5-Hydroxytryptophan

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INTRODUCTION

5-Hydroxytryptophan (5-HTP) is the immediate precursor for the neurotransmitter, serotonin. 5-HTP is produced by extraction from the seeds of the African plant *Griffonia simplicifolia* and, in the United States, is commercially available for purchase, under the Dietary Supplement Health and Education Act of 1994. 5-HTP crosses the blood–brain barrier and readily converts to serotonin and, because of this, it is thought that 5-HTP may be taken orally to boost serotonin levels. Therefore, the precursor may be used to modulate physiological processes mediated by serotonin (Fig. 1). This chapter updates our earlier work, dating 2004, on 5-HTP; particular interest continues to be on studies performed on humans using 5-HTP for therapeutic purposes. Trials using animal models will be referenced when no available information was found in human studies.

This article provides an overview of the pharmacokinetics and metabolism of 5-HTP and highlights several clinical areas in which 5-HTP has been suggested to be at least partially therapeutic. First, we describe the utilization of 5-HTP for its purported anorectic effect and potential application in obese individuals. Second, we discuss studies on depression, from the early studies of the 1960s, which provided the first indications that 5-HTP could have antidepressant properties to a meta-analysis published in 2002. The use of combination/augmentation therapy (5-HTP with antidepressants) will also be addressed. Third, we examine the use of 5-HTP in several neurological disorders including different kinds of headaches, sleep disturbances, fibromyalgia, and its controversial use in cerebellar ataxias. Fourth, we study

the use of radiolabeled 5-HTP as an imaging modality in the diagnosis of neuroendocrine tumors. Fifth, we assess the side effect profile of 5-HTP, its frequency, severity, and duration, including the infrequent but potentially lethal condition, eosinophilia–myalgia syndrome. Finally, we provide a summary and recommendations regarding the use of 5-HTP.

PHARMACOKINETICS AND METABOLISM

Serotonin is a monoamine neurotransmitter produced endogenously from the essential amino acid L-tryptophan. Serotonin cannot cross the blood–brain barrier and thus neuronal serotonin levels are highly dependent on L-tryptophan, the immediate precursor for 5-HTP, and 5-HTP, the immediate precursor for 5-hydroxytryptamine (serotonin) synthesis (Fig. 2), both of which are capable of crossing the blood–brain barrier. The L-tryptophan uses a transporter to cross the blood–brain barrier to get intracellular access before it is metabolized by tryptophan hydroxylase-2 to produce central 5-HTP, a reaction that requires the cofactor L-erythro-tetrahydrobiopterin. The crossing of L-tryptophan and metabolism of 5-HTP are the rate-limiting steps in the production of neuronal serotonin. Importantly, the passage of L-tryptophan across the blood–brain barrier is a saturable process in which tryptophan competes with other amino acids, suggesting that elevated levels of the latter could inhibit/diminish tryptophan transport rates into the neuron. Conversely, 5-HTP does not require a transporter system and readily crosses the blood–brain barrier; thus, it may be taken as a dietary supplement to potentially boost 5-HTP levels in the central nervous system. Studies in rodents indicate that ~7% of ^{14}C -labeled 5-HTP from the arterial circulation is extracted by the brain (1).

The synthesis of 5-HTP is not limited to the brain. Indeed, 5-HTP is also synthesized outside the brain in significant amounts, via hydroxylation of tryptophan by tryptophan hydroxylase-1 (2). 5-HTP can then be converted to serotonin by the enzyme L-amino acid decarboxylase, present both in the periphery and the central nervous system (Fig. 2). It follows that ingested doses of 5-HTP may increase both peripheral and central serotonin levels. Studies have reported a dose-dependent serotonin release that could last for more than 2 hours following the administration of 5-HTP (3,4). Subsequent to oral intake of 5-HTP, the peak plasma concentration is observed at 2–3 hours and the bioavailability is ~50–70% (5,6). The

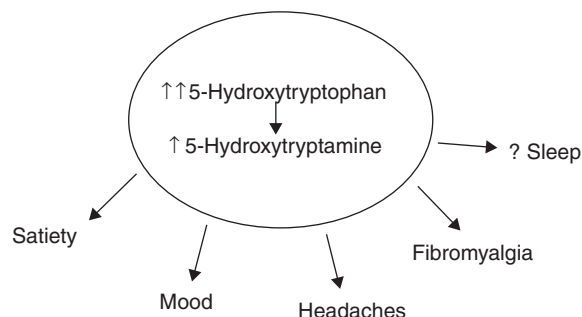


Figure 1 Possible therapeutic uses of 5-HTP in clinical medicine.

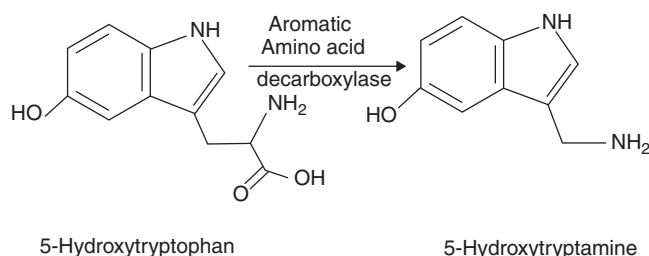


Figure 2 Chemical structure of 5-hydroxytryptophan and conversion to 5-hydroxytryptamine.

former may be substantially increased by pretreatment with carbidopa, a peripheral aromatic amino acid decarboxylase inhibitor. For example, a single dose of 5-HTP with carbidopa resulted in a 15-fold higher plasma peaks than did 100 mg without carbidopa (6–8), suggesting that if carbidopa is coadministered with 5-HTP, smaller doses of the later may be used. In addition, subject-to-subject variability in 5-HTP plasma half-life ranges from 2.5 to 7 hours after a single dose of 200 mg (6).

A recent double-blind, placebo-controlled, single-rising dose, four-way crossover trial by Smarius et al. examined the pharmacokinetics of 5-HTP in 15 healthy male volunteers ages 19–29 years. Study participants were given placebo, 100, 200, or 300 mg 5-HTP with carbidopa. The results indicated a clear dose response with 5-HTP $t_{1/2}$ of 3.0 hours and an oral clearance with an interindividual coefficient of variation of 31% (9). The model used by the authors did not allow for the trace of variability (absorption vs. elimination), but the values are virtually identical to those reported earlier at the 100-mg 5-HTP dose (8). Importantly, the pharmacokinetics and pharmacodynamics of ingested 5-HTP will be modulated by the aromatic L-amino acid decarboxylase gene expression and activity, as well as by the adequate amounts of pyridoxal phosphate, a key cofactor for the synthesis of serotonin. Clearly, future studies on pharmacogenomics will be valuable to better understand the wide range response to oral 5-HTP supplementation; however, this subject is beyond the scope of this work.

Little is known about intracerebral 5-HTP and the effects of carbidopa on brain concentration of 5-HTP in humans. The development of positron emission tomography (PET) and new tracers has emerged as the dominant methodology to assess neurotransmission in humans. In the early 1990s, Agren et al., at Uppsala University, Sweden, were the first to use PET and demonstrate ^{11}C -labeled 5-HTP uptake across the blood–brain barrier in eight healthy volunteers, with most of the tracer accumulating in the striatum and in the prefrontal cortex. The authors suggested that after intravenous injection, ^{11}C -5-HTP crosses through the blood–brain barrier by a simple diffusion mechanism prior to subsequent decarboxylation to ^{11}C -serotonin (10). In a subsequent study, the same investigators showed that administration of the peripheral L-aromatic amino acid decarboxylase inhibitor benzeride is an efficient method to raise levels of intracerebral 5-HTP uptake (11).

CLINICAL USE OF 5-HTP

Obesity

It has long been known that brain serotonin systems contribute to the modulation of food intake and satiety (12) and that an increase of intrasynaptic serotonin tends to reduce food consumption. One might consider that individuals taking 5-HTP will experience increased satiety and weight loss over a period of time. The few studies on the effects of 5-HTP on obesity all suggest an anorectic effect of 5-HTP (13–16).

A 5-week, double-blind, crossover study, without any dietary modification, found that oral administration of 300 mg of 5-HTP before meals (i.e., 900 mg/day) led to a mild decrease of food intake and weight loss in obese subjects (13). A second study from the same group followed obese patients for 6 weeks while treated with placebo or the same 5-HTP dose with no dietary restriction, followed by an additional 6 weeks in which the patients continued placebo or 5-HTP treatment plus dietary restriction diet of 1200 calories. The cohort receiving 5-HTP during the first 6 weeks experienced a small but significant weight loss compared with the group receiving placebo. In addition, during the second 6 weeks, the patients taking 5-HTP experienced further weight loss compared with the first 6 weeks and the placebo group plus dietary restriction. Food records indicated that subjects on 5-HTP reduced their carbohydrate intake by about 50% by the end of the first 6 weeks, and that 100% and 90% of patients receiving 5-HTP during the first 6 weeks and the second 6 weeks, respectively, reported early satiety. Nausea was reported by 80% of the treatment group during the first 6 weeks and 20% during the second 6 weeks. The authors argued that the episodic nausea was unlikely to have been a major contributor to weight loss because it declined substantially over time as the weight reduction was enhanced. The authors suggested that optimal adherence to dietary prescription in the patients receiving 5-HTP resulted in early satiety and reduced carbohydrate intake (14).

In a third study, this group extended their findings to noninsulin-dependent overweight diabetics. The authors postulated that low brain serotonin levels may contribute to excess energy intakes of diabetic patients. This study included 20 overweight noninsulin diabetic patients, 9 of whom were treated with 5-HTP and 11 of whom were treated with a placebo. The 5-HTP group demonstrated a reduction in energy intake and a reduction in weight in a 2-week period, as demonstrated in previous studies. Based on the authors' previous studies, one might wonder whether there would have been further benefits with an additional 4–6 weeks of study (15). It remains to be established whether longer periods of time would lead to greater weight loss and declines in glycosylated hemoglobin.

A more recent study by Rondanelli et al. further demonstrated the efficacy of 5-HTP in achieving satiety and inducing weight loss in overweight individuals. This double-blind study included 27 healthy overweight women who were randomly assigned to a treatment group or a placebo group. The treatment group received three sprays of a sublingual spray formulation of 5-HTP (5-HTP-Nat Exts) five times a day for 8 weeks (each spray contained 39 mg of 5-HTP). Importantly, 5-HTP-Nat Exts

was composed of different plant extracts, not just *G. simplicifolia* (5-HTP), including *Centella asiatica*, *Taraxacum officinale*, *Cynara scolymus*, *Paullinia sorbilis*, and *Alga Klamath*. Satiety was assessed on a numerical scale and venous blood samples were drawn to determine bioavailability of the amino acids. The results of the study showed significantly higher satiety sensation in the treatment group, as compared with the placebo group, as well as significant changes in body composition of the treatment group as compared with the placebo group. The median body mass index (BMI) (kg/m^2) difference (between baseline and after treatment) in the treatment group was $-1.04 \text{ kg}/\text{m}^2$ as compared with $0 \text{ kg}/\text{m}^2$ in the control group, resulting in a statistically significant reduction in BMI in the treatment group as compared with the control group. No side effects were reported with the sublingual spray. The authors concluded that the sublingual spray was well tolerated by the treatment group and that the results indicate that the use of such a spray may be indicated in the treatment of appetite control in the overweight population (16).

Overall, the treatment of obesity and appetite control using 5-HTP supplementation has been shown to be somewhat effective in the overweight population.

Depression

The hypothesis that 5-HTP of the central nervous system is involved in the pathogenesis of depression was based on observations of lowered concentrations of 5-hydroxyindoleacetic acid (5-HIAA, a metabolite of serotonin) in cerebrospinal fluid of depressed patients. Based on these findings, it seemed reasonable to consider that exogenous administration of 5-HTP could increase endogenous brain serotonin synthesis, presumably boosting synaptic serotonin activity and treating depression (17).

Little is known about the pathophysiological mechanisms by which serotonin synthesis is reduced in depression. Several investigators have examined whether brain 5-HTP uptake in depression differs from healthy controls (10). A PET study showed that 5-HTP uptake across the blood-brain barrier was about 30% lower in depressed patients compared with that in healthy volunteers. This study described lower 5-HTP uptake in depressed patients, but the nature of this anomaly remains elusive. Similarly, decrease brain trapping of ^{11}C -5-HTP in women with premenstrual dysphoria, particularly in those with depressed mood, suggest a role for serotonin in premenstrual dysphoria (18). Although one might consider 5-HTP to mediate its effects through serotonin, there is evidence hinting to the possible stimulation of dopaminergic activity. For instance, Takahashi et al. administered 300 mg/day of oral 5-HTP daily for 2 weeks to 24 depressed patients, and cerebrospinal fluid (CSF) 5-HIAA and homovallinic acid (HVA) levels were measured. The authors reported significant increases in CSF 5-HIAA in responders and nonresponders, whereas rise in HVA was seen only in responders (30% of the patients) (19). This suggested that 5-HTP dosing may alter serotonergic and dopaminergic turnover, and that only those patients who experience increases in both CSF metabolites could expect antidepressant effects. This is consistent with the earlier observations that dopaminergic and noradrenergic neu-

rons possess transport sites for 5-HTP (20). The use of 5-HTP in the treatment of depression has been studied for at least 40 years with varied results (10,18–30).

Preliminary data collected during the 1960s and 1970s showed some evidence suggesting a possible antidepressant effect of 5-HTP. Initial success was reported in treating depressed patients with small amounts of intravenous 5-HTP combined with a monoamine inhibitor (21). However, in a subsequent study, these results were not replicated (22). van Praag et al. published the results of the first double-blind, placebo-controlled study of 5-HTP in depressed patients. Three out of five patients responded compared with zero out of five patients receiving placebo (23). In an open-label study, Sano administered 5-HTP (50–300 mg/day) to 107 depressed individuals and found 70% response rate, suggesting beneficial effects in the treatment group (24). Other studies (see Ref. 17 for review) were plagued by methodological issues, such as low number of patients, lack of adequate controls, including placebo treatment, and coadministration of other antidepressant agents, such as monoamine oxidase inhibitors.

During the 1980s, van Praag and de Haan reported that 200 mg/day of oral 5-HTP given with 150 mg of carbidopa was more effective than placebo in preventing relapses in formerly depressed unipolar and bipolar patients during a 12-month period. Specifically, during placebo treatment, 17/20 patients experienced relapse compared with 6/20 taking 5-HTP. The authors concluded that 5-HTP plus a peripheral decarboxylase inhibitor may be an effective prophylactic agent to prevent recurrent depression in unipolar and bipolar patients (25).

Van Hiele performed a clinical study of 5-HTP with 50–600 mg/day dosing (mean was 200 mg) plus 150 mg/day of carbidopa in 99 depressed patients who had previously failed to respond to psychotherapy, tricyclics, lithium, and electroconvulsive therapy. During the experimental period, many patients continued treatment with tricyclics, lithium, or other neuroleptics. Although the author reported that 51% of patients achieved partial or complete recovery, it is not possible to know whether the recovery from depression was due to the experimental treatment alone or due to the combination with agents patients began taking before the study. Some of the responders had been followed on 5-HTP and peripheral decarboxylase inhibitor for up to 3 years (26).

In an older review of the literature, most studies of the 1980s were short in duration, had small sample sizes, and used a wide range of oral doses of 5-HTP, from 200 to 3000 mg/day (27).

Despite these limitations, a careful evaluation of the older literature reviewed by several investigators suggests a 25–50% efficacy of 5-HTP in alleviating depression (17,27,28). The treatment response does not appear to be dose dependent (100–300 mg/day, and there is no evidence that the use of decarboxylase inhibitor increases the efficacy of 5-HTP (26–28). A more recent meta-analysis, published in 2002, reviewed the literature on 5-HTP and depression from 1966 to 2000 (29). The authors found that 5-HTP was more effective than placebo at alleviating depression. In this review, a large body of evidence was subjected to very basic criteria for assessing reliability and validity and was found to be largely of insufficient quality to inform clinical practice. The small size of the studies

and the large number of inadmissible, poorly executed trials cast doubt on the result from potential publication bias and suggest that they are insufficiently evaluated to assess their effectiveness. The authors suggested that well-designed studies are required before the true efficacy of 5-HTP is known.

The efficacy of 5-HTP to treat depression has been found to be similar to that of imipramine and clomipramine (27,28). Treatment with 5-HTP in combination with carbidopa potentiates the therapeutic effects of the tricyclic antidepressant clomipramine and of the monoamine oxidase nialamide (27,28). It is unknown whether this potentiating effect is observed with other antidepressants (28). It has been reported that patients resistant to fluvoxamine treatment, a serotonin reuptake inhibitor, fail to respond to subsequent 5-HTP (30). This should not have been an unexpected finding because both agents target the same neurotransmitter. Perhaps, the next step would be to cross over patients responsive to serotonin reuptake inhibitors to 5-HTP; a positive response would extend the findings of Nolen et al. and would suggest that serotonin-sensitive, depressed patients could be tried on 5-HTP (30).

Several studies suggest that 5-HTP might be efficacious in the treatment of depression in some patients, though additional studies should be performed to validate these results.

Neurological Disorders

Headaches

The hypothesis that serotonin might be involved in the mechanisms of chronic primary headaches was set forward in the late 1950s (31). By the 1970s, studies began emerging reporting the efficacy of 5-HTP in the treatment of headaches; one such study by Sicuteri et al. reported improved migraine headaches comparable with the therapeutic effect of methysergide, an ergot alkaloid, in 20 patients with the use of 200 mg/day of oral 5-HTP for 2 months (32). Other studies reporting the efficacy of 5-HTP in treating headaches emerged including a double-blind trial on a group of 80 patients suffering from common or classic migraine who were pretreated with placebo for 30 days and then randomized into groups receiving 1.4 mg/day of pizotifen (a serotonin antagonist and antihistamine) or 5-HTP (400 mg/day), respectively, for 60 days. The authors assessed headache severity using a 4-degree analogue scale and analyzed results based on headache severity. The 5-HTP treatment group showed significant improvement in the most severe headache subgroups (i.e., degrees 3 and 4) compared with placebo with the prophylactic effects being similar to those of pizotifen (33).

In a subsequent study, the same group of researchers set out to identify and describe responsive subgroups to 5-HTP treatment in 100 patients within similar categories of primary headaches. The subjects ingested 100 mg/day of 5-HTP at meal times for 4 months. Responders were defined as exhibiting a 60% or greater reduction in the pain total index (a composite of pain severity and pain duration). The study found 74% of patients improved with treatment, with the effect becoming noticeable by the sec-

ond month. Personal history of depression, age of onset younger than 20 years, and previous positive response to pizotifen were identified as having significant prevalence in the responder group. In addition, the responders were more likely to present with throbbing and anterior pain, whereas the nonresponders presented with generalized and posterior headaches (34).

Other investigators, however, could not reproduce the therapeutic effects of 5-HTP to the same extent (35,36). For example, in the study of De Benedittis and Massei, there was a 48% response rate in patients, which was not statistically significant, probably due to the relatively large and prolonged placebo effect in the patients with chronic primary headaches (35). This small discrepancy might be explained by the shorter duration (2 vs. 4 months) of the latter study compared with the former, and by the fact that 82% of the patient population had very frequent, severe, and long-lasting headaches refractory to previous prophylactic agents. A recent parallel, randomized, double-blind study found 5-HTP and placebo equally effective in patients treated for chronic tension headaches during an 8-week period (36). Less is known about the effects of 5-HTP on the management of headaches in children, though one study reported 70% effectiveness compared with a 10% placebo response in children and adolescents with recurrent headaches and parasomnias (37).

Although several studies have demonstrated a therapeutic effect of 5-HTP on headaches (32–34,37), other studies have been unable to replicate the results (35,36). More studies are needed to determine the true efficacy of 5-HTP in the treatment of headaches, especially in relation to different types of headaches as well as to the potential differing responses amongst adults and children.

Sleep Disturbances

In adults, there is an association between sleep and the serotonergic system. In the 1960s and 1970s, reports emerged demonstrating the effect of 5-HTP on sleep. One study, a case report, presented a single individual in whom progressive increases in the dosages of intravenous 5-HTP from 50 to 150 mg augmented the percentage of the night spent in rapid eye movement (REM) sleep from 22% to 30% (38). Another trial administered 40 mg of intravenous 5-HTP in six patients and reported a shortened period, from sleep onset to the first REM period in one of the patients (39). Yet another study examined the effects of 200 and 600 mg/day of 5-HTP administered orally to healthy volunteers during a five-night period, while electroencephalograms were performed throughout the night. Increases in REM sleep ranged from 5% to 53% for both patients receiving the 200 and 600 mg/day dose (40). Animal models have shown that the destruction of the raphe nuclei or the administration of the serotonin synthesis inhibitor *p*-chlorophenylalanine induces insomnia that is selectively antagonized/reversed by 5-HTP (41).

More recently, a group of investigators examined the efficacy of 5-HTP (2 mg/kg/day) on sleep terrors in a group of 45 children (42). Patients were considered responders if they showed more than 50% reduction in the number of sleep terrors with respect to their baseline value. A positive response to treatment was achieved in 93% of patients at 1 month; of these, 51%

showed complete remission of sleep terrors. Only 7% of patients receiving treatment were nonresponders; 71% of the untreated children remained with a similar number of sleep terror episodes. At 6-month follow-up, 77% of the children treated with 5-HTP remained sleep terror free. Importantly, no side effects were reported. The investigators collected blood samples monthly to screen for eosinophilia, aiming to avoid eosinophilia-myalgia syndrome. Despite the methodological limitations of this study (i.e., no placebo control, no crossover study), the reported therapeutic effects merit follow-up.

Studies on 5-HTP and sleep have demonstrated a possible therapeutic effect in both increasing REM sleep and reducing sleep terrors in children. Future studies in humans should explore the possible sleep-inducing effect of 5-HTP and, if so, the dose-response.

Fibromyalgia

Fibromyalgia is characterized by chronic aching of skeletal muscles, multiple tender joints, fatigue, morning stiffness, and disturbed sleep. There are also reports of reduced blood serotonin levels in this patient population (43). In a 30-day double-blind trial, Caruso et al. found 100 mg of oral 5-HTP three times a day more effective in improving fibromyalgia-related symptoms than placebo (44).

In a subsequent study, the authors were interested in examining the efficacy and tolerability of long-term orally administered 5-HTP at the same dose. Good clinical improvement in the clinical variable assessed (number of tender points, anxiety, pain intensity, quality of sleep, fatigue) was found in 50% of the patients as early as the 15th day and retained up to the 90th day of treatment; 5-HTP was well tolerated and its side effects were mild and transient (45).

Although only a limited number of studies have been performed looking at the effect of 5-HTP on fibromyalgia, these studies have indicated encouraging results for a therapeutic option. More studies need to be performed to validate the possible therapeutic effect of 5-HTP on fibromyalgia.

Inborn Errors of Metabolism and Other Neurological Disorders

The therapeutic use of 5-HTP has been exceedingly valuable in patients with selected inborn errors of metabolism affecting the synthesis or recycling of tetrahydrobiopterin, a cofactor for tryptophan hydroxylase, as well as other proximal enzymes of catecholamine synthesis. Tetrahydrobiopterin deficiency is an autosomal recessive disorder, most of the cases are accounted by 6-pyruvoyl-tetrahydrobiopterin synthase (PTPS) deficiency and by guanosine triphosphate cyclohydrolase deficiency. Patients, typically infants, present with seizures, hypersalivation, drowsiness, and developmental delay; laboratory evaluation reveals mild-to-severe hyperphenylalaninemia. PTPS deficiency seems to be approximately sixfold more prevalent in Taiwan natives than in Caucasians. Treatment includes oral tetrahydrobiopterin or a low-phenylalanine diet and correction of monoamine deficiencies with L-dihydroxyphenylalanine (L-DOPA)/carbidopa and 5-HTP. The therapeutic doses of 5-HTP ranges from 3 to 10 mg/kg/day, starting with

low dose and increasing slowly guided by the patients' response (46–49). The dose can be divided in three to four equal portions during the day (47).

There is some evidence that 5-HTP at doses of about 10 mg/kg/day for 4 months can improve postural equilibrium and dysarthria in patients with various inherited and acquired cerebellar ataxias, and particularly in those with lesions located precisely in the anterior lobe vermis (50). Improvements in coordination have been reported in patients with Friedreich ataxia; however, the effect is only partial and not clinically significant (51).

A report by Ramaekers et al. in 2001 described a group of five boys between ages 1 and 5 years presenting with floppiness in infancy followed by motor delay, hypotonic-ataxic syndrome, learning disability, and short attention span (52). All patients had 51–65% reduction of CSF 5-HIAA compared with age-matched median values, as well as decrease in urinary 5-HIAA excretion. The levels of tryptophan in CSF and serum were normal. Urine and CSF serotonergic metabolites were unaltered by tryptophan loading, but normalized following 5-HTP treatment. The authors stated that this new neurodevelopmental syndrome responsive to treatment with 5-HTP and carbidopa might result from an overall reduced capacity of serotonin production due to tyrosine hydroxylase gene regulatory defect/inactivation or selective loss of serotonergic neurons.

Functional Tumor Imaging

Neuroendocrine tumors, such as carcinoids, gastrinomas, and endocrine pancreatic tumors, have the capacity to take up amine precursors such as 5-HTP (53). Because of the inefficiency of ^{18}F -fluorodeoxyglucose PET in the localization of some neuroendocrine tumors (54,55) and the capacity of these tumors to take up 5-HTP (53), studies have emerged looking at radiolabeled 5-HTP as a possible imaging modality for neuroendocrine tumors. Multiple studies have been performed, beginning in the early 1990s, to assess the efficacy of ^{11}C -5-HTP PET as a diagnostic imaging modality as well as its potential efficacy in evaluating treatment of neuroendocrine gastrointestinal tumors (56–59).

A 1993 study by Eriksson et al. demonstrated the efficacy of ^{11}C -5-HTP PET in both visualizing neuroendocrine gastrointestinal tumors and following treatment results for these tumors. Seven patients with gastrointestinal neuroendocrine tumors (five mid gut carcinoids, one lung carcinoid, and one endocrine pancreatic tumor) were imaged using computed tomography (CT) and ^{11}C -5-HTP PET. ^{11}C -5-HTP PET proved to be a superior imaging modality to CT in four out of five cases and equal in one case (in one case, a CT was not available for comparison). In addition, four patients underwent systemic treatment for their disease and had subsequent imaging with ^{11}C -5-HTP PET. The ^{11}C -5-HTP PET imaging results correlated with treatment results for the three out of four patients (56).

Subsequent studies in 1995 and 1998 provided additional evidence to the efficacy of ^{11}C -5-HTP in the localization and assessment of treatment of neuroendocrine tumors. The 1995 study by Ahlstrom et al. included six

patients with neuroendocrine tumors whose tumors were imaged on CT, ^{11}C -L-dihydroxyphenylalanine (L-DOPA), and ^{11}C -5-HTP PET. This study showed that ^{11}C -5-HTP has better uptake than L-DOPA but was unable to demonstrate any clear advantage of PET imaging over CT. One major limitation to this study, however, was the lack of ^{11}C -5-HTP availability, limiting the number of patients who were able to undergo such imaging (57). The 1998 study by Orlefors et al. included 18 patients with neuroendocrine tumors who underwent ^{11}C -5-HTP PET and CT imaging to compare the two imaging modalities, and 10 patients, who were undergoing treatment for their disease, were imaged before treatment and at different time intervals during treatment to evaluate the correlation between PET uptake and treatment efficacy. Overall, this study indicated the usefulness of ^{11}C -5-HTP PET not only in the diagnosis of gastrointestinal neuroendocrine tumors but also its possible usefulness in evaluation of treatment (58).

A 2005 study by Orlefors et al. demonstrated the efficacy of ^{11}C -5-HTP PET in the localization of neuroendocrine tumors, especially in tumors that may be too small to be visualized on anatomical imaging such as CT. This study included 38 patients with known neuroendocrine tumors who underwent whole body ^{11}C -5-HTP PET, whole body CT, and somatostatin receptor scintigraphy (SRS). The results of this study indicated that 95% of patients had tumors visualized on ^{11}C -5-HTP PET. In addition, 58% of patients had more tumors visualized on ^{11}C -5-HTP PET than on CT or SRS, including several lesions in the 0.5–1.5 cm range. The study concluded that ^{11}C -5-HTP PET may be a good diagnostic imaging technique for neuroendocrine tumors, especially those that have positive biochemical studies but negative anatomical imaging results (59).

Overall, the above studies demonstrate the efficacy of ^{11}C -5-HTP PET imaging in both the diagnosis and evaluation of treatment of neuroendocrine tumors.

ADVERSE SIDE EFFECTS

The most common side effects of 5-HTP supplementation are nausea, vomiting, and fatigue/sleepiness. Adverse events appear to be dose related to some degree with studies using higher doses of 5-HTP are more likely to report side effects (24). Coadministration of L-aromatic amino acid decarboxylase inhibitors prevents the conversion of 5-HTP to serotonin in the periphery, which in turn increases the plasma concentration of 5-HTP, associating it to a higher incidence of nausea (8).

A recent pharmacokinetic study using 100, 200, and 300 mg 5-HTP with carbidopa has suggested that even though 5-HTP side effects are partly dose dependent, there is a subset of individual that are hypersensitive, manifesting side effects (nausea and vomiting) at 100 mg (9). A recent case report suggests that modest dosage (100 mg/day) of 5-HTP can lead to patients presenting with intermittent but persistent diarrhea or fatigue, both with elevated 5-HIAA, leading to unnecessary costly medical evaluations if the doctor is not aware of the 5-HTP supplementation (60,61). These underscore the critical need to specifically question patients about the use of dietary supplements.

In 1994, the dietary supplement industry was deregulated by passage of the Dietary Supplement and Health Education Act. As a consequence, the purity, safety, and efficacy of dietary supplements are not evaluated by the U.S. Food and Drug Administration. The potential dangers of ingesting contaminated tryptophan were demonstrated in 1989 with the outbreak of eosinophilia–myalgia syndrome, which affected more than 1500 people and caused approximately 30 deaths (62).

Eosinophilia–myalgia–like syndrome, characterized by an increase in eosinophil granulocytes that induces a flu-like neurological condition, has been described in individuals taking 5-HTP (63). The compound thought to cause this syndrome was structurally characterized as a 6-hydroxy-1-methyl-1,2,3,4-tetrahydro- β -carboline (63). The quantity of contaminant per dosage of 5-HTP varies between preparations, and a threshold to prevent eosinophilia–myalgia syndrome may not be established. Indeed, some investigators have questioned the validity of the low levels near the detection limits by sophisticated mass spectrometers, leading to undue speculation between 5-HTP links to eosinophilia–myalgia–like syndrome (64).

Another possible adverse effect of 5-HTP consumption is serotonin syndrome, characterized by altered mental status; autonomic dysfunction; and neuromuscular abnormalities. It is conceivable that high doses of 5-HTP could increase serotonin levels excessively, and theoretically it could lead to serotonin syndrome. To our knowledge, there are no published cases of serotonin syndrome linked to 5-HTP consumption.

Although there can be serious side effects associated with 5-HTP consumption, the majority of side effects are mild and dose dependent. It is important to note that administration of 5-HTP to individuals without known affective disorders may cause mood elevation and, at times, mania (24). It is also important for clinicians to always be aware of the supplements that their patients are taking to be able to give proper care.

Despite 5-HTP having its own side effects, a recent study by Wilkins et al. suggested that 5-HTP is being used to counter side effects from recreational psychotropic use. Specifically, a recent computer-assisted telephone survey in New Zealand indicated that 5-HTP is being used as a “recovery pill” following the use of “party pills” containing 1-benzylpiperazine and trifluoromethylphenylpiperazine, a legal alternative (in New Zealand) to ecstasy and amphetamines. As suggested by the authors, it is unclear to what extent 5-HTP could actually enhance recovery of recreational use of the “party pills,” because both would enhance serotonin levels and could actually enhance the risk of triggering serotonin syndrome (65). To date, as far as we know, there is no evidence of 5-HTP use linked to recreational drug use in the United States.

CONCLUSIONS

The serotonin precursor, 5-HTP, is commercially available over the counter in food supplement stores and via the Internet. It is sold in the United States for approximately US\$10–40 for 45–120 capsules/tablets of 50–100 mg, some of it comes in combination with vitamin B-6, and at least

one manufacturer (Natrol®) has on the market a 200-mg time-release formulation. Following oral administration of 5-HTP, plasma concentration peaks are observed at 2–3 hours and can be raised by pretreatment with L-aromatic amino acid decarboxylase inhibitors. Brain PET scans indicate most 5-HTP accumulates in the striatum and in the prefrontal cortex and that it can be decreased in women with premenstrual dysphoria. 5-HTP, as described above, appears to have therapeutic effect in the treatment of obesity, depression, and neurological disorders (such as headaches, sleep disturbances, fibromyalgia, inborn errors of metabolism, and neurological ataxias). In addition, radiolabeled 5-HTP can be used for diagnostic imaging of neuroendocrine tumors, especially those of gastrointestinal origin.

The literature suggests that 200–300 mg of 5-HTP three times a day with meals exerts an anorectic effect, which leads to weight loss and increased satiety in obese individuals, even without dietary modification. 5-HTP seems to preferentially reduce carbohydrate intake by as much as 50% in overweight/obese and noninsulin-dependent overweight diabetics.

There is reasonable evidence to suggest that gradual individually titrated dose of 50–300 mg/day taken with meals may reverse/attenuate depression in about 50% of patients. The treatment response does not appear to be dose dependent, and there is no evidence that the use of decarboxylase inhibitor increases the efficacy of 5-HTP. Of note, it is acknowledged that methodological issues plagued most studies, and further studies are necessary before appropriate clinical recommendations can be established.

Moderate-to-severe primary chronic headaches may be alleviated in patients receiving oral 5-HTP at 100 mg three times a day, particularly in patients presenting with anterior throbbing pain. Children may also respond well to treatment with 5-HTP. The therapeutic effect takes several months before a clear response is observed.

5-HTP at 100 mg three times a day may also be useful in about 50% of patients with fibromyalgia. Other possible therapeutic effects of 5-HTP on inducing sleep and alleviating the frequency of sleep terror in children, treating selected forms of ataxias, and treating inborn errors of serotonin metabolism have not been well studied. The bulk of the literature reviewed here is more than 20 years old; more clinical studies in most targeted areas are warranted before any formal recommendation can be made.

Functional imaging utilizing ¹¹C-5-HTP PET has been found to be very effective in diagnostic localization of neuroendocrine tumors as well as the evaluation of tumor treatment and disease progression. This functional imaging compound could potentially be used in the imaging of other tumors/cancers, and more studies should be conducted to determine the true efficacy of 5-HTP in diagnostic imaging.

Nausea, vomiting, and fatigue/sleepiness are the most common side effects of 5-HTP and appear to be somewhat dose related. The most serious side effect, albeit uncommon, is eosinophilia–myalgia syndrome. Its incidence is associated with the presence of a contaminant acquired during the commercial synthesis of tryptophan and possibly 5-HTP. Therefore, it is recommended that individuals choosing to take 5-HTP be alert to the de-

velopment of myalgias or cognitive/motor changes and make arrangements with their primary care physicians to follow eosinophil counts regularly.

5-HTP has been shown to have a wide range of effect in clinical medicine and there are many more potential areas of 5-HTP efficacies that have not yet been studied. For example, novel molecular genetic studies are unraveling polymorphism of tryptophan hydroxylase and other metabolic intermediates that could potentially alter the flux of tryptophan and 5-HTP to serotonin. There is a need to conduct more rigorous study designs, including, but not limited to, randomized, double-blind, controlled clinical trials. Similarly, hypothesis-driven proposals examining the mechanisms of action of 5-HTP could be of interest to private and federal funding agencies such as The National Center for Complementary and Alternative Medicine and other institutes within the National Institutes of Health. In addition, the Office of Dietary Supplements provides valuable resources to investigators. We hope that future studies will enhance our understanding on the wide range response to oral 5-HTP supplementation.

COMPENDIAL/REGULATORY ISSUES

Not applicable.

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Iron

Laura E. Murray-Kolb and John Beard

INTRODUCTION

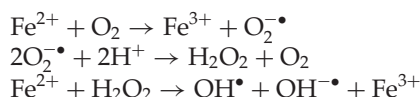
Iron is one of the essential micronutrients and, as such, is required for growth, development, and normal cellular functioning. In contrast to some other micronutrients such as water-soluble vitamins, there is a significant danger of toxicity if excessive amounts of iron accumulate in the body. However, a finely tuned feedback control system limits absorption of iron. We discuss here viable supplementation approaches and compounds as well as the risks associated with supplementation in some individuals.

COMMON AND SCIENTIFIC NAME

Iron is element number 26 in the periodic table and has an atomic weight of 55.85. It is the fourth most abundant element and the second most abundant metal in the Earth's crust. In simple aqueous solutions, iron exists in two principal oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}).

GENERAL DESCRIPTION

The two forms of iron in solution can be interchanged by the addition or subtraction of an electron. Many common reducing agents (ascorbic acid) convert ferric iron to ferrous iron, while simple exposure to oxygen in solution converts ferrous back to ferric iron. The amount of "free" iron within cells or in the fluid spaces of the body is quite low because iron can easily participate in redox reactions that underlie the inherent toxicity of excess free iron within cells. This well-known Haber–Weiss–Fenton reaction is illustrated below:



The hydroxyl radical, OH^{\bullet} , is capable of attacking most proteins, nucleic acids, and carbohydrates and initiating lipid peroxidation reactions (1). The vast majority of iron within cells of plants and animals is (i) stored within

large complex proteins such as hemosiderin or ferritin (1000 mg in an adult human, on average); (ii) contained as an essential component with proteins and enzymes and is critical for their functioning (300 mg in an adult human, on average); or (iii) contained in proteins of iron transport that move iron from one cellular organelle to another, from one cell to another cell, or between organs (2500 mg in an adult human, on average) (transferrin is an example of this iron protein complex). On average, adult humans have 2–4 g of iron with premenopausal women having lower values than men. The typical Western diet contains 15–20 mg of iron, of which 1–2 mg/day is absorbed. Thus, in addition to dietary iron absorption, recycling of iron from RBC turnover is essential for meeting the body's iron requirements. Certain forms of iron salts are highly insoluble in aqueous solutions, especially at neutral pH.

A number of different forms of iron salts are used as fortifications or supplements and vary greatly in their solubility and availability for absorption by enterocytes. A brief list is included in Table 1.

The choices that manufacturers face related to which form of iron to use in their preparations are those of not only cost but also the chemical properties of each form of iron and what other chemical or food components are also present in the preparation (2). The highly soluble sources of iron generally have a high bioavailability, but they are also more likely to participate in oxidation reactions with fats to form color reaction products and have a metallic taste. On the other hand, the less soluble forms of iron

Table 1 Iron Supplements Frequently Used and Their Relative Bioavailability

Iron source	Iron content	Relative bioavailability
Freely water soluble		
Ferrous sulfate	20	100
Ferrous gluconate	12	89
Ferrous lactate	19	100
Slowly soluble		
Ferric citrate	17	31
Ferric sulfate	22	34
Poorly soluble		
Ferrous fumarate	33	100
Ferrous citrate	24	74
Nearly insoluble		
Ferric orthophosphate	25	31
Elemental iron	97	13–90

Data are expressed as mean percentages.

Note: This chapter is dedicated to Dr. John Beard, who was its author for the first edition of the Encyclopedia. I have had the privilege of calling Dr. Beard my mentor, colleague, and friend and it is now my honor to update this chapter upon his passing. His contributions to the iron field were immense and his untiring drive to move the field forward will be forever appreciated.

have fewer organoleptic “problems” in general, but their bioavailability is quite variable.

Slight modifications of iron compounds such as altering the particle size of insoluble compounds or protecting highly soluble compounds are showing hopeful results in terms of iron absorption. Recent studies of soluble ferric pyrophosphate show it to be a potentially promising source of iron for food fortification and supplements because the magnitude of the effects of inhibitors of absorption appears to be smaller than on iron salts (3). Protection of soluble compounds through microencapsulation has received more attention recently. One example is the microencapsulation of ferrous fumarate in Sprinkles®—sachets like small packets of sugar that contain a blend of micronutrients in powder form. Such a preparation is easily sprinkled onto foods prepared in the home. Use of such products has been correlated with increased iron status in multiple studies.

DIETARY FORMS

Dietary iron occurs in two fundamental forms in the human diet: heme and nonheme iron (4). Heme iron refers to all forms from animal sources in which the iron molecule is tightly bound within the porphyrin ring structure, as is found in both myoglobin and hemoglobin. Nonheme iron refers to all other forms. Contaminant iron that is derived from dust and soil iron are relatively unavailable to the absorptive cells but may constitute a significant amount of iron intake in developing countries. There is substantial information that demonstrates that nearly all nonheme dietary iron mixes together in a luminal “pool” of iron in the upper gastrointestinal (GI) tract as a result of acidification in the stomach and subsequent exposure to pancreatic and GI enzymes. Inorganic iron is solubilized and ionized by gastric acid juice, reduced to the ferrous form, and kept soluble in the upper GI tract by chelation to compounds such as citrate and ascorbic acid. The type and amount of other materials such as ascorbic acid that can chelate iron to keep it in solution also determine the amount of nonheme iron in a soluble luminal pool. The number of “inhibitors” of nonheme iron absorption is substantial, with phytate, polyphenols, and tannins leading the list. These inhibitors typically bind either ferric or ferrous iron in a tight complex in the lumen of the gut and make it unavailable for the absorptive proteins. Thus, it should be clear that a diet containing a large amount of unrefined grains, nondigestible fibers, etc., will have poor iron bioavailability. In contrast, a diet that is highly refined has little roughage, and substantial portions of meat will have a greater iron bioavailability regardless of other factors. The American diet typically obtains about 50% of its iron intake from grain products, in which the iron concentration is between 0.1 and 0.4 mg per serving. Some fortified cereals, however, may contain as much as 24 mg of iron in a single serving. Heme iron is more highly bioavailable than nonheme iron, and its bioavailability is less affected by other components of the diet. Overall, dietary nonheme iron absorption is approximately 5–10% efficient compared with heme iron absorption at nearly 40%. Only about 10% of total dietary

iron intake is represented by heme iron in many Western countries. However, given its greater absorption efficiency, heme iron plays a significant role in an individual's iron status.

Regulation of Absorption

There are two fundamental regulators or determinants of the amount of iron absorbed in humans. The first is the total amount and form of iron compounds ingested (discussed earlier) and the second is the iron status of the individual (5). Thus, individuals with a high iron status will absorb proportionally less of any amount of iron consumed than will an iron-deficient individual, and individuals with a lower iron status will absorb more of any dietary intake. This process of selective absorption is the fundamental mechanism whereby humans regulate iron balance (6). The mechanism of this regulation is still not entirely clear although recent discoveries have added to our understanding.

Heme Iron Absorption (Fig. 1)

There are two prevailing hypotheses of heme iron absorption: (i) receptor-mediated endocytosis and (ii) direct transport into the enterocyte through recently discovered heme transporters. The hypothesis of receptor-mediated endocytosis is a long-standing hypothesis that originated after discovery of a heme-binding protein in the upper small intestine in pigs and humans (7). Once the heme is internalized into the enterocyte, it is degraded by heme oxygenase 2 inside the vesicles that releases nonheme iron and produces bilirubin. The iron that is released from heme then needs to be transported out of the vesicle to join the labile iron pool. The transporter responsible for this has not yet been identified, but divalent metal transporter-1 (DMT-1) has been suggested as a possibility (8). Once this iron joins the labile iron pool, it is processed in the same manner as dietary nonheme iron. The hypothesis that heme iron may be directly transported into the enterocyte originated after the recent discovery of heme transporters, proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) (9,10) and feline leukemia virus subgroup C receptor-related protein (FLVCR) (11). While the mechanisms are not completely understood, it is thought that heme iron in the intestinal lumen is taken up by HCP1 directly into the cytoplasm and then has one of two possible fates. It can be catabolized to nonheme iron and bilirubin by heme oxygenase 1 after which the nonheme iron joins the labile iron pool and is processed as nonheme dietary iron. Alternatively, intact heme may cross the enterocyte and be transported across the basolateral membrane by FLVCR. Ongoing research in this area will surely shed light on the exact mechanisms of heme iron absorption in the near future.

Nonheme Iron Absorption (Fig. 1)

There are several steps involved in nonheme iron absorption: (i) reduction of iron, (ii) apical uptake, (iii) intracellular trafficking or storage, and (iv) basolateral release. Most dietary iron is found in the ferric form and must be reduced to ferrous iron before it can be taken up by the enterocyte. This is accomplished by a ferric reductase, duodenal cytochrome B, or dietary-reducing agents such

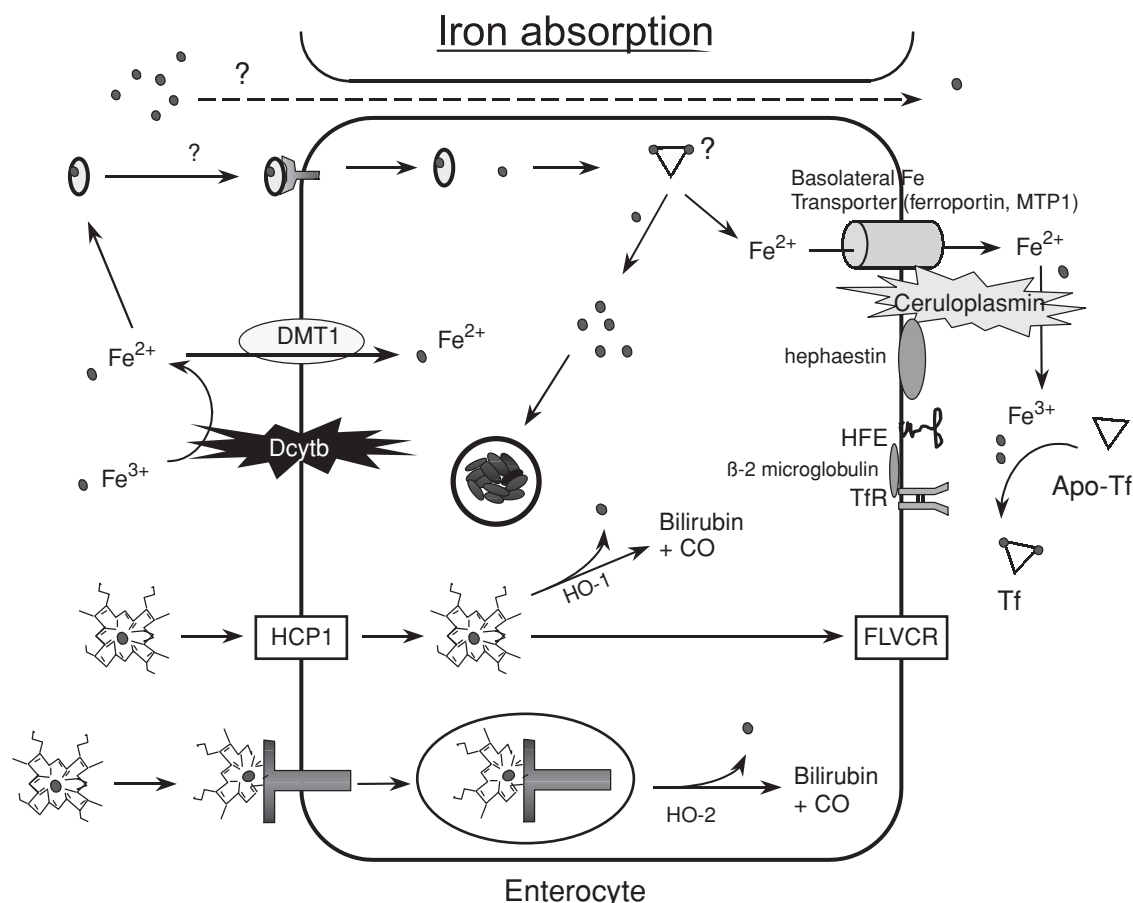


Figure 1 Putative mechanism for the absorption of iron in enterocytes of the upper GI tract in humans. On the left side of the diagram (illustrated from the bottom to the top) is a putative heme iron transporter, receptor-mediated endocytosis of heme iron, divalent metal transporter (DMT-1) mediated uptake of nonheme iron coupled to a ferroxidase, and a poorly described nonheme iron transporter independent of DMT-1. Soluble intracellular iron can be inserted into ferritin (center of the cell) or exported through the metal transport protein-1 (MTP-1) shuttle system located in close proximity to copper-containing hephaestin or ceruloplasmin. The hemochromatosis gene product, HFE, is likely to exert its influence at this site of iron export from the absorptive cell.

as ascorbic acid. The ferrous iron then enters the enterocyte via the DMT-1 where it can be stored as ferritin or be transported to the basolateral surface for entry into the circulation. The mechanism of intracellular iron transport is not known. Basolateral iron transfer likely occurs through the iron exporter ferroportin 1 (or metal transport protein-1), and the ferrous iron that is transported across the membrane is then oxidized by hephaestin before binding to plasma transferrin for transport throughout the body. Although specific mechanisms are still under investigation, it is thought that iron transfer across the basolateral membrane of the enterocyte is the rate-limiting step in iron absorption. Crypt endothelial cells receive a signal from the iron storage "pools," which then establishes a set point for iron absorption (12). The putative signal, hepcidin, is released from hepatocytes into the plasma pool. Hepcidin binds to ferroportin 1 causing its internalization and destruction (13), thus inhibiting ferroportin 1 mediated export of iron. In cases of excess dietary or supplemental iron exposed to the microvillus enterocyte, the excess iron can be stored in the form of ferritin within the enterocyte. The amount of ferritin that is synthesized by the entero-

cyte is under the regulation of the mRNA-binding protein, iron regulatory protein (IRP), which binds with high affinity at an iron response element (IRE) located in the 5'-untranslated end of the ferritin mRNA. There is also a similar set of IREs on the 3' end of the mRNA for transferrin receptor (TfR) and DMT-1 that allows for a reciprocal regulation of iron storage and iron uptake. This IRE-IRP system of regulation, however, is also susceptible to oxidative stress, because nitric oxide may alter the affinity of this regulator of protein translation (4). In situations of very high iron salt intakes, it is likely that an increased oxidative stress within these enterocytes will lead to altered iron storage and absorption (14).

ACUTE EFFECTS AND TOXICITY OF HIGH IRON

Acute high doses of iron salts may also lead to diffusion gradient movement of iron in a pericellular mechanism in which the normal regulatory machinery is bypassed. Near toxic intakes of iron salts such as ferrous sulfate, ferrous gluconate, and ferric chloride are known to cause

erosion of the enterocyte, cell death, increased flux of iron across the mucosa, very rapid increases in vascular iron, and iron accumulation in liver cells with subsequent liver pathology (6,14). An oral dose of iron in excess of 180 mg/kg of body weight is usually associated with mortality that is preceded by vomiting and diarrhea. If the normal cellular and extracellular mechanisms are overwhelmed, free iron will immediately be available for participation in redox-coupled reactions that are associated with cellular damage to proteins and lipids (1). Symptoms of excess iron accumulation become evident in some individuals when as little as 10–20 mg Fe/kg is consumed, with GI upset and constipation as the most common symptoms. These GI effects appear to be highly “case-sensitive” at lower doses, with some individuals being more affected by the absence of food in the stomach and others by the particular form of iron that is taken in the iron supplement. As the dose increases, however, an increasingly greater proportion of individuals report GI effects of increasing severity.

High iron supplement use appears to reduce zinc absorption and plasma zinc concentrations (15,16). This effect is most frequently seen when the molar ratio approaches 25:1 (17) and is lost if the absorption rate of zinc is measured in the presence of other food items (i.e., during a meal). Fortification of grains and cereals, infant foods, and weaning foods does not appear to affect zinc absorption (18).

IRON OVERLOAD

Iron overload diseases have received much attention in the last decade, especially in the last 5 years, with the discovery of the gene associated with hereditary hemochromatosis. Approximately 1 in 200–400 individuals of Anglo-Saxon ancestry is affected by an autosomal recessive gene mutation that results in this iron overload disease (5). The gene mutation of the HFE gene in position C282Y accounts for the vast majority of the cases of hemochromatosis in individuals of Celtic origin. Various other mutations have also been described, and, in most cases, the mutations are associated with a failure of the HFE protein to bind effectively with the β -2 microglobular protein and the TfR1 protein at the plasma membrane. The failure of the association is related to the lack of control of iron flux across the enterocyte. Hereditary hemochromatosis is characterized by hepcidin deficiency despite massive iron loading found in these individuals. This indicates that HFE most likely acts as a regulator of hepcidin synthesis. Hereditary hemochromatosis is thus characterized by a failure to control iron absorption in the enterocyte, with a resulting accumulation of iron in iron storage pools, primarily in cells of the reticuloendothelial system (RES). Clinical signs of iron toxicity occur in homozygous individuals without treatment in the fourth decade of life, or earlier (in the case of juvenile hemochromatosis), at which time their total body iron content is more than 20 g. Lack of treatment by chelation and phlebotomy results in cirrhosis of the liver, hepatocellular carcinoma, myocardial pathology, and damage to pancreatic function (19). This accumulation of iron in the liver and the accompanying hepatic fibrosis and cirrhosis appear to be causal in na-

ture. Thus, these individuals must be careful to avoid iron supplements. The evidence for iron accumulation in heterozygous individuals is less clear, and the role of dietary iron bioavailability in body iron accumulation in these individuals is still being debated (20). At the time, it appears prudent for known heterozygous individuals to limit their consumption of iron supplements.

Other forms of iron overload due to chronic excessively high iron intakes have been reported, but these intakes of approximately 200–1200 mg Fe/day for long periods of time are unusual. Bantu siderosis in Africa is an additional example of iron toxicity due to excessively high iron intakes for prolonged periods of time (6). Home brewing of beer in large iron pots is associated with an intake of iron in excess of 50–100 mg Fe/day, with a resulting iron overload disease. There is some evidence as well that there is a genetic component to this disease (6).

Transfusional iron overload may result from the accumulation of iron after senescent transfused red cells are metabolized in the RES. Because each unit of blood contains approximately 225 mg of Fe, there is a real danger of hepatic iron overload with repeated transfusions. Individuals with disorders in effective erythropoiesis who receive transfusions have the additional iron burden of excessive iron absorption. That is, iron accumulation in the RES occurs because of high rates of red cell turnover and iron absorption.

INDICATIONS AND USAGE

Supplementation

In 2001, the United States Food and Nutrition Board of the National Academy of Sciences released the current recommendations for dietary reference intakes (DRIs) (20). There are several categories of DRIs including recommended daily allowances (RDA), estimated average requirements, adequate intake, and upper limit (UL). The committee had the perspective that functional consequences of iron deficiency occur only when there are depleted iron stores and there is insufficient delivery of iron to the essential iron pools in all tissues. The erythroid mass has the largest essential iron pool in the form of hemoglobin, with bone marrow uptake responsible for more than 70% of the plasma iron turnover on a daily basis. In the evaluation of assessment of iron status, the review panel utilized the following indicators: (i) serum or plasma ferritin for iron storage pool size; (ii) plasma soluble TfR for adequacy of iron delivery to rapidly growing cells; (iii) plasma or serum transferrin saturation for iron transport; and (iv) hemoglobin concentration, hematocrit, or red cell counts for the existence of anemia. The need to utilize all of these indicators is well justified given the impact that acute and chronic infections have on the evaluation of iron status (6,14). The TfR is relatively new but appears to be insensitive to inflammation (although there is some suggestions that TfR is sensitive to malaria). Hence, there is great promise that it will prove to be a valuable indicator of iron status in complicated clinical and nutritional diagnosis.

The RDAs shown in Table 2 represent the required intakes to ensure adequate nutrition in 95–97.5% of the population and are an overestimation of the level needed for most people in any given group. Individuals who do

Table 2 Recommended Dietary Allowances (RDA)^a and Estimated Average Requirements (EAR)^a for Iron Consumption (in mg Fe/day)

Age	EAR	RDA
0–6 mo ^b		
7–12 mo	6.9	11
1–3 yr	3.0	7
4–8 yr	4.1	10
Boys		
9–13 yr	5.9	8
14–18 yr	7.7	11
Girls		
9–13 yr	5.7	8
14–18 yr	7.9	15
Men	6	8
Women		
19–50 yr ^c	8.1	18
51 yr	5	8
Pregnancy ^d	22	27
Lactation ^e	6.5	9

^a2001 United States Food and Nutrition Board, Institute of Medicine, National Academies.

^bOnly adequate intakes could be estimated in this age group. The AI was estimated as 0.27 mg/day.

^cValues are given as estimates for reproductive age women.

^dThis is an overall estimate throughout pregnancy, though iron requirements clearly vary greatly by trimester. In addition, this estimate is slightly higher for teenage pregnancy (EAR of 22.6 mg and RDA of 27 mg Fe/day).

^eThis estimate is slightly higher for teenagers who are lactating (EAR of 7 mg and RDA of 10 mg Fe/day).

not routinely consume the suggested level of iron from foods should be encouraged to supplement their diets with iron compounds.

Treatment of Iron Deficiency

Iron deficiency has traditionally been separated into iron deficiency anemia and tissue iron deficiency, also referred to as “depleted iron stores.” Iron deficiency anemia is diagnosed as a low serum transferrin saturation (<15%), a low serum ferritin concentration (<12 µg/L), and an elevated soluble TfR (>6 mg/dL) in the setting of microcytic anemia. However, anemia reflects a later stage of iron depletion, with earlier stages often evidenced by low serum ferritin and slightly elevated TfR levels. Because both serum ferritin and serum iron concentrations are acute phase reactants to inflammatory cytokines, the presence of inflammation must be considered in a diagnosis of iron deficiency (20). To date, there is no consensus on how to “correct” these values in the presence of inflammation, but ongoing work in this area appears promising.

After the diagnosis of true iron deficiency, rapid restoration of an iron-replete state can be achieved by administering 125–250 mg of ferrous sulfate orally per day. This dose of the salt will deliver 39–72 mg of highly bioavailable iron per day. There is some evidence that doses greater than 250 mg ferrous sulfate convey additional benefits, and it is still common practice in severe anemia of pregnancy to administer this dose twice per day. This, however, results in a high prevalence of complaints of GI distress, constipation, and blackened stools. Thus, compliance with these high doses drops considerably from prescribed amounts. Slow release formulas appear to cause fewer side effects and may prove helpful to

those who suffer from GI distress with iron supplementation. Once the iron deficiency is resolved, daily intake of iron based on the levels indicated in Table 2 should be maintained.

Preventative Iron Supplementation

One of the great concerns regarding iron deficiency anemia is the adequacy of iron intake during pregnancy (21). There is evidence that poor iron status in the first trimester of pregnancy is associated with prematurity, low birth weight, and small-for-gestational-age newborns. Because many women in developing and underdeveloped countries are not even aware that they are pregnant prior to the 10th to 12th week of pregnancy, there is a need to ensure that women “enter” pregnancy in an iron-adequate state. This has led to a reevaluation of the concept of “daily iron supplements” (22,23). At issue is the relative effectiveness of daily therapeutic doses of iron compared with the administration of lower “preventative intermittent iron” doses. The concept is this: because the GI enterocyte will “reset” its set point for iron absorption every 3–4 days as a new crop of crypt cells migrate up to the tip of the villus, the large doses of iron given on days 2 and 3 are wasted and may in fact result in oxidative damage and mucosal injury. To test this hypothesis, a number of studies in developing countries have compared the efficacy of daily doses with an intermittent dose in correcting iron deficiency anemia in children, adolescents, reproductive age women, and pregnancy (22). The daily dosage approach had a faster response, but with lower compliance, than did the intermittent oral iron dose. The end result in terms of correcting the anemia was similar in nearly all studies, with the exception of pregnancy, where daily iron therapy clearly provided a greater benefit (24).

Adverse Effects of Drugs on Iron Status

Iron balance is largely regulated at the level of the duodenal enterocyte. Hence, any clinical condition or administered drug that significantly alters the integrity of these enterocytes or the conditions needed for optimal bioavailability of iron (such as an acidic environment) has great potential to alter iron requirements and metabolism. Thus, antiulcer drugs such as Prevacid and Prilosec may reduce iron absorption by a reduction in the amount of acidification of the stomach contents. In addition, a number of antibiotics with structures similar to those of doxycycline and tetracycline have been related to decreased absorption of iron due to their ability to chelate iron in the upper bowel. In a similar fashion, biliary excretion of iron is the manner in which most iron is excreted from the body. Hence, drugs known to alter enterohepatic circulation are likely to increase iron requirements. For example, the popular antilipidemic drug cholestyramine interferes with iron absorption and enterohepatic circulation. Clearly, blood loss from the body is the single largest cause of increased or altered iron requirements. Bleeding ulcers, lesions, and leaking inflammatory states in the gut are all associated with a decreased iron status due to increased rates of blood and iron loss.

The administration of Epogen[®], the recombinant form of erythropoietin, leads to a rapid increase in red cell production and is of course related to an increase in iron

requirements, as iron is cleared from the plasma pool at a much greater rate. Thus, it is usually essential that patients also receive a supplemental supply of highly available iron to meet this increased demand.

Contraindications

Individuals with a demonstrated or suspected HFE gene mutation are clearly more at risk of iron overload pathology than they are of needing exogenous iron supplements. A family history is sufficient to warrant a thorough evaluation of iron status and possible genetic mutations. Interventions early in the iron accumulation process are effective in reducing pathology associated with iron accumulation. Similarly, a diagnosis of hemolytic or hemorrhagic anemia is a clear signal to avoid administration of iron. These anemias often lead to a dramatic increase in iron absorption and total body iron early in life due to both transfusional iron overload and the increased iron absorption (6).

PRECAUTIONS AND ADVERSE REACTIONS

It has long been known that iron supplementation may increase a person's susceptibility to infection, but the known benefits of iron supplementation were thought to outweigh the risks of adverse effects. However, recent results from a large-scale epidemiological study in Pemba, Tanzania, have caused the scientific community to rethink recommendations for iron supplementation. The Pemba study showed an increase in serious adverse events among children who were being supplemented with iron. These same results were not found in a large-scale study in Nepal. Pemba is an area of hyperendemic malaria, while the study area in Nepal was malaria free and hence, the adverse findings from the Pemba trial have been generally attributed to interactions between iron and malaria. It is important to note that the adverse findings in the Pemba study were found only in children who were iron replete, while beneficial outcomes were seen in children who were iron deficient. When weighing the evidence from iron supplementation trials in malaria-endemic areas, the results are equivocal. A recent review of 15 such studies found the following: 6 showing no effect of iron supplementation on malaria risk, 3 showing an increase in clinical malaria attacks, and 6 showing nonsignificant increases in malaria outcomes (25). One of the differences between studies reporting significant adverse malaria outcomes and those not reporting such outcomes is active treatment of malaria incident cases. Thus, treatment of malaria infections may curb adverse effects of iron supplementation. Large efforts are underway to understand the mechanism by which iron supplementation results in adverse outcomes in malaria-endemic areas. However, even in areas of high malaria endemicity, data suggest that iron supplementation is beneficial in iron-deficient individuals. Therefore, current recommendations are to adopt a cautious approach to supplementing children with iron by either screening children to determine those who are iron deficient or by instituting active treatment of malaria before administration of supplemental iron is commenced. Much more research is needed to truly understand the

relation between iron and malaria so that definitive recommendations can be reached.

Doses of iron above 180 mg may be lethal in adults (26). As described earlier, high acute intakes of iron may be associated with necrotizing gastritis and enteritis, pallor, lassitude, and frequent diarrhea. A rapid rise in the plasma iron concentration within 60 minutes to levels in excess of 500 $\mu\text{g}/\text{dL}$ is common and is likely related to the pathology. Rapid treatment with iron chelators, such as desferrioxamine, will rapidly decrease plasma iron concentration, and gastric lavage has improved recovery rates (6).

In 2001, the Food and Nutrition Board of the National Academy of Sciences recommended 45 mg as the UL for iron for adults 19 years and older, including pregnant and lactating women. An uncertainty factor of 1.5 was selected to extrapolate from a lowest observed adverse effect level to a now observed adverse effect level (NOAEL) using GI side effects as the outcome for adverse effects. ULs for infants and young children were estimated from an NOAEL of 40 mg/day and an uncertainty factor (UF) of 1.0, with a resulting UL of 40 mg/day. This UL remains for children 1–3, 4–8, and 9–13 years old, primarily due to a lack of data to suggest otherwise. As noted previously in the contraindications section, individuals or subpopulations may be at special risk of iron overload when it is consumed at these UL levels and would not be protected by these ULs. Importantly, the committee did not feel there was compelling evidence to utilize either cardiovascular disease or cancer as dependent variables for setting the UL.

COMPENDIAL/REGULATORY STATUS

Not applicable.

CONCLUSIONS

A number of iron compounds exist in the marketplace that can effectively and safely supplement dietary iron to bring individuals back into iron balance. There is risk, however, that overzealous use of supplements can lead to GI distress and, in some individuals with genetic mutations or certain infections, toxic iron overload, and even death. New understanding of the genetic causality of iron overload syndromes and the relation between iron and malaria will provide greater avenues for preventing accidental iron overload in the near future.

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Isoflavones

Mark Messina

INTRODUCTION

Isoflavones are a subclass of the rather ubiquitous flavonoids but by comparison have a much more limited distribution in nature. The primary dietary sources of isoflavones are soybeans and soyfoods. The literature is replete with declarations that many legumes, fruits, and vegetables contain isoflavones. Although these statements are technically correct, they are unintentionally misleading because the amounts in most of these foods are so small as to almost certainly be physiologically irrelevant (1). Over the past 20 years, an impressive amount of health-related isoflavone research has been conducted; in fact, approximately 1000 peer-reviewed papers on isoflavones are published annually and they are the primary reason there has been so much focus on soy in recent years. The sheer volume of research presents a challenge to correctly interpreting and synthesizing the findings. Complicating matters are the diverse experimental designs that have been employed in clinical studies—they have used markedly different isoflavone doses, interventions (products) that vary considerably in chemical composition and isoflavone profile, and involved subjects with different health statuses (e.g., healthy vs. at risk). These and other differences in experimental design make interpreting the scientific literature a difficult task.

In contrast to phytoalexins (substances that are formed by host tissue in response to physiological stimuli, infectious agents, or their products and that accumulate to levels that inhibit the growth of microorganisms), isoflavones are constitutive and are thus always present in significant quantities in soybeans because one of their primary functions is to stimulate nodulation genes in soil bacteria belonging to the genus *Rhizobium* (2). *Rhizobia* have the ability to induce the formation of nodules on soybean roots, which are required for the reduction of atmospheric nitrogen to ammonia, which the soybean can then use as a source of nitrogen for growth.

In total, there are 12 different soybean isoflavone isomers. These are the three aglycones: genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone), and glycitein (7,4'-dihydroxy-6-methoxyisoflavone) and their respective simple β -glycosides: genistin, daidzin, and glycitin, and the glycosides esterified with either malonic or acetic acid (Figs. 1 and 2).

Typically, there is somewhat more genist(e)in than daidz(e)in in soybeans and soyfoods, whereas glycit(e)in comprises only 5–10% of the total isoflavone content (3). In soybeans and in nonfermented soyfoods, isoflavones

R1	R2	Chemical Name	M.W.
OH	H	4',5,7-Trihydroxyisoflavone (Genistein)	270
H	H	4',7-Dihydroxyisoflavone (Daidzein)	254
H	OCH ₃	4',7-Dihydroxy-6-methoxyisoflavone (Glycitein)	284

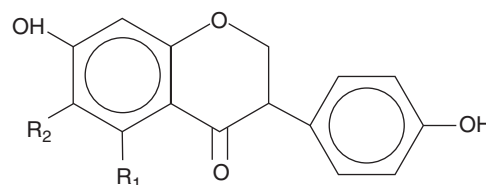


Figure 1 Chemical formulas and molecular weights of the soybean isoflavone aglycones.

are present primarily as their glycosides (<2% is present as the aglycone), whereas in fermented soy products, the isoflavones are present primarily in their aglycone form due to microorganism-induced fermentation and hydrolysis of the parent compounds.

Red clover (*Trifolium pratense*) also contains a rich supply of isoflavones and, along with soybeans, is used as a source for the production of isoflavone supplements. This plant is common throughout North America, Europe, and Central and northern Asia. The two predominant isoflavones in red clover are the methylated isoflavones formononetin (4'-methoxy-7-hydroxyisoflavone) and biochanin-A (4'-methoxy-5,7-hydroxyisoflavone) [see chapter 78, "Red Clover (*Trifolium pratense*)" for more detail].

Unfortunately, there is no uniform method for expressing isoflavone content, and molar concentrations are generally not used as information intended for the public. Consequently, there is some ambiguity regarding the amount of biologically active isoflavones in products and administered to subjects in clinical studies, even when total isoflavone amounts are declared. Because the molecular weight of the aglycone is approximately 60% that of the glycoside, an unqualified statement such as "100 mg isoflavones" can actually refer to between approximately 60 and 100 mg of biologically active (aglycone) isoflavone. In recent years, recommendations that

R3	R4	R5	Chemical Name	M.W.
OH	H	H	Genistein, 7- <i>O</i> -β-D-glucopyranoside (Genistin)	432
OH	H	COCH ₃	6'- <i>O</i> -Acetylgenistin	474
OH	H	COCH ₂ COOH	6''- <i>O</i> -Malonylgenistin	518
H	H	H	4',7-Dihydroxyisoflavone, 7- <i>O</i> -β-D-glucopyranoside (Daidzin)	416
H	H	COCH ₃	6''- <i>O</i> -Acetyldaizin	458
H	H	COCH ₂ COOH	6''- <i>O</i> -Malonyldaizin	502
H	OCH ₃	H	Glycitein, 7- <i>O</i> -β-D-glucopyranoside (Daidzin)	446
H	OCH ₃	COCH ₃	6''- <i>O</i> -Acetylgaitin	488
H	OCH ₃	COCH ₂ COOH	6''- <i>O</i> -Malonylgaitin	532

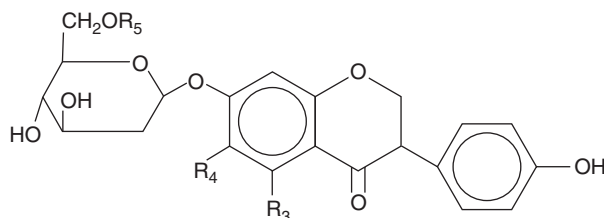


Figure 2 Chemical formula and molecular weights of the soybean isoflavone glycosides.

isoflavone content be stated as in the amount of aglycone have been made.

ISOFLAVONE CONTENT OF SOYFOODS

Isoflavones are relatively heat stable, as baking or frying at high temperature alters total isoflavone content very little or not at all. The isoflavone content (aglycone weight) of raw soybeans is approximately 1.0 mg/g, with a range of about 0.4–2.4 mg/g. Traditional soyfoods typically provide 0.2–0.4 mg/g of fresh weight product and about 2–4 mg/g protein. One serving [e.g., 3–4 oz of tofu or 1 cup (250 mL) soy beverage] of a traditional soyfood provides approximately 25 mg isoflavones. A general rule of thumb for estimating the isoflavone content of a traditional soyfood is to multiple the grams of protein in that food by 3.5 mg. This ratio does not hold for processed soy products because during processing, considerable isoflavone content can be lost. Isolated soy proteins (which, by definition are at least 90% protein) vary in isoflavone content (range, 0.5–2.0 mg/g), although the average is about 1 mg/g. As a result of processing losses, the isoflavone content of alcohol-washed soy protein concentrates, the most common type of concentrate, is only 5–20% that of the water-washed concentrates. The U.S. Department of Agriculture, in conjunction with Iowa State University, operates an online database (4) that provides the isoflavone content of foods.

In addition to soyfoods and supplements, isoflavones are used as food fortificants, being added to both soy and non-soy products. One brand of isoflavones, Novasoy[®], which is produced by the Archer Daniels Midland Company, has achieved GRAS status in the United States through self-affirmation and review by an outside panel of experts. It can be added to adult single meal replacements and health beverages and bars. Novasoy is an example of a supplement that is derived from soybean molasses (distilled ethanol extract from soy flakes) and that reflects the isoflavone profile of soyfoods (Fig. 3).

In contrast, a second source of isoflavone supplements, which is commonly referred to as soygerm, is derived from the hypocotyl portion of the soybean and in comparison to soyfoods, it is very low in genistein and high in glycitein (5).

As is the case with many types of supplements, analyses show that there is often a discrepancy between the isoflavone content of a product and the amount listed on the label; many supplements contain less than 20% of label value, whereas a smaller percentage contain considerably more than the stated amount (6).

ISOFLAVONE INTAKE IN ASIA

Initial values reported in the literature in the early 1990s greatly overestimated Asian isoflavone intake (7). There are, however, now quite good data on isoflavone

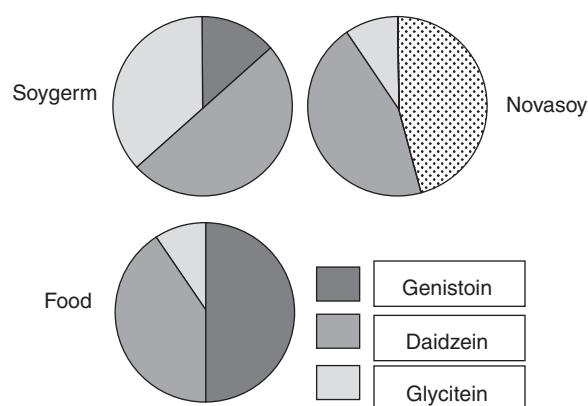


Figure 3 Relative isoflavone profiles of different sources of isoflavones.

exposure via the consumption of soyfoods, especially for Hong Kong, Japan, and selected Chinese cities such as Shanghai. In Japan, the Ministry of Health and Welfare has conducted a national household survey (NHHS) of food intake since 1946. The NHHS was conducted on a quarterly basis between 1946 and 1963 and has been done on an annual basis thereafter (8). It includes randomly selected prefectures comprising 10,000–20,000 subjects of all ages. This survey shows that absolute soy protein intake during the past several decades has remained relatively constant at about 9 g/day, but as a percentage of total protein has declined quite markedly, from about 13 to 10. In a comprehensive review of the literature published in 2006, Messina et al. (9) reported that among older adults in Japan and Shanghai, mean isoflavone intake ranged from about 25 to 50 mg/day, whereas intake in Hong Kong is about half of these amounts. More recent data are consistent with these findings (10).

Large cohort studies from Shanghai indicate that the mean isoflavone intake of individuals in the upper intake categories (upper quartiles and quintiles) is about 75 mg/day. The isoflavone intake of no more than 5% of this Chinese population exceeds 100 mg/day. These findings are similar to those for Japan. However, in Japan, westernization of the diet has resulted in soy/isoflavone intake among younger people being roughly half that of the older generation consuming a more traditional diet. Finally, in Japan, approximately half of isoflavone intake comes from the consumption of fermented soyfoods (in which the isoflavones are present to a large extent as aglycones), whereas fermented foods are consumed to a much more limited extent in China.

ISOFLAVONE METABOLISM

Isoflavones circulate in plasma mostly in the conjugated form, bound primarily to glucuronic acid, and less than 4% circulates in the free form. The absorption of isoflavones present in the intestine as glycosides first requires hydrolysis to the aglycone, a reaction that occurs via the action of brush border membrane glucosidases and bacterial glucosidases. During passage across the enterocyte, there occurs significant phase-II metabolism with conjugation

to glucuronic acid to form the isoflavone-glucuronides. Between the intestinal tract and liver, there is a massive capacity for conjugation so that circulating proportions of unconjugated isoflavones are maintained at relatively low concentrations.

Plasma isoflavone levels increase in relation to the amount ingested, although some evidence, but not all, suggests that there is a curvilinear relationship between the area under the plasma curve and isoflavone dose (11,12). Plasma isoflavone levels among Asians are approximately 500 nmol/L (13), but because measurements are typically taken after an overnight fast and the plasma half-life of daidzein and genistein is relatively short (generally observed to be between 5 and 10 hours (14)), fasting levels are much lower than postprandial levels. In response to the consumption of approximately 50–100 mg of isoflavones (the amount found in approximately two to four servings of traditional soyfoods), peak serum isoflavone levels can be expected to be approximately 2 μ mol/L (12). Because of the relatively short half-life, and because the efficiency of isoflavone absorption is inversely related to dose, for the highest sustained plasma levels, it is best to consume isoflavones throughout the day rather than at one sitting.

Isoflavones undergo enterohepatic circulation; nevertheless, most of the isoflavones absorbed are excreted from the body within 24 hours after a single ingestion. Studies indicate that no more than 50% of the isoflavone dose ingested is absorbed, but this varies according to the individual isoflavone. Most of the methylated isoflavones formononetin and biochanin-A, which are found in red clover, are converted by the liver to daidzein and genistein, respectively. Daidzein is metabolized primarily to the isoflavone equol and *O*-desmethylangolensin, whereas genistein is metabolized to dihydrogenistein and a number of other oxidative metabolites. It is, however, widely recognized that there is a huge interindividual variation in isoflavone metabolism such that in response to the ingestion of the same amount of isoflavones leads to dramatically different circulating levels of the parent isoflavones and their metabolites between individuals.

The conversion of daidzein to equol, which is accomplished by intestinal bacteria, may be particularly important because it has been proposed that equol is an especially beneficial compound and that those individuals possessing equol-synthesizing bacteria are more likely to benefit from soyfood consumption. Only approximately 50% of Asians and 25% of Westerners possess these bacteria; evidence indicates that equol-producing status is essentially a lifelong attribute with the exception of a temporary loss following exposure to antibiotics.

Overall, the evidence indicates that whether isoflavones are present in foods as glycosides or aglycones appears not to be critically important in regard to potential health effects, because the glycosides, although not absorbed intact, can be efficiently hydrolyzed *in vivo* (15). Initially, some data suggested that aglycones are absorbed to a greater extent than isoflavone glycosides, but more recent work shows that this is not the case (6,12). Isoflavones when ingested in aglycone form appear to be absorbed more quickly even though as noted, the total area under the curve is not affected. Also, in response to

large doses of isoflavones in aglycone form, it is possible that peak plasma unconjugated isoflavone levels may be higher than in response to a similar amount of isoflavones presented as glycosides, which may have a physiological impact, although this remains to be proven.

PHYSIOLOGICAL PROPERTIES

Isoflavones bind to estrogen receptors and affect estrogen-regulated gene products, although their binding affinity is lower than that of 17 β -estradiol. For this reason, isoflavones are often referred to as phytoestrogens. It is, however, probably more accurate to refer to the estrogen-like, rather than estrogenic, properties of isoflavones because isoflavones are much different from the hormone estrogen. Isoflavones have traditionally been considered to be weak estrogens, but for several reasons, it is difficult to arrive at a single estimate of their relative overall estrogenicity. In any event, because their circulating levels can exceed those of estrogen by 1000-fold, there is a reasonable expectation that isoflavones can exert physiological effects although this is often not apparent clinically. Perhaps even more importantly, isoflavones bind with much greater affinity to estrogen receptor beta (ER β) than estrogen receptor alpha (ER α) and are much more potent at triggering transcriptional activity when bound to ER β in comparison with ER α (16–18). Because these receptors have different tissue distributions, and different functions, isoflavone “estrogenicity” and actions will vary markedly from tissue to tissue.

The different tissue distributions of ER α and ER β and their greater binding affinity and transactivation of ER β in comparison with ER α have led to the classification of isoflavones as natural selective estrogen receptor modulators (SERMs) (mixed estrogen agonists/antagonists) (19). SERMs such as the breast cancer drug tamoxifen and the osteoporosis drug raloxifene have estrogen-like effects in some tissues but either no effects or antiestrogenic effects in other tissues in which estrogen receptors are present. Not surprisingly, research shows that isoflavones affect the expression of many genes differently than does estrogen (20).

Even categorizing isoflavones as SERMs does not fully describe the potential mechanisms by which isoflavones exert physiological effects because they (especially genistein) may affect signal transduction pathways by inhibiting the activity of enzymes (e.g., tyrosine protein kinase, mitogen-activated kinase, DNA topoisomerase) and regulating cellular factors that control the growth and differentiation of cells (21). It would be remiss also not to mention that isoflavones are frequently classified as endocrine disruptors, chemicals that alter the function of the endocrine system, and potentially cause adverse health effects (22). Consequently, isoflavones have become controversial although as discussed at the end of this article, the human safety-related data are very reassuring. In the evaluation of these data, it is important to recognize that the metabolism of isoflavones by rodents and monkeys, two species in which adverse health effects of isoflavones have been studied, differs dramatically from humans, making extrapolations of the findings difficult (23).

CHRONIC DISEASE PREVENTION AND TREATMENT

Cancer

The possible chemopreventive effects of isoflavones have been rigorously investigated for 20 years since the U.S. National Cancer Institute first funded research in this area. Initially, much of this research focused on cancer of the breast, in part because of the historically low breast cancer incidence rates in soyfood-consuming countries. There has also been much investigation of the role of isoflavones in reducing risk of prostate cancer, the rates of which are also low in Asian countries. Because these two cancers have been investigated most intensely, they are discussed later. However, it is worth noting that genistein inhibits the growth of a wide range of both hormone-dependent and -independent cancer cells *in vitro* (21). The *in vitro* concentrations required to inhibit cancer cell growth are typically much higher than serum isoflavone levels, but the animal data suggest that the *in vitro* anticancer effects may still be relevant (24). In fact, there is some evidence indicating that the *in vitro* data underestimate the *in vivo* anticancer activity of isoflavones (25).

Like all foods, the soybean contains many biologically active components including phytate, protease inhibitors, fatty acids, phytosterols, lunasin, and saponins. Many of these have been investigated for their chemopreventive properties; however, most evidence suggests that if, in fact, soy intake is protective against cancer, it is because of the presence of isoflavones (see discussions on breast and prostate cancer that follow).

In regard to breast cancer, a recently published meta-analysis by Wu et al. (26) that included only those studies with relatively complete assessment of dietary soy exposure in the targeted populations and appropriate consideration for potential confounders in the statistical analysis of the data found that there was a significant trend of decreasing risk with increasing soyfood intake. Compared with the lowest level of soyfood intake (≤ 5 mg isoflavones per day), risk was intermediate [odds ratio (OR) = 0.88, 95% confidence interval (CI) = 0.78–0.98] among those with modest (~ 10 mg isoflavones per day) intake and lowest (OR = 0.71, 95% CI = 0.60–0.85) among those with high intake (≥ 20 mg isoflavones per day). However, as recently reviewed by Messina and Wu (27) and Messina and Hilakivi-Clarke (28), there is an intriguing body of evidence suggesting that to derive protection against breast cancer, soy consumption has to occur during childhood and/or adolescence. All four epidemiologic studies that examined this hypothesis reported protective effects with reductions in risk ranging from 28% to 60%, whereas as discussed later, the clinical studies show that adult exposure does not favorably affect markers of breast cancer risk. In addition, in young rats, genistein exposure for just a few weeks reduces chemically induced mammary cancer by half, whereas exposure only during adulthood has no effect (29,30). Interestingly, adult exposure further reduces mammary tumor development in rats given genistein also when young.

Exposure to genistein causes breast tissue differentiation and reduces the number of terminal end buds, the anatomical structure within the rodent mammary gland that is the likely site of tumor development (29,31). There is evidence that these kinds of changes are also important

in humans (32). The notion that early isoflavone exposure is protective against later development of breast cancer is consistent with the school of thought that maintains early life events—such as becoming pregnant at a young age—profoundly impact breast cancer risk.

As noted previously, there is also interest in the isoflavone and prostate cancer relationship. In 2009, Yan and Spitznagel (33) systematically reviewed 15 epidemiologic studies on soy consumption and 9 on isoflavones in association with prostate cancer risk. The soy intake data yielded a combined relative risk (RR)/OR of 0.74 ($P = 0.01$). When separately analyzed, studies on non-fermented and fermented soyfoods yielded a combined RR/OR of 0.70 ($P = 0.01$) and 1.02 ($P = 0.92$), respectively. The isoflavone studies yielded a combined RR/OR of 0.88 ($P = 0.09$); however, when analyzed separately, the combined RR/OR for studies involving Asian and Western populations were 0.52 ($P = 0.01$) and 0.99 ($P = 0.91$), respectively. [Despite the ability to identify statistically significant associations between soy/isoflavone intake and health outcomes in epidemiologic studies involving typical Western populations, it is likely that because of the negligible intake, these associations do not have a causal basis (34).]

Clinical studies examining the effects of isoflavone-containing products on prostate specific antigen (PSA) levels show mixed results. In healthy men with normal PSA levels, isoflavones are without effect, whereas in prostate cancer patients who have been unsuccessfully treated for their disease, isoflavone exposure has been shown to slow down the rise in PSA levels in about half of the studies conducted. In addition, genistein markedly inhibits prostate tumor metastasis in mice (35), and a recent pilot study found that in prostate cancer patients, genistein exposure decreased levels of matrix metalloproteinase-2 transcripts in normal prostate cells from prostate cancer-containing tissue, suggesting genistein can inhibit cell invasion (36). Thus, although still very speculative, there is evidence that isoflavone exposure may both inhibit the development of prostate cancer and its spread. Because prostate tumors are slow growing and are typically diagnosed late in life, even modestly delaying tumor onset and/or growth can profoundly impact prostate cancer mortality.

Stroke and Coronary Heart Disease

In 1999, the U.S. Food and Drug Administration (FDA) approved a health claim for the cholesterol-lowering effects of soy protein (25 g/day was established as the threshold intake needed for cholesterol reduction) (37), although in late 2007, the FDA announced their intention to reevaluate the evidence in support of this claim because so much new data had been published since the claim was first approved. Current estimates of the hypocholesterolemic effects of soy protein are much lower than initially proposed, but there is relatively little evidence to suggest that isoflavones per se contribute to this effect or by themselves lower cholesterol. Nevertheless, there are preliminary but very intriguing data indicating that independent of effects on circulating cholesterol levels, isoflavones exert coronary benefits. If confirmed, these benefits will likely

prove to be of greater clinical value than of the cholesterol-lowering effects of soy protein.

Indirect support for the coronary benefits of isoflavones comes from several Asian epidemiologic studies showing that relatively modest soy consumption is markedly inversely related to risk of coronary heart disease and stroke. For example, a large prospective study involving nearly 65,000 women from Shanghai found that after a mean follow-up period of 2.5 years, the RR and 95% CI for nonfatal myocardial infarction was 0.14 and 0.04–0.48, respectively, for the highest versus the lowest soy protein intake quartile (38). Similarly, in Japan, a prospective investigation that included 40,462 women (40–59 years old, without cardiovascular disease or cancer at baseline) found that after 503,998 person-years of follow-up, the multivariable hazard ratios for the highest versus the lowest quintiles of isoflavone intake were 0.35 and 0.37 for cerebral infarction and myocardial infarction, respectively (39). The inverse associations were observed primarily among postmenopausal women. Finally, a recent case-control study in southern China that included 374 incident ischemic stroke patients and 464 hospital-based controls of both genders found that mean weekly soyfood intake was significantly lower ($P = 0.001$) among cases (89.9 g) than among control subjects (267.7 g) and that increased consumption of dried soybean, tofu, soymilk, and total soyfoods were associated with reduced risks of ischemic stroke after adjusting for confounding factors ranging from 0.18 to 0.56 (40).

Aside from the fact that in none of these studies was soy protein intake sufficiently high to, in theory, lower blood cholesterol, even if it was, the hypocholesterolemic effects of soy protein could account for only a small proportion of the protective effects observed. Admittedly, epidemiologic studies are not designed to show cause and effect relationships, and some of the observed protection could be due to the non-isoflavone soybean components; however, there are data (although inconsistent) showing that isoflavones might inhibit low-density lipoprotein cholesterol oxidation (41), enhance systematic arterial compliance (42) and flow-mediated dilation (43), reduce platelet aggregation (44), and inhibit smooth muscle cell proliferation (45). Of the many possible protective mechanisms, the effects of isoflavone-containing products on endothelial function have been studied clinically to the greatest extent, but the results of this research are quite mixed. Several explanations for this inconsistency have been proposed and two recent studies appear to have provided important insight in this regard. In one, benefits were observed only in those subjects with impaired endothelial function at baseline, and in the other, improvement was seen only when circulating isoflavones reached a threshold level that may not have been achieved in most studies. The results of a just-published meta-analysis support the former explanation (46).

Osteoporosis

The well-recognized skeletal benefits of estrogen therapy for postmenopausal women, both in terms of reducing bone loss and fracture risk, provide a theoretical basis for exploration of the possible skeletal benefits of isoflavones. The first rodent study demonstrating the soy isoflavone

genistein improved bone mineral density (BMD) was published in 1996 and the first clinical study showing isoflavone-rich soy protein favorably affected BMD was published 2 years later. Since then, more than 25 studies have examined the effects of isoflavone-containing products on BMD in postmenopausal women, although most trials were no longer than 1 year in duration. In general, it is recommended that such studies be conducted for 2–3 years. Also, many of the isoflavone studies were small in size involving fewer than 50 women per group.

An important exception to these design limitations was a 2-year trial conducted by Italian investigators. In this study, 389 postmenopausal osteopenic women were supplemented with vitamin D and calcium and either a placebo or 54 mg genistein (in aglycone form). At study end, spinal BMD increased by 5.8% ($n = 150$) and decreased by 6.3% ($n = 154$) in the active and placebo groups, respectively (47). Similar effects were noted at the hip. Although formally designed as a 2-year trial, approximately half of the subjects participated for a third year with the results showing even more pronounced differences between groups. However, in contrast to these, very impressive results are those from several recently published long-term trials that employed as the intervention product, either isoflavone supplements or isoflavone-rich soy protein. Most notable in this regard are the results of a 3-year trial by Alekel et al. (48) that included, in addition to the placebo group, two groups of postmenopausal women who were given either 80 or 120 mg/day isoflavones; only at the high dose did isoflavones exhibit even a modest benefit and only at the femoral neck. In addition, Weaver et al. (49) found, using novel ^{41}Ca calcium methodology, that high-dose isoflavone supplements very modestly decreased net bone resorption. Thus, at this point, the clinical evidence in support of the skeletal benefits of isoflavones is disappointing. There is no obvious explanation for the striking results observed by the Italian investigators in comparison with most other research, although it is notable that this trial is unique because the intervention product was isolated genistein (aglycone form), whereas the other trials used mixed isoflavones in glycoside form.

Finally, and considerably, more encouraging are the epidemiologic data. Both of the prospective studies to examine the relationship between soyfood intake and fracture risk among postmenopausal women found protective effects. In the Shanghai cohort, over the 4.5-year follow-up period, there were 1770 fractures among the 24,403 women (50), and in the Singapore cohort, there were 692 hip fractures among the 35,298 women over a period of 7.1 years (51). Interestingly, despite much lower isoflavone intakes in the latter study, both reported one-third reductions in risk when comparing high- with low-soy consumers. There are several possible explanations for the contrasting results between the epidemiologic and intervention studies including differences in exposure period (Asian adult soy intake assessed in the epidemiologic studies may reflect lifelong intake), soy components other than isoflavones may provide skeletal benefits, differences in response to isoflavones between Asians and non-Asians, and soy intake being a marker for a bone-healthy lifestyle. There is, however, no direct evi-

dence in support of these differences being contributing factors.

Menopausal Symptoms

Hot flashes are the most common reason given by women for seeking treatment for menopausal symptoms. For the majority of women who experience them, hot flashes begin prior to menopause and are severe and frequent in about 10–15% of these women (52). Although hot flashes usually subside after 6 months to 2 years (53), many women report having them for up to 20 years after menopause (54).

In 1992, Adlercreutz and colleagues (55) suggested that the low prevalence of hot flashes reported by Japanese menopausal women might be at least partially due to their high consumption of soyfoods. Speculation was that the estrogen-like effects of isoflavones might mitigate the drop in estrogen levels—one trigger for hot flashes—that occurs when women enter menopause. More than 50 hot flash trials evaluating the efficacy of isoflavone-containing products have been conducted. Over the past few years, several reviews and analyses of these data have been published, but with mixed conclusions. For example, a meta-analysis published in 2006 by Howes and colleagues (56) that included 11 trials found that isoflavones were modestly efficacious, whereas a more recent review by the Cochrane Collaboration (57) stated that there were no definitive data allowing such a conclusion to be reached, although five of the nine studies evaluated reported that the soy isoflavone intervention significantly alleviated hot flash frequency and/or severity. Similar conclusions were reached by Jacobs et al. (58) in their recent review.

It can be argued that even the inconsistent data are sufficiently encouraging to justify health professionals recommending the use of isoflavones because benefit can be subjectively determined and the overall improvement (including placebo response) in many studies in response to isoflavones is about a 50% reduction in the severity and frequency of hot flashes. This magnitude of response is likely to be viewed quite favorably by women seeking nonhormonal alternatives to estrogen for menopausal symptom relief. Several explanations for the mixed data have been proposed, including the variation in baseline hot flash frequency (59), interindividual differences in isoflavone metabolism, and the differing genistein content of the intervention products (see Fig. 1) (60).

In an attempt to provide some clarity about the effects of isoflavones on the alleviation of hot flashes, a team of investigators including those from the National Institutes of Health in Japan and the University of Minnesota conducted a systematic review and meta-analysis of the literature, although only studies evaluating the effects of isoflavone supplements derived from soybeans were considered. The overall results as presented at a recent meeting can be summarized as follows: seven of nine high-genistein isoflavone supplement studies and four of six low-genistein isoflavone studies reported significant benefit. High-genistein isoflavone supplements were more potent than low-genistein isoflavone supplements; the overall effect of the former was to reduce frequency and severity by about 19 and 32%, respectively,

beyond the placebo effect, which was approximately 50% greater than the effects of the low-genistein isoflavone supplements.

CONTROVERSIAL AREAS

Cognitive Function

The possibility that estrogen therapy prevents age-related declines in cognitive function and reduces risk of Alzheimer disease (AD) has spurred investigation of the cognitive effects of isoflavones. However, the role of estrogen in the etiology of AD has become less clear in recent years. One theory is that there is a window of opportunity to derive protection from estrogen therapy against AD; exposure in the early postmenopausal years is considered to be protective, whereas when exposure occurs during the later years, risk may actually be increased. With respect to soy, two epidemiologic studies have raised concern about possible detrimental effects.

One was a prospective study (begun in 1965 as the Honolulu Heart Study) published in 2000 that found that tofu consumption was associated with impaired cognitive function in Japanese men and women residing in Hawaii (61). More recently, an Indonesian cross-sectional study carried out in two rural and one urban (Jakarta) site among mainly Javanese and Sundanese elderly ($n = 719$; age range, 52–98 years) found that worse memory, as measured using a word learning test sensitive to dementia, was associated with high tofu consumption (measured by food frequency questionnaire, FFQ), whereas high tempe (a fermented whole soybean product) consumption was independently related to better memory, particularly in participants older than 68 years (62). The analyses were controlled for age, sex, education, site, and intake of other foods. In both studies, isoflavones were thought to be responsible for the observed effects.

In contrast to these two studies, however, are the results of a cross-sectional study conducted in Hong Kong involving 3999 men and women aged 65 years and older that found isoflavone intake was unrelated to cognitive function (63). Dietary intake was assessed using a 7-day FFQ, and cognitive function was assessed by the cognitive part of the community screening instrument for dementia. Furthermore, it is important to recognize the limitations of the Hawaiian and Indonesian studies. In the former, the intake of only 26 foods was assessed, questions about tofu intake were not consistent over the course of the follow-up period, and cognitive function was a secondary endpoint added near the end of the study. The results of the Indonesian study are unusual in that two isoflavone-rich soyfoods had opposite effects. The authors of this study suggested that the high folate content of tempe, but not tofu, was responsible for the differing findings. However, for several reasons, a more likely explanation, also discussed by the authors, is that tofu, but not tempe, contained formaldehyde—a known toxin shown to adversely affect memory in rodents. Formaldehyde is used as a preservative in Indonesia by the many small local manufacturers of tofu.

The three epidemiologic studies are clearly an insufficient basis for drawing conclusions about the effects

of isoflavones on cognitive function. Even if the epidemiologic evidence was more persuasive, definitive conclusions can only be reached on the basis of results from clinical trials. Since 2001, at least eight different trials have been published; most of these have involved postmenopausal women (64). These data provide some reason for optimism and certainly justify continued research in this area. Most trials found benefits in one or more aspects of cognition, but the data are not internally consistent, with studies often showing benefits in different aspects of cognitive function.

Soy Infant Formula

An estimated 22 million infants have used soy formula since 1960 (currently $\leq 15\%$ of U.S. infants use soy formula at some point in their development). Unquestionably, properly formulated soy infant formula promotes normal growth and development in term infants. Nevertheless, the estrogen-like properties of isoflavones have raised concerns about the effects of soy formula on longer-term development. Critics of soy formula argue that the possible untoward consequences may not be apparent until adolescence or early adulthood and point out that no studies have adequately evaluated this possibility. On a body weight basis, isoflavone exposure is 10-fold higher in infants consuming soy formula than it is in Japanese adults consuming a traditional diet; furthermore, Setchell et al. (65) found that mean plasma concentrations of genistein and daidzein in infants fed soy-based formulas were 684 ng/mL and 295 (61) ng/mL, respectively, level of magnitudes that are obviously higher than estrogen.

A comprehensive review published in 2004 accurately summarized views on the isoflavone content of soy formula by stating that “The evidence from laboratories showing biological activities at doses or tissue concentrations relevant to soy-fed infants is difficult to reconcile with the long record of uneventful use of these formulas” (66). This statement is similar to the current position of the American Academy of Pediatrics (AAP) that soy formula produces normal growth in term infants and that “. . . although studied by numerous investigators in various species, there is no conclusive evidence from animal, adult human, or infant populations that dietary soy isoflavones may adversely affect human development, reproduction, or endocrine function.” The AAP did, however, also emphasize that more infants are on soy formula than is medically justified.

Several governmental agencies have issued cautionary statements about the use of soy infant formula, although concerns are based exclusively on studies involving rodents, which likely poorly reflect the response of human infants especially when considering differences in isoflavone metabolism between the two species. Finally, a 14-member panel of independent scientists convened at the request of the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction and charged with evaluating the safety of soy infant formula concluded in late 2009 that there was minimal concern (the five levels of concern are negligible concern, minimal concern, some concern, concern, and serious concern) (67).

Breast Cancer Patients and Women at High Risk of Developing Breast Cancer

There is concern, based primarily on animal data, that the estrogen-like effects of isoflavones pose a risk to estrogen-sensitive breast cancer patients and women at high risk of developing this disease. The American Cancer Society stated in 2006 that the daily consumption of three servings of traditional soyfoods is unlikely to be harmful to breast cancer patients, but their review of this topic was quite cursory (68). It should be noted that the evidence in support of the hypothesis that estrogen-only therapy (as opposed to estrogen plus progestin therapy) increases breast cancer risk, or is harmful to breast cancer patients, is relatively weak (69). In any event, the human data are strongly supportive of the safety of isoflavones, regardless of whether they are derived from soyfoods or supplements. This evidence includes clinical studies showing that isoflavones lack effects—in contrast to combined hormone therapy—on breast tissue density and breast cell proliferation (69)—and have negligible impact on reproductive hormone levels (70). Furthermore, recently published epidemiologic studies indicate that soyfood consumption after a diagnosis of breast cancer either does not affect or improves the prognosis for patients and does not interfere with the efficacy of chemotherapeutic agents (10,71).

Thyroid Function

In *in vitro* and *in vivo* rat studies, isoflavones have been shown to partially inactivate thyroid peroxidase, an enzyme required for the synthesis of thyroid hormones. However, even though in rats, which are extremely sensitive to thyroid insults, thyroid peroxidase is inhibited, soy-containing diets allow normal thyroid function (72). Furthermore, and most importantly, a comprehensive review of the literature published in 2006 found that the clinical evidence clearly indicates that isoflavone exposure has no effect on thyroid function in euthyroid individuals (73). Studies published subsequent to this review support this conclusion (74). In many of these studies, isoflavone exposure was considerably higher than in typical Japanese adults consuming a traditional diet. In addition, although most studies were no more than 6 months in duration, several studies were 2–3 years long. Despite these findings, there are two relevant clinical situations related to soy and thyroid function, which has yet to be evaluated. One is the effect of isoflavone exposure in subjects with subclinical hypothyroidism—defined as elevated levels of thyroid-stimulating hormone but normal thyroxine and triiodothyronine levels. It is estimated that about 5% of U.S. postmenopausal women fall into this category. The second is individuals whose iodine intake is inadequate, as some animal data indicate that iodine deficiency could exacerbate any possible antithyroid effects of isoflavones.

The first situation can be fairly easily studied, but ethical concerns make study of the second unlikely. From a clinician's perspective, when individuals whose iodine intake is inadequate are identified, the appropriate recommendation is not to avoid isoflavones but to increase iodine intake. Finally, data suggest that soy protein may increase the dose of synthetic thyroid hormone needed to maintain therapeutic levels in hypothyroid patients, but

whether isoflavones contribute to this effect is unclear (75). This interaction can largely be avoided by separating the time at which the medication is taken from the consumption of soyfoods. Furthermore, the medication dose can be adjusted accordingly if necessary to accommodate any modest effect on absorption.

Fertility and Feminization

Although the large Asian populations seemingly argue against any antifertility effects, isoflavones have been shown to cause infertility in some animal species, such as the captive cheetah in North American zoos and the sheep in Western Australia grazing on a type of clover rich in isoflavones (76). However, problems in the former arose because felines are only poorly able to glucuronidate phenolic compounds, a major step in the bodily elimination of isoflavones in humans, which would lead to higher circulating level of total and aglycone isoflavones. In the case of the sheep, serum levels of equol far exceeded anything approaching human levels simply because daily isoflavone intake was estimated to be several grams.

More relevant to infertility concerns is the finding of a multivariate analysis from a pilot epidemiologic study involving 99 infertile men that subjects in the highest intake category of soyfoods had, on average, 41 million sperm/mL less than men who did not eat soyfoods ($P = 0.02$) (77). It is not clear that these findings have implications for fertility, however, because the inverse relation between soyfood intake and sperm concentration was more pronounced at the high end of the distribution (77). Furthermore, there were several weaknesses of this research that raise questions about the validity of the findings. For example, other than soy intake, no assessment of diet was made. In addition, much of the decrease in sperm concentration seems to have occurred because ejaculate volume increased which is why the total number of sperm was unaffected.

More importantly, the clinical data do not support this epidemiologic finding. Three clinical studies (78,79), one of which is available only as abstract (80), and one case report (81), have examined the impact of isoflavone exposure on sperm and semen parameters, and none have reported adverse effects even though isoflavone exposure ranged from that typical for Japanese men to 10 times this amount. Furthermore, the case report described an individual with oligospermia whose sperm concentration normalized after isoflavone treatment. In animals in which sperm and semen parameters had been adversely affected by isoflavones, circulating testosterone levels decreased dramatically. In contrast, a recent meta-analysis found that neither soy nor isoflavones affect circulating testosterone levels in men (82). Similarly, there is essentially no clinical evidence that isoflavone exposure increases estrogen levels (83). These hormone-related findings should help to allay concerns about both fertility and feminization.

Finally, soy and isoflavone intake have only minor and likely clinically insignificant effects on reproductive hormones in pre- and postmenopausal women (70). Although soy may increase the length of the menstrual cycle by approximately one day, ovulation is only delayed and not prevented. A fertility-related issue concerns the effect

of isoflavones on the developing fetus. Although rodent research has been conducted, this issue has not been directly examined in humans. However, it is noteworthy that the rodent fetus is exposed to relatively low endogenous levels of estradiol in comparison with humans; thus, any estrogen-like effects of isoflavones will likely be greatly exaggerated in the former compared with the latter.

RECOMMENDED INTAKES

No formal isoflavone intake recommendations from health agencies have been issued. In lieu of such, the findings from the clinical and epidemiologic studies as well as historical Asian intake can serve as a general guide. As noted, mean Japanese isoflavone intake from soyfoods among older individuals ranges from about 25 to 50 mg/day, whereas in Shanghai, intake may be somewhat higher. The mean intake is not necessarily the ideal, however, because as discussed, several epidemiologic studies measuring a variety of health outcomes show higher soy and isoflavone intake to be associated with lower disease risks. Isoflavone intake in the upper range is more than 50 mg/day. Clinical studies, such as those assessing effects on menopausal symptoms and endothelial function, generally have used isoflavone doses ranging between 50 and 100 mg/day. Because there is no historical precedent for consuming amounts above 100 mg/day, the totality of the evidence suggests that daily intakes between 50 and 100 mg are reasonable. Two to four servings of traditional soyfoods provide approximately this amount of isoflavones. Consuming this amount of soy is certainly consistent with the importance of eating a diverse diet that does not place undue emphasis on any one food.

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Isothiocyanates

Elizabeth H. Jeffery and Anna-Sigrid Keck

INTRODUCTION

Isothiocyanates (ITCs) are the hydrolysis products of a class of naturally occurring plant secondary metabolites called glucosinolates that are present in our diet in vegetables belonging to the plant genus *Brassica* (Family *Brassicaceae*, syn. *Cruciferae*), commonly known as crucifers or cruciferous vegetables. The term glucosinolate refers to a class of more than 100 S-glycosides that yield thiocyanate, nitrile, or ITC products upon enzymatic hydrolysis. Brassica plants have been used medicinally and as food for millennia. Medical conditions for which these plants have been used include gout, diarrhea, deafness, headache, wound healing, and mushroom poisoning (1). Today, the glucosinolates and their hydrolysis products are important because epidemiologic evidence and animal and cell culture studies indicate that a number of ITCs have potential for positive health effects, in particular for cancer prevention.

BACKGROUND

The plant family *Brassicaceae* is the single most common source of ITCs in our food supply; it embraces both vegetables, such as broccoli, and condiments, such as mustard (Table 1). The glucosinolate content, and therefore dietary ITC availability, varies greatly between different vegetable types, across cultivars and even across seasons and locations (Fig. 1).

The ITCs and their parent glucosinolates have gained significant attention, following their identification as the components likely responsible for lowered cancer risk in individuals consuming diets high in crucifers (2,3). The number of studies on the ITC sulforaphane, which is most abundant in broccoli, is far greater than that of any other ITC. Yet many other ITCs appear to have similar

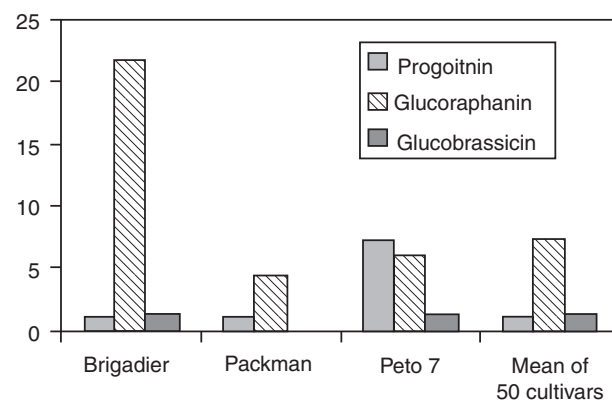


Figure 1 Variation in progoitrin, glucoraphanin, and glucobrassicin content ($\mu\text{mol/g}$ dry weight) in broccoli cultivars grown in one season. (View this art in color at www.informaworld.com.)

bioactivity to sulforaphane (4). As the health benefits of individual ITCs have become better recognized, extracts and concentrates of plant foods rich in glucosinolates or their ITC derivatives have started to appear on the market. However, there is little information supporting the efficacy of glucosinolates without conversion to ITC (5). Before safe and effective amounts of whole vegetables, sprouts, or ITC-containing dietary supplements can be determined, there is still important research to be performed on stability, bioavailability, metabolism, and mechanism of action.

BIOCHEMISTRY AND FUNCTION

In whole plant tissue, ITCs are found as inactive thioglycosides, termed glucosinolates. Over 100 different glucosinolates have been identified (6). Glucosinolates consist of a side chain or R-group, (Fig. 2) from one of several different modified amino acids and a β -D-thioglucose group linked to a sulfonated aldoxime moiety. The key amino acids methionine, phenylalanine, and tryptophan each give rise to a separate series of aliphatic, aromatic, and indolyl glucosinolates, respectively (6). In a few plant foods, such as white mustard (*Sinapis alba* L.), there is only one major glucosinolate. More commonly, plant species contain a characteristic mixture of several glucosinolates, although generally not more than three or four are present in substantial amounts (7). For example, broccoli contains

Table 1 Common Dietary Isothiocyanates, Their Parent Glucosinolates and Major Dietary Sources

Isothiocyanate	Parent glucosinolate	Major dietary source(s)
Allyl ITC	Sinigrin	Cabbage; brown mustard
4-hydroxybenzyl ITC	Sinigrin	White (yellow) mustard
Benzyl ITC	Glucotropaeolin	Garden cress, radish
Iberin	Glucobrassicin	Brussels sprouts, cauliflower, broccoli
Phenethyl ITC	Gluconasturtiin	Chinese cabbage, radishes, watercress
Sulforaphane	Glucoraphanin	Broccoli
Erucin	Glucorucin	Arugula

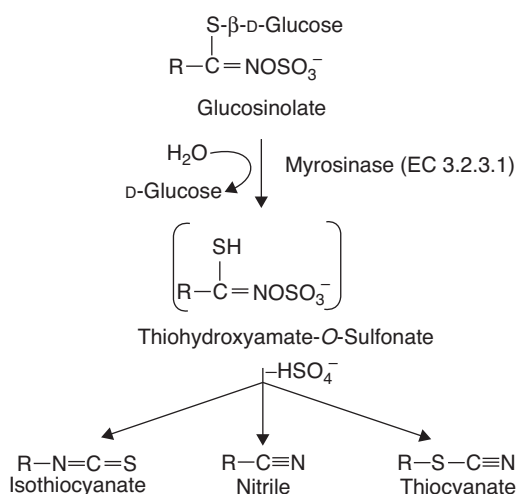


Figure 2 Bioactivation of glucosinolates. Hydrolysis of glucosinolates by the endogenous enzyme myrosinase to form glucosinolate hydrolysis products; nitriles, isothiocyanates, and thiocyanates.

at least a dozen glucosinolates, with the amount depending upon the variety; however, glucoraphanin, from which sulforaphane is derived, commonly makes up as much as 50% or more of the total glucosinolate content (7).

Intact glucosinolates are biologically inert, and ITCs must be released from their glucosinolate precursors to exert their activity. Glucosinolates are S-glycosides rather than the more common O-glycoside secondary metabolites found in many plants, and mammalian tissue has no thiohydrolase. However, both brassica plant tissues and mammalian gastrointestinal microflora contain thiohydrolase activity. In the plant, β -thioglucoside glucohydrolase, or myrosinase (EC 3.2.1.147), is physically separated from stored glucosinolates in specialized myrosin cells, while the glucosinolates occur in vacuoles in the plant cells. The two come into contact when the plant is crushed, such as during chewing (4). Myrosinase activity releases glucose along with an unstable intermediate, which can rearrange to form an ITC or one of several alternative products (Fig. 2). For example, when broccoli is crushed, glucoraphanin hydrolysis yields a mixture of bioactive sulforaphane and an inactive nitrile, depending upon the cultivar and conditions of growth and postharvest handling (8). A myrosinase cofactor, the epithiospecifier protein (ESP), supports nitrile formation by binding and removing the sulfur from the unstable intermediate (9). The ESP is more heat sensitive than myrosinase, and thus heating at 60°C can destroy ESP, leaving myrosinase to form only active sulforaphane (9). For the few glucosinolates, like progoitrin with a terminal unsaturation, the ESP can place this sulfur atom across the double bond, forming an epithionitrile. The ESP is absent from some plants that synthesize glucosinolates, so that no nitriles are formed upon hydrolysis (e.g., white mustard) (10).

Because myrosinase is heat sensitive, it can be inactivated by prolonged heating. Following ingestion of intact glucosinolates from boiled vegetables or heat-dried concentrates or extracts, the glucosinolates may be

hydrolyzed by gut microflora. Metabolites of ITCs that appear in the urine following ingestion of heat-inactivated vegetables attest to this activity (11). Studies have shown that oral antibiotics lower the production of urinary ITC metabolites, further confirming the role of the gut microflora in breaking the thioglucose bond (12). Some data suggest that although microflora support hydrolysis of dietary glucosinolates resulting in formation of ITC and enzyme induction in the colon, absorption of ITC from the lower gut may be insufficient to exert a systemic effect (13). Further details of hydrolysis by the gut microflora remain to be determined.

Cancer Prevention

Epidemiological studies strongly support an inverse association between dietary intake of fruits and vegetables and risk for cancer (14). Evidence for cancer prevention by ITC-containing cruciferous vegetables is stronger and more consistent than for fruits and vegetables in general, across many common cancers (2). A prospective study of diet and prostate cancer found a 41% lower incidence of prostate cancer in men consuming three or more servings of crucifers per week, compared with those consuming one or fewer servings (15). In an analysis of 7 cohort studies and 87 case-control studies, 67% of the cases studied showed an inverse association between total crucifer intake and cancer risk (2). Specifically, an inverse association between cancer risk and intake of cabbage, broccoli, cauliflower, and brussels sprouts was noted in 70%, 56%, 67%, and 29% of the studies, respectively. The cohort studies showed an inverse association between intake of cabbage, cauliflower, and broccoli and risk for lung cancer; total crucifer intake and risk for stomach cancer; and broccoli intake and risk for all cancers.

Cancer risk reduction by ITC in humans is supported by animal studies, using whole vegetables, juices, extracts, and pure ITCs, thus implicating ITC as the active component in epidemiological studies of whole vegetables (16,17). Administration of either sulforaphane or phenethyl ITC to rats protected against azoxymethane-induced colon cancer, given prior to or following the carcinogen (18). Similarly, a significant decrease in nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumor formation was seen when phenethyl ITC was fed to rats before and during the administration of NNK (19). More recently, in a study feeding rats a freeze-dried ITC-rich broccoli sprouts extract, there was a significant dose-dependent inhibition of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine-induced bladder cancer by 12 weeks (20). Rats given the extract showed decreased tumor development, and the number of tumors per rat dropped from 2 (control group) to 1.4 and 0.5 for the 40 and 160 $\mu\text{mol/kg}$ body weight groups, respectively.

Enhancing Elimination of Carcinogens

For many years, the primary cancer-preventative mechanism of ITCs was considered to be modulation of carcinogen metabolism, including suppression of bioactivation of carcinogens through inhibition of phase I detoxification enzymes (cytochromes P450) and enhancement of clearance of carcinogens through upregulation of phase II

detoxification enzymes. Today it is recognized that ITC can affect many systems (Table 2) in addition to the detoxification enzymes, although the upregulation of NAD(P)H:quinone oxidoreductase-1 constitutes a useful biomarker of efficacy of ITC. In cell culture studies, several ITCs, including phenethyl ITC and sulforaphane, have been found to inhibit activity of cytochromes P450 1A1, 2B1/2, 3A4, and others (21). Benzyl ITC has been shown to interact at the active site of P450 2B, to form a product that destroys the cytochrome (22). It is not clear if all ITC suppression of cytochromes P450 is by such a suicide mechanism. The upregulation of phase II enzymes has been studied in detail: most ITCs trigger upregulation of a battery of detoxification and host-defense genes, each bearing an antioxidant response element (ARE) in the promoter region (23).

The mechanism for upregulation of detoxification enzymes is via disruption of the interaction between cytosolic KEAP1 protein and the transcription factor nuclear factor-E2 p45-related factor 2 (Nrf2), through alkylation of KEAP1. This compromises the ability of KEAP1 to direct ubiquitination and degradation of Nrf2, thus allowing Nrf2 to accumulate in the nucleus, where it binds the ARE (32). Importantly, clinical studies confirm that broccoli sprouts, a rich source of sulforaphane, increase these detoxification enzymes (33,34).

A second response element, the xenobiotic response element (XRE), is associated with upregulation of an overlapping but distinct group of genes involved in detoxification, including cytochrome P4501A1. Hydrolysis products from glucosinolates derived from tryptophan (indolyl glucosinolates), but not from aliphatic glucosinolates, upregulate enzymes via the XRE (35). Indole-3-carbinol, the breakdown product of an unstable ITC formed from glucobrassicin that is present in most crucifers, may provide protection from estrogen-dependent cancers, through upregulating cytochromes P450, either directly or following acid condensation in the stomach to the potent metabolite diindolyl methane. In rats, administration of indole-3-carbinol decreased the risk for dimethyl benzanthracene-induced breast cancer and altered urinary estrogen metabolites (an increase in the ratio of 2-hydroxy to 16 α -hydroxy products) (36). Further-

more, crucifer-rich diets have been associated with similar changes in urinary estrogen metabolites in women (37). Clinical trials have been undertaken with purified indole-3-carbinol with at least partial success in causing regression of cervical intraepithelial neoplasia, a risk factor for cervical cancer (38). Other mechanisms possibly associated with protection against cervical intraepithelial neoplasia transitioning into cervical cancer include the loss of PTEN tumor suppressor gene function (39).

Mechanisms of Antiproliferation

In addition to enhanced clearance of carcinogens, cell culture studies suggest that ITCs slow proliferation, arresting the cell cycle and inducing cell death through apoptosis. For example, as little as 8 μ M benzyl ITC arrests cells in G₂/M, slowing the cell growth by 50% in BxPC-3 cells, a human pancreatic cancer cell line (40). In human colon cancer cells, sulforaphane arrested cells in G₂/M and increased cell death by apoptosis (41). Although ITCs appear to arrest the cell cycle in G₂/M in many cell types, there are significant differences among the cell-signaling pathways that ITCs modulate to slow proliferation and trigger apoptosis. For example, phenethyl ITC has been reported to induce apoptosis through a p53-dependent pathway in JB6 cells (42), but through an ERK-dependent pathway in PC-3 cells (43). In HT-29 cells, phenethyl ITC activated caspase-3 and c-Jun N-terminal kinase, resulting in increased apoptosis (44).

Some *in vivo* studies have also evaluated the effect of ITCs on the cell cycle and/or apoptosis. Rats given Brussels sprouts 48 hours after being administered the carcinogen 1,2-dimethylhydrazine exhibited increased apoptosis and decreased mitosis in colonic crypts (45). In a xenograft model, mice were implanted with PC-3 human prostate cells and given allyl ITC thrice weekly, which significantly inhibited tumor growth ($P = 0.05$), increased apoptosis, and decreased mitosis compared with controls (46). These findings suggest that ITCs cause increased apoptosis in whole animal models as well as in cultured cells.

Histone Deacetylase Inhibition

When histone deacetylase (HDAC) removes acetyl groups from nuclear histones, the result is a decreased ability to transcribe affected genes, therefore disrupting gene expression. HDAC overexpression has been implicated in some cancers (47) and thus inhibitors may be able to slow or prevent carcinogenesis. For example, HDAC inhibitors can trigger p21 expression, a regulator of p53 tumor suppressor activity and cell cycle progression. Modeling studies suggest that the cysteine conjugate of sulforaphane can slip into the active site of HDAC, causing competitive inhibition. Sulforaphane has been shown to inhibit HDAC activity in human colon (48), breast (49) and prostate (50) cancer cells, the APC-min mouse (27), and a mouse prostate xenograft model, as well as in human circulating peripheral blood mononuclear cells 3–6 hours after eating a single serving of 68 g broccoli sprouts (27). Feeding mice sulforaphane for 10 weeks resulted in an increase in global histone H3 and H4 acetylation and an increase in p21 (27). This latter study implies a long-term effect of HDAC inhibition, even though the mechanism proposed is of a competitive nature, and therefore

Table 2 Selected Enzymes Modulated by Isothiocyanates

Enzymes	Effect of isothiocyanate	References
Cytochrome P450 1A1/2	Decreased	21
Cytochrome P450 2B1/2	Decreased	21,22
Cytochrome 3A4	Decreased	21
Cytochrome 2E1	Decreased	21
Glutathione reductase	Increased	23,24
Glutathione S-transferases	Increased	16
γ -glutamylcysteine synthetase	Increased	23,25
Heme oxygenase 1	Increased	26
Histone deacetylase	Decreased	27
Multidrug resistance protein (MRP-1)	Decreased	28
NAD(P)H:quinone oxidoreductase	Increased	29
P-glycoprotein	Decreased	28
Thioredoxin reductase	Increased	30
Thioredoxin	Increased	31
UDP-glucuronosyltransferases	Increased	23

might be expected to be temporary. A very recent study has identified destabilization of the androgen receptor in prostate cancer cells and that this may occur due to acetylation of the androgen receptor chaperone HSP60, following HDAC inhibition (51).

A Coordinated Mechanism

For many years, ITCs were considered to be anticarcinogenic solely because they increased clearance of carcinogens. More recent developments show that ITCs disrupt the cell cycle and cause apoptosis, suggesting a role for ITCs in reversing or slowing carcinogenesis, not just blocking initiation. ITCs are electrophilic and able to bind reversibly to thiols. One possibility is that all the mechanisms described earlier are triggered by a common primary event, such as a change in redox status within the cell (52). Redox change is essential for passage through the cell cycle (53). Key players in cellular redox control are glutathione (and the ratio of reduced to oxidized glutathione) in cytosol and thioredoxin in the nucleus. Glutathione synthesis is induced, and expression of thioredoxin and thioredoxin reductase are both upregulated by ITC (25,30). Furthermore, two central transcription factors, AP-1 and NFkB (see later), are both redox sensitive and data are accumulating suggesting their inhibition by sulforaphane and other ITC (54,55). A recent publication shows that sulforaphane, given to rats prior to the chemotherapeutic drug cisplatin, attenuated cisplatin-induced renal dysfunction and structural damage, together with the associated oxidative/nitrosative stress. Glutathione depletion was attenuated, as was the loss of renal antioxidant enzymes (catalase, glutathione peroxidase, and glutathione-S-transferase), a clear indication of the ability of sulforaphane to counter oxidative damage (56). However, prior to initiating adjuvant therapy with sulforaphane, it will be necessary to determine how these changes in redox and glutathione availability have impacted the therapeutic effect of cisplatin.

Chronic inflammation is associated with increased risk for cancer (57). A number of studies have shown that sulforaphane can attenuate lipopolysaccharide (LPS)-induced inflammation in RAW264.7 cells (mouse macrophages), implicating the Nrf2 pathway and disruption of NFkB, resulting in depressed response to LPS in IL-6 and TNF- α release, inducible nitric oxide synthase (iNOS) activity, and cyclooxygenase (COX)-2 expression (58). Benzyl ITC has been shown to inhibit iNOS and COX-2 (59). Recently, sulforaphane was reported to suppress LPS-induced inflammation in primary rat microglia, implicating ITC in possible protection against cognitive deterioration of aging (60). Whether redox regulation and control of oxidative damage are central to prevention of inflammation, and indeed to all effects of ITCs, remains to be determined.

PHYSIOLOGY

Absorption and Metabolism

Few studies have evaluated the absorption of ITCs directly. In a clinical study where a jejunal segment was experimentally isolated in situ using a doubly cuffed gavage and intestinal perfusion, 74% of a dose of sulforaphane

given as an extract of broccoli was absorbed from the jejunum (61). In the absence of active myrosinase, hydrolysis of glucosinolates and release of ITC relies on colonic microbiota and apparently provides a far poorer yield. In a small clinical study feeding extracts of heated and unheated broccoli sprouts, the 24-hour ITC recovery in urine was 71% from a raw sprouts extract, but only 18% from the heated extract containing intact glucosinolates and no myrosinase (62). Similarly, 24-hour recovery of allyl ITC in human urine following a meal of raw and cooked cabbage was 36% and 15%, respectively (63). Also, consumption of fresh and steamed broccoli resulted in a 32% and 10% ITC recovery, respectively (11). More recently, ITC recovery from raw crushed broccoli was shown to be 35% versus 3% from cooked crushed broccoli (64). The peak plasma concentration was delayed in those receiving the cooked broccoli (peak at 6.1 hours postingestion) compared with those receiving raw (peak at 1.6 hours), suggesting that when hydrolysis is dependent on the gut microbiota, the ITCs released are both fewer and much later than when the plant myrosinase is active. Following hydrolysis, metabolism of ITC is by conjugation to glutathione and subsequent metabolism to mercapturic acid, the *N*-acetyl cysteine conjugate of ITC (65). As binding to glutathione is reversible, conjugation can also act as a transport system for bioactive ITCs, which, upon reaching peripheral organs, might be released, ready to induce enzymes that protect against carcinogenesis (66). Urinary ITCs and their *N*-acetylcysteine conjugates have been used successfully as biomarkers of crucifer intake (65).

Plasma and Tissue Concentrations

In humans consuming an extract of 3-day-old broccoli sprouts containing 200 μ mol ITCs, the ITCs were absorbed rapidly, and the peak concentrations were 2.0 ± 0.3 and 1.2 ± 0.4 μ M in plasma and red blood cells, respectively, at 1 hour postfeeding (67). Tissue ITC concentration declined with first-order kinetics and a half-life of 1.8 ± 0.1 hours. Renal clearance rate was 369 ± 53 mL/min, and the cumulative urinary excretion of total ITCs was 58% of the amount ingested. Consumption of 100 g watercress resulted in a peak plasma concentration of 0.93 ± 0.25 μ M phenethyl ITC, 2.6 ± 1.1 hours postconsumption (68). The half-life for phenethyl ITC was found to be 4.9 ± 1.1 hours. Whole glucosinolates are also absorbed, although they are mostly then excreted unchanged into the bile, for hydrolysis in the colon (69).

Variability Factors

A growing number of epidemiological studies show significantly decreased risk for cancer in individuals consuming a diet rich in crucifers. Yet, not all epidemiological studies show this effect. One cause for this may be nutrigenomic in nature: some individuals, because of their genetic profile, appear to respond particularly well to a diet rich in crucifers, whereas others do not respond as well. A recent study of breast cancer risk found no benefit associated with dietary crucifers in the full population, but the risk for breast cancer was cut virtually in half by high crucifer consumption in a subset of women who were missing the detoxification enzyme glutathione S-transferase M1 (GST-M1), and who are typically at far greater risk

for breast cancer than the general population (70). Polymorphisms of GST enzymes are common. These enzymes are responsible for glutathione conjugation of both carcinogens and ITCs. Therefore, it can be expected that an individual with a mutant form of the enzyme that lacks activity (null) will excrete fewer carcinogens and fewer ITCs, exaggerating effects of both (71). Epidemiological findings are consistent with this idea: People bearing null genotype for certain GSTs exhibit increased risk for a number of cancers, but also respond more positively to a high crucifer intake, reversing that risk more than for individuals with functioning GST enzymes. A study of lung cancer risk found a 64% decrease in risk for GST-M1 null individuals compared with the general population, and a 72% decrease in risk for those who were both GST-M1 and GST-T1 null (72). It is interesting to note that sulforaphane, one of the more potent ITC, is also one of the poorest substrates for GST, again suggesting that low GST metabolism may reflect prolonged duration in the body and greater bioactivity (73).

The variation in plant content of glucosinolates can be a confounding factor in epidemiological studies (Fig. 1) (7). Even though the profile of glucosinolates remains characteristic of a plant species, the absolute amount of individual glucosinolates in a given vegetable can vary with genotype (plant variety) and with environment (pre- and postharvest conditions). The variation in content of the aliphatic glucosinolates such as glucoraphanin is mostly described by genotype, whereas the variation in indolyl glucosinolates such as glucobrassicin is mostly described by environment (74). Seeds often have much higher concentrations of glucosinolates than other plant parts such as the leaf or floret (Table 3). This property has been harnessed to provide a high-glucosinolate dietary supplement of sprouted broccoli seeds (75). As the seedling grows, new tissue dilutes the existing glucosinolate pool, so that by 10 days of growth, the concentration is similar to that of the fully grown plant (75). There are no studies comparing the glucosinolate profile and yield of ITCs in differently aged plants. Processing and storage of whole

plants, concentrates, and extracts can also greatly affect the final content of glucosinolates and/or ITCs. For example, glucosinolates are water soluble and can be lost during cooking.

For all these reasons, the content of bioactive components in a crucifer meal is hard to predict. In contrast, use of a single genotype to produce specific age sprouts under set conditions overcomes much of this variability, allowing easier use in clinical trials (33). Also, although it would be possible to quantify one or two components in each lot of a dietary supplement, most ITC are highly reactive and thus cannot readily be stored. A further confounding issue is that ITCs may have greater bioavailability when provided within the original vegetable matrix than in purified form, or even when added back to a vegetable matrix (64). In light of this, sprouts or seedlings of a known variety that are grown under controlled conditions for a set period of time may be one of the most effective ways to provide a product with a consistent ITC content.

INDICATIONS AND USAGE

Food Sources

The source and amount of ITCs or their parent glucosinolates in human diets vary among cultures and countries. Primary dietary sources in North America include cabbage and broccoli (79). The Japanese consume substantial amounts of wasabi, a Japanese horseradish preparation, as a condiment with sushi, in addition to radishes and cabbage, and the Koreans consume kimchi, a fermented cabbage or cabbage/radish product with almost every meal (4). Per capita consumption of crucifers by the Japanese is four- to fivefold greater than by Americans. Consumption in Europe varies greatly, with the United Kingdom being similar to that of the United States, whereas consumption in Spain is minimal. An estimate of consumption can be gained from the United States Department of Agriculture–National Agricultural Statistics Service,

Table 3 Total Glucosinolates and Per Standard Serving in Various Plant Parts of Cruciferous Vegetables and Condiments

Plant	Range of total GS (mg/g fresh weight)	Plant part	Standard serving (g) ^a	Total GS/standard serving (mg)
Broccoli	0.45–1.48 (9) ^b	Floret	88	40–130
Broccoli sprouts	0.80–4.46 (3)	Seedling	17 ^c	14–76
Brussels sprout	0.60–3.90 (29)	Leaf	88	53–343
Cabbage, savoy	0.47–1.24 (7)	Leaf	70	33–89
Cabbage, red	0.41–1.09 (11)	Leaf	70	29–76
Cauliflower	0.27–0.83 (8)	Floret	100	27–83
Chinese cabbage	0.17–1.36 (18)	Leaf	70	12–95
Horseradish	5.00 (2)	Root	15	75
Mustard, brown	51.30–106.70 (6)	Seed	11.2	575–1195
Mustard, white	45.1–82.3 (7)	Seed	11.2	505–921
Radish, white	0.07–0.21 (6)	Root	116	8–24
Turnip	0.20–1.41 (6)	Root	130	27–183
Watercress	0.36–0.54 (76)	Plant	25	9–30

^aStandard serving, from USDA National Nutrient Database for Standard (77).

^b() Number of varieties analyzed.

^cNo serving size in the database; used 1/2 cup value for alfalfa sprouts.

Source: Adapted from Refs. 6,78.

based on disappearance of the vegetables from the store shelf. These data suggest that U.S. *per capita* consumption of crucifers for 2008 was 16.5 lbs: roughly one-third cup or 20 g/person/day (79). Glucosinolate content of crucifers commonly consumed in the United States varies from 0.5 to 2.0 mg/g fresh weight, giving an average daily consumption in the range of approximately 10–40 mg glucosinolates/day/person (6,7,78). A study of women in Shanghai revealed an average crucifer consumption of 98 g/day, primarily as bok choy, providing approximately 40–100 mg glucosinolate/day/person (70).

Optimum Intake

A number of clinical studies show that as little as three to five servings (3–5 cups fresh or 1.5–2.5 cups cooked) of crucifers a week may significantly lower the risk of developing cancers (15,80,81). A prospective dietary assessment study found that three or more servings of crucifers per week, as compared with one or fewer, lowered prostate cancer risk by 41% (15). In two other studies, five or more servings of crucifers per week, as compared with one or fewer, lowered bladder cancer risk by 51% (80) and compared with two or fewer servings per week, lowered non-Hodgkin lymphoma risk by 33% (81). These data suggest that the amount of ITCs needed for crucifers to prevent cancer is achievable in a normal healthy diet.

Range of Health Benefits from Isothiocyanate Consumption

Reviewing the literature on crucifers and cancer, the strongest data are for prevention of initiation of cancer, possibly because most studies are designed to evaluate prevention of initiation. Increasingly, studies show that ITCs have the ability to protect against cancer promotion and progression by slowing the growth of tumor cells, arresting the cell cycle, and increasing apoptosis.

There are a few studies of effects of ITCs on health outcomes other than cancer prevention. Sulforaphane may antagonize the growth of *Helicobacter pylori*, the bacterium considered responsible for stomach ulcers and progression to stomach cancer (82). In a spontaneously hypertensive strain of rat, feeding dried broccoli sprouts daily for 14 weeks decreased oxidative stress, increased glutathione levels, increased endothelial-dependent relaxation of the aorta, and significantly lowered blood pressure. There was little or no change in these parameters in healthy Sprague–Dawley rats (24). More recently, sulforaphane was found to protect against ischemic injury in isolated rat hearts (83). These data, as with the nutrigenetic data on GST described earlier, suggest that crucifers may provide the greatest efficacy to individuals at highest risk, by normalizing physiological parameters. Sulforaphane and phenethyl-ITC as well as the whole foods from which they are derived have generated interest as possible cancer therapies in addition to lowering risk. To be considered as therapies, they must undergo complete safety and efficacy studies, as with any drug under development. Published studies to date have focused almost exclusively on efficacy, with little focus on adverse side effects.

ADVERSE EFFECTS

In animals, very high dietary levels of glucosinolates and their ITC and nitrile hydrolysis products have been shown to have some toxic effects. Crucifers contain goiter-promoting, thyroid-enlarging compounds such as thiocyanate ion and the cyclized ITC from progoitrin hydrolysis, goitrin (5-vinylloxazolidine-2-thione) (6). The latter has not only proven a problem in livestock, but goiters were observed in Europeans between World Wars I and II, when in famine-stricken areas, crucifers were almost the only food available (84). Pigs and chickens fed chow containing 40% crushed rapeseed meal as the sole source of protein exhibited reduced growth and pancreatic, liver, and kidney damage. Rape and crambe meals can contain substantial amounts of an epithionitrile and the nitrile crambene from progoitrin hydrolysis, in addition to goitrogenic products. Rapeseed also contains erucic acid, a fatty acid that has been associated with cardiac toxicity. Erucic acid is present in a number of crucifer seeds, including broccoli seeds, although it is absent from seedlings and whole plants. However, controlled toxicity study has not been carried out using seed meal from cruciferous vegetables. Adverse effects have not been reported in healthy humans with balanced diets, even with high intake of crucifers. Feeding subjects with extracts of raw or cooked broccoli sprouts, equal to 4 or 50 g fresh weight, respectively, caused no adverse effects (62). The urinary bladder may be the most sensitive organ to ITCs: In rats, phenethyl ITC (0.1% in the diet, ~75 mg/kg/day) and benzyl ITC (~80 mg/kg/day) both caused preneoplastic changes in the urinary bladder in as little as 2 weeks, inducing bladder cancer when treatment was extended (85,86).

CONCLUSIONS

Preclinical and epidemiological data supporting an anticancer effect of crucifers and their bioactive ITCs are compelling. However, there is little concrete information from small, controlled clinical trials about effective dose or frequency of intake to gain optimal health benefits (87). Furthermore, ITCs are highly reactive and unstable and research is needed to develop stable ITC preparations. Studies on bioavailability, pharmacokinetics, and interaction between ITCs should accompany any new preparations. Extracts and concentrates from crucifers are appearing on the market as dietary supplements. Although the risk for adverse effects appears low, there are three possible areas for concern. One is that processing conditions may destroy the active ITCs; a second, related concern is that processing might favor production of nitriles and epithionitriles, compounds that typically lack the efficacious effects and may, in large quantities, be associated with toxicity in liver and kidney. A third concern is that many supplements on the market today contain intact glucosinolates without myrosinase present, and their efficacy has not been evaluated. In summary, data on efficacy of ITC in animal cancer prevention studies are strong, and small clinical studies are emerging. The time is right for clinical trials of purified and semipurified ITCs as well as whole vegetables.

COMPENDIAL/REGULATORY STATUS

Cruciferous vegetables and condiments containing ITCs have traditionally been part of our diet and have assumed GRAS (generally recognized as safe) status. Processing to form extracts or purified ITCs to be sold as dietary supplements is within the scope of the Dietary Supplement Health and Education Act of 1994. Glucosinolates, ITCs or freeze-dried plant material may have different regulatory status in the United States and elsewhere. For example, Canada might regulate freeze-dried broccoli sprouts in capsules as natural health products, whereas purified glucosinolates might need to go through a drug approval process. In the United States, any ITCs chosen for development as potential drugs will be required to undergo full preclinical and clinical toxicity testing. There is no evidence to suggest that ITCs change their bioactivity when extracted and purified. However, bioavailability may change, giving an apparent change in efficacy. It may be possible, through processing, to inadvertently favor nitrile or even epithionitrile formation during hydrolysis, leading at best to loss of health-promoting ITCs, at worst to potential liver or kidney damage.

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Kava

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INTRODUCTION

Kava, the common name of the plant *Piper methysticum* G. Forst., is native to many South Pacific islands. The term kava and the variant kawa are used for both the plant and the traditional beverage prepared from its roots. However, some other names or variants are also encountered. In Hawaii and other parts of Polynesia, it is known as *awa* or *ava* (which may mean “bitter,” “sour,” or “sharp”). The conventional method of preparing kava for human consumption is to make an aqueous extract of the lateral roots. Although some supplements are made in this manner, the majority is prepared through large-scale commercial extraction processes using organic solvents or supercritical fluid (CO₂) extraction. While the method of extraction changes the relative proportion and concentration of the chemical components responsible for kava’s therapeutic action, the components themselves remain the same and are referred to as kavapyrones, or, more commonly, as kavalactones.

There exists many legends and myths for the discovery of the ceremonial and medicinal use of kava by native populations. In the South Pacific, where kava is a native plant, it was, and still is, used ceremonially for its relaxing, mood calming effects and has long been utilized to promote dispute resolution in group settings. The kava custom has been so widespread throughout the South Pacific that it might be considered the one item in their material culture that linked together most of the peoples of this region of the world (1). Balick believes that the use of kava is an important cultural keystone practice in many Pacific islands, which defines and creates a deeply rooted history of the culture (2). Once it was introduced to Europe, it became the subject of intense scientific study and was soon adapted for use by the medical community. The most notable of kava’s medicinal qualities is its anxiolytic effects and, in higher concentrations, its extremely relaxing effects (3).

Kava preparations have been consumed for decades, and modern clinical trials have been conducted reporting its efficacy at treating anxiety disorders, as well as other trials reporting *some* adverse health effects *when kava is consumed in large quantities and depending on the quality of the source*. Over a 12-year period between 1990 and 2002, a number of cases of hepatotoxicity, some involving liver transplants, were associated with kava consumption. These reports prompted a number of countries to withdraw drug registrations related to kava and/or ban its sale. The causality of case reports of liver toxicity associated with kava consumption is still a matter of scientific debate and has been the subject of numerous

scientific meetings and publications. However, a toxicological review of kava-associated hepatic adverse event reports from Europe and the United States conclude that, based on all the available data, “there is no clear evidence that the liver damage reported in the U.S. and Europe was caused by the consumption of kava” (4). [Continuous updates on the most recent developments in kava research are available (5).]

BACKGROUND

Botany

A member of the family Piperaceae, kava is a long-lived, slow-growing shrub that can grow to more than 6 m in height. It has 10–15 or more upright branches that spread 2–4 m across. As the plant ages, the stems can reach 10 cm in diameter. The leaves are heart shaped; suborbicular-ovate to 25 cm long and somewhat narrower in width, to 20 cm; acuminate; and deeply cordate at the base, containing nine to thirteen radiating veins. The inflorescences are solitary spikes growing to 7.5 cm long. It does not produce seeds (sterile) and is generally propagated by planting of its stalks (Fig. 1). The part of greatest interest to humans is the roots because they are used to prepare beverages and extracts. The roots are stout brown structures that when split open are yellowish in color (2); they can be harvested after the plant reaches 2–3 years in age and grows to a height of approximately 2–3 m. The plant grows well in lowland and upland forests (Fig. 2).



Figure 1 Kava plant growing in the island of Pohnpei, Federated States of Micronesia. Source: Photo courtesy of Michael J. Balick.



Figure 2 Kava (*sakau*) growing in lowland agroforest in Pohnpei. Source: Photo courtesy of Michael J. Balick.

Consequently, local varieties (cultivars) with specific properties have been carefully selected and developed for ceremonial, medicinal, and commercial purposes throughout the Pacific Island societies. More than 100 distinct cultivars have been identified; Lebot and Levesque (1989) noted 82 cultivars recorded on Vanuatu, 11 on Fiji, 8 on Tonga, 14 on Tahiti, and 2 on Pohnpei. Often, these differ not only in their physical appearance but also in their effects when consumed by humans. Physical differences include the shade of leaf color and, more importantly, for taxonomic purposes, the color of the stem (purple to brown and presence of dark spots) and internode space between stem joints (Fig. 3). Desirable medicinal properties range from appropriate for daily consumption to limited use only for specific symptoms.

The authors, Balick and Herrera, have been part of a team that has carried a great deal of research on the importance and use of kava in Pohnpei, Federated States of Micronesia. The major kava (or *sakau* in the Pohnpeian language) cultivar in Pohnpei is known locally as *Rahmwahnger* and can be distinguished by a series of purplish or blackish spots on the stem. The second cultivar, known as *Rahmedel*, is characterized by a smooth, unspotted

stem. The most common variety of *sakau* in use today is *Rahmwahnger* (95% of all preparations), perhaps because the local people report that it is stronger, with the effects lasting much longer than the second cultivar. Root stock is currently harvested as a cash crop in many other locations, including the islands of Fiji, Hawaii, Samoa, Tonga, and Vanuatu.

Botanical Origin

Lebot et al. (3) suggested that cultivated *P. methysticum* derives from a wild progenitor, *Piper wichmanii* C. DC., a fertile *Piper* indigenous to New Guinea, the Solomon Islands, and Vanuatu. In other words, *P. methysticum* is a human creation (<2500–3000 years old) because it consists of sterile cultivar clones ultimately from *P. wichmanii* in an ongoing selection process, which was spread throughout the Pacific Islands through back migrations and voyages. Micronesia along with Papua New Guinea and Polynesia are suggested as sites of relatively late introductions.

Ethnobotany and Traditional Uses

There exist many different local legends and myths from all parts of the Pacific Islands in which kava is introduced—many of them relate to the plant's origin and ritualistic use as a beverage. Interested readers are directed to Refs. 1 and 2 for further reading.

Consumption of kava transports drinkers to the realm of ancestors and gods (3). Although the significance of kava varies from island to island within the Pacific Islands, everywhere it serves (or served) as a means of religious inspiration. Kava is (was) an integral part of each island's religious, economic, political, and social life.

An extensive review on the ceremonial and traditional history of use of kava in other Pacific islands is provided by Singh (1). According to Balick and Lee (2), *sakau* (kava) is a species so tightly woven into the traditional practices of Pohnpei that it has become an integral part of Pohnpeian culture, "with no palpable boundary between culture and plant." *Sakau* represents ultimate respect, higher than the highest chief and even higher than the most important of the ancestral spirits, both good and evil. *Sakau* brings peace and community to the island, holding the cultural traditions together in face of some of the destabilizing effects of globalization.



Figure 3 Two different cultivars of kava in Pohnpei: *Rahmwahnger* (distinguished by black spots on the stem) and *Rahmedel* (smooth-stemmed). Source: Photos courtesy of Michael J. Balick.

There is a broad range of traditional uses for *sakau* in Pohnpei. The principal use is for a beverage that is used ritually, ceremonially, and in contemporary times, recreationally. From an ethnomedicinal perspective, the leaves, stems, and roots are important local medicines used in traditional healing to treat both physical and supernatural (culture-bound) ailments for many different conditions. Preparing *sakau* for drinking can be done in many different ways, depending on the use, and it involves methodologies that are particular to the island, such as squeezing the freshly pounded roots with a press made from the inner bark of another plant, *Hibiscus tiliaceus* L. (*keleu* in Pohnpeian) (2).

The process begins with the harvest of the plant from the field. Larger and older plants are saved and harvested for special events such as a feast or tribute. The entire shrub is dug up and brought into the *nahs* (feast house). The roots are removed from the stem with a knife, split into smaller pieces, dipped in water, and carefully scrubbed with a brush to remove all of the remaining earth and contaminants. These are then placed to one side as the pounding stone is prepared. The stone is a special piece of basalt which is chosen for its shape, size, and tone—a special sound will resonate throughout the area when the pounding has started—and is found in a limited number of areas on the island and passed down from family to family. The smaller river stone is used to pound the roots while they sit on the larger, flat pounding stone. This rounded stone is made smooth by many years in a riverbed and is the size of an orange. It is selected to fit well within the palm of the hand for extended periods of pounding. In a more formal, ritualized setting, the pounding stone will have leaves of *Alocasia macrorrhizos* (L.) G. Don covering it and four leaves on the floor around it (2). The purpose of the leaves is to catch any pieces of root falling to the ground during the pounding ceremony, thus keeping it clean and “sacred.”

Another participant of the community cuts a stem of the *keleu* tree, peels off the inner bark, slices it into strips, and ties it into a press on which the pounded roots will be placed. The *keleu* used must have a rich slimy film of exudates on the inner bark to infuse into the beverage as it is squeezed from the press into half a coconut shell (~100–150 mL).

Sakau drinkers maintain that this potentiates the effect of the *sakau*, making its effect stronger and longer lasting. The first four cups are presented to the highest-ranking elders in the room as a sign of respect. Guests from outside the island usually are recognized for their presence and can be accorded one of the first cups. Persons presented with the cup accept it with both hands, close their eyes, raise it to their lips, and drink a portion. Pohnpeians believe that when the eyes are closed, the spirits of the *sakau* cannot enter the body through the eyes, nor can the vapors from the drink cause injury to this sensitive part of the body (2).

Drinking can last for several hours, during which time the conversation, jokes, and even singing begin, eventually tapering off to silence and deep thought as the effects of the *sakau* take hold of the drinkers. A kilogram or so of root will yield 20 or more cups of “stone” *sakau*, while that found in the market is usually much more diluted, depending on the vendor. At current prices on Pohnpei

(2008), a clump of fresh roots sells for about \$2.50 per lb (2).

Interestingly, the former method of preparing kava beverages elsewhere in the Pacific involved chewing the freshly harvested roots and spitting them into a bowl. The mixture was then diluted with a small amount of water, divided, and served. However, that practice has given way to soaking the finely ground root with water or coconut milk for several hours, followed by filtering prior to consumption. Because the active constituents are more soluble in organic solvents, commercial preparations are generally obtained by extracting the roots with alcohol, acetone, or supercritical carbon dioxide.

Ethnomedicine

Hawaiians used kava to counteract fatigue, sooth the nerves, reduce weight, induce deep relaxation and sleep, treat asthma and rheumatism, and for congestion in the urinary tract. In addition, throughout the Pacific, kava has a long history of use as a treatment for urinary tract infections and gonorrhea. It was widely believed that drinking the extract would not only relieve the symptoms associated with gonorrhea but ultimately result in a cure (1). Moreover, kava extracts have also long been used as a natural remedy for the relief of symptoms associated with menopause and for the relief of pain and inflammation associated with arthritis, chronic gout, menstruation, and toothache (1). In Pohnpei, *sakau* has been used to treat stingray stings, cuts, as well as a culture-bound syndrome called *soumwahu en insensuwed* (Pohnpeian for “sadness” or “depression”) (2).

The Effects of Sakau

According to Balick and Lee (2), the effects of drinking *sakau* “can be divided into two main phases: the initial stage, where a person is uplifted, relaxed, conversational, and communal, and the latter stage, where the person turns inward, becomes quiet, tired, of unsteady gait, and surrenders to an overwhelming feeling of hyperrelaxation and sleep.” The physiological effects of the *sakau* experience starts with a numbing of the lips and tongue, as soon as the beverage is first sipped from the cup, manifesting its rapid anesthetic property. Then, “conversations, jokes, and singing” (2) become evident, as it has anxiolytic properties. *Sakau* is also a potent muscle relaxant, and in the later hours of continued consumption, “there is loss of muscle control—some people, not all, have great difficulty standing up and walking home—and vision can also be affected. However, consumers report that their minds and thought processes become very clear. Eventually, the person goes to sleep (sedative property)” (2).

In Pohnpei, alcohol is commonly consumed after drinking *sakau*, a practice known as *kapohpo*. People will drink a few cans of beer or a six-pack, noting that it helps to settle the stomach, gives them strength, and helps with the kava dermatopathy that comes with much *sakau* drinking (2). Alcohol is a central nervous system depressant, and its use is not advised when medicinal preparations of *sakau* are taken for therapeutic purposes (4). Balick and Lee (2) spoke to physicians in Pohnpei who suspect that the practice of *kapohpo* may be linked to a sudden death syndrome that they have recorded among people who drink *sakau*.

Chemistry and Preparation of Products

The interest in kava extracts in Western medicine began following its first description in 1777 by naturalist Johann George Forster. Forster, then 18 years old, and his father, Johann Reinhold Forster, served as naturalists aboard the H.M.S. Resolution during Captain James Cook's second voyage to the South Pacific from 1772 to 1775. Within 4 months of Cook's return to England, the younger Forster's description of kava and its medicinal effects were published and broadly circulated in the scientific community of Europe (1). These reports generated a significant number of scientific studies related to the pharmacological properties and chemical constituents of kava. The first extensive research into the active constituents was conducted by the French scientists Gobley (1860) and Cuzent (1861). By the early 1900s, pills, extracts, and tinctures derived from kava were widely available in Europe, and in 1914, it was included in the *British Pharmacopoeia* (1).

More than 40 different chemical compounds have been isolated or identified in *P. methysticum* (2). Isolated compound classes include alcohols, alkaloids, chalcones, steroids, and long-chained fatty acids. The chemical constituents responsible for the observed effects when consuming the beverage are a group known as substituted alpha-pyrones (although they are more often called kavalactones) and chalcones. There are 18 identified kavalactones; however, only 6 of these are recognized as the major chemical compounds responsible for the therapeutic activities: kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin (Fig. 4) (6). These kavalactones are found in the resin.

Also, three chalcones have been identified from this plant: flavokawain A, flavokawain B, and flavokawain C (3).

While Gobley and Cuzent had been able to isolate independently an active constituent in the mid-1800s, it was not until the early 1900s that the complete chemical structures of the active constituents were described. Borsche and Blount published more than a dozen articles describing the structure and isolation of the active kava constituents (7). It was not until 1950 that the correct chemical structure of yangonin was published by Macierewicz (8). The complete spectroscopic data of the nine most abundant kavalactones have only been recently published (9).

The dried rhizome can contain kavalactone levels of up to 17%, but ranges of 3–7% are more typical (10). Of the total lipid extract, more than 96% is made up by the six primary kavalactones. The relative proportion of the six principal kavalactones varies considerably from cultivar to cultivar (Fig. 5), and this has been used to develop a chemotaxonomic coding scheme by which different cultivars could be identified (11). In addition to the cultivar type, the nature of the solvent used to extract the raw root material has a dramatic effect on both the amount and proportion of kavalactones present in the extract (12). For example, extraction with water yields primarily kavain and dihydrokavain with very small amounts of methysticin and yangonin. However, if it is performed with an organic solvent, such as acetone, quantitative recovery of all the major kava pyrones can be achieved.

Several studies describe significant differences in the concentration and ratio of kavalactones present in the stems, leaves, and roots (13,14). These reports generally

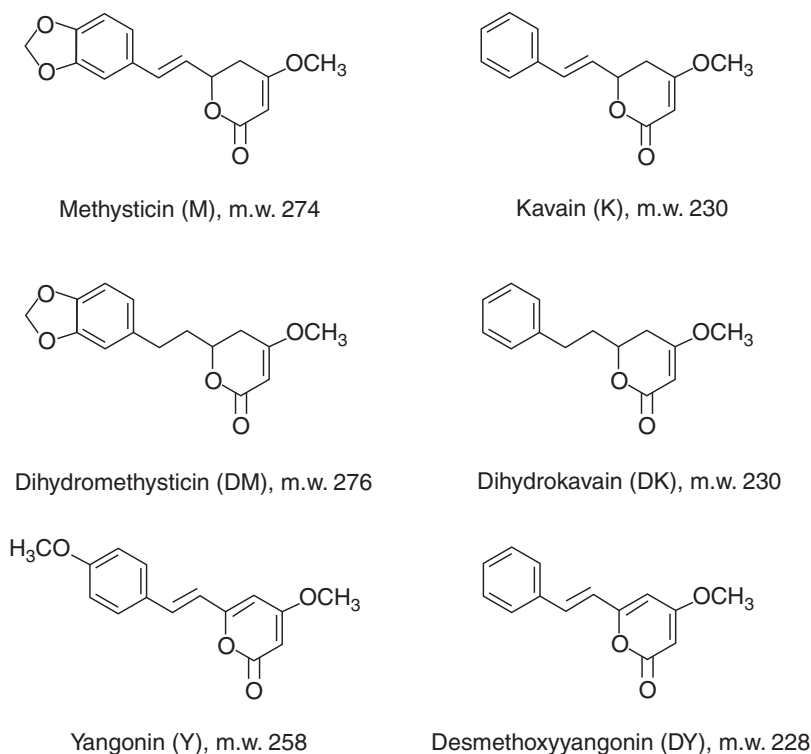


Figure 4 Chemical structures of the most abundant active components isolated from kava.

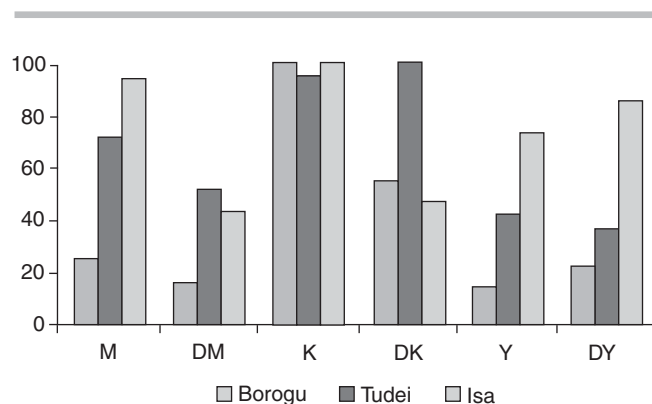


Figure 5 Percentage of relative composition of the six primary kavalactones in three different cultivars. Cultivars Borogu and Tudei were obtained from Vanuatu, while the Isa cultivar was from Hawaii.

agree that overall kavalactone levels are at the highest in the roots and lowest in the leaves. Also, kavain and methysticin are present in greater quantity in the roots, while the leaves contain more of dihydrokavain and dihydromethysticin. However, a recent analysis of roots, stumps, and stems from a number of different cultivars indicated that both the relative ratio and amount of kavalactone present in the extract were comparable (15). Furthermore, while cultivar type was found to have the largest effect on kavalactone composition, cultivar age, up to plant maturity at 2–3 years, and seasonal variations were also found to affect its chemical composition.

Because the six major kavalactones comprise more than 96% of the active components in an extract, accurate measurement is important for quality control of manufactured products containing kava root extracts. Recognizing this need, analysis of the individual kavalactones along with other chemical components has been an ongoing scientific endeavor since Forster first disclosed its medicinal properties. As a result, numerous chromatographic methods have been applied to the separation of the active constituents. The first successful demonstration of a mechanism capable of separating all the six primary kavalactones employed thin layer chromatography with UV detection (16). Although gas chromatography (GC) can be used to successfully separate and quantitate the abundant kavalactones, it must be performed carefully to avoid decomposition of methysticin in the hot injection port (17). In part because of the possibility of decomposition of the components during GC analysis and need for a more convenient sample preparation method, high-performance liquid chromatography has become the preferred analytical method for the quantitation of kavalactones (18–20). The complete analysis, including the separation of 12 kavalactones, can be accomplished in less than 20 minutes using the appropriate combination of solvent, column, and column temperature.

More recently, the isolation of the alkaloid, pipermethystine, a major constituents of leaves, but also present in small amounts in the roots and stems, has been a source of the hepatotoxicity conflict with kava (see preclinical studies section).

Preparation of Products

The traditional methods used in the preparation of kava are discussed in the section of ethnobotany earlier. However, because the active constituents are more soluble in organic solvents, commercial preparations are generally obtained by extracting the roots with alcohol, acetone, or supercritical carbon dioxide.

The dried kava root powder can contain kavalactone levels of up to 17% (10).

Standardized extracts are extracts that have been concentrated and standardized to contain a particular amount of a specific component. For the production of herbal tinctures, a 25% ethanol to 75% water mixture is used as the solvent, so commercially available kava tinctures contain one part kava root to three parts solvent. For commercially available herbal extracts of kava, the solvents used are either 60% or more of ethanol or 60% or more of acetone; these high concentrations of solvent ensure that the kavalactones are extracted (about 30–70% kavalactones) (21). Most standardized kava extract powders sold in herbal and natural stores contain 30% kavalactones, which have been prepared by using an extraction mixture of 25% ethanol to 75% water (22).

More critically, commercially available kava caplets are commonly prepared by ethanolic or acetonic (60% or more) extraction of the full kava plant, and it has been suggested that pharmaceutical companies sometimes add a racemic mixture of synthetic kavain to boost the activity of the kava extract (23).

In the last years, carbon dioxide gas has become an important and attractive chemical in the innovative supercritical fluid drug formulation technology because it is a benign replacement for toxic solvents. Perrut and Clavier (2003) have combined online super critical fluid extraction and impregnation of a kavalactone-rich extract into the short-chained starch, maltodextrin, to create microspheres (5–50 μm), which patients can then administer by inhaling or by other noninvasive methods (24).

Preclinical Studies

While the intoxicating, sedative qualities of kava extracts were obvious from Forster's description of kava's traditional use, it was not until the early 1900s that serious investigations into the pharmacological properties were made (25). These early studies clearly demonstrated that the active components of kava extracts existed in the lipophilic portions. Although aqueous extracts had similar activity, they were much less potent. Because the traditional method of preparing kava involved extensive chewing of the root, Schubel and many others postulated that enzymatic activity in the saliva was required to increase the potency of the extracts. However, this has since been proven incorrect, as the chewing action merely creates an emulsion that aids in the extraction of the lipophilic components. Van Veen was able to demonstrate that both the lipophilic extract and the purified form of dihydrokavain caused pigeons and monkeys to become sleepy (26). At high doses, the animals lost control of their limb and quickly entered into long periods of sleep. These results, in addition to loss of righting reflex, were confirmed by Meyer et al., following oral administration of dihydromethysticin and dihydrokavain to mice, rats,

rabbits, and cats (27). As a result of the continued study, Meyer found that both kava extracts and individual pure chemical constituents produced centrally acting smooth muscle relaxation and antagonized strychnine-induced convulsions (28). Interestingly, none of the individual kavalactones exhibited an equivalent potency as that induced by extracts, causing a number of investigators to propose a synergistic action of the natural combination of compounds.

The increasing interest in the use of kava not only for its well-documented effects as a sleeping aid, but also for its anxiolytic qualities, has prompted numerous investigations into its effectiveness as a treatment for anxiety disorders and mild forms of depression. Neuropharmacological trials of the binding of individual kavalactones to gamma-amino butyric acid (GABA) and benzodiazepine receptors in isolated rat and mouse synaptosomal membranes found only weak binding to GABA_A receptors and no significant binding to GABA_B or benzodiazepine receptors (29). The weak receptor binding of the individual kavalactones and kava extract did not correlate with the observed centrally acting pharmacological properties of kava in animal and human studies. More recently, it was shown that the kava extract produced anxiolytic-like behavioral changes and sedation in mice that are not mediated through the benzodiazepine binding site on the GABA_A receptor complex, and the authors suggest that both effects of kava are mediated through another process, which remains to be determined (30).

In addition, the individual effects of the six main kavalactones as well as the whole root extract (containing at least 15% dihydrokavain) were tested on a chick social-separation-stress model. It was found that kava produces an apparent decrease in chick distress vocalizations and causes sedation in a dose-dependent manner; however, it does not affect ventral recumbency; dihydrokavain is necessary and sufficient in mediating the anxiolytic effects of kava, but not the sedation effects (31). Furthermore, Herrera and Balick together with Dr. Maryam Bamshad from Lehman College (City University of New York) have demonstrated using prairie voles (*Microtus ochrogaster*) that the aqueous root extract lowers anxiety levels and alters their normal social and affect behaviors. These results will allow for neurohistochemistry studies to pinpoint the molecular mechanism of kava action (Herrera et al. 2010, unpublished data).

Other experiments suggest the mechanism of action to involve sodium- and calcium-gated channels along with inhibition of noradrenalin uptake (32,33). Another evaluation indicates that central nervous system receptor binding of indicated components other than kavalactones may be responsible for the anxiolytic activity in kava extracts. Leaf and stem extracts contained components with potent binding to GABA_A, dopamine D₂, opioid, and histamine receptors, which were unrelated to the concentration of kavalactones (34).

A number of animal studies have been carried out to examine the metabolism of kavalactones. In rats, metabolism was extensive and consisted primarily of hydroxylation of the benzene ring and hydrolysis of the pyrone ring (35). Analysis of human urine following ingestion of a traditionally prepared kava beverage detected the

known kavalactones along with a complex mixture of hydroxylated metabolites (36). Other metabolism included the demethylation of the methoxy substituent on the pyrone ring, as well as reduction of the 3,4-double bond of the pyrone ring.

Although kava had not previously been shown to be hepatotoxic, recent reports of liver failure associated with consumption of kava-containing products have prompted new studies related to its metabolism. Because one well-known pathway by which natural products and drugs can cause hepatotoxicity is through the inhibition or modulation of cytochrome P450 (CYP450) enzymes, the ability of individual kavalactones to inhibit specific CYP450 enzymes was investigated. Although kavain was associated with little or no inhibition, methysticin, dihydromethysticin, and desmethoxyyangonin were all found to cause significant inhibition of the isozymes CYP2C9, CYP2C19, and CYP3A4 (37). More importantly, this study showed that methysticin and dihydromethysticin form irreversible metabolic complexes following incubation with these CYP450 isozymes. Because the affected enzyme systems are responsible for the metabolism of more than 90% of all drugs, there is a significant risk of adverse reaction associated with kava consumption and conventional drug therapy. This would not be unexpected because drug/natural product combinations have been associated with adverse reactions involving a number of natural products, such as grapefruit juice and St. John's wort.

The delayed onset and low incidence of hepatotoxicity associated with kava products suggested an idiosyncratic reaction similar to those observed for some drug products. In many cases, these reactions are often associated with bioactivation to reactive electrophilic metabolites. Further study into the chemical reactivity of the principal chemical constituents of kava extracts was conducted to evaluate their ability to generate reactive metabolites in the presence of human liver microsomes and hepatocytes. The results of these investigations showed significant and extensive formation of glutathione reactive metabolites with several of the kava pyrones (38). Characterization of the glutathione conjugates pointed to a mechanism involving the generation of *ortho*-quinones as the reactive intermediates. Although it is often difficult to link reactive metabolite formation to a specific toxicity, identification of the reactive metabolites has provided important mechanistic insights for future toxicological studies. For example, should toxicity be mediated through this mechanism, rats could not be used because they do not have the ability to metabolize methysticin and dihydromethysticin to the same reactive metabolite observed in humans (Fig. 6).

Because cases of hepatitis similar to those reported for kava extracts have often been associated with the direct action of a chemical contaminant on the liver, numerous studies are underway to evaluate other chemical constituents present for the same activity. One such investigation into direct acting hepatotoxic contaminants present in kava extracts has identified an epoxide form of pipermethystine (39). The epoxide (Fig. 7) was isolated from the outer stem peelings of a kava cultivar from Papua New Guinea known as Isa. Peelings from 10 other cultivars, including another variety from Papua New Guinea, were also included in the analysis, but they did not have

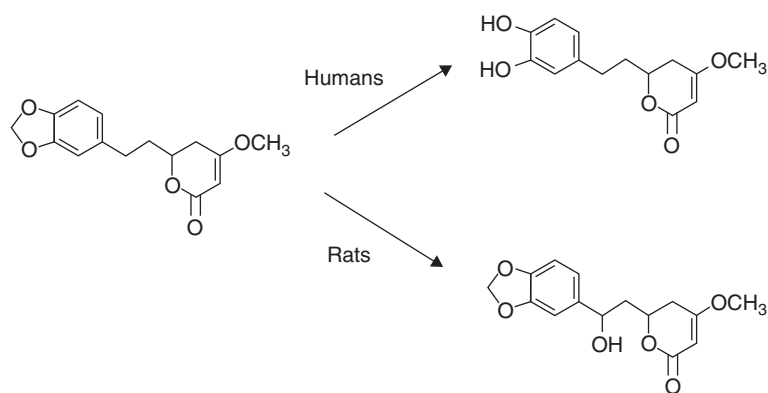


Figure 6 Schematic representation of the difference in primary metabolism of dihydromethysticin and methysticin in rats and humans. Demethylation of methysticin and dihydromethysticin can lead to the formation of a reactive *ortho*-quinone species.

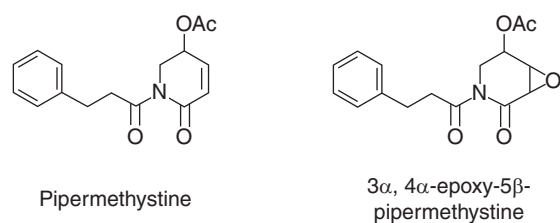


Figure 7 Chemical structures of possible contaminants of kava extracts, pipermethystine and the *in vitro* hepatotoxic epoxide form of pipermethystine.

detectable levels of the epoxide. This new chemical compound was found to be toxic in *in vitro* cell cultures of hepatocytes. Because stem peelings are not used traditionally, and have only been recently used to fill the enormous demand for kava, the authors speculate that this alkaloid may be responsible for some of the observed hepatotoxicity. Some support for this hypothesis may be found in the data generated from the Port Import/Export Reporting Service, which records more than 100 ton of kava root peelings being imported to the United States prior to the year 2001, presumably for use in kava preparations. While the toxicity of the pipermethystine epoxide remains to be proven, it would not explain the hepatotoxicity observed with a patient taking only kavain, implying that there may be multiple pathways of hepatotoxicity.

CLINICAL STUDIES

Although there are a number of potential medicinal uses for kava root extracts, the principal purpose is for the relaxing, anxiolytic effects. Its pharmacological properties have led to a continuous, worldwide increase in use as an over-the-counter dietary supplement or a medicinal herbal preparation. The German Commission E monographs permit daily doses of 60–120 mg of total kavalactones for the treatment of anxiety, stress, and restlessness. While the mechanism of action may not be completely understood, a recent review of clinical trials conducted on

the effectiveness of kava for treating depression and anxiety disorders clearly suggests efficacy in comparison with placebo treatment (40).

The review included meta-analysis of data collected from seven double-blinded, randomized clinical trials. One of the most recent clinical trials included in the meta-analysis was that conducted by Volz and Kieser (41). It included 101 patients and employed a carefully formulated dose of kava equivalent to 210 mg of kavalactones per day. Using the Hamilton Anxiety Scale as a measure of efficacy, the study found a significant improvement over the control group following 8 weeks of treatment. Although adverse events were reported, they were confined to relatively minor complaints including headache, restlessness, and drowsiness.

Extracts have been shown to produce both short-acting local anesthetic action and longer lasting pain relief. The former effect has been likened to that produced by lidocaine (42) and has not been attributed to the individual kavalactones but to other components present in the extract. The individual kavalactones have been found to produce analgesic action similar to aspirin. An investigation into the mechanism of the anti-inflammatory response to the kavalactones found that all six of the primary kavalactones, in addition to flavokawain, had inhibitory effects on cyclo-oxygenase (COX) enzymes COX-1 and COX-2 (43). Because the mechanism of action of nonsteroidal anti-inflammatory drugs, such as ibuprofen, is attributed to their ability to inhibit the COX enzymes, the finding that kavalactones, in particular dihydrokawain and yangonin, also perform a similar function is a likely explanation for their activity.

Some supporting scientific evidence for this traditional use is found with the observed broad spectrum of antimicrobial and antifungal effects of kava extracts (44).

Indeed, clinical evaluation of the extract for the treatment of menopausal symptoms does conclude that some efficacy is observed (45). One study evaluated the combined effectiveness of hormone replacement therapy along with a daily dose of 100 mg kava extract containing 55% kavain (46). Results of this trial found considerable relief of psychological symptoms associated with menopause when kava was combined with traditional hormone replacement therapy.

The kavalactone, kavain, has antithrombotic properties on platelets, perhaps because it inhibits COX and decreases the production of thromboxane 2 (47).

Toxicity

Kava has been used for centuries with little but isolated or circumstantial reference to human toxicity. Prior to numerous recent reports of hepatotoxicity, reported human toxicity was limited to a scaly dermatopathy, also known as kava dermatopathy, which is observed with chronic, high consumption rates. The condition is described as an ichthyosiform eruption (with "fishlike" scales on the surface of the skin) and is believed to result from inhibition of cholesterol metabolism (48). Singh and Blumenthal suggested that two pigmented compounds, flavokawain A and flavokawain B, may explain the skin's reaction to excessive consumption (49). Kava dermatopathy disappears following suspension of kava consumption. People in Pohnpei treat this skin condition by applying on the affected area a mixture of coconut (*Cocos nucifera*) and ylang-ylang (*Cananga odorata*) oils (2).

Responses to the question of what people on Pohnpei considered to be negative effects that follow *sakau* drinking, with no qualification as to frequency, by a 2001 survey that Balick and collaborators performed in Pohnpei with 180 participants, resulted in the following: hang-over (14.5%); tiredness or weakness, specially the next day (14.5%); sickness, such as nausea, vomiting, chest pain, and feelings of sickness (6.5%); headaches (4.9%); stomach problems (4%); insomnia (3.2%); and skin problems (1.6%) (2).

Clinical observations on an Australian Aboriginal community described greatly increased levels of gamma-glutamyl transferase, along with decreased levels of bilirubin, plasma protein, and urea in all frequent kava users (50). Although many of the individuals in this study consumed very high levels of kava, with most exceeding 100 g/wk and some exceeding 400 g/wk, no reports of hepatotoxicity were observed. A number of other epidemiological investigations have also been conducted on the carcinogenicity of kava extracts. However, no study has shown any causal link with cancer.

The toxicity of kava extracts to humans is a matter of fierce debate. Prior to 2000, kava was among the top 10 best selling botanical supplements in the United States, and worldwide millions of doses were consumed annually. However, based on a number of reported cases of severe hepatotoxicity, some involving liver transplant, sales have been banned in a number of countries including Germany, Switzerland, and United Kingdom. In a summary of 29 cases of kava-implicated hepatotoxicity reported in Germany between 1990 and 2002 (51), nine people developed fulminant liver failure, and eight of them received liver transplants. The use of the aerial part of the plant by certain producers of kava caplets is also suspected to lead to toxicity of the extract, because the cytotoxic alkaloid pipermethystine has been isolated from this part of the plant (23). It is also noteworthy to mention that many of the individuals exceeded the German Commission E recommended daily dose of 120 mg (4).

So far, a definitive mechanism for kava-induced hepatotoxicity has not been identified, but it could include

both idiosyncratic and immunoallergic mechanisms. The most likely mechanism of toxicity could be related to individual metabolic idiosyncratic reaction, because the affected individuals generally demonstrated a long latency period and rarely showed evidence of autoimmune response. A recent study of the metabolic profiles of kava-sensitive individuals showed that subjects with CYP2D6 deficiency seemed to be at risk for developing kava-related hepatotoxicity (52). Although those with this deficiency are known to be sensitive to a broad range of drugs, there is insufficient evidence to come to this conclusion.

The American Botanical Council suggests that kava should not be used during pregnancy or while nursing (4).

Drug Interactions

The American Botanical Council states that "simultaneous consumption of kava with alcohol, barbiturates, psychopharmacological drugs, or other substances acting on the central nervous system (CNS) may potentiate inebriation or the CNS depressant effect" (4).

Research discussed in the preclinical studies section related to interactions with P450 (CYP450) enzymes showed that methysticin and dihydromethysticin form irreversible metabolic complexes on incubation with some CYP450 isozymes. Because the affected enzyme systems are responsible for the metabolism of more than 90% of all drugs, there is a significant risk of adverse reaction associated with kava consumption and conventional drug therapy. Moreover, drinking kava may potentiate effects of other anxiolytics and can increase Parkinson symptoms by reducing levodopa's effects. There is one case of "coma" associated with the combined intake of kava and Xanax[®] (alprazolam), Tagamet[®] (cimetidine), and Hytrin[®] (terazosin) (4).

Consumption Patterns: The Case of Pohnpei

In Pohnpei, *sakau* (kava) drinking was once limited to the chiefs and others with high titles; it is now consumed by both men and women regardless of their rank or title and sold by the bottle as a beverage in the marketplace. This type is referred to as "market *sakau*" or simply "market" and sold in 1-L bottles and served in individual cups; it is somewhat diluted through the addition of more water compared with the one made in the home or feast house.

In 2001, Balick and collaborators developed a four-page questionnaire, translated into Pohnpeian, and orally administered to 180 people, to gather information on *sakau* consumption patterns. Of the total population sampled, 69% reported that they drink *sakau*, while 31% said they do not drink it. Of the *sakau* drinkers, 83.6% are men and 53.2% are women. Men reported drinking 8.9 cups (one cup is half of a coconut shell) per sitting and spending on average 5.4 hr/wk drinking *sakau*. Women reported consuming 7.7 cups per sitting and spending 4.3 hr/wk drinking *sakau*.

An important question in this same survey was "why do people on Pohnpei drink *sakau*?" The highest number of people (44%) noted that the primary reason for drinking *sakau* was social, whether at a ritual ceremony, friends' house, or *sakau* bar. The second major reason for drinking was the feeling that *sakau* provided, with 32% stating this as their primary reason. Boredom was

suggested as a major reason for drinking by 17%, while help in sleeping was the fourth major reason, with 15% of people stating this reason. A few people reported that it helped them to forget their problems or anxieties. The feelings reported during drinking were the following: relaxation (44%), sleepiness (27%), dizziness (18%), feeling good (11%), heaviness (4%), numbness (4%), drunkenness (3%), weakness (2%), and tiredness (1%).

CONCLUSIONS

Kava extracts have a well-documented, effective pharmacological action in inducing muscle relaxation and sleep and reducing mild cases of anxiety. They are also used extensively as a substitute for alcohol, and numerous kava bars are present in Hawaii and elsewhere in the South Pacific and United States. Because of all the incomplete or generally unavailable data reported from cases of hepatotoxicity, the consumption of most properly prepared kava-containing products is thought to be safe but should not be taken for more than 3 months without medical supervision (4).

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Lactobacilli and Bifidobacteria

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INTRODUCTION

The lower human gastrointestinal tract hosts complex and diverse microbial communities that have been the most extensively studied body habitat in attempts to define microbial diversity in normal health and predisposition to disease. The distal gastrointestinal tract is estimated to harbor in excess of 1×10^{12} bacterial cells and approximately 800–1000 distinct bacterial species, the vast majority of which are unculturable microbes (1,2). To elucidate the full range of diversity of the human microbiota estimated at 100 trillion microbial inhabitants for all body habitats, it is essential to define normal states and the role that disruptions in microbiota play in pathogenesis. As is true for other body habitats, the gastrointestinal microbiota is distinct and consists primarily of obligate anaerobes that, in homeostasis, appears to maintain low rates of translocation and generally remains sensitive to antimicrobial agents likely due, in part, to coevolution and adaptability with our host immune system (3). Despite the fact that microbial cells across body habitats outnumber human cells by ten to one, the impact on human physiology, pathogenesis, immunity, and nutrition is largely unknown and is an active area of research. Metabolic activity of the microbiota is further influenced by host immune response, antibiotics, nutrient availability, and competition between individual species or strains in these microbial communities (4).

Taxon: Bacteria, Firmicutes, Lactobacillales, and Lactobacillaceae

Lactobacillus acidophilus NCFM, *Lactobacillus brevis* ATCC 367, *Lactobacillus casei* ATCC 334, *Lactobacillus casei* BL23, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365, *Lactobacillus fermentum* IFO 3956, *Lactobacillus gasseri* ATCC 33323, *Lactobacillus helveticus* DPC 4571, *Lactobacillus johnsonii* NCC 533, *Lactobacillus plantarum* JDM1, *Lactobacillus plantarum* WCFS1, *Lactobacillus reuteri* DSM 20016, *Lactobacillus reuteri* JCM 1112, *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* Lc 705, *Lactobacillus sakei* subsp. *sakei* 23 K, *Lactobacillus salivarius* UCC118, *Pediococcus pentosaceus* ATCC 25745.

Taxon: Bacteria, Actinobacteria, Actinobacteridae, Bifidobacteriales, Bifidobacteriaceae

Bifidobacterium: *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium animalis* subsp. *lactis* AD011, *Bifidobac-*

terium animalis subsp. *lactis* BI-04, *Bifidobacterium animalis* subsp. *lactis* DSM 10140, *Bifidobacterium longum* DJO10 A, *Bifidobacterium longum* NCC2705, *Bifidobacterium longum* subsp. *infantis* ATCC 15697.

Member strains of *Lactobacillus* and *Bifidobacterium* genera are found naturally in the human and animal gastrointestinal tracts and other body sites. It has long been known that *Bifidobacterium* spp. are early colonizers of the breast-fed colon, and preferentially utilize human milk oligosaccharides abundant early in lactation, that may afford *Bifidobacterium* spp. a selective growth advantage (1). DNA hybridization and gene sequencing analysis supports earlier evidence that *Bifidobacterium* strains bind to human milk oligosaccharides and possibly epithelial cell surface glycans that may represent a competitive strategy unique to breast-fed infants (5).

An ever-increasing number of *Lactobacillus* and *Bifidobacterium* strains are currently marketed in food products (Federal Food, Drug and Cosmetic Act, 1938) and as dietary supplements (Dietary Supplement Health and Education Act, DSHEA, 1994). A substantial number of strains containing members of genera *Lactobacillus* and/or *Bifidobacterium* also are in development in the United States as potential investigational biotherapeutic products. The most widely accepted definition from an earlier FAO/WHO workgroup states that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (6). *Lactobacillus* and *Bifidobacterium* genera, comprising the most commonly known probiotic strains, are anaerobic or microaerophilic, nonmotile, nonspore-forming prokaryotes. *Lactobacillus* are nonbranching, rod-shaped bacteria (Fig. 1), whereas *Bifidobacterium* spp. are branching, rod-shaped microbes. A distinctive feature characterizing prokaryote microorganisms is that important genes (antibiotic resistance, DNA transfer, and stability systems) are encoded by plasmids (extrachromosomal DNA that can potentially transfer horizontally from one organism to another). By contrast, the genetic repertoire in most eukaryotes is divided into linear bundles of organized chromosomes (7).

Organic compounds (lactic and/or acetic acid) produced by commensal *Lactobacillus* and *Bifidobacterium* spp. increase acidity of the gastrointestinal tract that may mediate antagonistic effects and overgrowth of pathogenic bacteria (8). *Lactobacillus* spp. are primarily homofermentative (produce only lactic acid from sugar fermentation) or heterofermentative (produce lactic and other

organic acids and CO₂ from sugar fermentation). *Bifidobacterium* spp. are heterofermenters of both acetic and lactic acid.

As described further on in this chapter, a range of postulated defense mechanisms are under active in vitro and in vivo investigation. The most extensively characterized probiotic mechanism of action of *Lactobacillus* and *Bifidobacterium* spp. is the evidence of immunomodulatory activity; more recent studies have also demonstrated potential anti-inflammatory and immunostimulatory properties. Increasing focus is also on postulated protective mechanisms against disruption of mucosal barrier function and inhibitory effects of antimicrobial compounds in preventing translocation of pathogens to the host mesenteric lymph nodes and circulatory system.

Commensal *Lactobacillus* and *Bifidobacterium* Spp.

The evolutionary patterns detected in phylogenetic trees obtained from sequence typing analysis have revealed lineage clusters for both *Lactobacillus* and *Bifidobacterium* strains that may exhibit high specificity to particular hosts. The relative lengths of branching observed in evolutionary tree dendrograms (Fig. 2) indicate that there can be deep phylogenetic divisions and that *Lactobacillus* and *Bifidobacterium* lineages have likely been associated with respective host species throughout long evolutionary time spans. This may suggest host directed evolution of bacterial selection and diversification. To identify candidate genes contributing to deep phylogenetic diversification, DNA microarray and more recent metagenomic analyses have identified lineage-specific clusters that may account, in part, for differences in genome content. Although speculative, it is reasonable that genes from different bacterial strains present in different mammalian hosts increase ecological fitness of the host gastrointestinal tract or other mucosal site and contain redundant or unique functional properties (e.g., polysaccharide formation, bac-

teriocin production, and protein secretion) important for host adaptability (2,3).

Autochthonous members of human *Lactobacillus* and *Bifidobacterium* strains are part of commensal microbial communities in the gastrointestinal tract of breast-fed infants and adults. Food and alcohol, use of antibiotics, and other disturbances in host physiology may alter the long-term composition of the entire microbiota and the fermenting metabolism of individual species (10). Discovery of new *Lactobacillus* and *Bifidobacterium* strains may be acquired transiently in the lower gastrointestinal tract from food or dietary supplements, as well as from the oral cavity. While *Lactobacillus* and some *Bifidobacterium* strains typically grow well in the vagina and urethra, a wide range of other bacterial species are also present (11).

Among the microbial communities of commensal bacteria that coexist in the colon, the lactic acid-producing bacteria utilize lactose as their primary source of carbon for energy biogenesis. *Lactobacillus* and *Bifidobacterium* strains are a rich source of lactase, the enzyme required to digest milk sugar. Fructo-oligosaccharides are naturally occurring carbohydrates that cannot be digested or absorbed by humans (12). Matching host carbohydrate synthesis with the capacity of these anaerobes to produce glycosidases, oligosaccharide outer chains in mammalian glycans have been postulated to play an important role in the developing intestine.

16S rRNA and Metagenomic Methods

Culture-independent approaches to profile the landscape of microbial communities using advanced DNA sequencing technology, including the NIH Roadmap Human Microbiome Project and global funding initiatives, have spawned a new area of research termed metagenomics. Advances in this emerging field permit genomic analysis of entire environmental and human microbial communities in parallel, providing comprehensive studies of microbiota structure and function. Traditionally, culture methods were used to isolate *Lactobacillus* and *Bifidobacterium* spp. from biological samples and then identify the organisms using commercial API test systems, single-nucleotide polymorphic DNA analysis, and DNA fingerprint typing via pulsed field gel electrophoresis methods.

Culture-independent methods have been developed in recent years as an alternative to characterize whole bacterial communities by direct extraction of DNA without prior cultivation. Culture-independent approaches, typically based on amplification of the 16S ribosomal RNA (rRNA) gene permits identification of members of the community either through direct shotgun variant sequencing or by hybridization to oligonucleotide probes that target unique regions of the 16S rRNA gene specific to individual or closely related species. The partial and full sequencing of 16S rRNA genes are rapidly becoming a standardized, albeit narrow marker to define microbial diversity at the species level. This approach has been used extensively to catalog the major species of microbes within the microbiota, but it fails to discriminate between highly homologous species. For example, while the 16S rRNA biomarker is an excellent target to broadly define microbial diversity, due to the high level of 16S rRNA sequence identity between members of *Lactobacillus* and

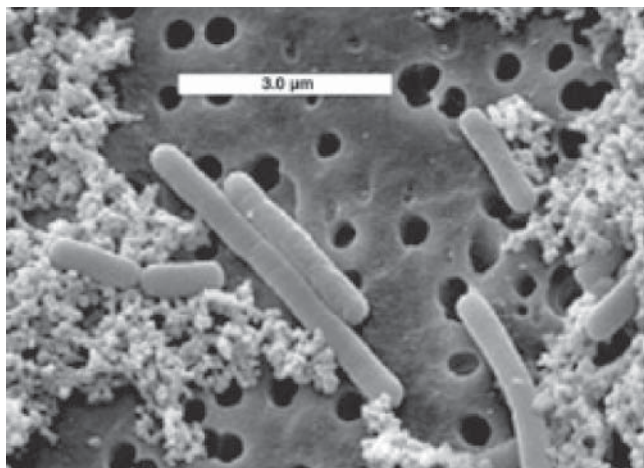


Figure 1 *Lactobacillus casei* are gram-positive, facultatively anaerobic, nonmotile and nonspore-forming, rod-shaped [cell size range = (0.7–1.1) × (2.0–4.0) μm] members of the industrially important lactic acid bacteria. Source: Photo courtesy of Jeff Broadbent, Utah State University.

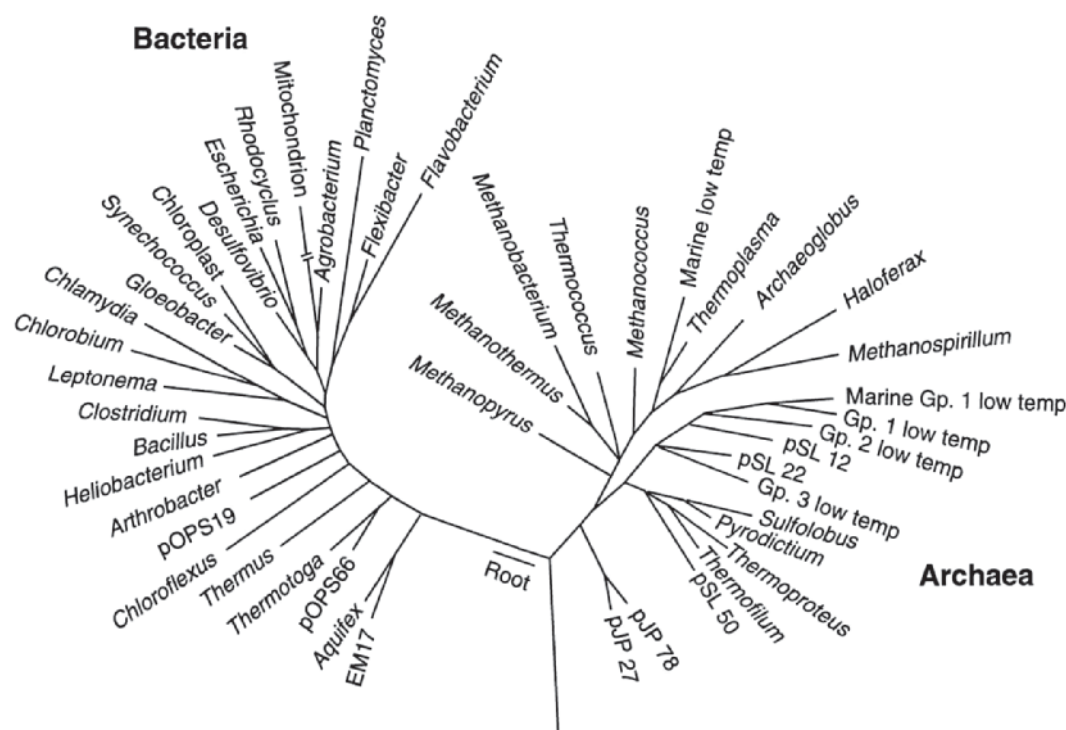


Figure 2 The phylogenetic tree of life based on comparative ssrRNA sequencing. From Ref. 9.

Bifidobacterium spp., it is not ideal for distinguishing closely related species within, for example, the *Lactobacillus casei* or *Bifidobacterium lactis* species or for its quantitative assessment in the whole microflora, because the copies of ribosomal DNA per genome can vary. Individual species variation can be highly diverse and lead to significant changes in phenotype and functional traits. As an alternative to 16S ribosomal gene-based analysis, single gene analytical techniques (including *pheS* genes for *Lactobacillus* and *tuf* for *Bifidobacterium* spp.) can provide an additional tool with discriminating properties for greater differentiation at the subspecies level (13). Strain differentiation within species using metagenomic and metabolomic technologies as single-nucleotide extension probes for rRNA analysis, microarray and phylogene chip technologies, and barcoded pyrosequencing and deeper 454 sequencing will need to be comparatively evaluated and validated on functional genomic platforms (14).

Functional profiling of the comparative microbial communities may be performed by high-throughput sequencing of cDNA generated from mRNA extracted from samples (metatranscriptomic analysis) or by profiling the protein (metaproteomics) or metabolites (metabolomics) produced by members of the community. These approaches are currently being used to determine the range of structural and functional microbial profiles that constitute both healthy and disease states in humans, providing insights into the core repertoire of microbes and their products that define human health. With these advances in culture-independent technologies, the molecular mechanisms by which probiotic species elicit a health benefit

in the context of the host microbiome may now be fully elucidated.

The culture-independent metagenomic analyses provide a significantly broader view of the diversity of organisms present in a microbial community and how that community changes in response to environmental signals, stress, nutrients, antibiotics, and the introduction of probiotic microbes. Transcriptional, proteomic, and metabolomic approaches are being used to investigate which genes are turned on and off in the bacteria comprising the microbiota and determine the products generated and altered by the entire community habitat at large. What this also means is that the field of functional genomics can now investigate the underlying mechanisms driving the health effects of probiotic microbes at the level of the bacteria, the host, and the interactions between the two. The complexity of these relationships has only begun to be studied in the human gastrointestinal tract (2,15,16).

Metagenomic and metabolomic technologies using single-nucleotide extension probes for rRNA analysis, microarray and phylogene chip technologies, and barcoded pyrosequencing and deeper 454 sequencing extends beyond 16S RNA data but awaits cross-validation of meta-sequencing techniques on principal metabolic functions (14).

Characterizing complex population shifts in the microbiota can also be investigated in real time after various disturbances, that is, presentation of nutrient antigens, antibiotics, etc. Moreover, following these perturbations, changes that restore individual species, strains, and functions may help to broadly define the composition of a "healthy" microbiota community (17).

Mechanisms of Action in *Lactobacillus* and *Bifidobacterium* Spp.

The mechanisms of action of *Lactobacillus* and *Bifidobacterium* spp. are incompletely understood and the evidence available suggests key mechanistic pathways that may be redundant or vary from organism to organism. General effects are postulated to include improvement of intestinal barrier function, immunomodulation and suppression of growth, epithelial binding, or invasion by pathogenic bacteria, such as *Salmonella* or *Shigella* species, enterotoxigenic *Escherichia coli*, and *Vibrio cholera*. There is also increasing interest in the role supplemented probiotic species may play in modifying the gastrointestinal microbiota, enhancing, restoring, and/or maintaining microbial communities and beneficial activity without harm to the host. Suggested mechanisms include direct effects, such as reduction in intestinal pH, production of organic acids and gut protective metabolites, as well as binding and metabolism of toxic metabolites.

Mechanistic pathways by which specific members of the gut microbial community influence immunomodulatory responses may include modulation of the microbiota itself, improved barrier function with consequent reduction in microbial exposure, and direct effects of bacteria on different epithelial and immune cell types. Evidence for direct effects was reported, for example, in a recent comparative analysis of the gut microbiota of isogenic mice provided by two different suppliers, where differential numbers of Th17 cells using the 16S rRNA PhyloChip identified a specific species, segmented filamentous bacterium was present in dramatically altered abundance between the two groups of mice. This species was subsequently shown to induce Th17 cell populations in the gastrointestinal tract of mice that protected the animals against infection by *Citrobacter rodentium* (18). Earlier, extensively studied models examined host-microbe cross-talk and signaling pathways (e.g., toll-like receptors) that continue to elicit enormous research interest. Although the failure to definitively elucidate the mechanism(s) of probiotic activity is a major impediment to the field, pre-clinical immunogenicity studies in animals have provided important insights of postulated activity as well as safety. In vivo dose escalating and metabolic activity studies in animals are particularly important for establishing estimates of effective dose (in 50% of animals administered ED₅₀) in candidate strain development.

Probiotic Mechanisms Involving Host Defense Systems

Various mechanisms have been proposed to explain the effects of probiotics on human health. An intact and functional gut mucosal surface is essential to the maintenance of intestinal health, and the intestinal microbiota play an important role. Probiotics may promote and/or maintain the gut defense barrier through both immunologic and metabolic pathways (19). When pathogenic microorganisms, antibiotics, chemicals, radiation therapy, or even dietary substances perturb either the intestinal epithelium or the normal microbiota, the host's gut defense can be compromised and predispose to disease.

Commensal strains of *Lactobacillus* and *Bifidobacterium* may be able to stabilize the gut intestinal barrier by way of not totally understood defense strategies, for exam-

ple, to survive under conditions of gastric acidity and/or adhere to intestinal cells. There is, for example, evidence of probiotic maintenance of tight junctions by enhancing occluding protein expression. In a recent set of experiments, *Shigella*-induced rats showed reduced expression of tight junction transmembrane proteins. Pretreatment with polymicrobial *L. rhammosus* and *L. acidophilus* spp. significantly prevented membrane damage (20).

Lactobacillus and *Bifidobacterium* spp. may provide stability to the gut intestinal barrier by immunomodulatory activities that protect against invasion by pathogens. Breakdowns in barrier function may contribute to inflammatory metabolic disturbances. Both in vitro and in vivo studies have measured microbial interactions on cytokine production and the role played in the inflammatory and immune responses to various antigens. Although there has been a wide range of cytokine responses observed including upregulation of some cytokines and downregulation of others, it has been hypothesized that probiotic *Lactobacillus* and *Bifidobacterium* strains may mediate the balance between proinflammatory and anti-inflammatory cytokines and potentially influence whether the human host is allergic or nonallergic (21). In addition, lactic acid bacterial enzymes may modify foreign dietary antigens to render them less immunogenic, which could also prove to be beneficial in development of allergy in infants (22).

Cytokine (IFN- γ , TNF- α , TGF- β , interleukin 2, IL-4, IL-10) studies in pigs, mice, and rats and in limited human investigations are beginning to shed light on how the gut microbiota influences tolerance to dietary, as well as other antigens in the microenvironment, and reactivity to pathogens. Animal models and a small number of human clinical investigations have shown that probiotics are well tolerated, and survival of probiotic *Lactobacillus* and *Bifidobacterium* strains as they transit through gastric acidity and bile to intestinal adherence also have been reported in vitro and in vivo (23). In addition, there have been a number of interesting observations made recently with *Lactobacillus* and *Bifidobacterium* strains in the production of mucous by stimulating mucous gene inhibition of epithelial cell apoptosis induced by cytokine production and interaction with toll-like receptors.

Dendritic cells also appear to play an increasingly important role and it is reasonable that pathogens have evolved strategies to evade this host-defense mechanism, as well. Recent findings suggest that carbohydrate polysaccharides of bacterial commensals can be taken up from the intestine by dendritic cells and presented to T cells in the mesenteric lymph nodes. This observation implies that polysaccharides of the bacterial microbiota play an even more intricate role in the balance between health and disease than previously considered (24).

In the oral cavity, the ability of oral bacteria to cluster within a biofilm appears to be essential for their survival. Recent experiments have tentatively shown that *Actinomyces* promote growth of key *Lactobacillus* strains in a biofilm. Community assembly patterns influencing growth of microbes in biofilm-like plaques may impact inflammatory cellular responses, although the presence of individual bacteria in biofilm formation is not necessarily an indicator of either survival or pathogenic potential in a complex multi-species biofilm (25). Use of functional

genomics to determine molecular factors related to the ecology of biofilm formation that may play a significant role in the interaction of enteric pathogens is an area of great interest in the rapidly expanding field of probiotic biology.

Implications for Vaccine Development

Vaccines are administered to healthy adults and children. From a public health point of view, probiotics that might, in the future, be promoted as adjuvants or as complementary modalities to vaccine (e.g., influenza) in the United States will likely require extensive preclinical safety studies, including local reactogenicity and systemic toxicity testing, and substantial data supporting the rationale for clinical benefit and dose.

Nonpathogenic bacteria in the gut may directly influence the intestinal epithelium of the host to limit immune activation caused by exposure to various antigens. Commensal organisms acquired postnatally are essential for the development of tolerance to luminal antigens (26), and immune responses are potentially attenuated if there has been prior exposure to gut antigens. In healthy hosts, such tolerogenic responses must be efficient in downregulating overactive responses to dietary antigens and commensal microbes, yet remain capable of differentiating and mounting effective protective responses to pathogenic organisms.

This conceptual framework raises the notion that suitable carrier organisms for live oral vaccines could use attenuated mucosal pathogens. Commensal bacterial strains may offer better control, prevent reversion to virulence and optimization of expression systems to be used in live oral vaccines. Genetic manipulation of probiotic strains might also lead to promising approaches to presentation of antigens, although such strategies will need to be closely scrutinized and would not be appropriate for vulnerable risk groups, particularly immunocompromised subjects, unable to tolerate live attenuated vaccine administration (27). Probiotics could possibly provide adjuvant effects as well to available vaccines, for example, via activation of toll-like receptors (TLR9) (28).

REGULATORY STATUS OF PROBIOTICS

Pharmaceutical research expanded in the latter part of the twentieth century due, in part, to extensive screens of soil microorganisms for new antibiotics following the earlier discovery of penicillin. Hence, the idea that probiotic bacteria as natural products may be useful in biotherapeutics—specifically “prevention, treatment, mitigation, or cure of disease”—is intriguing and of much current interest. However, probiotic products promoted in foods, dietary supplements, or as biologic drugs represent a challenging issue for regulatory agencies globally (29,30). Food terms do not apply to biological products. Consequently, more product characterization of safety, potency, and efficacy of biologic products are needed for premarket approval of probiotics promoted for preventive and biotherapeutic health claims.

Microbial food products do not need to meet the same characterization requirements to be marketed. Microbial food products, including dietary supplements,

may promote general health, nutritional, or structure–function claims (31). The U.S. FDA Center for Food and Applied Nutrition regulates dietary supplements under the Dietary Supplement Health and Education Act of 1994 (DSHEA) differently than safety for food ingredients. In brief, the dietary supplement manufacturer is responsible for ensuring that a dietary supplement is safe before it is marketed, whereas the FDA regulatory role is primarily responsible for postmarketing surveillance. In the United States, for example, lactic acid–producing proprietary strains approved as food additives or considered generally recognized as safe status are used in the production of yogurt and other food products. For probiotics considered dietary supplements, the regulations are intended to ensure compositional quality (identity, purity, and strength) of probiotic supplements in the U.S. market. In contrast, a probiotic intended for investigational study of an unapproved indication, that is, used to “diagnose, cure, mitigate, treat, or prevent disease,” is defined as an investigational new drug (IND) and biologic product. Currently, the U.S. FDA Office of Vaccines Research and Review at the Center for Biologics Evaluation and Research has regulatory jurisdiction over probiotic products for clinical use and classifies such products as “live biotherapeutics,” subject to the premarket safety and regulatory provisions of the Public Health Service Act and the Federal Food, Drug and Cosmetic Act under the Code of Federal Regulations (21 CFR, parts 600 to 680). Manufacturers and sponsors promoting these products must comply with regulations as INDs (IND 21 CFR, part 312) and current Good Manufacturing Practices (cGMP) (21 CFR, parts 210, 211.9) (32). Safety-related data of biologic cGMPs are required to be implemented prior to initiation of a phase I clinical study in humans.

Chemistry, manufacturing, and control (CMC) data is an essential component of an IND to support a probiotic biologic product and significantly overlaps with similar process testing needed for vaccine submissions. CMC data can be obtained from the manufacturer and submitted as part of an IND, or the information can be submitted separately by the manufacturer in a Master File (e.g., when the same product is evaluated under multiple INDs). In the actual IND application, the description of the product manufacturing process (including complete characterization of the source and quality of starting materials, in-process testing, and lot-release specifications, i.e., identity, purity, sterility, general safety, and potency) must include detailed standards of procedure for each manufacturing component. Safety and product consistency in-process testing, as well as lot-release and potency testing, also require inclusion of quality control assays that can meet sensitive, specific, and reliable validation testing requirements (33).

Regulatory concerns worth special consideration for advancing candidate probiotic strains as investigational biologic drug products include the need for improved (i) consistency of taxonomic identification and terms; (ii) manufacturer and sponsor familiarity with biologic product manufacturing safety and consistency testing requirements that exceed those for dietary supplements and food ingredients; (iii) standards for how potency should be defined and evaluated; (iv) pathogenicity and transmissibility data of virulence and antibiotic resistant genes; and

(v) need for clear and complete CMC data to support use of a live biotherapeutic in a phase I/II clinical investigation under an IND.

Despite the range of DNA-based fingerprinting and sequencing methods, the varied and inconsistent identification of organisms at the species and strain level, including for *Lactobacillus* and *Bifidobacterium* products, represent a potential cause of probiotic mislabeling and an additional reason for heightened efforts to tighten probiotic claims of potency in the United States (34). Labeling has been criticized for overstating the level of viable bacteria, for inaccurately indicating the species of probiotic bacteria present, and for the presence of species of bacteria not listed on the label. In addition, recent reports from independent testing laboratories have yielded discrepancies in the number of viable cells than are reported. Accurate labeling is critical to standardizing and establishing uses and recommendations for specific probiotic strains.

Pharmacology of Probiotic *Lactobacillus* and *Bifidobacterium* Spp.

Pharmacologic studies generally focus on testing absorption, distribution, metabolism, and excretion of chemical substances, whereas the approach for probiotic studies of gut-related health focus on the transit through the upper gastrointestinal tract (ability to survive in the presence of stomach acid and bile) as well as distribution and metabolism in the lower gastrointestinal tract—the prime site of immunologic and digestive physiologic activity (22).

Resident *Lactobacillus* and *Bifidobacterium* genera have been identified that synthesize bacteriocin-like antimicrobial compounds. These inhibitory properties are an exciting pathway for combating pathogenic infection, possibly avoiding collateral damage from antibiotics to surrounding microbial commensal communities. Multiple classes of bacteriocins are produced by food-grade lactic acid-producing species and, given that they are generally heat stable, have many food microbiology applications (35). However, bacteriocin compounds often must function as two-component systems to display full activity that can disappear upon separation techniques.

Of particular concern, probiotic strains that have easily transferable resistance genes on plasmids may increase the risk that the resident commensal bacteria will acquire the antimicrobial resistance from the probiotic strain. Serious concern has been raised that *Enterococcus* strains that have displayed risk of acquiring vancomycin resistance plasmids in humans could be transferred to other commensal bacteria. For this reason, *Enterococcus* strains or lactic acid bacteria exhibiting confirmed, transferable resistance genes should not be used (36).

Clinical Indications and Safety of Probiotic *Lactobacillus* and *Bifidobacterium* Spp.

As rapid, inexpensive molecular sequencing is now available, it is theoretically possible to elucidate metabolic pathways and pattern changes of the microbiota in different body habitats in adults and children following probiotic supplementation. Ideally, source and history of candidate

strains including phenotypic and genotypic characterization would be of human commensal origin, be non-pathogenic and nontoxic, contain a sufficient number of viable cells, be capable of surviving and metabolizing in the gut, remain viable during storage and use, remain active in the presence of certain antibiotics, and sensitive to at least two as rescue medications should an infection occur, be antagonistic to pathogens, include full analysis of residual virulence and antimicrobial resistance profiles, and, most important, exert unequivocal and demonstrated beneficial effects on the host. In addition, marketed probiotic products need to be correctly labeled with the accurate amount of viable strain-specific organism(s). Probiotic strains of bacteria consumed as dietary supplements are measured by the amount of viable colony forming units per dose. However, dosage requirements may vary by individual, product to product, and by clinical indication. It is debatable how often or whether probiotics should be ingested regularly to enhance persistent health effects. Use of viable organisms is still preferred over heat-inactivated products in which the potential for adverse effects is largely unknown. In addition, cGMP lot-release testing requirements are rigorous, and live biotherapeutic products must be able to meet higher quality testing standards for safety and consistency (Fig. 3).

A detailed discussion of the safety and efficacy results on the large number of clinical indications studied in the published literature is beyond the scope of this review. Despite extensive information from preclinical studies in animals, results from IND regulated, randomized controlled trials are still awaited in the United States before there is clear consensus regarding definitive recommended guidelines as clinical best practice for probiotic use. Unfortunately, many of the earlier clinical studies reported in the literature are problematic because complete CMC data was not necessarily available as part of IND phase I/II clinical investigations, tracking of adverse events were vaguely reported as were definitions of the indications and study outcomes, and studies were further weakened by small sample size and high dropout rates. There are a surprising number of meta-analyses and systematic reviews of completed clinical trials for treatment of a range of gastrointestinal illnesses such as infectious diarrhea in children, prevention of antibiotic-associated diarrhea and *Clostridium difficile* infections, traveler's diarrhea, as well as inflammatory bowel disease (ulcerative colitis, pouchitis, and Crohn disease) and irritable bowel syndrome. There are also several trials and meta-analyses regarding use of *Lactobacillus* and other probiotics for pediatric atopy and atopic dermatitis, neonatal sepsis, and necrotizing enterocolitis. VSL#3 that contains four *Lactobacillus* and three *Bifidobacterium* strains, as well as *Streptococcus thermophilus*, has reported efficacy in the prevention and treatment of pouchitis (38). Adjuvant studies and formulations are complex to study and there is no data currently available to justify a recommendation.

There are also an increasing number of candidate strains with new applications such as for the treatment of obesity, hypertension, hypercholesterolemia, and cancer, although there is insufficient data or clinical trials under regulatory IND to support any recommendation

at this time. Clear and complete CMC data contributes greatly to the success of clinical investigations under IND. Clinical investigations that follow regulatory guidelines and include an evidence-based minimum of consolidated standards of reporting trials (CONSORT), complete CMC, safety and activity, as well as adverse events reporting including severity grading scales are especially important. As indicated later, use of probiotics is not without risk in certain vulnerable populations.

Adverse Effects

Appropriate use and safety of probiotics in critically ill patients has received attention following the recent randomized clinical trial reporting increased bowel ischemia and mortality after probiotic administration in patients in an acute phase of severe pancreatitis (39). The risk of increasing oxidative stress in the critically ill state has led to the general recommendation that probiotics are contraindicated in patients with severe acute pancreatitis and other life-threatening conditions. This is complemented by numerous case reports of invasive infection with probiotic organisms in patients in ICUs, particularly those with central venous catheters and in immune-compromised states. In light of the available evidence, probiotics are not recommended in critically ill patients.

Preterm infants represent a unique, vulnerable group for developing bacterial translocation from their gastrointestinal tract, should overgrowth of pathogenic, endotoxin-producing microbes occur. Administration of lactobacilli- or bifidobacteria-containing probiotics may reduce neonatal sepsis and possibly reduce risk of necrotizing enterocolitis, but the safety of this strategy remains unclear. Overgrowth of *Clostridium* species and other virulent toxin-producing organisms in the immature preterm gut provides increasing support for the underlying rationale that prophylactic administration of commensal probiotics may alleviate the incidence and severity of this life-threatening condition in preterm neonates (23).

Although the available literature suggests that *Lactobacillus* and *Bifidobacterium* strains rarely cause infection in humans, there are more case reports of serious invasive infections with the number of probiotic products on the market and underscores the need for tighter oversight of these products. Rare cases of endocarditis, meningitis, deep abscesses, and bacteremia have been reported for selected strains in subjects with serious underlying health conditions (40). Most rare cases of infection with probiotic *Lactobacillus* (consumed organism matched the infectious isolate by molecular typing) have occurred in patients with predisposing conditions. On the other hand, recent reports suggest that the French and other European populations with long dietary and prophylactic consumption patterns of probiotic lactic acid bacterial products have not reported increases in infection due to these organisms (41).

Long-term effects of probiotic use especially in children are unknown. In one recent study, risk of eczema was reduced in infants administered a *Lactobacillus* probiotic, although asthma symptoms were more frequent in the probiotic group after 7-year follow-up (42).

In development of new candidates and modified probiotic strains, there is recognition that both beneficial and potential adverse effects of probiotics should be considered as strain specific, especially to prevent any chance of potential virulence or horizontal transfer of antibiotic resistance genes. This understanding has also brought attention to the need to improve polyphasic taxonomic identification, combining genomic technologies in assessing biosafety of individual *Lactobacillus* and *Bifidobacterium* strain effects for human consumption. There is growing support for phylogenomic efforts to compile a phylogeny-driven genomic encyclopedia of bacteria (43) and cell-culture banks. It is essential to elucidate metabolic pathways and new diagnostic tests with these approaches. In addition, safety and adverse events reporting will increasingly depend on rapid adoption of DNA-based cross-validation approaches, adherence to regulatory policies, improvement in sentinel monitoring systems, and agreement on international harmonization standards.

Although preclinical studies in animal models, including gnotobiotic studies in pigs and rats/mice, remain critical components in evaluation of safety for any probiotic, their relevance is insufficient to determine safety in humans. Studies of whether long-term colonization with administered probiotics can occur and the safety of colonization over long periods needs intensive study in well-designed, randomized controlled trials that include functional genomics where possible to do so and effects on microbiota and metabolic processes. There is also an important need to more closely assess whether antibiotic resistance can be transferred from administered candidate strains to other commensal organisms, and this will likely require general adoption of metagenomic and visualization tools to do so. This topic is already receiving close attention in the European Union funded "Biosafety Evaluation of Probiotic Lactic Acid Bacteria for Human Consumption" (PROSAFE) (44) and "Assessment and Critical Evaluation of Antibiotic Resistance Transferability in the Food Chain" (ACE-ART) projects (45).

Most bacteria studied demonstrate some degree of intrinsic antibiotic resistance, and, therefore, antibiotic resistance in most members of *Lactobacillus* and *Bifidobacterium* genera is not considered, in itself, a cause of unusual concern. However, to provide normative values for clinical breakpoints and to assure that probiotic strains do not horizontally transfer antibiotic resistance genes to other commensals, the range of antibiotics tested may need to be broadened and applied to many, closely related strains discussed in the European Union funded PROSAFE and ACE-ART projects (44,45).

Source and history of safety including in vitro and in vivo testing should include strain-specific evidence that strains are sensitive to relevant antibiotics and do not contain plasmids for genes encoding transmissible resistance to antimicrobials prior to use in humans or animals. Candidate strains under consideration should not promote virulence factors, including toxicity or bacterial translocation; or produce adverse metabolic activities, including bile salt deconjugation and biogenic amines (e.g., histamine, tyrosine, or phenylethylamine); express hemolytic potential; or promote D-lactate production, deleterious enzymatic activity, or mucin degradation (44,46).

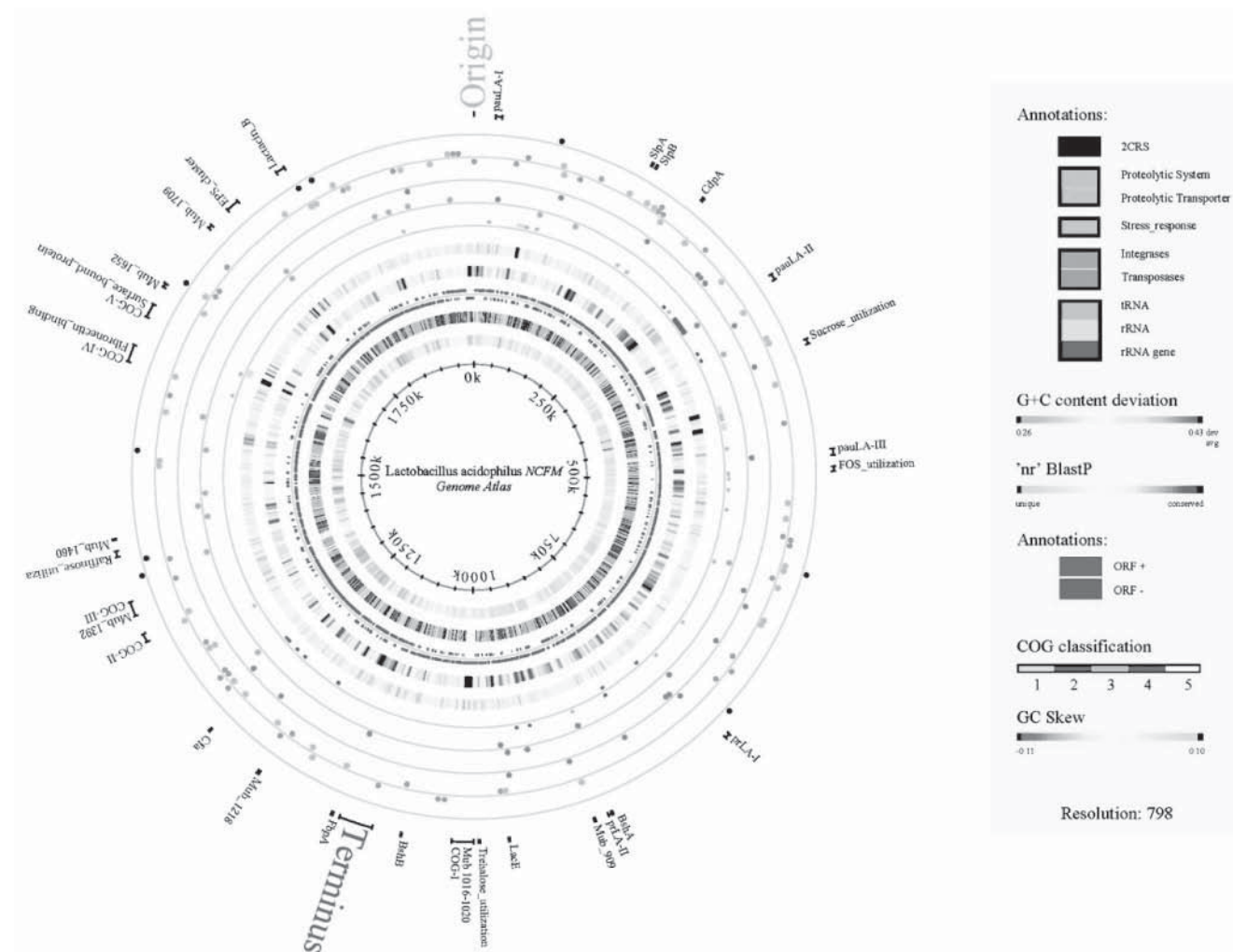


Figure 3 Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. From Ref. 37.

CONCLUSIONS

A large body of research conducted over the past century strongly supports the notion that members of *Lactobacillus* and *Bifidobacterium* genera likely impart health benefits when they populate the gastrointestinal tracts of breast-feeding infants, children, and adults. However, definitive evidence that probiotic *Lactobacillus* and *Bifidobacterium* strains demonstrate specific health benefits is inconclusive, largely due to insufficient CMC data available, sparse number of IND-regulated investigations, and the lack of methodological rigor under which most studies have been conducted (29,31,47,48). Exciting new avenues include the potential for candidate strains to deliver nano-engineered drugs, antimicrobials, and vaccine antigens. To effectively move forward, product development in the field of probiotic biology requires interdisciplinary collaboration across many fields, including clinical medicine, molecular epidemiology, genetics, microbiology, regulatory health policy, evolutionary biology and infectious disease, gastroenterology, immunology, nutrition, microecology, biochemistry, computational biology, and food science tech-

nology. Metagenomic tools and capabilities, in vitro and animal models with germfree, knockout, and conventional animals and rigorous design of randomized controlled trials under IND in humans are beginning to provide critical information on host-microbial relationships in distinct and interconnected body habitats, providing a clearer understanding of the microbial landscape and what comprises a normal microbiota. With this arsenal, it is now possible to complete cross-validated testing on what constitutes safe and efficacious, biotherapeutic use of probiotic organisms (3,31,48).

In a coordinated effort to address probiotic and prebiotic research topics and issues, as well as challenges and safety concerns in the field, the National Institutes of Health (NIH) formed the Prebiotic and Probiotic Working Group (PPWG) in 2006 to work with the NIH institutes, centers, and offices and with affiliated federal agencies in collaborative efforts. The PPWG seeks to facilitate initiatives and collaborations across the NIH and encourage research opportunities (e.g., the Human Microbiome Project).

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Licorice

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INTRODUCTION

Licorice (*Glycyrrhiza glabra* and other related species) is one of the most widely used medicinal plants, employed in traditional formulas since antiquity. It is a perennial herb native to the Mediterranean region and central and south-western Asia, and is now cultivated in temperate and subtropical regions of the world. The extract is obtained from the dried roots and stolons of the plant. Present-day uses of licorice include applications in medicine and food products. Licorice is commonly utilized in foods as a natural flavoring agent. It is reported to possess soothing, anti-inflammatory, and antitussive properties and is used to treat respiratory and gastric diseases and primary adrenocortical insufficiency. Activity against bacteria and several viruses [including hepatitis, HIV, and SARS-associated coronavirus (SARS-CV)] has been reported too.

Triterpene saponins constitute the major chemical components of licorice. Key among these is glycyrrhizin. This glycoside gives licorice root its sweet taste. The other compounds vary from species to species depending on the provenance of the plant. Several flavonoids, as well as other phenolic constituents, are found in licorice; amines, amino acids, sterols, sugars, and starch are also present.

Individuals vary in their sensitivity to licorice. In general, undesirable symptoms (such as hypokalemic hypertension) appear only on long-term consumption of moderate to large quantities, and casual use does not produce side effects. However, even small amounts over short periods of time may lead to adverse consequences in some.

BACKGROUND

- Family: Fabaceae (Leguminosae)
- Genus: *Glycyrrhiza*
- Species: *G. glabra*: *G. glabra* L. var. *typica* Regel & Herd. (Italian licorice, Spanish licorice), *G. glabra* L. var. *glandulifera* (Waldst. & Kit) Regel & Herd. (Russian licorice), *G. glabra* L. var. *violacea* (Boiss. & Noe) Boiss. (Persian licorice)
- Other species: *G. uralensis* Fischer (Chinese licorice), *G. inflata* Batalin, *G. lepidota* Pursh (American licorice)

Glycyrrhiza Glabra

G. glabra grows to a height of 1–2 m. It has pinnate leaves with pairs of narrow leaflets (Fig. 1), pea-like purple-blue flowers, and short and flat pods (1–3 cm long and 6 mm wide) containing small, reniform seeds. The root system presents highly developed stoloniferous roots and rhi-

zomes that spread out just under the soil surface. The parts used for medicinal and food purposes are the dried unpeeled roots and stolons (underground stems). Cultivated roots are harvested after 3–4 years of growth (1).

The varieties of *G. glabra* yielding most of the commercial material are *G. glabra* var. *typica* (Italian licorice, Spanish licorice), which is grown in Italy, Spain, United Kingdom, France, Germany, and the United States; *G. glabra* var. *glandulifera* (Russian licorice), abundant as a wild plant in Russia; and *G. glabra* var. *violacea* (Persian licorice), a wild variety from Iran and Iraq. Other commercially available species are *G. uralensis* (Chinese licorice), a perennial herb 30–100 cm high found in northern China, Mongolia, and Siberia, and *G. inflata* (from China). *G. lepidota* (American licorice) is a wild species native to North America and is found from western Ontario to Washington and south to Missouri, Texas, and Mexico.

Typical licorice roots consist of straight pieces 14–20 cm or more in length and 5–20 mm in diameter (Fig. 1). The unpeeled root has a brownish-grey cork, while the peeled root presents a yellow fibrous exterior. The fracture is fibrous (Fig. 1), the odor is characteristic, and the taste is sweet (the name licorice is derived from the Greek *glükos*, “sweet,” and *ríza*, “root”).

Historical Notes

The first documented use of licorice dates back to Assyrian and Egyptian times—about 2500 years ago. The plant was used in ancient Greece, and the Greek botanist Theophrastus (IV–III century BC) reported its use as a remedy for dry cough, asthma, and other respiratory diseases (2). Among the Romans, Pliny the Elder (I century AD) mentioned the properties of licorice in reducing hunger and thirst and its efficacy against asthma and sterility in women. During the Middle ages, licorice root was used in Arab medicine. The famous *Canon* of Avicenna (AD 980–1037), considered to be an important recapitulation of the medicine of Hippocrates and Galen as well as the philosophy of Aristotle, reports licorice as a remedy for wounds and ulcers and for diseases of the respiratory tract, stomach, kidneys, and bladder. Avicenna also summarized the art of use of licorice root extracts, developed in oriental medicine. In the traditional Chinese *Materia Medica* as well as in Tibetan medicine, licorice has been used against respiratory irritative diseases and for gastrointestinal spasms. In India, licorice has been used in traditional ayurvedic medicine.

Uses of Licorice

Licorice is widely available and used in food applications and medicine. The Council of Europe indicates that it can



Figure 1 *Glycyrrhiza glabra*: leaves (left), dried roots (top right), and the typical aspect on fibrous fracture of the dried root (bottom right).

be added to foodstuffs in small amounts as a flavoring agent, and in the United States, it is listed as “generally recognized as safe” (3). Pharmacopeias and traditional medicine describe licorice as a demulcent, anti-inflammatory, and expectorant, and it is used in cases of bronchial disease, gastritis, peptic ulcer, and primary adrenocortical insufficiency (4).

Excessive ingestion of licorice may produce unwanted and potentially harmful side effects. For example, it can cause pseudohyperaldosteronism, which is characterized by hypertension, edema, and hypokalemia (see adverse side effects section) (5,6). The European Union’s Scientific Committee on Food recommends an upper limit of 100 mg/day for regular ingestion. This is considered to provide a safe quantity of the main active constituent of licorice, glycyrrhizic acid.

CHEMISTRY AND PREPARATION OF PRODUCTS

The main chemical constituents of licorice are triterpene saponins, of which glycyrrhizin is the major component. It is present in amounts ranging from 1% to 24% (1,7). Glycyrrhizin is a glycoside, occurring as a mixture of calcium, sodium, and potassium salts of glycyrrhizinic acid (also called glycyrrhizic acid) (Fig. 2). On hydrolysis, it releases two molecules of D-glucuronic acid and the aglycone 18 β -glycyrrhetic acid (otherwise known as glycyrrhetic acid), a pentacyclic triterpene derivative of the β -amyrin type (1). Glycyrrhizin is considered the most important constituent of licorice root and is responsible for its sweet taste: Its sweetness is about 50 times that of sucrose (6). Upon hydrolysis, the glyco-

side loses its sweet taste. Hydroxy- and deoxytriterpenoid acids related to glycyrrhetic acid, such as liquiritic acid (a C-20 epimer of glycyrrhetic acid), licorice acid, glycyrrhetol, glabrolide, and isoglabrolide (7), have been described. Other components are dependent on species and geographical location. Several flavonoids are present in licorice (about 1%), including liquiritin, liquiritigenin (aglycone of liquiritin), isoliquiritin, isoliquiritigenin (aglycone of isoliquiritin), rhamnoliquiritin, rhamnoisoliquiritin, isoliquiritoside, licoisoflavonol, licoisoflavones A and B, licoisoflavanone, genistein, licoisofuranone, licoricidin, glabrin, glabrol, glabrone, glyzarin, glisoflavone, and glycyrrhisoflavone (1,7). Other phenolic constituents (such as coumarin compounds glycyrol, glycyrin, glycyrcoumarin, herniarin, umbelliferone, licopyranocoumarin, licoarylcoumarin, and licocoumarone), amines (1–2%: asparagine, betaine, and choline), amino acids, sterols (stigmasterol and β -sitosterol), and sugars (5–15% as glucose, sucrose, and mannitol; starch can represent about 20% of the dried root) are also found (1).

Formulations

- *Licorice root liquid extract*: It is an aqueous extract containing 10–20% glycyrrhizinic acid (8), obtained from licorice root with boiling water after maceration. An alcoholic extract (ethanol 70% vol/vol) is also described (9), containing 3–5% glycyrrhizinic acid.
- *Succus liquiritiae*: It is a dried aqueous extract obtained from the roots of licorice, containing 15% glycyrrhizinic acid.
- *Dried aqueous extract or block juice*: It is obtained by boiling the roots of licorice in water and evaporating the

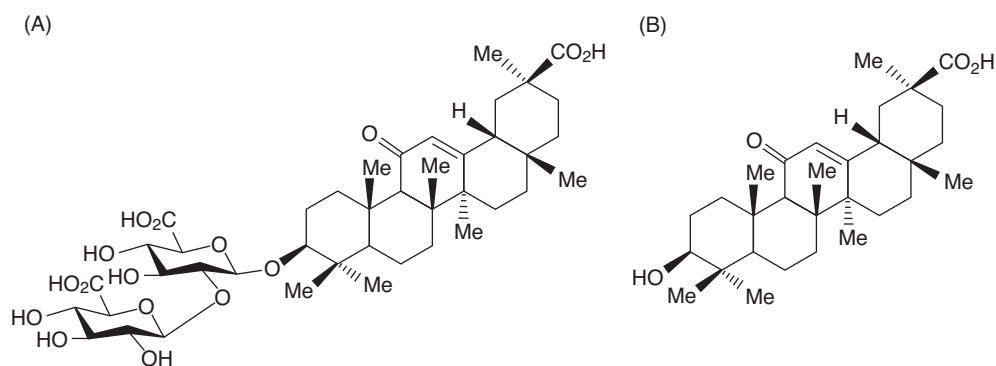


Figure 2 Chemical structures of glycyrrhizinic acid (A) and glycyrrhetinic acid (B).

liquid to dryness. It contains 4–25% glycyrrhizinic acid (6). It is also available as spray-dried powder.

- *Powdered licorice extract*: It is prepared from comminuted licorice extracted with water, or solvents (alcohol, water, or mixtures), containing not less than 6% glycyrrhizinic acid (8).
- *Enzymatically hydrolyzed licorice extract*: It is used as a sweetener in Japan.
- *Deglycyrrhizinated licorice*: This has been developed to obtain some of the therapeutic effects of licorice, with a reduced risk of side effects (1).
- *Licorice root*: It consists of unpeeled, dried roots, rhizomes, and stolons. The content of glycyrrhizic acid must be not less than 2.5% (8) or 4.0% (9) calculated on a dry weight basis.
- *Powdered licorice*: This comprises dried and ground roots of licorice (8), reduced to a fine or very fine powder, containing not less than 2.5% glycyrrhizic acid.
- *Ammoniated glycyrrhizin*: It is prepared from the water extract of licorice root by acid precipitation followed by neutralization with diluted ammonia.

Analysis

Chemical assay of glycyrrhizin (glycyrrhizinic acid or glycyrrhizic acid) can be done by liquid chromatography (9) or thin-layer chromatography (8,9).

PRECLINICAL AND CLINICAL STUDIES

Besides claims for therapeutic effectiveness from traditional use since antiquity against diseases, such as digestive and respiratory tract diseases and inflammation, licorice effects and safety have been extensively investigated in vitro and in vivo, and also in clinical trials. Some clinical reports were uncontrolled studies, but other considered double-blind with crossover design. An extensive review on pharmacology and toxicology of licorice has been published (10).

Mineralocorticoid Activity

Prolonged administration of licorice is associated with side effects such as hypertension, sodium and water retention, and potassium depletion. Clinically, these symp-

toms can be seen in the framework of apparent mineralocorticoid excess (AME) syndromes (6,11); for a review of pathogenetic mechanisms, see Ref. 12. Glycyrrhizinic acid and glycyrrhetinic acid have been reported to bind to mineralocorticoid receptors (or type 1 corticosteroid receptors). The affinity of glycyrrhizinic acid for the receptors is four orders of magnitude lower than that of aldosterone, but it is sufficient to explain, at least in part, the mineralocorticoid-like side effects when large amounts of licorice are consumed (13).

In the kidney, licorice produces a significant change in cortisol metabolism, indicating a strong competitive block (with K_i 5–10 nM) (14) of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) type 2, the enzyme that converts cortisol (active as mineralocorticoid) to cortisone (inactive) (2,13,14) by blocking the cortisol–cortisone “shuttle.” Glycyrrhetinic acid in vitro is 200–1000 times more potent an inhibitor of 11 β -HSD than glycyrrhizic acid (15). Hepatic Δ -4,5- β -steroid reductase, which inactivates glucocorticoids and mineralocorticoids, is also inhibited by glycyrrhetinic acid (12). Licorice constituents may also displace cortisol from its binding to transcortin (16). Plasma aldosterone and renin levels in serum may be reduced by suppression of the renin–angiotensin system, as indicated by a clinical study on volunteers (13).

Glucocorticoid and Antiglucocorticoid Activity

Several authors have reported the usefulness of licorice and glycyrrhizic acid in the treatment of Addison disease (for review, see Ref. 12). This effect is evident when even a small amount of cortisol is produced by the adrenals. In these clinical studies, the authors found that licorice can enhance the action of cortisone in the treatment of Addison disease and concluded that licorice is safe, effective, and free from undesirable side effects, in addition to being convenient, palatable, and inexpensive. The mechanism for its action was clarified after demonstration of the effect of licorice in blocking 11 β -HSD type 2, allowing more cortisol to be available at the level of target tissues.

A measurable affinity of glycyrrhetinic and glycyrrhizic acid for kidney glucocorticoid receptors was found in kidney cytosol, demonstrating a direct glucocorticoid activity (2,13). Chronic fatigue syndrome was successfully treated with licorice through enhancement

of glucocorticoid action (anecdotal evidence reported in Ref. 17).

Glucocorticoids play a crucial role in regulating the distribution and function of adipose tissue. The activity of cortisol in fat cells is modulated by 11 β -HSD type 1, which catalyzes the conversion of hormonally inactive cortisone to active cortisol. This enzyme is expressed in both adipocytes and stromal cells (preadipocytes). Glycyrrhetic acid inhibits its activity and consequently reduces the local availability of cortisol and the accumulation of lipids in adipocytes. This mechanism can partially explain the antiglucocorticoid activity of glycyrrhetic acid in adipose tissue. In a double-blind clinical study, oral consumption of licorice reduced the total fat mass in healthy subjects; topical application of glycyrrhetic acid could be used for the treatment of local fat excess (18).

Activity On Sex Hormones

Estrogenic Activity

Licorice roots are rich in flavonoids and isoflavones, which exhibit estrogen-like activity because of their structural similarity to endogenous estrogens. This configuration could enable them to bind to and activate estrogen receptors (2).

Glycyrrhizin and glycyrrhetic acid have a very weak affinity for estrogen receptors and appear to possess an antiestrogenic effect. Licorice has been demonstrated to influence estrogen metabolism, with opposite effects depending on estrogen concentrations (inhibition at high and enhancement at low concentrations). An estrogenic effect has been reported for the isoflavone components of licorice.

Antiandrogen Activity

Studies in vivo and in vitro have demonstrated that glycyrrhetic acid blocks 17-HSD and 17,20-lyase in the ovary. This effect could partially explain the usefulness of licorice in the treatment of sterility and excess of androgens as in polycystic ovary syndrome (2). In these patients, the mineralocorticoid properties of licorice can reduce the prevalence of side effects related to the diuretic activity of spironolactone, as demonstrated in a cohort prospective clinical study (19). An antiandrogenic effect has been documented in healthy male and female volunteers (20,21). Licorice causes increase of 17OH-progesterone and decrease of testosterone levels probably by inhibiting 17-HSD and 17,20-lyase as mentioned earlier, reducing the conversion of 17OH-progesterone to androstenedione and androstenedione to testosterone. Mean testosterone values have been shown to decrease by 26% after 1 week of treatment. Licorice can also affect 5 α -reductase, thus inhibiting the transformation of testosterone into dihydrotestosterone. Glycyrrhetic acid also binds sex hormone-binding globulin, thus increasing free testosterone concentration. It has, however, been shown that the reduction in testosterone is partially blunted by the increase of luteinizing hormone. Another mechanism involved in the regulation of testosterone during licorice consumption is the inhibitory action of glycyrrhetic acid on 11 β -HSD type 1 in Leydig cells.

Chronic use of licorice can also cause hyperprolactinemia (2).

Antiulcer Activity

Licorice extracts reduce gastric secretion and inhibit gastric ulcer formation in ulcer models in rats (22). Glycyrrhizin and the aglycone glycyrrhetic acid show anti-inflammatory activity on gastric mucosa and increase mucus secretion. Also, deglycyrrhized licorice is able to heal ulcers induced in animals and humans (23). The mechanism of the antiulcer activity is reported to be increased mucus secretion and increased synthesis of glycoprotein in the gastric mucosa, together with antipepsin action (22). Double-blind clinical studies with deglycyrrhized licorice have suggested that its cytoprotective effect may be helpful for patients with gastric ulcer (23,24).

Anti-Inflammatory Activity

Licorice exerts anti-inflammatory and antiallergic effects (25) due to the corticosteroid-like activity of its constituents, which enhance (indirectly) the effects of corticosteroids (26).

In a prospective, randomized, single-blind study, licorice gargle performed 5 minutes before anesthesia was found to be effective in attenuating the incidence and severity of postoperative sore throat (27).

Antitumoral Activity

Licorice and its constituents or derivatives may protect against carcinogen-induced DNA damage and may be tumor-suppressive agents as well (28). Glycyrrhizic acid may exert antitumoral activity because it is an inhibitor of lipoxygenase, cyclooxygenase (COX), and protein kinase C and downregulates the epidermal growth factor receptor.

Polyphenols contained in licorice induce apoptosis in cancer cells (28). Recently, licorice flavonoids have been shown to inhibit the growth of prostate cancer in vitro. This is probably related to an estrogenic effect (29). More recently, we have demonstrated that glycyrrhetic acid, when added to rat liver mitochondria at micromolar concentrations, induces swelling, loss of membrane potential, pyridine nucleotide oxidation, and release of cytochrome c and apoptosis-inducing factor. All these observations indicate that glycyrrhetic acid is a potent inducer of mitochondrial permeability transition and can trigger the proapoptotic pathway. These observed nongenomic effects can be involved in the antitumoral effect of licorice (30).

A chalcone oligoglycoside, the isoliquiritin apioside named "Rlicca" isolated from *G. glabra* (31), was studied for modulatory effect in genotoxicity using in vitro SOS chromotest on *Escherichia coli*, and on human peripheral blood lymphocytes using the single-cell gel electrophoresis Comet assay (31). The results showed marked modulatory effect on the genotoxicity, suggesting further studies to evaluate the chemopreventive potential of the compound.

Glycyrrhizin, alone or in combination with matrine, an alkaloid extracted from the traditional Chinese herb *Sophora flavescens*, showed significant hepatoprotective

and anti-hepatocarcinogenic effect in a rat model in vivo (32). The combination showed a strong nonspecific anti-inflammatory effect and reduced the incidence of sodium and water retention, without causing significant adverse effects.

Inhibition of the enzyme 11 β -HSD type 2 by glycyrrhizic acid tested in a mouse in vivo model reduced COX-2 activity in tumor, tumor growth, and metastasis through a glucocorticoid-mediated suppression of the COX-2 signaling pathway (33), without the adverse effects associated with NSAIDs and selective COX-2 inhibitors. The data suggested a possible future novel approach for colorectal cancer chemoprevention and therapy.

Antimicrobial Activity

The antibacterial activity of licorice is due to the saponin fraction with glycyrrhizin and glycyrrhetic acid, as well as the flavonoid fraction. The flavonoids glabridin, 3-hydroxyglabrol, 4-O-methylglabridin, hispaglabridin A, and glabrol showed activity against *Staphylococcus aureus* and *Mycobacterium smegmatis*, with minimum inhibitory concentrations of a few micrograms per milliliter. The coumarin derivatives, glycyrol and glycyrin, exhibited strong antibacterial activity against *Streptococcus mutans*. Glycycoumarin and licocoumarone inhibited the growth of gram-positive bacteria (34). The isoflavone licoricidin exhibited inhibitory activity against upper respiratory tract bacteria such as *Streptococcus pyogenes*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Three coumarin derivatives, glycyrol, glycyrin, and glycycoumarin, also showed antibacterial activity (34). Glabridin from *G. glabra*, licochalcone A from *G. inflata*, and licoricidin and glyasperin D from *G. uralensis* exhibited activity against methicillin-sensitive and -resistant *S. aureus* (35).

The in vitro growth of the *Candida albicans* strains was markedly reduced, in a pH-dependent manner, by relatively low doses ($\mu\text{g/mL}$ range) of 18 β -glycyrrhetic acid. The compound has been suggested as a promising biological alternative for the topical treatment of recurrent vulvovaginal candidiasis (36).

Anti-*Helicobacter pylori* Activity

The positive effect of licorice on peptic ulcer might be due, in part, to its inhibition of *Helicobacter pylori*. Among the many chemical constituents of licorice, the flavonoids glabridin and glabrene from *G. glabra*, licochalcone A from *G. inflata*, and licoricidin and licoisoflavone B from *G. uralensis* suppressed the growth of *H. pylori*, including clarithromycin- and amoxicillin-resistant strains (37). Also, glycyrrhizin, glycyrrhetic acid, and the lipophilic acetylated derivative glycyrrhetic acid monoglucuronide showed bactericidal activity against *H. pylori* (including clarithromycin- and metronidazole-resistant strains) (37).

Antiviral Effects

Screening investigations with licorice extracts have revealed that glycyrrhizin and glycyrrhizic acid are active against several viruses. In hepatitis B, the virustatic effect depends on inhibition of intrahepatic transport and sialylation of the hepatitis B virus antigen.

In Japan, a formulation containing glycyrrhizin (SNMC, Stronger Neo-Minophagen C[®], composed of 40 mg glycyrrhizinic acid, 20 mg L-cysteine, and 400 mg glycine) has been used for treating chronic hepatitis C, particularly in patients without response to α -interferon. The mechanism by which glycyrrhizin reduces the progression of liver disease without clearing the virus is unknown. A few in vitro and animal (rat) studies suggest that glycyrrhizin inhibits lipid peroxidation, thereby protecting the hepatocytes (38). It has been shown that glycyrrhizin inhibits immunomediated cytotoxicity toward hepatocytes and murine NF κ B activity in liver injury induced by CCl₄-ethanol. A double-blind, randomized, placebo-controlled clinical study conducted on hepatitis C patients, although providing amelioration of serum alanine aminotransferase concentrations, did not induce any effect on hepatitis cRNA levels (39,40).

It is suggested that the anti-HIV-1 effect of glycyrrhizin may be involved in the selective inhibition of the human casein kinase II-mediated stimulation of HIV-1 reverse transcriptase at the cellular level. The antiviral effects of glycyrrhizin may depend on interferon gamma and members of cellular signaling pathways, such as protein kinase II and casein kinase II, and transcription factors such as activator protein 1 and nuclear factor κ B (41).

Glycyrrhizin, albeit at high concentrations, inhibits SARS-CV replication (41). In addition to reducing virus replication, glycyrrhizin inhibits adsorption and penetration during the early steps of the virus replicative cycle. The mechanism of activity of glycyrrhizin against SARS-CV is unclear. Besides affecting several cellular signaling pathways, glycyrrhizin upregulates the expression of nitric oxide synthase, leading to increased production of nitric oxide, which correlates with the inhibition of virus replication (41).

Further antiviral effects and future possibilities of clinical uses are described in detail in a recent review (42).

Effect on Oxidative Stress

In a recent study, Calò et al. (43) have demonstrated that incubation of mononuclear leukocytes with aldosterone or glycyrrhetic acid enhances the protein expression of genes involved in oxidative stress, as well as plasminogen activator inhibitor-1 (PAI-1) and the subunit of NADPH oxidase (p22^{phox}). This effect is genomic, as it is blocked by incubation with aldosterone receptor antagonist canrenone.

PHARMACOKINETICS

Glycyrrhizinic acid is mainly absorbed after presystemic hydrolysis as glycyrrhetic acid (15,44). Because glycyrrhetic acid is as much as 1000 times more potent an inhibitor of 11 β -HSD than glycyrrhizinic acid, the pharmacological and toxicological aspects of its kinetics should be carefully considered (15,44). Following the administration of glycyrrhetic acid (130 mg/day for 5 days), a twofold increase in the cortisol/cortisone ratio in 24-hour urine was observed as a consequence of inhibition of 11 β -HSD. The ratio remained elevated for 4 days after cessation of treatment.

After absorption, glycyrrhetic acid is transported to the liver, where it is metabolized to glucuronide and sulfate conjugates (15,44), which are transported to bile. After outflow of the bile into the duodenum, the conjugates are hydrolyzed to glycyrrhetic acid by intestinal bacteria, and glycyrrhetic acid is reabsorbed, thus reducing its plasmatic clearance (15,44). The gastrointestinal transit rate largely influences the reabsorption of glycyrrhetic acid conjugates. In subjects with prolonged gastrointestinal transit times, glycyrrhetic acid might accumulate on repeated licorice consumption, thus increasing the health risk to this specific subgroup (15,44). The established relationship between the pharmacokinetics of glycyrrhetic acid and its inhibitory effect on 11β -HSD, evidenced by the urinary cortisol/cortisone ratio, suggests that this ratio might serve as a noninvasive marker to identify individuals at risk for excessive glycyrrhizic acid consumption.

CLINICAL EFFICACY

Licorice is considered safe for use as a flavoring and sweetening agent (3). The root is available as a herbal supplement; according to pharmacopeias and traditional medicine, it has demulcent and expectorant properties, and it has been used in cough preparations (45) as suggested by herbal practice. Licorice has ulcer-healing and cytoprotective properties (23), which may result from stimulation of mucus synthesis. Deglycyrrhized licorice has a low mineralocorticoid activity and has been used, in combination with antacids, for the treatment of peptic ulcer disease (46,24). Some clinical studies, however, have reported that the capsule formulation of deglycyrrhized licorice may have poor bioavailability (46), and other studies, such as the double-blind trial (43), have suggested that the product's efficacy in ulcer healing is not superior to that of placebo (47). In recent years, with the advent of powerful specific antiulcer drugs, licorice formulations are no longer used in the treatment of peptic ulcer.

As reported previously, licorice has mild anti-inflammatory and mineralocorticoid properties associated with the presence of glycyrrhizic acid and its metabolite glycyrrhetic acid, which is an inhibitor of cortisol metabolism. However, no evidence for clinically rational mineralocorticoid use is available at present.

The clinical evidence for most of the beneficial and detrimental effects of licorice extracts is contradictory, which is, in part, due to the individual sensitivity of subjects to the different components of the root, the varying content of components other than glycyrrhizic acid in the roots of different species, and the individual metabolism of these components. Clearer data have been obtained using pure glycyrrhetic acid to study potential wanted and unwanted effects; these are more homogeneous. Glycyrrhetic acid is actually the subject of several studies for its potential in blocking type 1 and type 2 11β -HSD and other enzymes involved in steroidogenesis.

ADVERSE SIDE EFFECTS

In evaluating the undesirable symptoms induced by the consumption of licorice, the purity and concentration of

the active ingredient must be considered. Another common and important aspect that needs to be kept in mind is the great variability in sensitivity to licorice among individuals (15). In most cases, it is necessary to consume large or moderately large amounts of licorice for a prolonged period of time to induce unwanted effects. Certain subjects, though, express these effects at low doses consumed over relatively limited time periods. In general, the sporadic consumption of licorice does not produce side effects.

In this regard, it is important to underline the rapid reversibility (1–2 weeks) of these effects following suspension of licorice consumption. Yet, sometimes, after prolonged use, persistent hypertension remained after stoppage of use, even in cases with normalized serum potassium, renin, and aldosterone levels, in a clinical study on volunteers (13).

A daily dose of 10 g of pure licorice dry extract (containing about 7% glycyrrhizic acid) can lead to significant side effects (principally hypertension and fatigue), but even 7 g, which constitutes normal consumption, can bring about nontrivial unwanted effects (13). The most frequent complication of chronic ingestion of licorice is acquired AME syndrome (6,11,12,48–50), which is characterized by retention of sodium and fluids, edema, headache, abdominal pain, depletion of potassium, weakness, nausea, cardiac irregularities, and cramps.

Hypokalemic paralysis with rhabdomyolysis and arterial hypertension following large amount of licorice intake was recently reported in a 47-year-old man (51). A recent case report of a 49-year-old woman physician with peripheral edema, weight gain, and relative hypertension following consumption of licorice candy cigars raised attention to the fact that public is likely not generally aware of the toxic potential of glycyrrhizic acid contained in significant amounts in many licorice products, such as black licorice, chewing gum, herbal teas, soft drinks, tobaccos and herbal remedies for cough, stomach ailments, and constipation (52).

Particularly in elderly people, licorice contained in herbal formulations can cause pseudoaldosteronism, characterized by hypokalemia, rhabdomyolysis, and respiratory impairment, as described in case reports of Refs. 53 and 54.

In a random, population-based urban cohort initially comprising 1049 infants, for whom maternal overexposure during pregnancy to glycyrrhiza as licorice confectuary was reported, children developed decrements in verbal and visuospatial abilities and in narrative memory and significant increases in externalizing symptoms, attention and aggression problems (55). The effect was attributed to inhibition of placental 11β -HSD type 2, the fetoplacental "barrier" to higher maternal levels of cortisol, leading to higher glucocorticoid levels.

Observed Drug Interactions

Several drugs can interact adversely with licorice (56).

- Concurrent use of licorice and thiazide diuretics may cause increased risk of hypokalemia and reduced effectiveness of the diuretic (57,58).
- Combination of licorice with corticosteroids may result in enhanced and prolonged effect of the latter due to

inhibition of their metabolism and delay in their excretion (59,60).

- A case report and a small trial on volunteers taking licorice with oral contraceptives suggest an increased risk of elevated blood pressure and fluid retention (58,61). Licorice itself contains flavonoids with estrogenic activity.
- Licorice may enhance the effect of mineralocorticoids.
- Simultaneous use of licorice and potassium supplements may result in reduced effectiveness of the latter.
- Licorice can reduce the effectiveness of antihypertensive drugs.
- Pharmacokinetic interactions may occur when licorice is taken together with other commonly used drugs, such as warfarin, ibuprofen, aspirin, and deoxycholic acid.
- Flavonoids, such as those found in grapefruit juice, inhibit 11 β -HSD type 2, and therefore licorice can enhance the mineralocorticoid-like effect of grapefruit, as suggested in a clinical study on six male volunteers (62).

Licorice consumption is thus dangerous in patients with hypertension, a family history of hypertension, or diseases of the cardiovascular system, bile tract, kidney, or adrenal gland. It is also unsafe in hyperaldosteronism (primary and secondary), in untreated hypothyroidism, in association with oral contraceptives, during pregnancy, and in diabetic patients.

CONCLUSIONS

Licorice is one of the most used plants, having been utilized since ancient times, and is now increasingly considered to have potential for the treatment of some clinical diseases. Studies using the pure principal component, glycyrrhetic acid, have been supported by clinical and research studies both in vivo and in vitro. The recent evidence on the blocking of 11 β -HSD has inspired further research on the physiological importance of the two isoforms of this enzyme. Glycyrrhetic acid has made possible the evaluation of the mechanism of action of aldosterone in the kidney and in the genesis of pseudohyperaldosteronism. Data also point to the involvement of glycyrrhetic acid in the metabolism of fat and its putative use in reducing triglyceride accumulation in adipocytes. Most surprising are the positive effects on viral infections, particularly hepatitis and SARS.

Studies using root extracts do have some limitations due to differences in components in different species of the plant and in particular in the content of flavonoids and other minor components. The interactions between glycyrrhizinic acid and these other components, which sometimes could have opposing effects, make validation of the clinical evidence and the scientific studies on licorice difficult.

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α -Lipoic Acid/Thioctic Acid

Donald B. McCormick

INTRODUCTION

Lipoic acid, also called thioctic acid, has been known as an essential growth factor for certain microorganisms for half a century and for most of this time, identified as a covalently linked cofactor for α -keto acid decarboxylating enzymes. With humans and other animals that biosynthesize lipoate from fatty acid, it is known that mitochondrial dehydrogenase complexes exist wherein there are lipoyl transacylases that receive acyl moieties from the thiamin pyrophosphate-dependent decarboxylase subunits and vector the acyl group to coenzyme A. A more recent variation of this is the similar function of a lipoyl residue within the glycine decarboxylating system that vectors an aminomethyl function. The dihydrolipoyl enzyme subunits generated in the foregoing cases are then reoxidized to the lipoyl enzymes by FAD-dependent lipoyl dehydrogenase subunits that generally couple with NAD^+ and electron-to-oxygen transfers.

Within the past quarter century, a greater understanding of the antioxidant properties of α -lipoate and its dihydro form has led to commercial use of lipoate in supplements, which are purported to insure better health, including protection against oxidant stress conditions that may be associated with certain diseases. It is the intent of this entry to examine reports that relate the supplement and therapeutic uses in humans, as have been derived from and relate to antioxidant as well as cofactor functions of lipoate. Most literature bearing on the subject occurs within the past 25 to 30 years, with a fairly rapid expansion of reports in the last decade.

CHEMISTRY

α -Lipoic (thioctic) acid is chemically recognized as a 1,2-dithiolane-3-pentanoic acid or 6,8-dithiooctanoic acid. The asymmetric carbon at position 6, using the latter chemical name, is found in nature only as the *R*-(+)-enantiomer, shown in Figure 1, though a racemate of both *R*-(+)- and *S*-(-)-stereoisomers is the result of usual chemical synthesis before resolution as optically active base salts. The polar carboxyl and, to some extent, the disulfide groups allow moderate water solubility of this compound, which is soluble in common organic solvents as usual for fatty acids. The sulfurs within the dithiolane ring provide reactive nucleophilic centers so that electrons in the p_z orbitals readily react with electrophilic functions. Addition to a sulfur with concomitant breaking of the disulfide bond results in covalent adducts that serve as intermediates in transfer of acyl or less frequently alkyl groups.

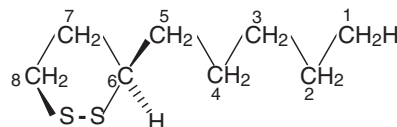


Figure 1 Structure of α -lipoic acid (with numbering as 6,8-dithiooctanoic acid).

Oxidative attack on the dithionyl sulfurs occurs readily to form monoxides and even dioxides. The disulfide function of lipoate is an effective scavenger for hydroxyl radicals. It is also readily reduced to dihydrolipoate, which is the 6,8-mercapto-or(6,8-dithiol)octanoate. The oxidation-reduction (redox) potential for the disulfide-disulfhydryl interconversion is -0.32 V, which is well poised to function within a range of biological redox systems. The pK_a of lipoic acid is 5.4, so that it is the salt form that occurs at physiological pH. Both lipoate and its dihydro form are fairly effective chelators of divalent metal ions, some of which are toxic to most organisms.

METABOLISM AND DYNAMICS

Biosynthesis of lipoate involves a nonheme iron-containing synthase capable of inserting S atoms into positions 6 and 8 of octanoate for the dithiolane ring system (1). Lipoate is fairly readily absorbed *in vivo* even at therapeutic levels. Single oral doses of 200 or 600 mg of racemic lipoate or 200 mg IV were given to 12 healthy subjects (2). The area under the curve following oral and intravenous administration of the 200 mg doses was approximately 47 and 158 $\mu\text{g min/mL}$, respectively; after the 600-mg oral dose, it was also approximately 159 $\mu\text{g min/mL}$. There was no significant difference in mean half-time for plasma concentrations. An absolute bioavailability of 29% was determined after the 200-mg dose. The lack of difference in total plasma clearance indicates nonsaturable kinetics. Oral supplements are better absorbed on an empty stomach than with food (3). Another study in which 50 to 600 mg of the racemate was given orally to healthy volunteers showed that maximal plasma concentrations of the natural *R*-(+)-enantiomer were 40% to 50% higher than those of the *S*-(-)-enantiomer (4).

A metabolic event that occurs with lipoate in humans as well as most organisms is that uptake at cellular level results in much being reduced to the dihydro form. The reduction of free lipoate (and lipoamide) at rather high

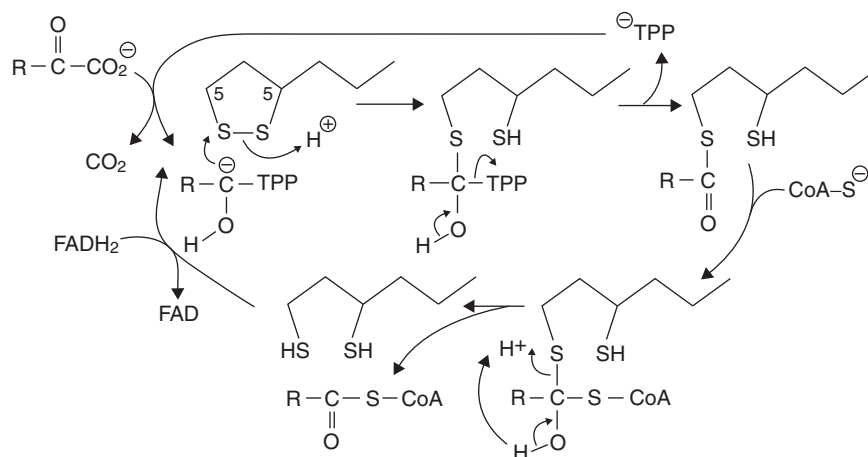


Figure 2 Function of the lipoyl moiety covalently attached to enzymes involved in transacylations following α -keto acid decarboxylations. In multienzyme dehydrogenase complexes in which lipoyl residues function, there is transfer of an acyl moiety from an α -hydroxyalkyl-thiamin pyrophosphate to the lipoyl group of a transacylase core and then to CoA. This results in formation of the dihydrolipoyl group, which is cyclically reoxidized by the FAD-dependent (dihydro) lipoyl dehydrogenase.

levels has been attributed mainly to NADPH-dependent thioredoxin reductase (5). Some essential lipoate is routed through lipoyl-AMP to form lipoylated holoproteins wherein the attachment is at the ϵ -amino function of lysyl residues. The two reactions involved are catalyzed in mammals by separate mitochondrial enzymes. The gene for the second enzyme, lipoyl transferase, has been shown to be located on chromosome 2q11.2 in humans (6). The subsequent operation of both lipoate and dihydrolipoate is summarized in Figure 2. It has been reported that the lethal syndrome of metabolic acidosis found in an infant reflected suppression of the mitochondrial dehydrogenases for pyruvate, α -ketoglutarate, and branched-chain keto acids (7). The decreased activities were attributable to inability to normally utilize lipoate as cofactor, because addition of lipoate to a medium containing the patient's fibroblasts markedly improved conversions of leucine and valine. A severe acidosis found in an 8-month-old boy was attributed to a deficiency of lipoamide (lipoyl) dehydrogenase, which was improved by oral administration of 25 to 50 mg/kg of lipoate (8). Less clear is the case of primary biliary cirrhosis in which antimitochondrial antibodies are reported to be present against the transacylase of the pyruvate dehydrogenase complex (9).

Catabolic events with α -lipoate (10), elucidated in microbes (11-15) and mammals (16,17), are shown in Figure 3, as are the more recently discovered catabolites of dihydrolipoate, which are the methylated and sulfoxidized compounds found in plasma and urine after high oral intakes of lipoate (18,19).

SUPPLEMENT USES AND CLAIMS

Investigations and reviews of the noncofactor nature of α -lipoate have burgeoned in the past two decades. The focus has been on the antioxidant, generally thiol, na-

ture of the lipoate-dihydrolipoate interconversion in cells. In most cases, a therapeutic effect, real or potential, is stated. For example, a review, "The pharmacology of the antioxidant lipoic acid" (20), lists four antioxidant properties of lipoate including its metal-chelating capacity, its ability to scavenge reactive oxygen species (ROS), regenerate endogenous antioxidants, and repair oxidative damage. Dihydrolipoate, formed by the reduction of lipoate, has the capacity to regenerate the antioxidants vitamins C and E as well as glutathione. It can also provide peptide methionine sulfoxide reductase with reducing equivalents. Other reviews on thiol-based antioxidants suggest therapeutic potential for *N*-acetyl-L-cysteine and lipoate but point out that an advantage of the latter is that it is readily recycled in the cell (21,22). Interestingly, some proponents of the use of lipoate as an antioxidant also recognize the pro-oxidant activities of lipoate and dihydrolipoate (23).

Among effects reported for lipoate is that it inhibits the *in vitro* glycation of albumin by glucose (24). The decrease in advanced glycation end products has been extended by studies of lipoate with endothelial cells (25,26) and erythrocytes (27). Incubation of lipoate with Jurkat T (human leukemic T-lymphocyte) cells was reported to inhibit nuclear factor kappaB (NF- κ B) activation (28). Lipoate treatment of these cells also potentiates caspase 3 activation, which leads to Fas-mediated apoptosis (29). The myeloperoxidase-dependent activation of caspase and apoptosis in human HL-60 leukemic cells is protected against by incubation with lipoate as well as dehydroascorbic acid, both of which act via their reduced forms, viz. dihydrolipoate and ascorbate, respectively (30). Reduction of NF- κ B activity, which regulates production of many inflammatory cytokines and adhesion factors, occurred when lipoate was incubated with Mono Mac 6 (a human monocyte) cells (31). Such inhibition of NF- κ B was also reported for lipoate with human aortic endothelial cells (32). Given the presumption of the

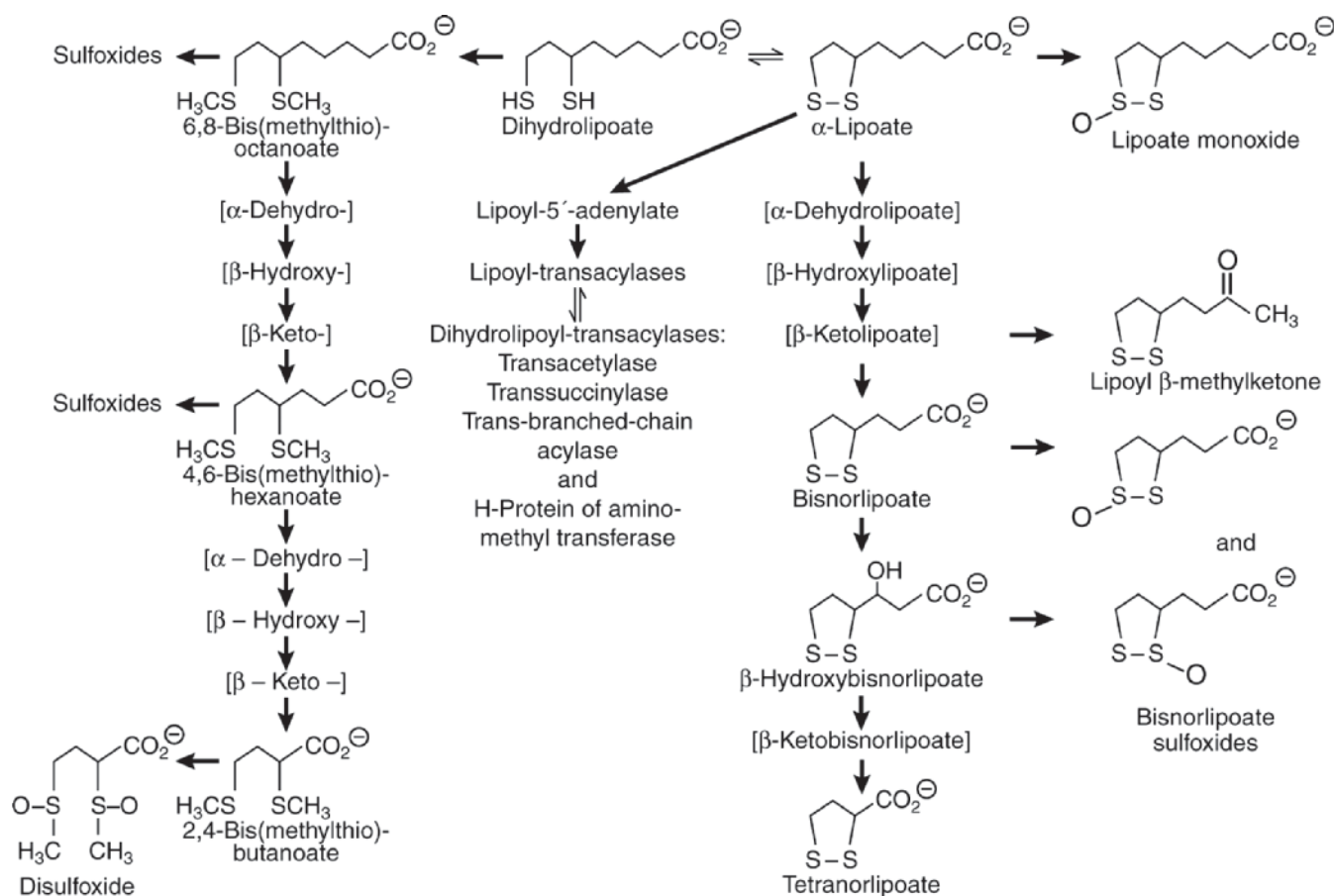


Figure 3 Metabolic conversions of α -lipoate. Intermediates in brackets, that is, the α -dehydro-, β -hydroxy-, and β -keto-compounds are transitory, and in-line-metabolites that must exist with β -oxidation; other compounds and enzymes named have been isolated and/or chemically identified/synthesized.

investigators in these studies of NF- κ B that it is the activation by ROS that is sequestered by lipoate, it is of interest that recent analyses have questioned the role of oxidative stress in activation of NF- κ B (33). Further along lines of the antioxidant potential of lipoate with cells, there have been reports of it leading to an increase in glutathione biosynthesis by improving cystine utilization in several cell types (34). Nitric oxide synthesis in human aortic endothelial cells is stimulated by lipoate as well as vitamin C independent of glutathione status (35). In a comparison of the effects of α -lipoate and α -tocopherol given in high oral doses (600 mg/day lipoate; 400 IU/day α -tocopherol) to healthy adults for 2 months separately and then 2 months in combination, it was determined that lipoate functions as an antioxidant because it decreases plasma oxidation, LDL oxidation, and urinary isoprostanes. However, no additional benefit was seen with the combination of lipoate plus vitamin E (36).

In connecting lipoate (and other antioxidants) with the ability to decrease such reactive oxygen intermediates as are thought to cause "oxidative stress," including that in aging, there are reports that pharmacologic levels of lipoate given orally to rats (37), mice (38), and beagle dogs (39) can prevent or partially reverse memory loss. Even the suggestion has been made that α -lipoic acid may

offer a treatment option for Alzheimer's-type dementia (40), but confirmatory evidence is lacking. A couple of recent reports using rodents as models suggest that α -lipoic acid may remediate age-associated impairment of the Akt pathway in rat hepatocytes (41) and is as effective as caloric restriction in decreasing transcriptional markers of aging in mice cerebellums (42). Some reviewers have suggested lipoate to be "a highly promising thiol antioxidant supplement" (43). Generally, the amounts currently available in over-the-counter or over-the-net sales are in the 30 to 200 mg range. These levels are hundreds of times higher than available in foods (44) that largely contain lipoyl lysyl residues in proteins. Animal tissues that have been estimated to contain significant lipoyl lysine include kidney, heart, and liver; spinach and broccoli are relatively good plant sources.

THERAPEUTIC USES AND CLAIMS

The therapeutic uses of lipoate, in part stimulating the use of supplements, range over several diseases and disorders. One of the prime uses of high-dose (usually 600 mg/day or greater) lipoate is with diabetics, particularly those evidencing neuropathy. Use of lipoate as an adjunct therapy

in diabetes has been in practice in Germany for a generation. One of the earlier groups (in Düsseldorf) reporting benefit from administration of lipoate to Type 2 diabetics with polyneuropathy reviewed 15 clinical trials in a multicenter study and concluded that doses of 600 mg/day or greater given for weeks to months caused some improvement of neuropathic deficits (45). However, in reporting a 7-month, multicenter, randomized, control trial involving 509 outpatients (46), the conclusion reached by the Düsseldorf group was "Findings indicate that a 3-week intravenous treatment with alpha-lipoic acid, followed by a 6-mo oral treatment, had no effect on neuropathic symptoms distinguishable from placebo to a clinically meaningful degree." Yet, the surmise was that this treatment was associated with a favorable effect on neuropathic deficits without causing significant adverse reactions. Ongoing reviews of the literature on lipoate therapy of diabetics include the overviews of reported improvement in insulin sensitivity attributable to intravenous infusions (47) and lessening of the impact of oxidative damage caused by dysregulation of glucose metabolism (48). In a recent review of botanicals and dietary supplements available in the putative treatment of the peripheral neuropathy of diabetics, lipoate is considered with other components that continue to receive attention. However, a concluding statement is that "further studies are needed to confirm their efficacy" (49). Recent studies with animals offer conflicting views. Oral lipoate was reported to significantly correct blood lipids and insulin disorders in hyperlipidemic New Zealand rabbits (50), but intraperitoneal lipoate aggravated energy imbalances in streptozotocin-treated diabetic rats (51).

Among other reports of possible benefit from lipoate therapy are effects seen with *liver disease*, where success was sometimes claimed for *Amanita* (mushroom) poisoning, metal toxicity, carbon tetrachloride toxicity, and alcohol-induced damage (52), though the last had already been refuted by a randomized, double-blind trial (53); *hepatitis C*, where three patients treated with a combination of three "antioxidants," that is, lipoate, silymarin, and selenium, seemingly recovered (54); *cancers*, where functional defects were improved in peripheral blood mononuclear cells from advanced stage patients (55) growth was suppressed in head and neck squamous cells (56), and levels of ROS were decreased in advanced cancer patients with tumors at different sites (57); and *mitochondrial enzyme defects*, where cases of infantile acidosis due to a defect in lipoamide (lipoyl) dehydrogenase were improved specifically (8) and energy levels in brain and skeletal muscle improved in a progressive external ophthalmoplegia due to a mitochondrial cytopathy (58). With *burning mouth syndrome*, which is an idiopathic dysgeusia, earlier reports suggested amelioration by lipoate (59–61), but a recent double-blind, randomized, placebo-controlled study refutes any benefit from lipoate in this disorder (62). In *smell dysfunction following viral infection*, there may be modest benefit but, as pointed out by the authors, "the outcome of double-blind, placebo-controlled studies in large groups of patients must be awaited" (63). Other suggestions for therapeutic use of lipoate, largely based on its ability to decrease some oxidative stress due to ROS, are in HIV infection (64), though no effect on cognitive function in AIDS patients was found (65), in ataxia-telangiectasia, based on cell studies (66), and in genetic anemias (67). The possible

benefit of lipoate as a protective agent against cardiovascular disease has been considered based on work with experimental animals (68). However, any benefit that may accrue to humans must be weighed against the lack of knowledge regarding most appropriate form, dose, and method of administration as well as stage of disease.

As reported by the Linus Pauling web site (69), which generally favors above-mentioned RDA amounts of micronutrients, doses of racemic lipoate as high as 600 mg/day IV for 3 weeks (70) or 1800 mg/day orally for 6 months (71) or 1200 mg/day for 2 years (46) did not result in serious adverse effects when used to treat diabetic neuropathy. Two minor and one major anaphylactoid reactions have been reported following intravenous administration (72). People taking high oral doses note malodorous urine (73).

One finding that should instill caution in those who would promulgate the use of lipoate and perhaps other micronutrients well beyond the nutritional requirement levels is the report of the enhancement of pathogenicity of organisms that depend on the host for the nutrient. Specifically, *Listeria monocytogenes*, a gram-positive intracytosolic pathogen that causes severe disease in pregnant and immunocompromised individuals, depends on host-derived lipoate, which may be critical for in vivo replication of this pathogen (74).

CONCLUSIONS

In consideration of over a hundred publications retrieved electronically, which cover the relatively current knowledge base on lipoic (thioctic) acid use, it is apparent that many are collations derived from studies in vitro, that some of the studies in vivo (particularly with a clinical aim) lack optimal controls, and that a number of these publications are simply reviews that sometimes cite suggestions and suppositions as if they were facts. Nevertheless, the extent and weight of evidence is that there is an additional, noncofactor role of lipoate that may be useful in suppression of "oxidative stress" as encountered in certain diseases. It is less clear whether or not supplement use for otherwise healthy individuals confers any benefit, including delay of aging. More carefully controlled scientific studies with larger numbers of subjects over longer periods will be needed to clarify the possibility that the sale of this compound is justified on a nutritional basis.

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Lutein

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INTRODUCTION

Lutein [(3R,3'R,6'R)- β , ϵ -caroten-3,3'-diol] is a polar lipophilic dihydroxycarotenoid with a molecular weight of 568.871. It contains a conjugated polyene chain and two hydroxylated ionone rings. The compound is biosynthesized by hydroxylation of α -carotene in plants, photosynthetic algae, and some bacteria and fungi. The major function of lutein within these organisms is as an antiphotosensitizing agent and accessory photosynthetic pigment. Lutein is a main constituent of retinal macular pigment and acts as a filter of light at wavelengths capable of inducing photochemical damage and generating reactive oxygen intermediates (ROIs). Lutein intake is associated with decreased likelihood of age-related macular degeneration (AMD), cataract, heart disease, and certain cancers. It may modulate processes or factors involved in immune response. Humans do not have the capacity for de novo biosynthesis of lutein and are thus dependent on dietary sources.

BIOCHEMISTRY, BIOPHYSICS, FUNCTIONS, AND ACTIONS

Of approximately 600 carotenoids identified in nature (1), 50 in the human diet (1), and 20 in human serum (2) only two forms of dietary xanthophylls, lutein and zeaxanthin [(3R,3'R,6'R)- β , β -caroten-3,3'-diol], are present in human macular pigment (3). *meso*-Zeaxanthin [(3R,3'S)- β , β -caroten-3,3'-diol] is the third major macular xanthophyll (4). Figure 1 shows the chemical structures of these compounds. Lutein represents approximately 36% of all retinal carotenoids; zeaxanthin and *meso*-zeaxanthin each represent about 18%. We have applied the term macular xanthophyll to represent these three compounds when discussing ocular structure and function; elsewhere, it represents 3R, 3'R lutein, and 3R, 3'R zeaxanthin.

Lutein and zeaxanthin are found in the same food sources with green leafy vegetables being major sources of lutein and corn and corn products being major food sources of zeaxanthin (5). The food sources, metabolism, and tissue storage of lutein and zeaxanthin are similar. Separation and quantitation of carotenoids are most frequently achieved through high-performance liquid chromatography (HPLC) and spectrophotometric detection with peak integration. The topographical distribution of macular xanthophylls has been determined by spectrophotometry, a microdissection technique with HPLC, heterochromatic flicker photometry, orthogonal compari-

son of polarized light extinction, lipofuscin fluorescence, and Raman spectroscopy (6).

Most major carotenoids in the human diet and serum are internally symmetrical, with one or two cyclic rings at the terminals of a conjugated carbon chain. These compounds can be characterized by the formula $C_{40}H_{56}O_n$; n ranges from 0 to 6. Xanthophylls are carotenoids with one or more oxygen atoms. The chemical formula for lutein is $C_{40}H_{56}O_2$. Carotenes, or hydrocarbon carotenoids (e.g., lycopene and α - and β -carotene), do not contain oxygen.

Lutein contains an allylic hydroxyl group at the 3' carbon of an ϵ -ionone ring (double bond at C4'-C5') and a secondary hydroxyl group at the 3 carbon of a β -ionone ring (double bond at C5-C6). The ionone rings are connected to the conjugated polyenic hydrocarbon chain at the 6' and 6 carbons, as they are for zeaxanthin and most other dietary carotenoids. Zeaxanthin differs from lutein in that it contains two β -ionone rings and thus an additional conjugated bond. There are eight stereoisomeric forms of lutein and three of zeaxanthin (7). In retinal tissue and commonly consumed fruits and vegetables, these xanthophylls exist mainly in a single isomeric (all-*trans*) configuration (5). This suggests that tissue stores are of dietary origin. Within the central macula, *meso*-zeaxanthin exists in concentrations equal to those of all-*trans* zeaxanthin, but it is virtually absent in plants of the human food supply. The relatively lower concentration of lutein within the central retina has led to speculation that *meso*-zeaxanthin may be metabolized from oxidized lutein via a cone-photoreceptor-specific enzyme (2,7-9). Conclusive evidence for this comes from studies in rhesus monkeys with life-long xanthophyll exclusion from the diet. Supplementation with pure lutein or zeaxanthin found that in the 4-mm macular area of the lutein-fed monkeys, approximately half the xanthophyll content was *meso*-zeaxanthin, whereas no *meso*-zeaxanthin was detected in animals fed zeaxanthin (10).

The natural tissue distribution and biochemical and biophysical characteristics of lutein provide a reasonable basis for speculating that this nutrient acts in biological systems as (i) an important structural molecule within cell membranes; (ii) a short-wavelength light filter; (iii) a modulator of intra- and extracellular reduction-oxidation (redox) balance; and (iv) a modulator in signal transduction pathways (7,11-14).

Structural Interactions with Membrane Phospholipids and Transmembrane Proteins

Lutein and zeaxanthin interact with lipids and transmembrane proteins as structural molecules in bioactive

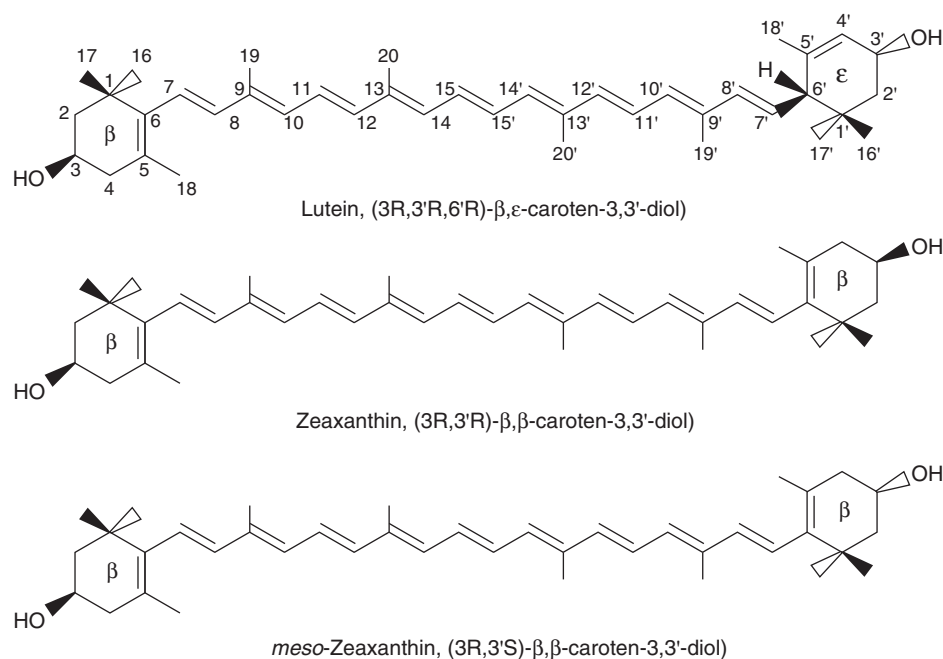


Figure 1 Chemical structures of major macular xanthophylls. Source: Adapted from Ref. 15.

phospholipid membranes. The unique hydroxyl moieties of these carotenoids are responsible for their polar properties and affect solubility, aggregation, and reactivity in membranes. Macular xanthophylls are most soluble in nonpolar or dipolar solvents (15). In primates, xanthophylls are preferentially accreted in lipophilic tissue, but may align within hydrophilic aspects of phospholipid molecules in areas adjacent to the aqueous compartment of cells (12).

The stereochemical configuration of lutein results in orientation of the ϵ -hydroxyl group with the equatorial plane of its ionone ring; the β -hydroxyl group is oriented in an axial direction relative to its ring plane. For zeaxanthin, both β -hydroxyl groups are oriented in an axial direction relative to the ring structures. The stereochemical configuration of zeaxanthin thus constrains the C5–C6 and C5'–C6' double bonds to $\sim 40^\circ$ at the plane of the conjugated polyene chain. The functional implication of this condition is that the ionone ring system can operate independent of the polyene chain (7,15). Structural differences in the orientation of the hydroxyl groups in lutein and zeaxanthin impart specific stereochemical properties to each of these xanthophylls and may affect their recognition by transmembrane and binding proteins (12). In model liposomic membrane systems, lutein has been observed to exist in orientations orthogonal and parallel to the membrane surface (13). Zeaxanthin exists mainly in an orthogonal orientation (11,16).

Lutein binds to β -tubulin, a cytoskeletal protein involved in maintenance of cell shape. It has been suggested that this structural complex may stabilize the dynamic volatility of tubulin within the retina (17,18). More recently, a lutein-binding protein in the human retina has been isolated from the human retina that

meets many of the criteria needed for a specific lutein-binding protein including copurification of the protein with endogenous lutein in a multistep biochemical purification, saturable and specific binding with exogenously added carotenoids, and spectral alterations characteristic of carotenoid–protein interactions (19).

Absorption and Attenuation of Short-Wavelength Radiation

Lutein acts as a filter of short-wavelength light associated with photochemical damage and the generation of ROIs (20). Photochemical retinal injury induced by short-wavelength light (~ 440 nm) affects retinal photoreceptor outer segments and the retinal pigment epithelium (RPE), a layer of the retina that supports the photoreceptors. Photoc damage is maximized at irradiation levels between 400 and 450 nm. At 440 nm, the intensity of light energy required to produce retinal damage is 1/20 that required around 533 nm (21). Macular pigment has a peak spectral absorbance of 460 nm, a range of absorption from approximately 390 to 515 nm, and may filter 40% to 90% of incident “blue” light (15).

The number of conjugated double bonds in the polyene chain and characteristics of the ionone rings determine the peak spectral absorption of a carotenoid. Lutein contains 10 conjugated double bonds; zeaxanthin contains 11. In both of these compounds, nine of the bonds are fully conjugated (7). Subtle differences in the interaction of unsaturated bonds within the polyene hydrocarbon chain with those of the ionone rings lead to stereochemical differences in these compounds; such relationships are manifested as differences in the spectral absorption parameters. The wavelength of maximum

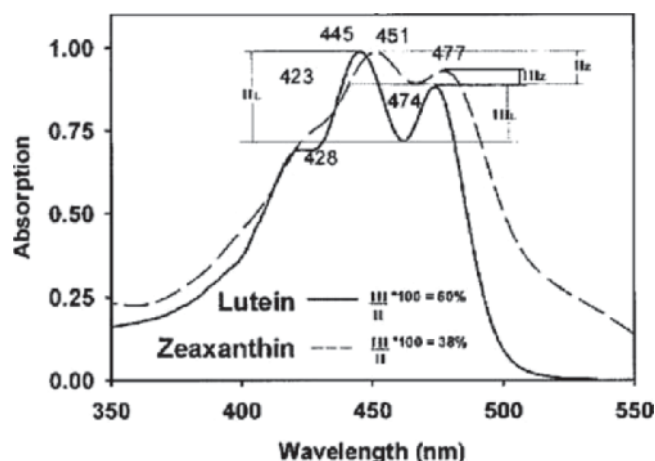


Figure 2 Absorption spectra of lutein and zeaxanthin in ethanol. Source: From Ref. 7.

absorption for lutein is 445 nm. For zeaxanthin, the value is 451 nm (Fig. 2).

Lutein may act against the effects of photochemical retinal injury in its capacity to operate as (i) a biochemical filter of electromagnetic energy in the inner retina (22) and (ii) a sink for ROIs generated through acute or chronic tissue irradiation within the photoreceptors and RPE (23). In the first case, lutein would attenuate the rate of short-wavelength-induced radical generation, and the subsequent potential for peroxy-radical-propagated reactions. In the second case, it may reduce the potential for short-wavelength-induced photosensitization and subsequent generation of singlet oxygen.

Oxidative Stress and Balance of Cellular Reduction–Oxidation

Lutein has the capacity to act as an electron donor to ROIs. Its specific distribution within the macula has led to the speculation that it may operate there to scavenge oxidants and re-reduce oxidized macromolecules (24). Xanthophylls have the capacity to quench triplet excited states of photosensitizers and singlet oxygen through electronic energy transfer to the triplet state of the xanthophylls. Subsequently, this abundant energy is thermally dispersed. Xanthophylls exhibit the capacity to neutralize peroxy radicals and nitric dioxide by forming resonance-stabilized carbon-centered radical adducts and then reacting with vitamin E or vitamin C. The structural characteristics of these xanthophyll-radical cations provide the potential to accept unpaired electrons, thereby possibly conferring pro-oxidant properties to the compound. Under physiological conditions, lutein and other carotenoids are more likely to demonstrate antioxidant properties. Recent reviews provide detailed commentary on these issues (24,25).

With the exception of their ability to quench singlet oxygen, there is a paucity of evidence demonstrating an *in vivo* antioxidant function of carotenoids (15). Although there is biological plausibility for the idea that lutein and zeaxanthin act as macular antioxidants, the ox-

idation products of these xanthophylls that are found in macula (2) may be a result of high oxidant stress (12).

Key factors that may affect the anti- or pro-oxidant activities of carotenoids in biological systems include molecular structure and form; site of action within the cell; concentration; interaction with dietary anti-oxidants; and partial pressure of oxygen (25).

Molecular Structure

The conjugated polyenic system on the carotenoid hydrocarbon chain facilitates electron transfer reactions and quenching of singlet oxygen. The polyene chain length allows the position of the excited molecular state to shift to a less reactive configuration within the carotenoid radical. The position of the hydroxyl groups on the ionone rings affects the xanthophyll's ability to react with molecular O_2 as well as its positioning within the membrane. Lutein shares electron-quenching potential with other carotenoids, but the stereoconfiguration of its OH moiety may increase chemical quenching potency.

Cellular Site of Action

Carotenoids exist in biological systems strongly bound within protein or lipoprotein complexes. This alters the absorption spectrum relative to those of monomeric (free) xanthophyll chromophores. The nature of a membrane and its composition affects carotenoid interactions and thus influences thermostability and membrane fluidity. The orientation of xanthophylls that span the membrane allows reactions with the polyene chain across its depth. Such a configuration would affect penetration of reactive oxygen metabolites to the highly susceptible fatty acid domain of the membrane. The two orientations of lutein would place the nutrient in close contact with the aqueous and lipid-dense compartments of the cell and allow interaction with both water- and lipid-soluble compounds involved in attenuating oxidative stress.

Interaction

Carotenoids may re-reduce oxidized vitamins that have the capacity to modulate redox state (26). Xanthophylls interact with vitamins C and E *in vitro*. The polar nature of xanthophylls may permit a more effective interaction with vitamin C than that of β -carotene, because they have the capacity to bind closely to membrane phosphate head groups at the aqueous interface of the cell. There may be a synergistic response between carotenoid-radical cations and carotenoids. In this capacity, lutein seems to work most effectively with other carotenoids, but β -carotene is unable to reduce lutein or zeaxanthin cations.

Partial Pressure of Oxygen

Carotenoid radicals may react in the presence of high molecular O_2 to form carotenoid-peroxy radicals through autoxidation. These compounds may then react readily with unsaturated fatty acids within cell membranes. Lutein is less sensitive to O_2 tension than β -carotene and is less likely to react with this type of radical. Carotenoids may also react with the lipid-peroxy radicals (ROO^\bullet) via addition to any area within the polyene chain. This reaction yields a resonance-stabilized carbon-centered ROO -carotenoid $^\bullet$ adduct with the capacity to inhibit the

chain-propagating step in lipid peroxidation. Adduct formation is an O₂-dependent process, and at high partial O₂ pressure, there is a risk of reversible reaction with molecular O₂ to yield secondary peroxy radicals. Carotenoids may react via electron transfer from the polyene chain to a radical that subsequently may yield carotenoid cation, anion, or alkyl radicals. The carotenoid cation radical has the capacity to react with ascorbate (7) to re-reduce the carotenoid to its original form. This provides the basis for suggesting that carotenoids may prevent irreversible oxidation of proteins, nucleic acids, and unsaturated fatty acids. Also, carotenoids may react via allylic hydrogen abstraction to yield carotenoid radicals (15,24).

Lutein may exert an antioxidant capacity against ROO• and singlet oxygen molecules (7). It may react with ROO• by direct electron addition to generate a more stable carotenoid radical. This less reactive radical is more likely to exist long enough to react with reducing agents (e.g., vitamin E, vitamin C) within the tissue. Lutein may also interact with singlet oxygen and convert, via energy transfer, to a triplet state carotenoid that “is able to harmlessly relax through vibrational transitions and collision without destructive bond breaking.”

Signal Transduction Cascades—Interactions with Signaling Molecules

Xanthophylls may modulate key factors and processes in cell-signaling pathways (27). Lutein has been suggested to influence expression of the connexin gene. This gene codes for signaling molecules involved in intercellular gap junctional communication, a process that modulates cellular proliferation in human tumor systems. Xanthophylls also bind with lipocalin proteins. Certain lipocalin proteins have been implicated as immunomodulators.

PHYSIOLOGY

Tissue Distribution

Lutein is a major carotenoid of serum and some lipophilic tissues. Approximately 90% of all carotenoids in the body is stored in tissues; less than 10% is found in circulation (24). Lutein is circulated nonpreferentially by high-density lipoprotein (HDL) and low-density lipoprotein (LDL) (28). Transport in the fasted state is conducted mainly by HDL, with a smaller amount carried by very-low-density lipoprotein (VLDL) (29,30). Table 1 displays the concentration range of lutein within a number of tissue types. Liver, adrenal, adipose, pancreas, kidney, and breast tissue usually contain higher levels than most other tissues. Of these, adipose has the highest proportion of total carotenoids as lutein and zeaxanthin. Furthermore, among adipose tissue sites (abdomen, buttocks, and thigh), abdominal concentrations of lutein are the greatest (31). Interindividual variation in tissue stores is substantial and has been attributed to demographic, environmental (diet based and lifestyle based), and genetic factors (32).

There is a selective accretion of lutein and zeaxanthin in the retina (15), and specific binding proteins are now under investigation (19,34). The concentration is approximately 1 mM in the human fovea (the cone-photoreceptor-dense area of the central macula that processes fine pattern information). Retinal concentrations may be 1000 times

Table 1 Concentration Range of Lutein within Various Tissues

Tissue	μmol/L (serum)/ μmol/g (tissue) ^a	μg/dL (serum)/ μg/g (tissue) ^b
Serum	0.10–1.2	0.10–0.66
Adrenal	—	0.28–5.73
Adipose	—	0.29–2.70
Pancreas	—	0.08–1.21
Liver	0.10–3.0	0.10–0.66
Kidney	0.04–2.1	0.02–0.06
Lung	0.10–2.3	—
Outer segments	100	—
Retina	100–1000	—

^aSource: From Ref. 12.

^bSource: From Ref. 33.

higher than those in serum and other tissue (Table 1). The macula also contains the highest proportion of lutein and zeaxanthin as total carotenoids. Within the macula, the cellular distribution of lutein is believed to be highest in photoreceptor axons and interneurons of the inner plexiform layer (35). In eccentric retinal areas, lutein is believed to be present mainly in the rod photoreceptor outer segments (23,36).

The distribution of total and individual macular xanthophylls varies with retinal eccentricity (7–9). The average mass of lutein and zeaxanthin per unit retinal area was 1.33 and 0.81 ng/mm² at the foveal center and at an eccentricity of 1.6 to 2.5 mm, respectively. The lutein/zeaxanthin ratio at 0° to 5° was 1.0:1.6 in one study. At 5° to 19°, the ratio was 1.4:1.0. At 19° to 38°, it was 2.0:1.0. Research groups examining the distribution of macular xanthophylls have observed similar patterns, with relatively more zeaxanthin in the fovea (36). The distribution of xanthophylls may have implications for light processing and tissue protection, as subtle differences in the structure and physical form of lutein and zeaxanthin lead to positional differences in phospholipid membranes.

Snodderly et al. (35,37,38) have examined the distribution of macular xanthophylls in nonhuman primates. Figure 3 shows the macaque retina in cross section and the distribution of macular pigment within layers, relative to the fovea.

Plasma, Serum, Cell, and Macular Concentrations

Lutein and zeaxanthin are among the six carotenoids that contribute 60% to 70% of total plasma carotenoids under fasting conditions. These xanthophylls are commonly reported as a single value, as their elution profiles are similar; when grouped as such, they follow lycopene to represent the second highest concentration of carotenoids within total plasma composition (1). The plasma lutein/zeaxanthin ratio is 4 or 5:1.

Landrum and Bone (7) present the current knowledge on serum response to lutein and zeaxanthin/serum lutein and zeaxanthin levels of people consuming common Western diets are substantially lower than those attainable with supplementation; higher serum concentrations of lutein and zeaxanthin metabolites are found with supplementation; *meso*-zeaxanthin is produced from lutein; within the central macula, *meso*-zeaxanthin attains highest concentrations and represents approximately half of the zeaxanthin present in this region.

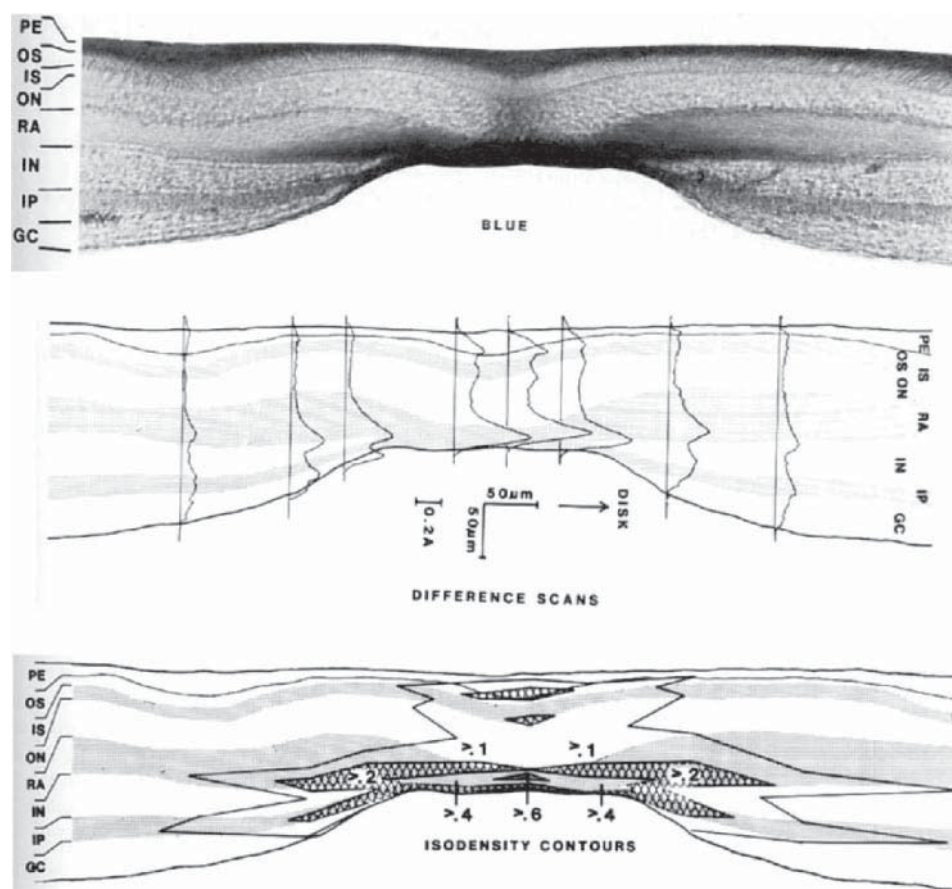


Figure 3 Distribution of macular pigment within layers of the macaque retina. Top panel: Photograph of a 45- μ m section through the fovea of a female macaque in blue light. Middle panel: Graphs of macular pigment density by retinal layer and eccentricity. Units are absorbance units relative to green light. The scale bar represents 0.2 absorbance units. Bottom panel: Isodensity contours with values of absorbance relative to those produced by green light. The highest pigment density within the macular region was observed in the nerve fiber layers. *Abbreviations:* PE, retinal pigment epithelium; OS, photoreceptor outer segments; IS, photoreceptor inner segments; ON, outer nuclear layer; RA, photoreceptor axons; IN, inner nuclear layer; IP, inner plexiform layer; GC, ganglion cell layer. *Source:* From Ref. 34.

In experimental populations fed with a low-carotenoid diet, plasma carotenoid levels decreased at a rate proportional to the concentration of nutrient in the diet for 2 to 4 weeks. Thereafter, the rate declined slowly to a plateau. Initial half-lives of lutein/zeaxanthin were 12 days in a 30-day feeding study and 19 days in a 64-day study (39). The mean plasma depletion half-life of lutein/zeaxanthin is estimated to be between 33 and 61 days from a metabolic ward study on a 13-week vitamin C-free diet providing 0.4 mg carotenoids/day (40). Serum concentrations were approximately 45% to 50% of baseline in 68- and 90-day feeding studies using low-carotenoid diets (40,41).

Stable isotopes have been used to characterize xanthophyll plasma response across a 528-hour period. ^{13}C -labeled lutein was fed as part of a low-carotenoid meal and showed a rapid serum response, with a unimodal distribution peaking at 16 hours (42). Figure 4 shows the results from this study. It is important to note that the 3-mg (5.3 mmol) lutein dose was dissolved in high-oleic safflower oil and represented a physiological level attainable through diet. A monophasic 16-hour peak was also re-

ported in a feeding study of an ethyl acetate form of lutein in tocopherol-free corn oil (0.5 mmol/kg body weight, 3.84 mmol/L) with samples taken across an 840-hour period (43).

A number of intervention studies with lutein supplements and/or feeding regimens have been developed to characterize plasma and serum lutein responsiveness. Although ability to compare across studies is constrained by differences in study design, implementation, and analysis, a number of consistencies emerge in the results: The serum or plasma response to nearly equal intakes of lutein was approximately two times higher in the groups receiving lutein in supplement form; and diets with ≤ 2 mg lutein/day had negligible effects on serum and plasma responses across study periods ranging from 1 week to 1 year.

A number of studies have demonstrated macular tissue response to dietary lutein and zeaxanthin intake. In a 140-day supplementation study on two subjects consuming 30 mg lutein/day, macular pigment increased 21% in one subject and 39% in the other (44). In a carotenoid-rich-food-based intervention, 9 of 13 subjects

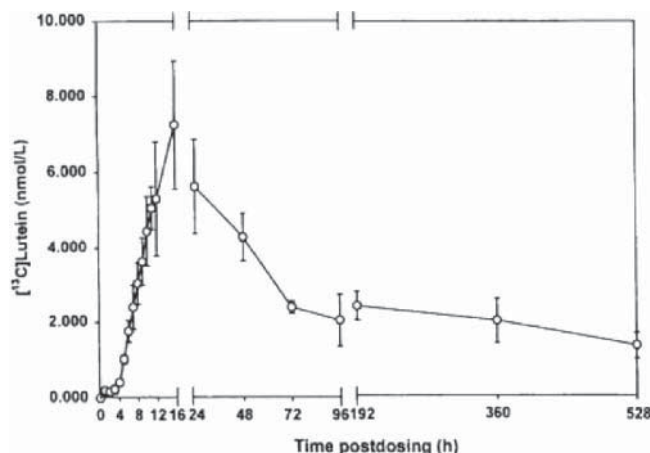


Figure 4 Plasma response to [^{13}C]lutein across a 528-hour period in subjects consuming a 3-mg oral dose of per-labeled [^{13}C]lutein. Values are mean \pm SEM; $n = 4$. Source: From Ref. 41.

responded to the diet with an average 20% increase in macular pigment density (45). Quantification of macular xanthophylls is possible with a number of in vivo imaging technologies (7,15).

Bioavailability

Bioavailability is the fraction of a consumed nutrient available for use in normal physiological functions or stor-

age (46). Deming and Erdman (28) present the current knowledge on fundamental pathways of carotenoid absorption and metabolism in mammalian species. Figure 5 displays the pathway of carotenoid absorption and metabolism.

Xanthophylls must first be released from the food matrix; this is the major factor modulating bioavailability of carotenoids. Bioavailability of xanthophylls from cooked dark-green leafy vegetables is not different from lutein supplements, whereas the bioavailability from egg yolk is higher than these sources (47). Most lutein from dark-green leafy vegetables is sequestered and noncovalently bound in protein–oxycarotenoid complexes within chloroplasts and organelle matrices. It is present within a matrix of fiber, digestible polysaccharides, and proteins. Prior to absorption through the mucosal cells into the portal or lymph systems, esterified xanthophylls must be hydrolyzed. Dietary lipids are essential for solubilization of lutein esters, as well as sufficient secretion of pancreatic esterases and lipases. As a diester, lutein has stronger hydrophobic properties than the monoester form, and is thus more difficult to solubilize.

Mild heating or grinding may enhance bioavailability by dissociation of these protein complexes. At this stage in the pathway, the stereochemical properties of the xanthophyll may also hinder the efficiency of nutrient release. Once released from the carrier matrix, xanthophylls are transferred to and incorporated within lipid micelles in the small intestine. The polar nature of xanthophylls results in micelle surface binding. Xanthophylls are then absorbed via passive diffusion to the intestinal mucosa, where they are incorporated into chylomicrons.

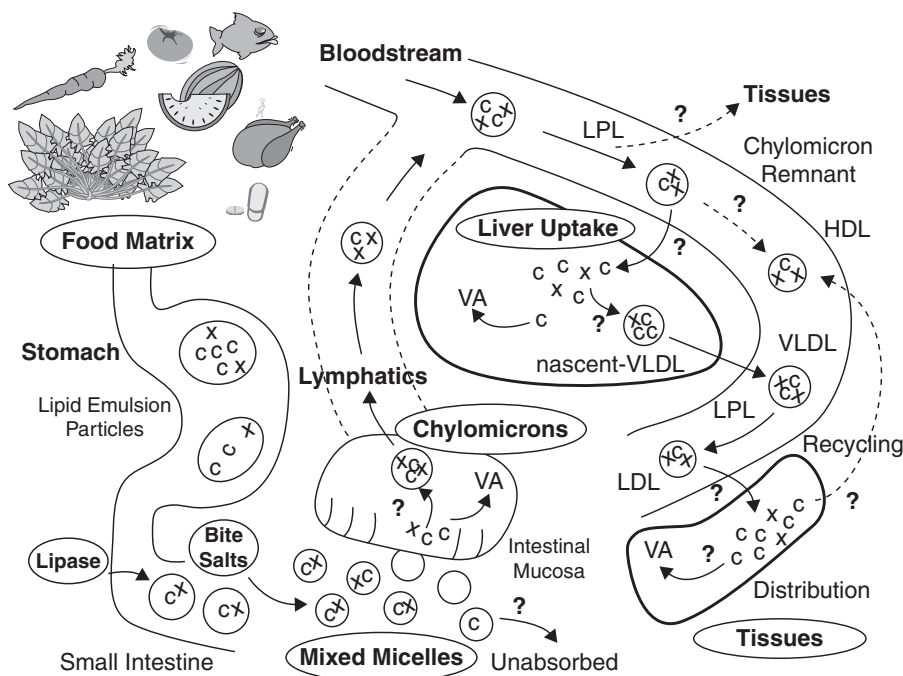


Figure 5 Pathways of carotenoid absorption and metabolism in mammals. Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; LPL, lipoprotein lipase; C, carotenes; X, xanthophylls (lutein and zeaxanthin); VA, vitamin A. Source: From Ref. 28. (View this art in color at www.dekker.com.)

The orientation and alignment of xanthophylls on micelle surfaces are thought to lead to more efficient enterocyte processing than that of the nonpolar hydrocarbon carotenoids. In a carotenoid-feeding study, lutein was present in chylomicrons at 2-hour postconsumption, while β -carotene and lycopene were observed at 4 to 6 hours. Xanthophylls contained in human chylomicrons may exist exclusively in a hydrolyzed form and are believed to occupy space near the surface of the structures. As chylomicrons are secreted into the lymphatic system en route to the liver, they are transformed into remnants by lipoprotein lipase. Once processed by the liver, the xanthophylls are circulated on the surface of VLDL. The surfaced-based positioning of the xanthophylls increases the potential for exchange across lipoprotein classes. Under normal conditions, there is nonpreferential binding of xanthophylls with LDL and HDL. These lipoproteins then transport xanthophylls to lipophilic tissue, where they bind to cell-surface receptors and are degraded by lipoprotein lipase. The mechanism of lutein uptake in the retina has not been fully elucidated, although a specific mammalian retinal receptor has been identified (34). Transport to retinal tissue also may occur via passive disposition.

In most dietary intake studies, lutein bioavailability ranged between 30% and 45% [appendix of Ref. (48)]. In a study using supplements, the bioavailability was approximately 80%. There are no standardized or validated methods for quantitative assessment of carotenoid bioavailability. Castenmiller and West (49) suggest that whole plasma pharmacokinetic studies are limited in their ability to represent bioavailability, as values produced represent carotenoid exchange from tissue storage and excretion, in addition to dietary carotenoid absorption. Postprandial carotenoid levels in chylomicrons of triglyceride-rich lipoprotein plasma fraction are considered a more appropriate measure of bioavailability, as they reflect uptake of newly absorbed carotenoids. This marker has been used for lutein determination (50).

Castenmiller and West (48) provide an extensive review of theory and research on nine key factors that may influence carotenoid bioavailability. These include carotenoid species (configurational or geometrical isomers, xanthophylls or hydrocarbon moieties); molecular linkage (free vs. esterified moieties); amount of carotenoid ingested; food or supplement matrix containing the carotenoid; compounds operating as effectors of absorption (nutrients, bioactive compounds, gastric pH); nutrient status of the individual; genetic factors affecting intake and status; host-related factors affecting postabsorption metabolism (sex, age, general health status, and health-related behaviors); and synergistic effects. We discuss selected factors below.

Species

Hydroxylated carotenoids appear to be more bioavailable than carotenes. When the area under the absorption curve for plasma is used to estimate relative rates of absorption, lutein appears to be absorbed two times as good as β -carotene (39). The relative bioavailability of carotenoids has also been assessed through chylomicron response to a natural carotenoid supplement. The proportion of lutein in chylomicrons was substantially higher than that of

other carotenoids relative to carotenoid composition of the supplement (51).

Linkage

Circulating carotenoids are noncovalently bound to lipoproteins and not homeostatically controlled; as such, plasma concentrations are highly dependent on intake (39). Xanthophylls must be de-esterified (hydrolyzed) prior to absorption. The natural form of most xanthophylls is that of esters with long-chain fatty acids or as glycosides. Supplemental formulations of lutein exist in suspended crystalline and esterified forms. Bioavailability from supplements has been estimated to be not different from food sources (47).

Amount

When ingested at levels normally present in the Western diet, carotenoids do not exhibit selective inhibition of bioavailability. Highly concentrated carotenoid supplement intake may affect the plasma response of xanthophylls and carotenes (52).

Matrix/Effectors

The bioavailability of lutein is reduced in the presence of certain forms of fiber, sucrose polyester (a dietary fat substitute), and pharmacological levels of β -carotene. Dietary fiber has been shown to reduce bioavailability by approximately 40% to 75% (53); the mechanism of action in this case has been linked to lipid metabolism pathways. Higher intake of dietary fiber is associated with increased fecal excretion of bile acids; this reduces the absorption of lipids and lipid-soluble compounds.

Dietary fat is required for the transfer of xanthophylls to lipid micelles in the small intestine. More than 3 g of dietary fat consumed simultaneous with lutein esters was required for the solubilization and induction of pancreatic enzymes that mediate the process of de-esterification (54). Likewise, dietary lipid analogs, phytosterols, and pharmaceuticals that alter lipid absorption all reduce carotenoid uptake. Plasma lutein/zeaxanthin response to intake of 6 mg of carotenoids was 17% lower in a group of women consuming 30 g alcohol/day, for three menstrual periods, relative to levels during a similar period in which alcohol was not consumed.

Nutrient Status

Plasma lutein is lower in children with falciparum malaria when compared with age-matched peers. Malnutrition negatively affects carotenoid absorption, possibly through its effects on lipid absorption.

Host-Based Factors

Smokers have concentrations of carotenoids of the order of approximately 20% to 30% less than nonsmokers (55). Other important factors have been discussed by Rock, Thornquist, and Neuhouser (56).

Potential Variable Factors

Serum response to lutein may be affected by nutrient interactions with dietary carotenoids, fat, and fiber, and alcohol; food source; variability in food lutein content; and food processing/preparation techniques (Table 2). When carotenoids are consumed at physiological levels, there are

Table 2 Predictors of Lutein/Zeaxanthin Intake and Lutein Tissue Status

Factor	Lutein/zeaxanthin intake	Lutein tissue status
<i>Demographic</i>		
Higher age	↑	↑
Female sex	↑	↑
Non-White race	↑	↑
Higher education	↑	↑
<i>Diet based</i>		
Total energy intake	↑	↓
Body mass index	↓	↓
Lutein/zeaxanthin intake		↑
Lipid intake		↑
Alcohol intake		↓
Fiber intake		↓
<i>Biomedical</i>		
Serum cholesterol		↑
Diseases affecting lipid metabolism		↓
Tx lipid storage and metabolism		↓
<i>Health-related behaviors</i>		
Exercise	↑	
Smoking	↓	↓
Sun exposure	↑	

Note: Regression coefficients reported in Ref. 55. Tx = "treatment for conditions affecting."

no significant interactions affecting absorption, distribution, metabolism, or excretion. Pharmacological doses of β -carotene lower the bioavailability of lutein. Carotenoid interactions in human supplementation studies are reviewed by Van den Berg (52).

INDICATIONS AND USAGE

Food Sources

Lutein is most highly concentrated in dark-green leafy vegetables. Some commonly consumed sources of lutein in the Western diet are broccoli, spinach, collard greens, kale, corn, and peas. Release 16 of The United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference contains detailed information on lutein/zeaxanthin content on a wide variety of foods and is available online (57). USDA also provides reports of the USDA-NCC Carotenoids Database of Foods containing lutein/zeaxanthin (58). In these databases, the food content of lutein is combined with zeaxanthin. Recently, major food contributors of lutein and zeaxanthin have been analyzed for their separate concentrations of these xanthophylls (5). Table 3 lists lutein/zeaxanthin values of selected foods. Sommerburg et al. (36) report the lutein content of foods; in this sample, relative to total carotenoids, corn had the highest proportion (60%). Approximately, half of all carotenoids is lutein in kiwi fruit, zucchini, and pumpkin. The ratio of dietary lutein to dietary zeaxanthin in Western diets is 7:1 to 4:1 (7).

Lutein is commercially available in free or diesterified forms as nutritional supplement. The main source of the nutrient is marigold (*Tagetes erecta*). Concentrations usually range from 6 to 25 mg/capsule. A list of commercially available lutein-containing supplements, the nutrient composition of these supplements, and the supplement manufacturers is present at the Natural Medicines

Table 3 Amount of Lutein/Zeaxanthin (mg) Per 100 g in Selected Foods

USDA-NCC #	Food	Amount
11236	Kale, frozen, cooked	19.70
11234	Kale, fresh, cooked	18.25
11464	Spinach, frozen, cooked	15.69
11457	Spinach, fresh, raw	12.20
11458	Spinach, fresh, cooked	11.31
11164	Collards, frozen, cooked	10.90
11313	Peas, frozen, cooked	2.40
11642	Summer squash, cooked	2.25
20112	Spinach egg noodles, cooked	2.23
11477	Zucchini, raw	2.13
11090	Broccoli, fresh, raw	1.69
11101	Brussels sprouts, frozen, cooked	1.54
11091	Broccoli, fresh, cooked	1.52
11093	Broccoli, frozen cooked	1.50
11423	Pumpkin, cooked	1.01
09148	Kiwifruit, raw	0.12
11912	Corn, frozen, cooked	0.05

Note: From the USDA-NCC Nutrient Database for Standard Reference, Release 16.

Comprehensive Database (59). When this work went to press, this database listed 1002 products.

Actions in Relation to Concentration

Tissue concentrations required to support the blue light absorption and attenuation action of xanthophylls are not currently known. This capacity and those related to redox balance may be dependent upon coexistence of vitamins E and C. In vitro, higher concentrations of β -carotene were associated with increased risk of autooxidation and generation of ROIs. This was not the case for zeaxanthin.

Possible Benefits of Lutein Consumption

Mares-Perlman et al. (32) have reviewed evidence on the relationship of lutein and zeaxanthin with health and chronic disease. Consistent protective associations of these nutrients in high- versus low-intake categories were observed for prevalence of nuclear cataract and cataract extraction. Protective relationships were also observed between dietary intake and certain forms of age-related macular degeneration, although such associations were not observed in all studies. Although existing evidence is insufficient to establish a diet-disease relationship, the distribution composition, and concentration of macular xanthophylls give strong basis for speculation. The role of lutein and zeaxanthin supplementation on AMD progression is being tested in the current AREDS extension trial (AREDS 2) (60).

Research on lutein intake and immune function is equivocal. In one case, a 15-mg supplement given for 26 days was ineffective in increasing monocyte surface molecules. In another, diets of 0.4% and 0.04% lutein/zeaxanthin decreased short-wavelength-light-induced cellular proliferation and acute inflammation in hairless mice fed for 2 weeks. Kim and colleagues used lutein-feeding regimens in canine (61) (0 to 20 mg lutein/day for 12 weeks) and feline (62) (0 to 10 mg lutein/day) populations to demonstrate that this nutrient is capable of stimulating aspects of cell-mediated humoral immune responses.

Protective associations of higher levels of dietary and serum lutein with cardiovascular risk have been reported in large epidemiological studies (32). In vitro studies demonstrate the capacity of lutein to affect the dynamics of cell adhesion molecules operating within key pathogenic pathways of atherosclerosis. Because lutein coexists with other carotenoids and macromolecules-sharing antioxidant and vasoregulatory properties, it is difficult to attribute an independent effect to this compound.

The evidence for a xanthophyll–cancer relationship is suggestive for premenopausal breast cancer, colon cancer, and skin cancer, although the question of whether carotenoids or components of carotenoid-rich foods confer benefit is still in question (32). This argument can be made for any of the carotenoid–disease relationships.

Dietary Reference Intakes (DRIs)/Prevention of Deficiency

The Food and Nutrition Board (FNB) of the U.S. National Academy of Sciences does not consider existing scientific evidence on the relationship of carotenoids with health and disease in *in vivo* systems to be strong enough to establish DRIs.

Humans lack the capacity to biosynthesize carotenoids. As such, macular xanthophyll tissue status is modifiable by and dependent upon intake of the preformed compounds. The FNB has not issued a statement defining carotenoid or xanthophyll deficiency. The mean intake of lutein/zeaxanthin was 3.8 mg/day among the 25% of The Third National Health and Nutrition Examination Survey (NHANES III) participants who were consuming the number of vegetable servings recommended by The Dietary Guidelines for Americans.

Mares-Perlman, Fisher, and Klein (63) have investigated habitual patterns of lutein/zeaxanthin intake in NHANES III. Median intake of lutein/zeaxanthin ranged from 0.32 mg/day in the lowest quintile to 9.7 mg/day in the highest. Average intake was 1 to 2 mg/day. Other groups have reported averages of 2 to 4 mg/day. Median serum levels ranged from 0.19 mmol/L in the lowest quintile to 0.79 mmol/L in the highest.

Because lutein and zeaxanthin values from food are commonly represented within food composition databases as a composite value, it has been difficult to determine the relative proportions of each compound within a typical intake pattern. Schalch, Dayhaw-Barker, and Barker (6) cite studies in European populations with values of 0.1 and 0.3 mg/day for zeaxanthin and 1.0 to 2.3 mg/day for lutein. Based on results of epidemiological studies on age-related eye diseases, a number of commercial-sector groups have suggested that 6 mg/day of lutein/zeaxanthin is associated with a decreased risk of cataracts and AMD (64–66).

ADVERSE EFFECTS

The FNB reports that chronic exposure to carotenoids is not associated with teratogenic, mutagenic, or carcinogenic effects in experimental animals. Kruger et al. (67) concluded likewise in a review of a crystalline lutein product. The only known adverse effect of high lutein intake

from diet is carotenoderma, a benign and reversible condition characterized by change in skin color. A public document from the U.S. Food and Drug Administration (FDA) references a report from the Cognis Corporation stating that existing studies “show no toxic or adverse effects from the consumption of lutein esters and that long-term consumption of lutein esters is well tolerated.” The reference to the Cognis report describes a 112-day study in which participants consuming a mixed lutein ester product of 30 mg/person/day developed carotenoderma. A 90-day study at doses of 40 mg/person/day did not result in carotenoderma among the study participants (68).

Two randomized clinical trials testing pharmacological levels of β -carotene intake have been terminated early due to increased incidence of adverse events among subjects receiving the active agent (see chap. 16, “ β -Carotene”). In model systems, high concentrations of hydrocarbon carotenoids have led to autoxidation of the polyene chain and a targeting of lipid membranes by subsequently generated radical species. Within model membranes, the biophysical and biochemical properties of xanthophylls may allow membrane stabilization that prevents lipid–peroxyl radical chain propagation and preserves membrane integrity (24,25,27). Higher cellular concentrations of zeaxanthin were associated with decreasing amounts of DNA damage.

COMPENDIAL/REGULATORY STATUS

Certain lutein products have been considered as substances generally recognized as safe (GRAS) in accordance with the FDA-proposed regulation (62 FR 18938). Independent, private GRAS panels have reviewed a lutein ester product in Xangold[®] (Cognis Corporation, LaGrange, Illinois, USA) and a crystal-line product in FloraGLO[®] (Kemin, Des Moines, Iowa, USA). Cognis states that the acceptable daily intake of the lutein ester product, for its intended purposes, is 40 mg/person/day (GRAS notice no. GRN 000110). Kemin states that the level for the inclusion of its product in medical foods is 20 mg/person/day.

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Lycopene

Rachel Kopec, Steven J. Schwartz, and Craig Hadley

INTRODUCTION

Observational and experimental evidence strongly implicates dietary variables as critical determinants of human health and disease risk. Among the many dietary variables concerning the promotion of optimal health, a diet rich in a variety of fruits and vegetables appears to be a major contributing factor. Much of the evidence derived from human epidemiologic studies suggests that an increased intake of fruits and vegetables is associated with a lower risk of cardiovascular diseases (1,2) and many types of cancer (3–5). Modern epidemiologic techniques employing detailed diet assessment tools have allowed investigators to further define the potential health benefits of specific fruits and vegetables suggested by observational epidemiologic studies and laboratory investigations. Numerous epidemiological studies have shown that the consumption of tomatoes and tomato-based foods containing lycopene are inversely associated with the risk for certain cancers (6) and cardiovascular disease (7). There are numerous phytochemicals in these foods that are hypothesized to be responsible for the potential health benefits observed in these studies; however, a majority of the research has focused on lycopene.

DIETARY AND SUPPLEMENT SOURCES

It is estimated that more than 80% of lycopene consumed in the United States is derived from tomato-based products (8), although apricot, guava, watermelon, papaya, and pink grapefruit also provide a dietary source (Table 1) (9–11). The presence of lycopene in human plasma and tissues primarily results from the consumption of tomatoes and a variety of tomato-based products, such as spaghetti sauce, salsa, tomato soup, and ketchup. The lycopene content of tomatoes can vary considerably with variety and ripening stage. Concentrations in the red strains approach 50 mg/kg compared with only 5 mg/kg in yellow varieties (9).

Although the vast majority of lycopene consumption is from dietary sources, supplements provide an additional vehicle. Recently, the pigment has been added to vitamin/mineral supplements, including some Centrum products (Wyeth, Madison, New Jersey, USA) and One-A-Day Men's Health (Bayer, Leverkusen, Germany). The amount of lycopene contained in one pill of these products is from 0.3 to 0.6 mg. Interestingly, these levels are nearly 50 to 100 times lower than that contained in a single can of condensed tomato soup (i.e., approxi-

mately 30 mg). Lycopene has also been added to Similac Advance EarlyShield infant formula (Abbott Nutrition, Columbus, Ohio, USA). In addition, supplemental forms such as LycoVit[®] 10% (BASF, Ludwigshafen, Germany) and Lyc-O-Mato[®] (LycORed Natural Products Ltd., Beer-Sheva, Israel) deliver lycopene as gelatin beadlets and/or soft-gel capsules. LycoVit 10% contains synthetic lycopene, whereas Lyc-O-Mato is a tomato extract that contains lycopene and other carotenoids. Lacto-lycopene[™] (Nestle, Vevey, Switzerland) is sold in Ateronon supplements (Cambridge TheraNostics, Cambridge, UK) and is also used in Innéov skin supplements marketed in Brazil, Russia, and parts of Europe. Lacto-lycopene is provided with whey proteins as carriers to assist in lycopene absorption and has been shown to have similar bioavailability to lycopene from tomato paste (59).

CHEMISTRY AND BIOCHEMISTRY

Carotenoids are natural pigments synthesized by plants and microorganisms (see chapter on "Carotenoids"). The most established roles of carotenoids in plants are to protect cells against photooxidation and to serve as light-absorbing pigments during photosynthesis (14). Approximately 700 carotenoids have been characterized and share common structural features, such as the polyisoprenoid structure and a series of centrally located conjugated double bonds (Fig. 1) (15–17). The color and photochemical properties of each one are determined by its structure (16,17). Lycopene is responsible for the characteristic red color of tomatoes and tomato-based foods. The structure also contributes to the chemical reactivity of carotenoids toward free radicals and oxidizing agents, which may be relevant to *in vivo* biological functions in animals (16,17). Lycopene is a 40-carbon (C₄₀H₅₆) acyclic carotenoid with 11 linearly arranged conjugated double bonds. Because of the highly conjugated nature of lycopene, it is particularly subject to oxidative degradation and isomerization. Chemical and physical factors known to degrade lycopene, in addition to other carotenoids, include exposure to light, oxygen, elevated temperature, extremes in pH, and active surfaces (18,19,20).

Some dietary carotenoids, such as β -carotene, provide an important source of vitamin A. The presence of a β -ionone ring structure contributes to the provitamin A activity of various carotenoids. Lycopene, however, lacks the β -ionone ring structure and is therefore devoid of provitamin A activity.

Table 1 Common Food Sources of Lycopene

Food	Type	Amount per serving		Serving size
		(mg/100 g wet wt.)	(mg)	
Apricot	Fresh	0.005 ^a	0.007	140 g
Apricot	Canned, drained	0.065 ^a	0.091	140 g
Apricot	Dried	0.86 ^a	0.34	40 g
Chili	Processed	1.08–2.62 ^a	1.40–3.41	130 g
Grapefruit	Pink, fresh	3.36 ^a	4.7	140 g
Guava	Pink, fresh	5.40 ^a	7.56	140 g
Guava juice	Pink, processed	3.34 ^a	8.35	240 mL (~250 g)
Ketchup	Processed	16.60 ^a	3.32	1 tbsp. (~20 g)
Papaya	Red, fresh	2.00–5.30 ^b	2.8–7.42	140 g
Pizza sauce	Canned	12.71 ^a	15.89	125 g
Pizza Sauce	From pizza	32.89 ^c	9.867	Slice (~30 g)
Rosehip puree	Canned	0.78 ^a	0.47	60 g
Salsa	Processed	9.28 ^d	3.71	2 tbsp. (~40 g)
Spaghetti sauce	Processed	17.50 ^d	21.88	125 g
Tomato	Red, fresh	3.1–7.74 ^c	4.03–10.06	130 g
Tomato	Whole, peeled, processed	11.21 ^c	14.01	125 g
Tomato juice	Processed	7.83 ^c	19.58	240 mL (~250 g)
Tomato paste	Canned	30.07 ^c	9.02	30 g
Tomato soup	Canned, condensed	3.99 ^c	9.77	245 g
Vegetable juice	Processed	7.28 ^c	17.47	240 mL (~250 g)
Watermelon	Red, fresh	4.10 ^a	11.48	280 g

^aUSDA 1998. USDA-NCI Carotenoid Database for U.S. Foods. Nutrient Data Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville Human Nutrition Research Center, Riverdale, Maryland.

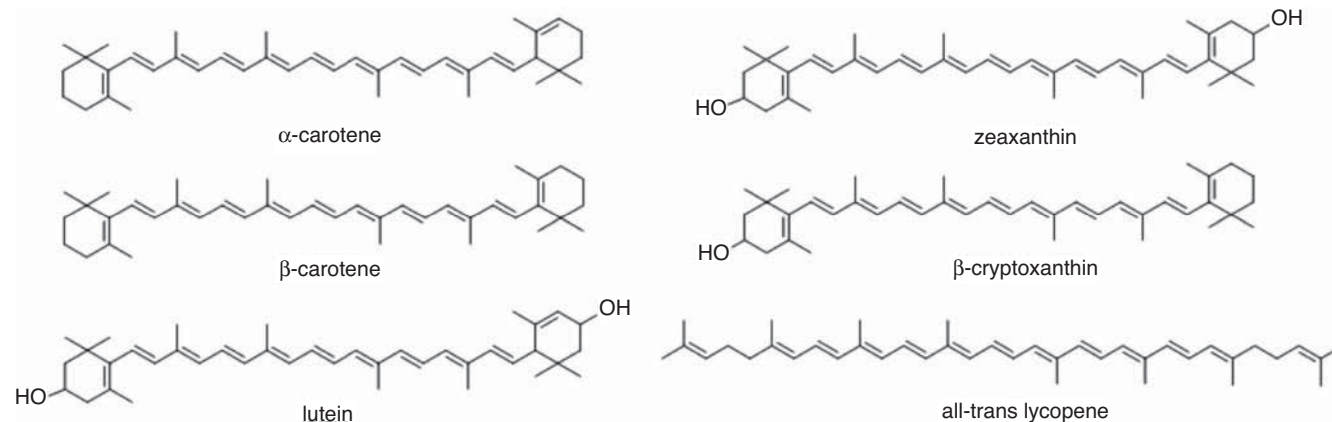
^bSource: From Ref. 10.

^cSource: From Ref. 12.

^dSource: From Ref. 13.

With very few exceptions, lycopene from natural plant sources exists in the all-trans form, which is the most thermodynamically stable configuration (20–22). As a polyene, lycopene readily undergoes cis–trans isomerization. As a result of the (11) conjugated carbon–carbon double bonds in its backbone, lycopene can be theoretically arranged in 1056 different geometric configurations (Fig. 2). Although a large number of geometric isomers are possible for lycopene, Pauling (23) and Zechmeister

(20) have found that only certain ethylenic groups of a lycopene molecule can participate in cis–trans isomerization reactions because of steric hindrance. Interconversion of isomers is thought to take place with exposure to thermoenergy, absorption of light, or by involvement in specific chemical reactions. Cis isomers of lycopene have chemical and physical characteristics distinctly different from those of their all-trans counterpart. Some of these differences include lower melting point, decreased color

**Figure 1** Representative structures of common carotenoids.

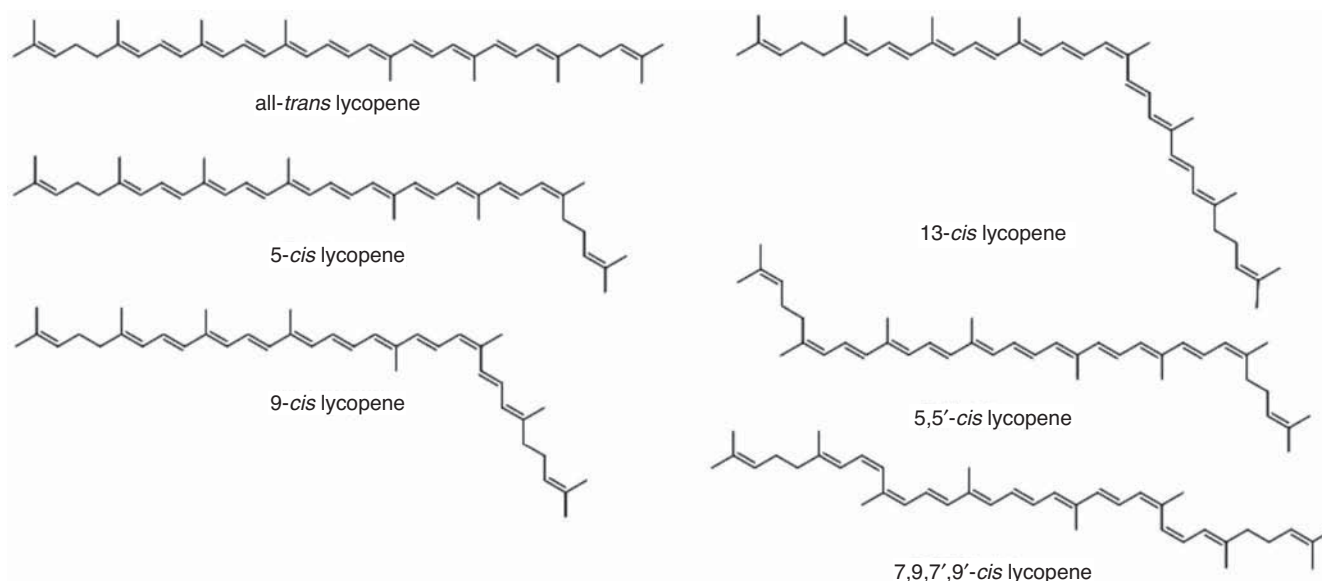


Figure 2 Structures of selected lycopene isomers.

intensity, a shift in the λ_{\max} , smaller extinction coefficient, and the appearance of a new maximum in the ultraviolet spectrum (24). To avoid underestimating the quantitative measurement of lycopene cis isomers, the appropriate wavelength maximum and molar absorptivity values should be applied. Unfortunately, extinction coefficients have been published for very few lycopene isomers. Consequently, quantitative data for isomer content of biological samples are generally estimated values.

ANALYTICAL ADVANCES AND ISOMER CHARACTERIZATION

High-performance liquid chromatography (HPLC) is the most commonly used method for the separation, quantitation, and identification of carotenoids found in plasma and biological tissues. Lycopene is separated from other carotenoids by using HPLC with reversed-phase C_{18} columns. Compared with conventional C_{18} reversed-phase and silica normal-phase columns, reversed-phase C_{30} columns are frequently used to achieve superior selectivity of lycopene isomers (25,26). The polymerically synthesized C_{30} columns not only provide excellent separation of the all-trans lycopene isomer from the cis counterparts, but also display extraordinary selectivity among the individual cis isomers (26,27). An HPLC method using multiple columns in series has also been shown to resolve cis and trans lycopene isomers (28). Variations in the properties of the silica-packing material in terms of carbon load, particle size, porosity, end-capping technique, and polymerization can also significantly alter the selectivity and sensitivity of lycopene analysis (25,29–31).

Similarities in the structural characteristics of carotenoids cause difficulty when trying to adequately

identify individual carotenoids using only fixed wavelength or retention time data. The use of photodiode array detection, allowing for the collection of spectral data across a wide range of wavelengths, has improved our ability to more accurately characterize individual carotenoids. Measurements of retention time, peak resolution, and spectral data for individual absorbing species, and the use of authentic standards for comparison of UV/vis spectra and retention times are required (32).

The ability to detect and quantify very low levels of carotenoids in biological samples is greatly improved with coulometric electrochemical (EC) detection and tandem mass spectrometric (MS–MS) detection. These methods are especially useful for analysis of tissues and chylomicron fractions. The limit of detection (LOD) of lycopene by using an HPLC–EC method has been reported at 50 fmol (33). In contrast, Fang et al. (126) have reported an LOD of 11 fmol for an HPLC–MS–MS method. These methods of detection are 10 to 100 times more sensitive than UV/vis (33,126). Mass spectrometric and tandem mass spectrometric analyses also provide molecular weight and characteristic fragmentation patterns. This additional information increases our confidence in the identification of various carotenoids (32). Historically, electron impact and fast atom bombardment were used in mass spectrometric analysis of carotenoids (34–36). Recently, HPLC systems have been interfaced with MS detectors via electrospray and atmospheric pressure chemical ionization methods for both identification and quantification of carotenoids.

Identification and structure elucidation of isomeric carotenoids have been facilitated with the aid of high-resolution nuclear magnetic resonance (NMR) spectroscopy. Hengartner, Bernhard, and Meyer (37) reported the use of 1H - and ^{13}C -NMR, and UV/vis, mass, and infrared spectroscopy to fully characterize 15 (*E/Z*)-isomeric forms

of lycopene. Likewise, Fröhlich et al. have isolated and characterized some of the most biologically relevant lycopene isomers by ^1H NMR, ^2D NMR, UV/vis, and MS (114). The continuing improvements in analytical technology will significantly impact future investigations designed to elucidate the biological impact of lycopene and its isomers on tissues and organs. Investigators ranging from epidemiologists and clinical scientists to those involved in animal studies will be able to more precisely quantitate lycopene and its isomers in extremely small biological samples.

STABILITY AND ISOMERIZATION

Consumers use the intensity of the red color as an index of quality for tomato-based products. Therefore, reducing the loss of lycopene throughout the production process and during storage has always been an important issue for the food-processing industry. Exposure to thermal treatments during food-processing operations can cause changes in the physiochemical stability of carotenoids. Boskovic (39) and Cano et al. (40) observed that processing and extended storage of dehydrated tomato-based products resulted in a loss of all-trans lycopene content by up to 20%. Food-processing techniques, such as canning and freezing, led to a significant reduction in lycopene and total carotenoid content in papaya slices. In contrast, many studies have found that hydrocarbon carotenoids such as lycopene, α -carotene, and β -carotene in processed fruits and vegetables are fairly heat resistant (41,44). According to Khachik et al. (41), most of these carotenoids remain stable after bench-top food preparation. In addition, no major changes in phytofluene, phytoene, and ζ -carotene were observed during the processing of tomatoes (43). Saini and Singh (44) reported that thermal processing had no effect on the lycopene content in juices made from several high-yield tomato hybrids. Zanori et al. (45) reported that despite the oxidative and thermal severity of the drying process, reflected in the 5-hydroxymethyl-2-furfural and ascorbic acid values, lycopene displayed high stability during drying of tomato halves. In addition, Nguyen and Schwartz (12) reported that thermal processing does not have a significant effect on the stability of lycopene, independent of product type, moisture content, container type, tomato variety, and severity of heat treatments.

Although lycopene may be fairly stable during standard food-processing procedures, less is known about the impact of heat on isomerization. Some studies have shown that heating tomato juice and bench-top preparation of a spaghetti sauce from canned tomatoes increases cis isomer concentrations (28,46). In contrast, Khachik et al. (42) observed that common heat treatments during food preparation, such as microwaving, steaming, boiling, and stewing, did not significantly change the distribution of carotenoids in tomatoes and green vegetables. Other studies have also reported low levels of lycopene cis isomers in thermally processed tomato-based products (14,27). Nguyen, Francis, and Schwartz (48) reported that typical cooking of tomatoes did not affect the thermal isomerization of all-trans lycopene, all-trans δ -carotene, all-trans γ -carotene, or prolycopene. Observations in our laboratory indicate

that lycopene is quite stable in its native matrix within tomato tissue. However, extensive physical and thermal processing that disrupts cell wall constituents can expose lycopene to degradative reactions. In addition, food products containing lipids or supplements formulated with oil will partially solubilize lycopene, leading to isomerization reactions. Further heating such as cooking of foods in the presence of oils and fats will facilitate solubilization, isomerization, and oxidation reactions. Difference in formulation and the extent of processing treatments may account for discrepancies in literature reports on the stability and isomerization susceptibility of lycopene. In addition, once extracted into organic solvents, lycopene is very labile, and analysis should take place immediately without exposure to heat, light, or oxygen. Additional information needs to be gathered on the thermal behavior of lycopene before definitive answers can be offered regarding its physical state and stability during processing and cooking. Very little data is available with regards to the stability of lycopene in supplemental form. Nevertheless, it is evident that the pigment is more stable in native tomato fruit matrices than in isolated or purified form due to the protective effects of cellular constituents (49).

BIOAVAILABILITY

Phytochemicals present in tomatoes and tomato-based products must be readily bioavailable for absorption to mediate their hypothesized beneficial health effects. Bioavailability is defined as the fraction of an ingested nutrient that is accessible to the body through absorption for use in normal physiologic functions and for metabolic processes (50). Differences in the bioavailability of lycopene may account, in part, for the relatively poor correlation between blood lycopene concentrations and estimated dietary intake.

Carotenoid absorption first involves micelle formation in the small intestine (Fig. 3). Micelles are composed of bile salts, cholesterol, fatty acids from the meal, and lipophilic compounds present in the digesta (110). Historically, carotenoids were believed to pass from the micelle into the enterocyte via passive diffusion (62,63). However, recent research has demonstrated that the carotenoids lycopene, β -carotene, and lutein are at least partially transported into the enterocyte by scavenger receptor class B, member 1 (SR-B1) (111–113). After absorption, lycopene is incorporated into chylomicrons, which are released into the lymphatic system and secreted into blood (110). Little is known about how lycopene in chylomicrons is subsequently accumulated by the liver and other tissues, repackaged in lipoproteins, and returned to the circulation. Lycopene is carried in the plasma entirely by lipoproteins, and no other lycopene-specific binding or carrier proteins have been identified thus far (53,54). It is hypothesized that highly lipophilic carotenoids, such as lycopene, are present within the hydrophobic core of the lipoprotein particle.

Details of how hepatocytes, the initial source of circulating lipoproteins, transfer lycopene into specific secreted lipoproteins, and how this process may be regulated are unclear. However, it is likely that dietary and

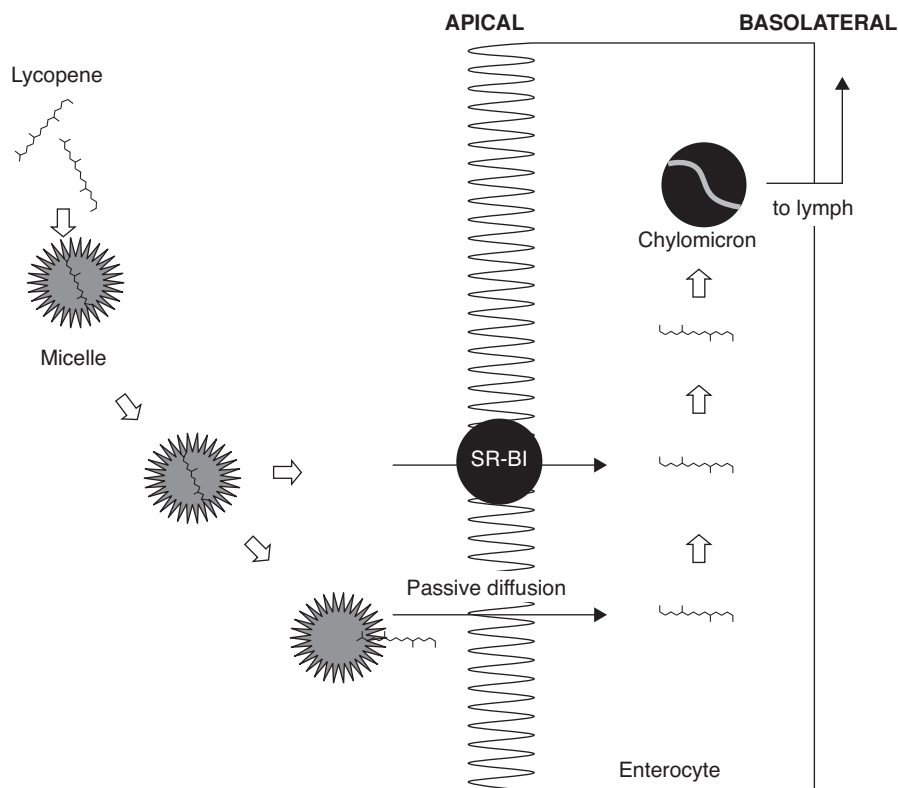


Figure 3 Intestinal absorption of carotenoids. Source: From Ref. 115.

pharmacologic agents that affect lipoprotein metabolism will also impact circulating lycopene concentrations.

Many factors affect bioavailability of carotenoids (see chapter on “Carotenoids”). Carotenoids can be present in crystalline form or strongly bound to intracellular macromolecules in many foods, and absorption may therefore be limited unless they are released from the food matrix (51,116). Heating tomato juice was shown to improve the uptake of lycopene in humans (46). Likewise, lycopene appears to be more bioavailable from processed tomato-based products than raw tomatoes. In one study, a single serving of tomatoes or tomato paste (each containing 23 mg of lycopene) was fed to study subjects and lycopene in the chylomicron fraction was monitored (57). Tomato paste was found to yield a 2.5-fold greater total all-trans lycopene peak concentration and a 3.8-fold greater postprandial response area under the curve than fresh tomatoes. These observations seem to be the result of thermal weakening and disruption of lycopene–protein complexes, rupturing of cell walls, and/or dispersion of crystalline carotenoid aggregates (46). In addition, various food-processing operations such as chopping and pureeing, which result in a reduction in physical size of the food particle, will also enhance lycopene bioavailability (55,56). van het Hof et al. (58) studied the effect of mechanical homogenization and heating on the bioavailability of multiple carotenoids from canned tomatoes. Interestingly, homogenization enhanced triglyceride-rich layer (TRL) and plasma lycopene

response (TRL fraction: 31% and 62% higher for mildly and severely homogenized tomatoes, respectively, vs. no homogenization; plasma: 16% and 21% higher, respectively; $P < 0.05$). Additional heating also increased lycopene responses in TRL fraction ($P = 0.14$) and plasma ($P = 0.17$). Interestingly, similar effects were found for β -carotene. These observations support the conclusion that food processing, homogenization, and cooking enhance lycopene bioavailability. When compared to these food matrix effects, microencapsulation, and solubilization in oleoresin may provide a more bioavailable form of most carotenoids. However, in studies measuring the plasma response to lycopene intake from supplement sources versus processed tomato-based foods, lycopene bioavailability does not appear to be statistically different (59,60).

Digestive processes will also certainly affect carotenoid bioavailability (see chapter on “Carotenoids”). Several factors affect initial carotenoid release from the physical food matrix and transfer and distribution into lipid droplets within the stomach and proximal duodenum (61,53). Dietary lipids appear to serve a critical role in the dissolution and subsequent absorption of very hydrophobic carotenoids (e.g., lycopene). Dietary lipids are required for the release of pancreatic lipases and bile salts, which are necessary to act upon carotenoid-containing lipid droplets entering the duodenum to form micelles (53). The co-consumption of fat with a meal has been shown to increase carotenoid bioavailability. In one study,

salad was consumed with and without full-fat dressing (117). Consumption of salad with full-fat dressing resulted in higher blood lycopene levels, whereas no measurable lycopene uptake occurred when salad was consumed with no-fat dressing (117). Similarly, a study by Unlu et al. found a 4.4-fold increase in lycopene absorption when tomato salsa was consumed with avocado as compared to tomato salsa consumption without avocado (118). This increase was primarily attributed to the lipids present in the avocado fruit (118). The three-dimensional configuration of lycopene also appears to have an impact on bioavailability. Multiple studies in ferrets (121) and humans (46,118,119,120) have demonstrated that *cis* isomers of lycopene are more bioavailable than all-*trans* lycopene. *In vitro* works suggest that this may be due to better micellization of *cis* isomers (122). Other factors, such as fatty acid chain length (123), fiber (124), probiotics (125), and other food components, also affect the carotenoid content of micelles and subsequent mucosal transfer (53). In addition, drugs that interfere with cholesterol absorption (64,111) and nonabsorbable fat analogs, such as sucrose polyesters (65), have been reported to reduce carotenoid absorption.

PLASMA AND TISSUE DISTRIBUTION

Serum lycopene levels can range from 50 to 900 nmol/L. It is well known that between 10 and 20 *cis* isomer peaks are typically observed in human blood and together account for the majority of lycopene in serum (47,66). Interestingly, we observed that the ratio of *cis* to *trans* lycopene isomers changes in those on a lycopene-free diet. According to Allen et al. (67), plasma lycopene isomer concentrations exhibit a 61:39 ratio for *cis*/*trans* at the start of a lycopene-free diet, whereas after 2 weeks, the ratio shifts to 70:30, which was significant. In a study by Hadley, Schwartz, and Clinton (68), the percentage of *cis*-*trans* lycopene isomers increased from 56:44 to 61:39, following a 1-week washout phase. Studies suggest that the all-*trans* lycopene content of serum is maintained through continuous dietary intake and that mobilization of all-*trans* lycopene from liver or other tissues, or reconversion of *cis* isomers to *trans*, cannot maintain the *cis*/*trans* ratio. In addition, it is plausible to hypothesize that there is a biological preference for certain lycopene isomers to be cleared from serum, to be distributed to tissues, or to participate in reactions that cause degradation.

Although data are still limited, it is apparent that carotenoids are not uniformly and equally dispersed in human tissues (46,69–71). The tissue-specific carotenoid patterns reported thus far suggest a process whereby certain carotenoids may exert unique biological effects in one tissue but not in another (Table 2). Presently, there is no evidence for a specific receptor or enzymatic process that mediates lycopene uptake by the tissue. We must therefore assume that uptake in the tissue is related to lipoprotein metabolism.

Lycopene has been shown to exist in over 15 different geometrical configurations in human prostate tissue, where the *cis* isomer content is even greater (at 80–90%) than that observed in serum (47). The chemical and physiological processes that account for the high

proportion of *cis* isomers in tissue remain speculative. An intriguing hypothesis is that isomerization reflects the participation of lycopene in antioxidant reactions within the prostate. Isomerization changes the structure of lycopene in a fashion that could alter its intracellular distribution within organelles and membrane structures that in turn could affect biological processes. These are hypotheses that will need additional investigation.

ROLE IN CHRONIC DISEASE

Multiple mechanisms of action for lycopene's purported beneficial health effects have been proposed. As reviewed by Heber and Lu (77), the pigment has been shown to display antioxidant, antiproliferative, and prodifferentiation activities at biologically relevant concentrations. In addition, other potential methods such as stimulation of xenobiotic metabolism, modulation of cyclooxygenase pathways, and inhibition of cholesterol synthesis and/or inflammation are discussed. Although these mechanisms may participate collectively to impart lycopene's health benefits, much of the research has been on the antioxidant activity of lycopene. Lycopene has also been implicated in ultraviolet (UV) photoprotection (128,129).

It has been widely postulated that oxidation may contribute to the damage of cellular DNA, proteins, and lipids that initiate or enhance the progression of cancer. Much research has focused on the role of reactive oxygen species (ROS) or free radicals that are produced from exogenous and endogenous factors. Mammals have developed multiple defenses against reactive oxygen. Nutritional substances such as vitamin E, vitamin C, and carotenoids (78) are thought to be important complements to other cellular systems, such as antioxidant enzymes (glutathione peroxidase, catalase, CuZn- and Mn-superoxide dismutase) and antioxidant quenchers (ceruloplasmin, transferrin, ferritin, Cd/Hg/Zn/Cu metallothioneins), which participate in the free radical defense system and provide protection against oxidative damage. As a result of having an extensive chromophore system of conjugated carbon-carbon double bonds, lycopene can accept energy from various electronically excited species. This characteristic gives lycopene the ability to quench singlet oxygen (1O_2) formed by energy transfer from a metastable excited photosensitizer (82). Singlet oxygen (1O_2) is a very reactive high-energy and short-lived oxygen species produced in biologic systems that can react with biomolecules. Lycopene may also interact with ROS such as hydrogen peroxide and nitrogen dioxide (83–85).

Research has also suggested that lycopene may play a role in skin photoprotection. In one study, tomato paste (containing approximately 16 mg of lycopene) was consumed daily with olive oil by a Caucasian control group ($n = 9$), while the placebo group ($n = 10$) consumed olive oil alone (128). UV light was used to induce a mild sunburn. Subjects were irradiated with a solar simulator at weeks 0 and 10, and chromametry was used to evaluate the redness of the skin (as measured by the *a*-value). *a*-Values in the treatment group were 32% lower in week 10 as compared to week 0. In addition, *a*-values at week 10 were 40% lower in the treatment group as compared to the

Table 2 Lycopene Concentrations in Human Tissue

Tissue	Concentration (nmol/g wet wt.)				Others
	Kaplan, Lau, and Stein (69)	Schmitz et al. (70)	Nierenberg and Nann (71)	Stahl and Sies (46)	
Adipose	1.30			0.20	
Adrenal	21.60			1.90	
Brain					2.55 ^a
Breast			0.78		0.43 ^b
Cervix					0.18 ^b
Colon			0.31		
Kidney	0.39	0.62		0.15	
Liver	2.45	5.72		1.28	0.65 ^b
Lung		0.57	0.22		0.56 ^b
Ovary	0.28			0.25	
Prostate					0.12 ^c , 0.24 ^d , 0.36 ^e , 0.53 ^c , 0.63 ^f , 1.60 ^h
Skin			0.42		
Stomach					0.20 ^g
Testes	21.36			4.34	

^aSource: From Ref. 72.^bSource: From Ref. 73.^cSource: From Ref. 74.^dSource: From Ref. 75.^eSource: From Ref. 76.^fSource: From Ref. 47.^gSource: From Ref. 66.^hSource: From Ref. 127.

control group. This study suggests that the tomato paste (which contains lycopene) was protective against acute UV damage (128). In another study (36), healthy Caucasian adults consumed either synthetic lycopene alone, a soft-gel encapsulated tomato extract, or a tomato beverage for 12 weeks (129). At weeks 0, 4, and 12, UV light was used to irradiate dorsal skin to induce a mild sunburn. Subjects consuming the synthetic lycopene had a 25% decrease in sunburn at week 12 as measured by *a*-value. In contrast, subjects consuming the tomato extract and tomato drink had a 38% and 48% decrease in *a*-value at week 12, respectively (129). These results suggest that the regular consumption of lycopene (especially lycopene-containing foods) may reduce sunburn severity and damage in light-skinned individuals. However, more research needs to be done before specific clinical recommendations can be made.

METABOLISM

The ability of lycopene to act as an antioxidant and scavenger of free radicals has historically been considered the most likely mechanism to account for the hypothesized beneficial effects on human health (79–81). However, researchers have recently suggested that lycopene may be metabolized into bioactive “lycopenoids” (130,131), analogous to the mechanism by which β -carotene is cleaved to produce retinal and converted into retinoic acid, both of which have important physiologic roles in humans (see chapter on “Carotenoids”). An enzyme known as β -carotene oxygenase 2 (BCO2) was first identified in humans by Keifer et al. (132) in 2001, and in vitro studies have demonstrated that BCO2 acts on lycopene (132,133).

A few lycopenoids have been identified in the lung tissue of ferrets (133) and the liver of rats (134) consuming a lycopene containing diet. We recently identified a series of lycopenoids (apo-6'-, -8'-, -10'-, -12'-, and -14'-lycopenal) in the blood plasma of humans who consumed 300 mL of tomato juice daily for 8 weeks (135).

Interestingly, past studies have reported lycopenoids in raw tomatoes (136) and tomato paste (137). We have also observed additional lycopenoids in lycopene-containing fruits and tomato-based products (135). Future research will need to be done to determine whether lycopenoids identified in humans are the products of enzymatic cleavage by BCO2 in vivo, or whether they are absorbed from lycopene-containing foods present in the diet.

Very limited research has been reported on the biological effects of lycopenoids. The in vitro growth of BEAS-2B (human bronchial epithelial cells) is inhibited by apo-10'-lycopenoic acid (138). Lung tumor multiplicity of A/J mice is also reduced with the feeding of apo-10'-lycopenoic acid in a dose-dependent manner (139). More research will need to be done to elucidate the potential role of lycopenoids in human health.

CANCER

In recent decades, we have seen an accumulated body of evidence strongly supporting the conclusion that diets rich in fruits and vegetables are associated with a lower risk of many malignancies. A comprehensive review of the epidemiological evidence regarding lycopene-containing tomatoes and tomato-based products and cancer risk was published by Giovannucci (6). Nearly 80% of the (72)

studies reported in the review revealed evidence of a protective association between consumption of tomatoes, tomato-based products, or carotenoids provided by these foods and the risk of cancer at several sites. In more than 60% of these trials, the inverse associations were statistically significant. The observed inverse relationship was strongest for lung, stomach, and prostate cancer and was supportive for cervical, breast, oral cavity, pancreatic, colorectal, and esophageal cancer. In addition, epidemiologic investigations of colon (86), upper aerodigestive (87), prostate (88–91), and lung cancer (92) further support the concept that lycopene-containing tomato-based products have cancer-preventive properties.

Prostate cancer is the most common noncutaneous malignancy in American men and is the second leading cause of cancer-related deaths (93). Epidemiologic studies have shown that an increase in lycopene consumption as well as serum lycopene concentrations is inversely correlated with the risk for prostate cancer. In 1991, Le Marchand et al. (94) studied a multiethnic Hawaiian cohort and found no association between estimated lycopene intake and prostate cancer risk. However, in one of the largest and most comprehensive ongoing epidemiologic studies in adult men, Giovannucci et al. (95) investigated the relationship between the risk of prostate cancer and estimated intake of various fruits, vegetables, retinol, and carotenoids in nearly 48,000 men in the Health Professionals' Follow-Up Study (HPFS). Dietary intake of lycopene (80% of which was derived from tomatoes and tomato-based products) was inversely related to risk when the highest quartile (>6.4 mg lycopene/day) was compared with the lowest quartile (<2.3 mg lycopene/day, $RR = 0.79$, 95% $CI = 0.64–0.99$, $P = 0.04$ for trend). A few years later, a case-control study of 797 men in New Zealand found a weak, nonsignificant trend between lycopene intake and prostate cancer incidence when comparing the lowest quartile (<663 $\mu g/day$) of lycopene intake to the highest quartile (>1994 $\mu g/day$) [$OR = 0.76$, 95% $CI = 0.53–1.26$, $P = 0.30$ for trend; (90)]. Interestingly, in this study, the estimated median intake of lycopene was less than half of the median in the HPFS cohort (1.2 vs. 3.4–4.6 mg lycopene/day, respectively). Recently, updated data from the HPFS for the period from 1992 through 1998 confirmed the earlier findings. Lycopene intake was associated with a decreased risk for prostate cancer [$RR = 0.84$ for high compared to low quintiles; 95% $CI = 0.73–0.96$; $P = 0.003$ for trend; (96)].

Several reports have investigated the relationship between blood concentrations of lycopene and prostate cancer risk. In a study conducted at the Memorial Sloan-Kettering Cancer Center from 1993 to 1997, Lu et al. (88) showed that when plasma carotenoid levels from men in the highest and lowest quartiles were compared, inverse associations for prostate cancer risk were statistically significant for plasma lycopene, zeaxanthin, lutein, and β -cryptoxanthin levels. Hsing et al. (97) evaluated serum obtained in 1974 from 25,802 persons in Washington County, Maryland, and reported lower mean serum lycopene concentrations in prostate cancer cases compared with controls. A 50% reduction in the relative risk for prostate cancer was observed when cases in the highest serum lycopene quartile were compared

to more in the lowest quartile. A study conducted by Nomura et al. (98) in a cohort of 6860 Japanese-American men examined from 1971 to 1975, however, showed no association between several plasma micronutrients and carotenoids, and prostate cancer risk. A nested case-control investigation was undertaken, which involved the analysis of carotenoids in blood samples from men enrolled in the Physicians' Health Study (a randomized, placebo-controlled trial of aspirin and β -carotene). In this study, subjects in the highest quintile (>580.1 ng/mL) of serum lycopene levels had a significantly lower risk of prostate cancer compared with those in the lowest quintile (≤ 261.7 ng/mL, $OR = 0.56$, 95% $CI = 0.34–0.92$, $P = 0.05$). The inverse association between serum lycopene and aggressive prostate cancer was particularly significant for men who were not consuming β -carotene supplements [$OR = 0.40$ for highest quintile vs. lowest quintile, 95% $CI = 0.19–0.84$, $P = 0.006$ for trend; (99)].

Several laboratories have conducted studies on lycopene and prostate carcinogenesis in rodents. An investigation using the DMAB and PhIP-induced rat prostate cancer models failed to detect a chemopreventive effect of lycopene provided as an extract of 99.9% purity from Lycopodium (100). In another study, two different doses of a lycopene-rich tomato oleoresin were fed to *lacZ* mice to study the effects on short-term benzo[a]pyrene (BaP)-induced and long-term spontaneous *in vivo* mutagenesis in the colon, prostate, and lungs (101). Spontaneous mutagenesis was inhibited in prostate and colon tissue at the higher dose of tomato oleoresin. In addition, BaP-induced mutagenesis in the prostate was also slightly inhibited in mice fed with tomato oleoresin. Boileau et al. (102) completed a large rat study evaluating the ability of lycopene or freeze-dried tomato powder to inhibit survival in the *N*-nitrosomethylurea-androgen-induced prostate cancer model. In this system, a very small beneficial trend for lycopene and a significant benefit of tomato powder were reported, suggesting that tomato consumption may provide additional benefits.

There are few human intervention studies investigating the role of lycopene on processes that are related to the development of prostate cancer. The most provocative observations have been published by Kucuk et al. (76). The study involved 26 men diagnosed with presumed localized prostate cancer who were scheduled to undergo a radical prostatectomy. The subjects were randomized to consume 30 mg of lycopene/day from two tomato oleoresin capsules (Lyc-O-Mato; Lycopodium Natural Products Industries) or to continue their normal diet for 3 weeks prior to surgery. Postsurgical prostate tissue specimens were then compared between the two groups. Men consuming the lycopene supplement had 47% higher prostatic tissue lycopene levels than the control group (0.53 ± 0.03 ng/g vs. 0.36 ± 0.06 ng/g, $P = 0.02$). However, plasma lycopene levels were not significantly different between the groups and they did not change significantly within each group. Those who consumed the lycopene supplement were less likely to have involvement of surgical margins (73% vs. 18% of subjects, $P = 0.02$). In addition, they were less frequently found to have high-grade prostatic intraepithelial neoplasia in the prostatectomy specimen (67% vs. 100%, $P = 0.05$). Furthermore, the

intervention group was found to have smaller tumors, a greater reduction in prostate-specific antigen (PSA) over the 3-week study period, and a higher expression of connexin 43. However, none of these differences were statistically significant. A case-control study by Chen et al. (140) investigated the effects of daily tomato sauce consumption (30 mg/day lycopene) for 3 weeks in 32 prostate cancer patients before a radical prostatectomy. Analysis of the prostate tissue after surgery revealed a decrease in DNA damage in the treatment group as compared to controls, as measured by the ratio of 8-hydroxy-2'-deoxyguanosine (a marker of oxidative DNA damage) to 2'-deoxyguanosine. A ratio = 0.76 was observed in the treatment group, as compared to 1.06 in the controls ($P = 0.03$) (140). In addition, a 20% decrease in serum PSA levels was observed in the treatment group ($P < 0.001$). Another recent study examined the effects of tomato and tomato product consumption with and without soy protein isolate in men ($n = 41$) with recurrent, asymptomatic prostate cancer (141). Subjects were split into two groups, with one group asked to consume tomato-based products for the entire 8-week study and the other group asked to consume tomato-based products for the final 4 weeks of the study. Subjects were asked to consume enough tomatoes and tomato-based products to meet a target of at least 25 mg/day lycopene. On the completion of the study, serum PSA levels were reduced in 34% of the subjects (141).

CARDIOVASCULAR DISEASE

Epidemiologic studies investigating the relationship between lycopene exposure and the risk for vascular diseases are beginning to emerge. Consumption of tomatoes and tomato-based products has been associated with a reduced risk of cardiovascular disease. In one study of 38,445 women, evaluation of highest and lowest quintiles of intake revealed that higher levels of tomato-based product intake were associated with a reduced risk of cardiovascular disease ($RR = 0.71$, $P_{\text{trend}} = 0.029$) and myocardial infarction ($RR = 0.39$, $P_{\text{trend}} = 0.033$) (7). Tissue and serum concentrations of lycopene have also been found to be correlated with a reduced risk for coronary heart disease (CHD) in several case-control studies. A multicenter case-control study was conducted to evaluate the relationship between adipose tissue concentration of antioxidants (i.e., α - and β -carotene and lycopene) and acute myocardial infarction (103). Cases and control subjects were recruited from 10 European countries to ensure maximum variability in exposure. Upon simultaneous analyses of the carotenoids and adjustment for other variables, lycopene was the only carotenoid associated with protection against acute myocardial infarction ($OR = 0.52$ when the 10th and 90th percentiles were compared, 95% $CI = 0.33$ – 0.82 , $P = 0.005$ for trend). Similarly, lower serum lycopene concentrations were found to be related to an increased risk of and mortality from cardiovascular disease (CVD) in a concomitant cross-sectional study evaluating Swedish and Lithuanian populations displaying diverging mortality rates from CHD ($n = 210$) (104). Klipstein-Grobusch et al. (105) investigated the

relationship between serum concentrations of the major carotenoids (i.e., α -carotene, β -carotene, β -cryptoxanthin, lutein, lycopene, and zeaxanthin) and aortic atherosclerosis as determined by the presence of calcified plaques of the abdominal aorta. A subsample of the elderly population of the Rotterdam Study consisting of 108 subjects with aortic atherosclerosis and controls was used for the case-control analysis. A 45% reduction ($OR = 0.55$, 95% $CI = 0.25$ – 1.22 , $P = 0.13$ for trend) in the risk of atherosclerosis was observed for the highest versus the lowest quartile of serum lycopene. When adjustments for smoking status were made, the inverse association was greatest for current and former smokers ($OR = 0.35$, 95% $CI = 0.13$ – 0.94 , $P = 0.04$ for trend). No associations were observed with any of the other serum carotenoids studied. A report on men (aged 46–64 years; $n = 725$) from the Kuopio Ischaemic Heart Disease Risk Factor Study indicated that those in the lowest serum lycopene quartile had a 3.3-fold (95% $CI = 1.7$ – 6.4 ; $P < 0.001$) increased risk of acute coronary events or stroke when compared to the others (106). In addition, subjects in the lowest quartile of serum lycopene had a significant increment in both mean intima-media thickness of common carotid artery wall (CCA-IMT) ($P < 0.006$ for difference) and maximal CCA-IMT ($P = 0.002$) as compared with others. In a cross-sectional analysis of 520 men and women from the Antioxidant Supplementation in Atherosclerosis Prevention Study, low plasma lycopene levels were associated with an 18% increase in IMT in men when compared with those with plasma lycopene levels higher than the median [$P = 0.003$ for difference; (107)].

Short-term dietary intervention studies have also supported a relationship between tomato-based products and tomato extract supplements on positive improvements in lipid biomarkers and a reduction in biomarker oxidation (142–143).

SAFETY AND ADVERSE EFFECTS

Safety assessment of phytochemicals from fruits and vegetables or supplements is necessary to ensure efficacy without toxicity in future trials. In rats, the consumption of 3 g lycopene/kg body weight/day for up to 13 weeks was reported to have no adverse physiologic effects or abnormalities in the animals (145). The safety of multiple acute doses of lycopene in humans was studied by Diwadkar-Navsariwala et al. (108). A Phase I study in healthy male subjects, using a physiological pharmacokinetic model, was conducted to study the disposition of lycopene, administered as a tomato beverage in five graded doses (10, 30, 60, 90, or 120 mg). The subjects reported no signs of toxicity at any level of intake. However, long-term consumption of these doses was not evaluated.

Consumption of extreme amounts of lycopene or lycopene-containing tomatoes and/or tomato-based products over an extended period of time can have adverse effects. La Placa, Pazzaglia, and Tosti (109) described a case study of a 19-year-old Italian girl who had consumed four to five large red tomatoes and pasta with tomato sauce daily for 3 years. She displayed yellow-orange discoloration of the skin and abdominal pain.

Upon investigation of the abdominal pain, a hepatic ultrasound revealed a digitate area that was relatively hypoechogenic, measuring 2 cm in diameter, in the upper portion of the parenchyma, consisting of deposits of lycopene. These clinical features and dietary history suggested the diagnosis of lycopopenia.

Additional studies are required to assess the safety of varying levels of lycopene from multiple sources for long periods of time. These experiments will enable future scientists to identify the optimum combination of intake and time to maximize the benefits without adverse effects. Therefore, caution should be exercised when recommending sources and amounts of this carotenoid.

WHOLE FOODS VERSUS SUPPLEMENTS

Although lycopene has received a great deal of attention as an important phytochemical from tomatoes and tomato-based foods, it is premature to suggest that lycopene alone is responsible for the reported beneficial health effects of these foods. As briefly discussed earlier, a study was conducted by Boileau et al. (102) to evaluate the effects of tomato powder or lycopene beadlet consumption on prostate carcinogenesis in *N*-methyl-*N*-nitrosourea (NMU) and testosterone-treated rats. This investigation showed that consumption of tomato powder but not lycopene alone inhibited prostate carcinogenesis, suggesting that tomato-based products contain compounds in addition to lycopene that modify prostate cancer development and/or progression. In addition, the study by Aust et al. (129) indicated greater photoprotection conferred from the consumption of a tomato extract or tomato beverage versus synthetic lycopene.

Care should be taken not to make the assumption that all the health benefits brought about by fruit and vegetable consumption are attributed to a single component such as lycopene. Because tomato and tomato-based product consumption is the primary source of lycopene in the North American diet, other compounds in tomatoes and tomato-based products may be responsible for positive effects observed in epidemiological studies. When the Food and Drug Administration (FDA) reviewed a proposed health claim on tomatoes, lycopene, and cancer, they concluded that there is "no credible evidence to support an association between lycopene intake and a reduced risk of prostate, lung, colorectal, gastric, breast, ovarian, endometrial or pancreatic cancer," (146). However, the FDA found, "very limited evidence to support an association between tomato consumption and reduced risks of prostate, ovarian, gastric, and pancreatic cancers" (146). The limited number of high-quality clinical studies in the literature was one of the main reasons cited in their decision. Additional studies are needed to determine the differences between lycopene supplementation and lycopene-containing diets on biologic processes related to chronic disease.

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Maca

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INTRODUCTION

Maca is a dietary supplement derived from the processed tuberous root of *Lepidium meyenii* Walpers (family Brassicaceae; common names: Peruvian ginseng, maka, mace, maca-maca, maino, ayak chichira, ayuk willku, pepperweed) (1). The genus *Lepidium* contains approximately 150 to 175 species (2). This plant was first described by Gerhard Walpers in 1843 and domesticated in the Andean mountain at altitudes from 3500 to 4450 m above sea level in the *puna* and *suní* ecosystems (3). It is arguably the highest altitude plant in cultivation. The genus probably originated in the Mediterranean region, where most of the diploid species are found (2,4); information about its origin and distribution are sketchy. Maca is an important staple for the Andean Indians and indigenous peoples and was domesticated during the pre-Inca Arcaica period sometime around 3800 B.C. It is the only species cultivated as a starch crop (5) and is rich in sugars, protein, starches, and essential minerals, especially iodine and iron.

Based on a long history of traditional use of maca in Peru and elsewhere, a wide array of commercial maca products have gained popularity as dietary supplements throughout the world for aphrodisiac purposes and to increase fertility and stamina. Limited research has been carried out during the past two to three decades by academia and the industry, including isolation and identification of several potentially bioactive constituents, as well as evaluation of biological activities, mainly focused on its aphrodisiac and nutritional properties. Here, we present a comprehensive review of the published literatures on maca, which includes morphological descriptions, traditional uses, nutritional status, chemical constituents, biological activities, cosmetic uses, and standardization.

BACKGROUND

Classification

L. meyenii (Fig. 1) is a herb or subshrub belonging to the Brassicaceae family (4,5). Chacon recommended changing its name to *L. peruvianum* because herbarium specimens from Bolivia and Argentina were classified as *L. meyenii* but had no resemblance in shape to maca in many cases (6). It has been suggested that the cultivated maca of today is not *L. meyenii* but the new species, *L. peruvianum*. Although most maca sold in commerce is still referred to as *L. meyenii*, it is *L. peruvianum*.



Figure 1 Dried tuberous root and above-ground parts of maca (*Lepidium meyenii*). (View this art in color at www.dekker.com.)

Cultivation

Unlike many other tuberous plants, *L. meyenii* is propagated by seed, and 7 to 9 months is required to produce the harvested root. It is cultivated on rocky soil on rough Andean terrain under intense sunlight, high wind, and fluctuating temperatures between -20°C and 20°C . The soil used for cultivation is acidic clay or limestone with a relative humidity of approximately 70%, and the plant can grow without shade or in semishade. Maca is sown from September to October at the beginning of the rainy season, and harvesting starts from May to July after a vegetative phase of 260 to 280 days. The yield is variable, from 2 to 16 ton/ha depending on the cultivation practices, fertilization, and pest control. Well-formed hypocotyls are selected and transplanted to fertilized seed beds for seed production. After a 100- to 120-day generative phase, seeds are harvested (5). Maca seeds represent centuries of cumulative selection by indigenous farmers, but it is only recently that scientists and governments have been growing out, testing, and saving them. The plantation area for maca has expanded drastically because of the increased demand, both domestically and for export. In 1994, less than 50 ha

of maca was cultivated in Peru; by 1999, production had increased 24-fold to 1200 ha, and it now stands at more than 2000 ha.

Traditional and Medicinal Uses

Maca was grown for food by the Pumpush, Yaros, and Ayarmaca Indians. Conquistadors fed the baked or boiled root powder to animals for fertility problems at high altitudes, and the Chinchaycochas Indians used it in bartering. Maca was also used to make beverages, to which hallucinogenic products were also added, which were consumed during dances and religious ceremonies. The tuberous root of maca is generally consumed fresh or dried and has a tangy taste and an aroma similar to butterscotch. Dried roots are brown, soft, and sweet, with a musky flavor, and retain their flavor for at least 2 years, and a 7-year-old root still has 9% to 10% protein. In South America, the sweet aromatic porridge of dried maca is consumed under the name mazamorra. In Huancayo, Peru, maca jam and pudding are popular, and maca is often made into a sweet, fragrant, fermented drink called maca chichi.

According to folk belief, maca can enhance male sexual drive and female fertility in humans and domestic animals. The Spanish conquerors found "well-fed babies and tall adults" in the high Andes, which they attributed to a diet based on maca (3). It is also reputed to regulate hormonal secretion, stimulate metabolism, and improve memory, and is touted for antidepressant and anticancer properties, as well as for curing anemia, leukemia, and AIDS. However, these properties have not been substantiated by scientific research. Due to its wide spectrum of putative qualities, maca is also known as Peruvian ginseng (3). In Peruvian herbal medicine, it has been used as an immunostimulant, for anemia, tuberculosis, menstrual disorders, menopause symptoms, stomach cancer, and sterility, and for other reproductive and sexual disorders, as well as to enhance memory.

CHEMISTRY

Nutritional Constituents

Maca is very nutritious, with 60% to 75% carbohydrates, 10% to 14% protein, 8.5% fiber, and 2.2% lipids (7,8). The dried root contains approximately 13% to 16% protein and is rich in essential amino acids, whereas the fresh root is unusually high in iodine and iron. It contains approximately 250 mg of calcium, 2 g of potassium, and 15 mg of iron in 100 g of dried root, and sterols (0.05–0.1%), minerals, and vitamins. Maca contains 3.72% fatty acids, including caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid (9). In addition, a new acyclic keto acid, 5-oxo-6*E*,8*E*-octadecadienoic acid [1] has been isolated from the tubers (10). Yellow maca has been found to have higher lipid and carbohydrate content compared to the red and black varieties (8). Evaluation of the nutritional property of maca in albino Swiss mice has shown that the serum values for content of total proteins and albumin are statistically higher for mice eating cooked maca than for those consuming raw maca, with no sign of malnutrition or overweight in any of the groups (11).

Secondary Metabolites

The major secondary metabolites present in *L. meyenii* can be classified into four groups: (a) essential oils; (b) glucosinolates; (c) alkaloids; and (d) macamides. In addition, the presence of malic acid and its benzoate ester [2] (12) as well as five sterols (7) and catechins, are also reported (Fig. 2)

Essential oils

A total of 53 essential oil components have been identified, using retention indexes and mass spectral data (13). Among the constituents, phenylacetone nitrile (85.9%), benzaldehyde (3.1%), and 3-methoxyphenylacetone nitrile (2.1%) are the major components of the steam-distilled oil.

Glucosinolates

The glucosinolates are a class of secondary metabolites found in 15 botanical families of dicotyledonous plants, notably including the Brassicaceae. Over 100 have been reported to date from plant sources. Glucosinolates are present at approximately 1% in fresh *L. meyenii* root, but no novel ones have been reported so far in maca. The presence of two main glucosinolates, glucotropaeolin [3] and *m*-methoxybenzylglucosinolate [4], have been reported from maca (12,14,15), and their combined presence in *L. meyenii* may be used as a chemotaxonomic marker, because the combination of 3 and 4 does not occur in other members of the Brassicaceae (14). Glucosinolates and their derived products have received increasing attention due to their biological activities; examination of glucosinolate degradation products in the hexane extract has revealed the presence of benzyl isothiocyanate [5] and its *m*-methoxy derivative [6] (15). The former reported to be present in the range of 0.1% to 0.15% in standardized maca product (9).

Several maca products derived from processed hypocotyls of *L. peruvianum* and other organs have been assessed by high-performance liquid chromatography (HPLC) for glucosinolate content. The most abundant glucosinolates were found to be 3 and 4 in fresh and dry hypocotyls and leaves. The richest sources of glucosinolates are seeds, fresh hypocotyls, and sprouts, in that order. Maca seeds and sprouts differ in profile from hypocotyls and leaves due to the presence of several modified benzylglucosinolates, including 5-methylsulfinylpentylglucosinolate [7], indolyl-3-methylglucosinolate [8], pent-4-enylglucosinolate, 4-methoxyindolyl-3-methylglucosinolate, glucolepigramin, and 4-hydroxybenzylglucosinolate, whereas the liquor and tonic contain sinigrin [9] (12). A HPLC method was reported for the quantification of benzyl isothiocyanate [5] released by the action of the thioglucosidase enzyme on the substrate [3], the predominant glucosinolate of maca hypocotyls (16).

Alkaloids

Qualitative detection of alkaloid like compounds in *L. meyenii* was first reported by Dini et al. (7) and a further detailed chemical analysis of the tubers by Muhammad et al. (10) reported the benzylated derivative of 1,2-dihydro-*N*-hydroxypyridine, named macaridine [10]. From the methanol extract of the tuber, (1*R*,3*S*)-1-methyltetrahydro- β -carboline-3-carboxylic acid [11], and uridine [12] and its

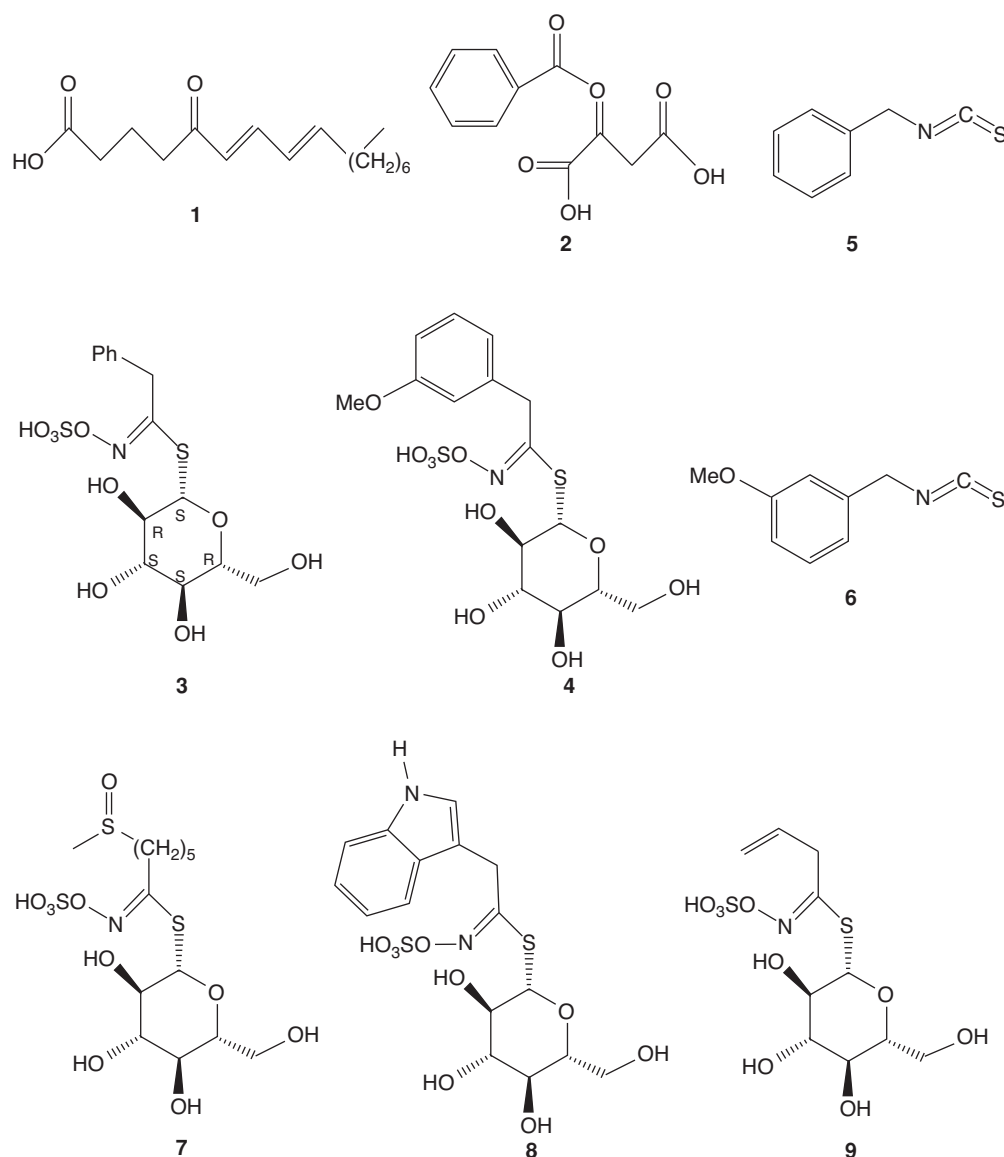


Figure 2 Selected structures of compounds isolated from maca.

benzoyl derivative have been isolated (15). Two new imidazole alkaloids, lepidiline A [13] and lepidiline B [14], were isolated from root extracts (17) (Fig. 2) and their 1,3-dibenzylimidazolium chloride derivatives were patented for treating proliferative diseases (18).

Macamides

Maca contains novel polyunsaturated acids and their amides, called macaene and macamide as reported by Zheng et al. (9). From purified standardized products of maca, three new macamides, *N*-benzyloctamide, *N*-benzyl-16-hydroxy-9-oxo-10*E*,12*E*,14*E*-octadecatrienamide, and *N*-benzyl-9,16-dioxo-10*E*,12*E*,14*E*-octadecatrienamide, have been isolated and identified by HPLC. (9) In addition, 17 other analog of macamide and macaene have been reported, but their chemical identity has not been disclosed. From maca tubers, seven additional

alkamides, *N*-benzyl-5-oxo-6*E*,8*E*-octadecadienamide [15], *N*-benzylhexadecanamide [16], *N*-benzyl-9-oxo-12*Z*-octadecanamide [17], *N*-benzyl-9-oxo-12*Z*,15*Z*-octadecadienamide [18], *N*-benzyl-13-oxo-9*E*,11*E*-octadecadienamide [19], *N*-benzyl-15*Z*-tetracosenamide [20], and *N*-(*m*-methoxybenzyl)hexadecanamide [21] have been isolated (10,19). In addition, *N*-benzylhexadecanamide, *N*-benzyl-(9*Z*)-octadecanamide, *N*-benzyl-(9*Z*,12*Z*)-octadecadienamide, *N*-benzyl-(9*Z*,12*Z*,15*Z*)-octadecatrienamide and *N*-benzyloctadecanamide were identified by using HPLC–UV–MS/MS (20).

COMMERCIAL PREPARATIONS AND STANDARDIZATION

A wide array of commercial products, including soft drinks, pills, and capsules, are currently processed and

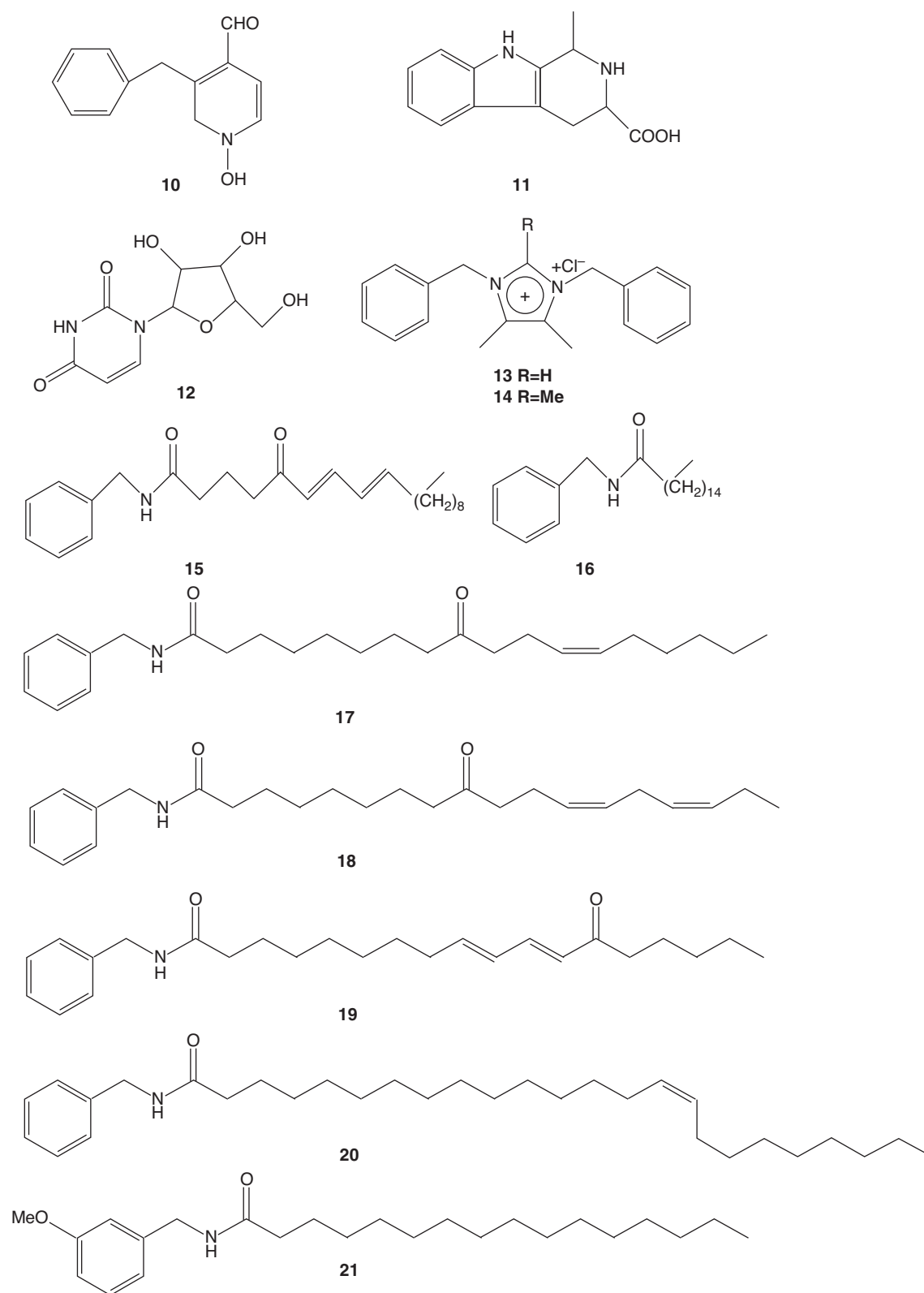


Figure 2 (Continued)

distributed by various companies throughout the world. These products are sold in markets and drug stores in South America, including Peru, and many of these are exported abroad. Today, maca is advertised as an aphrodisiac, stamina builder, and fertility promoter in the world market and is available for purchase through the World Wide Web as a dietary supplement.

Ganzer et al. (21) have reported an analytical method for the determination of the main macamides and macaenes of *L. meyenii*. The analysis of several commercially available maca products reveals a similar qualitative pattern for macamides and macaenes, but significant differences in the quantitative composition. The purified standardized product of maca has been analyzed by HPLC (9), and three new macamides and 17 other analogs of macamide and macaene have been reported. Several products (such as pills, capsules, flour, liquor, tonic, and mayonnaise) derived from processed maca (*L. peruvianum*) have also been analyzed and profiled by HPLC for glucosinolate content (12). Total macamides have been quantified by HPLC–UV in plant material from different vendors using *n*-benzylhexadecanamide as an external standard. The amount of macamides in the dried plant material ranged from 0.0016% to 0.0123% (20). Through chromatographic techniques, β -sitosterol was identified and quantified (22). Chen et al. investigated the polysaccharide in maca, and a colorimetric method was developed for its determination (23,24). FT-IR, TLC, and GC/MS techniques were employed for the identification of maca or maca products in the market (25).

PRECLINICAL STUDIES

Fertility-Enhancing and Aphrodisiac Activities

The aphrodisiac activities of maca have been reported by several research groups. Oral administration of the purified lipid extract decreased the latent period of erection in male rats with erectile dysfunction, as well as enhanced the sexual function of mice and rats by increasing the number of complete intromissions and the number of sperm-positive females in normal mice (9).

The effects of maca on mouse sex steroid hormones and embryo implantation were investigated. Progesterone and testosterone levels increased significantly in mice that were treated with maca. However, there were no marked changes in blood levels of 17β -estradiol or the rate of embryo implantation (26).

Bogani et al. conducted an investigation to test whether maca contains testosterone-like compounds, is able to bind the human androgen receptor, and can promote transcription pathways regulated by steroid hormone signaling. The results showed that the maca extracts (obtained with different solvents, such as methanol, ethanol, hexane and chloroform) were not able to regulate GRE (glucocorticoid response element) activation (27).

The root of maca has been used to help alleviate the symptoms of menopause. The effect of ethanol extract of maca on osteoporosis in ovariectomized rat was studied. The findings derived from the basis of bone mineral density, biomechanical, biochemical and histopathological parameters indicated that higher dose of ethanol extract of maca was effective in the prevention of estrogen-deficient

bone loss (28). Maca has also been traditionally used to increase fertility. A study on the effects of maca on several fertility parameters of female mice at reproductive age showed that administration of aqueous extract of yellow maca to adult female mice could increase the litter size. Moreover, this treatment could increase the uterine weight in ovariectomized animals (29).

Oral administration of an aqueous extract of maca roots resulted in an increase in the weights of the testis and epididymis, but not seminal vesicle weight, and the root invigorated spermatogenesis in male rats by acting on the initial portions of the seminiferous tubules, where mitosis occurs (30).

To determine the acute and chronic effects of maca on male sexual behavior and to examine chronic administration of maca on anxiety, maca (25 and 100 mg/kg) was orally administered to male rats for 30 days. Ejaculatory and mounting behavior and postejaculatory interval were monitored. An elevated plus maze, locomotion, and social interaction with another male were used for anxiety tests. The investigation showed that maca treatment did not produce large changes in male sexual behavior. However, an increase in ejaculation latency and postejaculatory interval was observed after both acute and 7 days of treatment. After 21 days of treatment maca had no effect on sexual behavior. Chronic administration of maca did not increase locomotion or anxiety (31).

Antagonistic effect of red maca (RM) on prostatic hyperplasia induced with testosterone enanthate (TE) in adult mice was investigated (32). Testosterone and oestradiol levels, as well as prostatic stroma, epithelium, and acini were measured. It was found that RM reduced prostate weight at 21 days of treatment. Weights of seminal vesicles, testis, and epididymis were not affected by RM treatment.

Cicero et al. reported that the subacute oral administration of a lipophilic hexane extract improved sexual performance parameters most effectively in sexually inexperienced male rats (33,34).

Effect of aqueous extract of maca on spermatogenesis in male rats was investigated to test the hypothesis that maca can prevent high altitude-induced testicular disturbances. The data showed that altitude reduced spermatiation (stage VIII) to half and the onset of spermatogenesis (stages IX–XI) to a quarter on days 7 and 14 but treatment with maca (666.6 mg/day) prevented these changes (35). The same group also investigated the effects of maca extracts on spermatogenesis in rodents, following spermatogenic damages induced by lead acetate and organophosphorous pesticide malathion (36,37). Assessment of the relative length of stages of the seminiferous epithelium showed that maca treatment resulted in rapid recovery of the effect of malathion. Administration of maca to rats treated with lead acetate resulted in higher lengths of stages VIII and IX–XI with respect to lead acetate-treated rats. Moreover, treatment with maca to lead acetate-treated rats resulted in lengths of stages VIII and IX–XI similar to the control group.

Maca has different ecotypes described according to the color of its hypocotyls (38). Gonzales et al. studied the different biological properties among different varieties of maca. They reported that black maca presented the greatest effect on sperm production in male rats and

on latent learning in ovariectomized female mice in comparison with yellow and red (39–41); whereas red maca reduced ventral prostate size in normal and TE treated rats (42,43). However, the difference of active secondary metabolites present in different maca varieties is still unknown.

Cytotoxic and Chemopreventive Activities

Glucosinolates appear to have little biological impact by themselves. However, release of biologically active products such as isothiocyanates, organic cyanides, oxazolidinethiones, and ionic thiocyanate (SCN^-) upon enzymatic degradation by myrosinase, which is typically present in cruciferous plants as well as in the gut microflora of mammals (44), is responsible for the observed activities. Natural isothiocyanates derived from glucosinolate are effective chemoprotective agents that detoxify carcinogens and prevent several types of cancer in rodent models. Isothiocyanates apparently induce mammalian Phase 1 and 2 drug-metabolizing enzymes and their coding genes, resulting in decreased carcinogen–DNA interactions (45). Benzyl isothiocyanates, most importantly, have been reported to be potent cancer inhibitors of mammary gland and stomach cancers (46) and of liver cancer (47) in rats treated with carcinogens. The above-mentioned work suggests that the type of glucosinolate and total concentration have important implications with respect to overall biological activity, including chemoprevention, in both human and animal nutrition. However, no tests on the chemopreventive activity of maca itself have been reported so far.

Valentova et al. studied the biological activity of methanolic and aqueous extracts of maca on rat hepatocytes and human breast cancer MCF-7 cells (48). Cytotoxicity in hepatocyte primary cultures was not observed up to 10 mg/mL of the extract concentration as measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] viability test, and lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) leakage. Moreover, after 72 hours, extracts inhibited LDH and AST leakage from the hepatocytes. Both methanolic and aqueous extracts showed estrogenic activity comparable with that of silymarin in MCF-7 cell line, but weak antioxidant activity in the α, α -diphenyl-2-picrylhydrazyl (DPPH)-radical scavenging test with IC_{50} values of 3.46 ± 0.16 and 0.71 ± 0.10 mg/mL, for aqueous and methanolic extracts, respectively.

Other Biological Activities

Maca has the capacity to scavenge free radicals and protect cells from oxidative stress. The antioxidant activity of maca was assessed, and the IC_{50} values for scavenging DPPH and peroxy radicals were found to be 0.61 and 0.43 mg/mL, respectively (49). Deoxyribose protection by maca (1–3 mg/mL) against hydroxyl radicals was of the order of 57% to 74%. Maca (1 mg/mL) protected RAW 264.7 cells against peroxynitrite-induced apoptosis and increased ATP production in cells treated with H_2O_2 (1 mM).

The oil of *L. meyenii* was selectively toxic toward the cyanobacterium *Oscillatoria perornata*, a blue-green alga

that causes off-flavor in commercial catfish production, compared with the green alga *Selenastrum capricornutum*, with complete growth inhibition at 100 $\mu\text{g/mL}$ (13). Mortality of the Formosan subterranean termite, *Coptotermes formosanus*, was numerically, but not significantly, higher in tests conducted on filter paper treated with maca oil. At 1% (wt/wt), maca oil appeared to act as a feeding deterrent to termites. Several minor components of the essential oil, including 3-methoxyphenylacetone nitrile and benzyl thiocyanate, were significantly active against the termite (13).

Rainbow trout fry fish were fed with maca and different extracts obtained from four different solvents to study their effects on growth performance, feed utilization, and survival of the fish (50). The fish were fed eight casein-based semipurified isonitrogenous and isocaloric diets containing 15% wheat flour (control, diet 1), 15% maca meal (diet 2), 12.5% maca meal residue after extraction (diet 3), mixture of 4 maca meal extracts (diet 4), hexane extract (diet 5), dichloromethane extract (diet 6), ethyl acetate extract (diet 7), and methanol extract (diet 8). After 14-week feeding, the fish fed diet 2 had the highest growth rate among all the dietary treatments. Fish fed diets 2, 3, and 8 had higher growth than the fish fed with the other diets. Feed intake was higher in fish fed with diets 2, 3, and 8 than in fish fed with diets 1 and 5. Feed conversion ratio and protein efficiency ratio were also improved in fish fed with diets 2 and 3 versus fish fed with diets 1, 5, 6, and 7. Survival was higher in fish fed with diet 2 versus 1, 5, and 6.

The effects of maca on lipid, antioxidative, and glucose parameters in hereditary hypertriglyceridemic rats were investigated (51). Maca was administered to rats as a part of a high-sucrose diet for 2 weeks. Rosiglitazone (0.02%) was used as a positive control. Maca significantly decreased the levels of VLDL (very-low-density lipoproteins), LDL (low-density lipoproteins), and total cholesterol, and also the level of TAG (triacylglycerols) in the plasma, VLDL, and liver. Maca, as well as rosiglitazone, significantly improved glucose tolerance, as the decrease of AUC (area under the curve) of glucose showed, and lowered levels of glucose in blood. The activity of SOD (superoxide dismutase) in the liver, the GPX (glutathione peroxidase) in the blood, and the level of GSH (glutathione) in liver increased in all cases significantly.

COSMETIC USES

There are patent claims that compositions containing papain-treated papaya (*Carica papaya*) powders, papain-treated maca (*L. meyenii*) powders, papain, and substantially water-free powders or oils are useful as face cleansers, packs, and bath preparations that show skin-conditioning effects (52). A face cleanser has been prepared from mannitol 50.0, soap 30.0, kaolin 10.0, talc 3.0, olive oil 1.0, papain 2.0, papain-treated papaya powder 2.0, and papain-treated maca powder 2.0 wt.%. Addition of polyols, mucopolysaccharides, sugars, and/or amino acids to the extract is claimed to improve the skin-moisturizing effect (53). Water-extracted maca is a desirable hygroscopic material, probably because it exhibits relatively good hygroscopic properties under

conditions of varying humidity and has high-moisture retention capacity even in dry silica gel desiccators (53). Interestingly, a water extract of *L. meyenii* inhibited tyrosinase, a key enzyme in the production of the skin pigment melanin, with an IC_{50} of 150 $\mu\text{g/mL}$ (54).

Two maca extracts (0.13 mg/mL), one obtained after boiling and the other without boiling, were administered on the dorsal surface of male Holtzman rats to study their protecting function against ultraviolet radiation (UVR) (55). The rats were exposed to UVR once a week during 3 consecutive weeks. A commercial sunscreen was used as a positive control. The results showed that UVR caused significant increase in skin epidermal thickness. The epidermal height in animals treated with maca was similar to those who did not receive UVR. The aqueous extract of maca after a boiling process had better effect than maca extract without a boiling process. A dose-response effect was observed with increasing doses of aqueous extract of maca after a boiling process.

CLINICAL STUDIES

Fertility-Enhancing and Aphrodisiac Activities

Gonzales et al. reported that maca does not affect serum reproductive hormone levels in adult men, but rather improves sperm motility and sperm production in a dose-dependent manner (56). In a similar protocol, Gonzales et al. (57) demonstrated the improvement of sexual desire after 8 weeks of treatment. Maca demonstrated an effect on sexual desire at 8 and 12 weeks of treatment, and this effect was independent of Hamilton depression and anxiety scores as well as serum testosterone and estradiol levels (57).

A randomized, double-blind, placebo-controlled, crossover trial was performed over 14 postmenopausal women. The women completed the Greene Climacteric Scale to assess the severity of menopausal symptoms, and the blood samples were collected for the measurement of estradiol, follicle-stimulating hormone, luteinizing hormone, and sex-hormone-binding globulin. In addition, aqueous and methanolic maca extracts were tested for androgenic and estrogenic activity by using a yeast-based hormone-dependent reporter assay. No differences in serum concentrations of estradiol, follicle-stimulating hormone, luteinizing hormone, and sex-hormone-binding globulin between baseline, maca treatment, and placebo ($P > 0.05$) were observed. The findings showed that maca reduced psychological symptoms, including anxiety and depression, and lowered measures of sexual dysfunction in postmenopausal women independent of estrogenic and androgenic activity (58).

A double-blind, randomized, pilot dose-finding study was carried out to determine whether maca is effective for selective-serotonin reuptake inhibitor (SSRI)-induced sexual dysfunction, comparing a low-dose (1.5 g/day) to a high-dose (3.0 g/day) maca regimen in 20 remitted depressed outpatients (mean age 36 ± 13 years; 17 women) with SSRI-induced sexual dysfunction. The results demonstrated that maca root may alleviate SSRI-induced sexual dysfunction, and there may be a dose-related effect. Maca may also have a beneficial effect on libido (59).

A pilot investigation into the effect of maca supplementation on physical activity and sexual desire in trained male cyclists was conducted. Eight participants each completed a 40 km cycling time trial before and after 14 days supplementation with both maca extract and placebo, in a randomized crossover design. Subjects also completed a sexual desire inventory during each visit. The result showed that maca extract administration significantly improved 40 km cycling time performance compared with the baseline test ($P = 0.01$), but not compared with the placebo trial after supplementation ($P > 0.05$). Maca extract administration significantly improved the self-rated sexual desire score compared with the baseline test ($P = 0.01$), and compared with the placebo trial after supplementation ($P = 0.03$). Long-term clinical studies involving more volunteers are needed to further evaluate the efficacy of maca extract in athletes and normal individuals and to explore its possible mechanisms of action (60).

A small but significant effect of maca supplementation on subjective perception of general and sexual well-being in adult patients with mild erectile dysfunction (ED) was observed through a double-blind clinical trial on 50 Caucasian men affected by mild ED, randomized to treatment with maca dry extract, 2400 mg, or placebo (61). The treatment effect on ED and subjective well-being was tested administering the International Index of Erectile Function (IIEF-5) and the Satisfaction Profile (SAT-P) before and after 12 weeks. After 12 weeks of treatment, both maca- and placebo-treated patients experienced a significant increase in IIEF-5 score ($P < 0.05$ for both). However, patients taking maca experienced a more significant increase in IIEF-5 score than those taking placebo (1.6 ± 1.1 vs. 0.5 ± 0.6 , $P < 0.001$). Both maca- and placebo-treated subjects experienced a significant improvement in psychological performance-related SAT-P score, but the maca group higher than that of placebo group ($+9 \pm 6$ vs. $+6 \pm 5$, $P < 0.05$). However, only maca-treated patients experienced a significant improvement in physical- and social-performance-related SAT-P score compared with the baseline ($+7 \pm 6$ and $+7 \pm 6$, both $P < 0.05$).

Although the "Preclinical Studies" section as well as the "Clinical Studies" section suggests potential beneficial effects of maca, demonstration of efficacy in humans requires the conduct of clinical trials using randomized, double-blind, placebo-controlled protocols, and administering standardized maca extracts.

CONCLUSIONS

Maca has been established as a nutritionally valuable food and food supplement through decades of research. The commercial activity of maca has grown explosively with the passing of the Dietary Supplement Health and Education Act in 1994. As with other herbal dietary products, quality, safety, and efficacy have been the critical concern for the consumer and industry. Furthermore, the rapid expansion of demand and diversity of products has created critical problems, as the scientific base of the industry has failed to keep pace. Further studies are required for the accurate authentication of raw plant material, including *L. meyenii* (maca), prior to commercial use. The quality of the tuberous root may depend upon the cultivation of

maca by using good agricultural practice. This includes selection of maca-specific habitat areas (typically highlands), soil and climatic conditions, seed stock, and correct storage of tubers. The presence of herbicides, pesticides, and heavy-metal residues needs to be analyzed during the quality control of raw material to insure the safety of the products. Second, extraction, preparation, and standardization of commercial maca products should be carried out using validated analytical methods, including the chemical profiling of marker compounds (21). The secondary metabolites of maca, including alkaloids, glucosinolates, macamides, and sterols, are just some of the marker constituents that may provide desirable nutritional, biological, and therapeutic (such as fertility-enhancing, aphrodisiac, and chemopreventive) leads. Future research efforts should be directed toward the isolation of the active constituents and the study of their mechanisms of action. Finally, more clinical studies related to specific disease areas should be directed to ensure safety, including from side effects and toxicity, and efficacy.

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Magnesium

Robert K. Rude

INTRODUCTION

Magnesium is an essential nutrient and is vital for numerous biologic processes in the body. This entry reviews the biochemistry, physiology, and homeostasis of magnesium. Dietary magnesium intake and requirements as well as current dietary recommendations are discussed. Causes of and risk factors for magnesium deficiency are reviewed along with the clinical manifestations of moderate-to-severe magnesium depletion. As dietary magnesium intake falls below the recommended daily allowance, possible complications of this nutrition deficiency such as hypertension, cardiovascular disease, and osteoporosis are discussed.

BIOCHEMISTRY AND PHYSIOLOGY

Magnesium (Mg) is widely distributed in nature being the eight most abundant element on earth and the second most abundant cation in sea water (1). It has therefore been incorporated widely in biology and is the fourth most abundant cation in the body and the second most prevalent intracellular cation. Due to its positive charge, Mg binds to negatively charged molecules. Most intracellular Mg binds to ribosomes, membranes, and other macromolecules in the cytosol and nucleus. Mg provides specific structure and catalytic activity for enzymes as discussed later.

Enzyme Interactions

Mg is involved in more than 300 essential metabolic reactions (2). Mg^{2+} is essential for many enzymatic reactions and has two general interactions: (a) Mg^{2+} binds to the substrate, thereby forming a complex with which the enzyme interacts, as in the reaction of kinases with MgATP and (b) Mg^{2+} binds directly to the enzyme and alters its structure and/or serves a catalytic role. Overall, the predominant action of Mg is related to adenosine triphosphate (ATP) utilization. ATP provides high-energy phosphate and exists in all cells primarily as $MgATP^{2-}$ (MgATP). Mg therefore is essential for the function of many pathways and enzymes including the glycolytic cycle, citric acid cycle, protein kinases, RNA and DNA polymerases, lipid metabolism, and amino acid activation, as well as playing a critical role in the cyclic adenosine monophosphate and phospholipase C second messenger systems.

Structural Modification of Nucleic Acids and Membranes

Another important role of Mg is its ability to form complexes with nucleic acids. The negatively charged ribose phosphate structure of nucleic acids has a high affinity for Mg^{2+} ; the resulting stabilization of numerous ribonucleotides and deoxyribonucleotides induces important physicochemical changes that affect DNA maintenance, duplication, and transcription (2). Mg, calcium, and some other cations react with hydrophilic polyanionic carboxylates and phosphates of the various membrane components to stabilize the membrane and thereby affect fluidity and permeability. This thereby influences ion channels, transporters, and signal transducers.

Ion Channels

Ion channels constitute a class of proteins across the cell membrane, which allow passage of ions in or out of cells when the channels are open. Ion channels are classified according to the type of ion they allow to pass such as sodium (Na^+), potassium (K^+), or calcium (Ca^{2+}) (3). Mg^{2+} plays an important role in the function of a number of ion channels. A deficit of Mg results in cellular potassium depletion (4). Mg^{2+} is necessary for the active transport of K^+ out of cells by Na^+ , K^+ ATPase. Another mechanism for the K^+ loss is an increased efflux of K^+ from cells via other Mg^{2+} -sensitive K^+ channels as has been seen in skeletal muscle and in heart muscle. Therefore, a deficiency in Mg^{2+} leads to a reduced amount of intracellular K^+ . The arrhythmogenic effect of Mg deficiency, as discussed later, may therefore be related to its effect on maintenance of intracellular K^+ .

Mg has been called nature's physiological calcium channel blocker (3). During Mg depletion, intracellular calcium rises. This may be due to both an increase from extracellular calcium and release from intracellular calcium stores. Mg^{2+} has been demonstrated to decrease the inward Ca^{2+} flux through slow calcium channels. In addition Mg^{2+} will decrease the transport of Ca^{2+} out of the sarcoplasmic reticulum into the cell cytosol. There is an inverse ability of inositoltriphosphate to release Ca^{2+} from intracellular stores in response to changes in Mg^{2+} concentrations, which would also allow greater rise in intracellular Ca^{2+} during a fall in Mg^{2+} .

BODY COMPOSITION AND HOMEOSTASIS

Composition

The distribution of Mg in various body compartments of apparently healthy adult individuals is summarized in

Table 1 Distribution and Concentrations of Magnesium (Mg) in a Healthy Adult^a

Site	Percentage of total body Mg	Concentration/content
Bone	53	0.5% of bone ash
Muscle	27	9 mmol/kg wet weight
Soft tissue	19	9 mmol/kg wet weight
Adipose tissue	0.012	0.8 mmol/kg wet weight
Erythrocytes	0.5	1.65–2.73 mmol/L
Serum	0.3	0.88 ± 0.06 mmol/L
% Free	65	0.56 ± 0.05 mmol/L
% Complexed	8	
% Bound	27	
Mononuclear		2.91 ± 0.6 fmol/cell
Blood cells		2.79 ± 0.6 fmol/cell
		3.00 ± 0.4 fmol/cell
Platelets		2.26 ± 0.29 mmol/L ^j
[Mg ²⁺]		0.5–1.0 mmol/L
Cerebrospinal fluid		1.25 mmol/L
free 55%		
complexed 45%		
Secretions		
Saliva, gastric, bile		0.3–0.7 mmol/L
Sweat		0.3 mmol/L (38°C)
		0.09 mmol/hr

Note: 1 mmol = 2 mequiv = 24.3 mg.

^aTotal body: 833–1170 mmol or 20–28 g.

Table 1. Approximately 60% of Mg is in the skeleton of which two-thirds of it is within the hydration shell and one-third on the crystal surface (5), which may serve as a reservoir for maintaining extracellular and intracellular Mg. Only 1% of Mg in the extracellular fluid and the rest is intracellular.

Cellular Mg Homeostasis

As already stated, Mg is compartmentalized within the cell and most of it is bound to proteins and negatively charged molecules. Significant amounts of Mg are found in the nucleus, mitochondria, the endoplasmic and sarcoplasmic reticulum, and the cytoplasm (1,6). Total cell Mg concentration has been reported to range between 5 and 20 mM. Ninety to ninety-five percent of that in the cytosol is bound to ligands such as ATP, ADP, citrate, proteins, and nucleic acids. The remainder is free Mg²⁺, constituting 1% to 5% of the total cellular Mg. The concentration of free ionized Mg²⁺ in the cytoplasm of mammalian cells has ranged from 0.5 to 1.0 mM similar to circulating ionized Mg²⁺. The Mg²⁺ concentration in the cell cytoplasm is maintained relatively constant even when the Mg²⁺ concentration in the extracellular fluid is experimentally varied to either high or low nonphysiological levels. The relative constancy of the Mg²⁺ in the intracellular milieu is attributed to the limited permeability of the plasma membrane to Mg and to the operation of specific Mg transport proteins, which regulate the rates at which Mg is taken up or extruded from cells. Maintenance of a normal intracellular concentration of Mg²⁺ requires that Mg be actively transported out of the cell. Mg transport in or out of cells appears to require the presence of carrier-mediated transport systems. The efflux of Mg from the cell appears to be coupled to Na transport and requires extrusion of sodium by Na⁺, K⁺-ATPase. There is also ev-

idence for a Na-independent efflux of Mg (7). Mg influx appears to be linked to Na transport but by a different mechanism than efflux. It has been reported that at least seven transmembrane Mg²⁺ channels have been cloned (7). These include NIPA2 (8) and MagT1 and TUSC3 (9). Studies of human hereditary diseases (see later) have identified paracellin-1 (claudin 16) and two transient receptor potential channel family members, TRPM6 and TRPM7 (10). TRPM6 is expressed in the kidney and TRPM7 is constitutively expressed. Studies have demonstrated that tissues vary with respect to the rates at which Mg exchange occurs and the percentage of total Mg that is readily exchangeable. The rate of Mg exchange in heart, liver, and kidney exceeded that in skeletal muscle, lymphocytes, red blood cells, brain, and testis.

The processes that maintain or modify the relationships between total and ionized internal and external Mg are not completely understood. Changes in cytosolic Mg²⁺ regulate some channels (TRPM6 and TRPM7) (7). Mg transport in mammalian cells may be influenced by hormonal and pharmacological factors. Mg²⁺ efflux was stimulated after short-term acute exposure of isolated perfused rat heart and liver or thymocytes to α - and β -agonists and permeant cAMP. Activation of protein kinase C by diacyl-glycerol or by phorbol esters stimulates Mg²⁺ influx and does not alter efflux. Epidermal growth factor has been shown to increase Mg²⁺ transport into a vascular smooth muscle cell line. Insulin and dextrose were found to increase ²⁸Mg uptake by a number of tissues, including skeletal and cardiac muscle. The mechanism of insulin-induced Mg transport is likely due to an effect on protein kinase C. An insulin-induced transport of Mg into cells could be one factor responsible for the fall in the serum Mg concentration observed during insulin therapy of diabetic ketoacidosis. It is hypothesized that this hormonally regulated Mg uptake system controls intracellular Mg²⁺ concentration in cellular subcytoplasmic compartments. The Mg²⁺ concentration in these compartments would then serve to regulate the activity of Mg-sensitive enzymes.

BODY HOMEOSTASIS

Homeostasis of the individual with respect to a mineral depends on the amounts ingested, the efficiency of intestinal and renal absorption and excretion, and all other factors affecting them. A schema for Mg balance is given in Figure 1.

Dietary Intake

Mg is widely distributed in plant and animal sources but in differing concentrations. In terms of major food sources (11), vegetables, fruits, grains, and animal products account for approximately 16% each; dairy product contributes 20% in adolescents and 10% beyond the third decade. The 1994 U.S. Department of Agriculture Continuing Survey of Food Intakes by Individuals (CSFII) indicated that the mean daily Mg intake was 323 mg in males and 228 mg in females, which was similar to the NHANES III survey. These values fall below the current Recommended Daily Allowance (RDA) recommendation of approximately 420 mg for males and 320 mg for females (12). Indeed, it has been suggested that 75% of

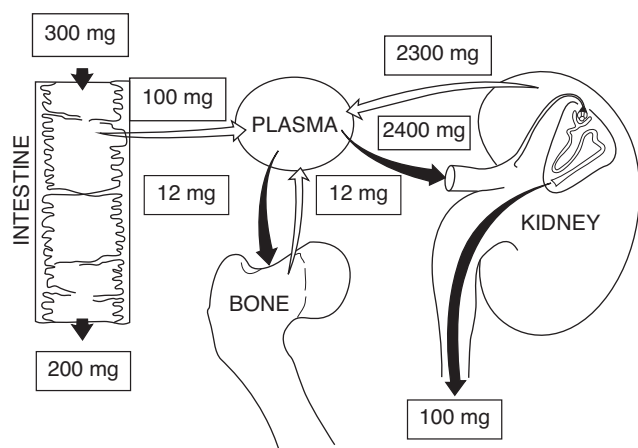


Figure 1 Mg homeostasis in man. A schematic representation of magnesium metabolism indicating (a) its absorption from the alimentary tract, (b) its distribution into bone, and (c) its dependence on the kidney for excretion. Homeostasis depends upon the integrity of intestinal and renal absorptive processes.

subjects in the United States have dietary Mg intake that falls below the recommended intake (see “Mg Requirements” section).

Intestinal Absorption

In humans, the primary site of intestinal Mg absorption is the jejunum and ileum although absorption can occur at other sites including the colon (13). Under a normal dietary Mg intake, 30% to 40% is absorbed. There exists both a passive paracellular mechanism and an active transport process for Mg absorption. The paracellular mechanism is dependent on transcellular potential difference generated by sodium transport and accounts for approximately 90% of intestinal Mg absorption (14). There exists a Mg-specific transport protein/channel, TRPM6, which accounts for the remainder of Mg absorption and may be influenced by a number of hormones (14). Net Mg absorption increases with increasing Mg intake however fractional Mg absorption falls; absorption fell progressively from approximately 65% to 70% with intake of 7 to 36 mg down to 11 to 14% with intake of 960 to 1000 mg (15).

Bioavailability: Influence of Other Dietary Factors

The fractional absorption of ingested Mg by healthy humans is influenced by its dietary concentration as discussed earlier as well as the presence of dietary components inhibiting or promoting Mg absorption (12). Long-term balance studies in healthy individuals, for the most part, indicate that increasing oral calcium intake does not significantly affect Mg absorption or retention. Increased amounts of Mg in the diet have been associated with either decreased calcium absorption or no effect. Some reports indicate decreased Mg absorption at high levels of dietary phosphate, whereas others found no consistent effect. Increased amounts of absorbable oral Mg have been noted to decrease phosphate absorption, perhaps secondary to formation of insoluble Mg phosphate. Increased intakes

of dietary fiber have been reported to decrease Mg utilization in humans, presumably by decreasing absorption. High dietary zinc intake decreased Mg absorption and balance whereas vitamin B₆ depletion was associated with negative Mg balance. The presence of excessive amounts of free fatty acids and oxalate may also impair Mg absorption.

Absorbability of Mg Salts

Multiple salts of Mg are available as dietary supplements including oxide, hydroxide, citrate, chloride, gluconate, lactate, and aspartate. The fractional absorption of a salt depends on its solubility in intestinal fluids and the amounts ingested (15). Absorption of enteric-coated Mg chloride is 67% less than that of the acetate in gelatin capsules. Mg citrate was found to have high solubility whereas Mg oxide was poorly soluble; better absorption of the citrate salt was demonstrated in humans. Little difference in absorption has been demonstrated among other salts however.

Regulation of Intestinal Mg Absorption

No hormone or factor has been described that regulates intestinal Mg absorption although several hormones may influence the TRPM6 channel as already discussed. Vitamin D and its active metabolites have been shown to increase intestinal Mg absorption in a number of studies (13). 1,25(OH)₂-vitamin D increases intestinal absorption in normal human subjects and patients with chronic renal failure. In balance studies, vitamin D increased intestinal Mg absorption but much less than calcium and mean Mg balance was not affected. In patients with impaired calcium absorption due to intestinal disease given vitamin D, only small increases in Mg absorption were observed compared with calcium. Mg was absorbed by individuals with no detectable plasma 1,25(OH)₂-vitamin D and, in contrast to calcium absorption, there is no significant correlation between plasma 1,25(OH)₂-vitamin D and Mg absorption.

Renal Mg Regulation

The kidney is the critical organ regulating Mg homeostasis. Mg handling is a filtration/reabsorption process (16). Approximately 2400 mg of Mg is normally filtered daily through the glomeruli in the healthy adult; of this only approximately 5% is excreted in the urine. The fractional absorption of the filtered load in the various segments of the nephron is summarized in Figure 2. Approximately 15% to 20% of filtered Mg is reabsorbed in the proximal convoluted tubule presumably by a paracellular mechanism. The majority, 65% to 75%, is reclaimed in the cortical thick ascending limb of Henle. The mechanism also appears to be paracellular transport. Paracellin-1 (claudin-16) and claudin-19 appear to mediate this transport (10). The distal convoluted tubule reabsorbs 5% to 10% of filtered Mg via an active transcellular pathway. Several proteins may be involved including the sodium chloride cotransporter (10). TMPR6 is also expressed in the distal tubule. Mutations of TRPM6 results in decrease intestinal Mg absorption and renal Mg wasting (10).

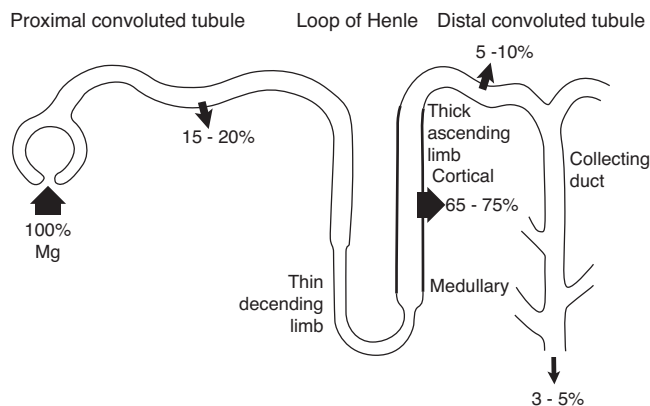


Figure 2 Fractional segmental reabsorption of filtered Mg in the nephron. The percentage absorption of filtered Mg^{2+} has been determined by micropuncture techniques in various laboratory animals as the Mg proceeds through the nephron. Approximately 15% to 20% of the Mg^{2+} is reabsorbed in the proximal convoluted tubule. The major site for Mg^{2+} reabsorption is the thick ascending limb of the loop of Henle, primarily in its cortical portion. Here, 65% to 75% of Mg leaves the lumen. In the distal convoluted 5% to 10% of Mg is reabsorbed. Source: From Cole DE, Quamme GA. Inherited disorders of renal magnesium handling. *J Am Soc Nephrol* 2000;11:1937-1947, with permission.

Hormonal and Other Regulatory Influences on Renal Mg Reabsorption

No hormone or factor has been described, which regulates renal Mg homeostasis. Micropuncture studies in rodents show that arginine, vasopressin, glucagon, calcitonin, and PTH, when added individually to the bath of mouse segments of the cortical thick ascending limb of loop of Henle and/or the distal convoluted tubule, significantly increased Mg absorption (16). The physiological significance of these observations however is unclear.

A number of conditions affect absorption, principally in the ascending thick limb. Inhibition occurs with hypermagnesemia and hypercalcemia (16). This is thought to occur because these cations binding to a calcium-sensitive receptor on the basolateral aspect of these tubular cells and decreasing transepithelial voltage and thereby decreasing the paracellular absorption of both Mg and calcium. Decreased Mg intake in experimental animals and humans rapidly decreases Mg excretion, even before serum/plasma Mg levels fall below the normal range, suggesting an adaption of the kidney to Mg insufficiency.

Tissue Sources

Extracellular, intracellular, and bone Mg fall during Mg depletion. Bone may serve as an important reservoir for Mg as human iliac crest Mg content fell an average of 18% with depletion (5). In young Mg-deficient rats and mice, approximately 30% of bone is lost (17).

Mg REQUIREMENTS

Assessment

For healthy older children, adolescents, and adults, the primary approach for assessing dietary Mg requirement

Table 2 Recommendations for Daily Intakes of Mg (mg)

Age (years)	Male	Female
0-0.5	30 ^a	30 ^a
0.5-1.0	75 ^b	75 ^b
1-3	80	80
4-8	130	130
9-13	240	240
14-18	410	360
19-30	400	310
31-50	420	320
51-70	420	320
>70	420	320
Pregnancy		
	≤18	400
	19-30	350
	31-50	360
Lactating		
	≤18	360
	19-30	310
	31-50	320

^aIntake from human milk by healthy breast-fed infants.

^bHuman milk plus solid food.

has been the dietary balance study. For infants and young children, the figures are based primarily on estimates of Mg intakes of milk and other foods that allow good development. Such data have been transmuted into the dietary reference intakes in the United States (12). The U.S. reference intakes for Mg are given in Table 2. These are estimates of intake that meet the needs of 97% to 98% of healthy individuals.

There are some problems with the study of balance. Laboratory analysis of foods revealed a Mg content 115% to 124% greater than those calculated by tables of food composition (18). A critique of previous RDAs for Mg noted that most of the published human balance data referenced in its various editions often did not meet the criteria for acceptable methodology (18). The balance studies (usually short term) were done mostly in adolescents and younger adults, and balance data presented for pregnant women were less than adequate. Published data about the elderly were meager. The need was pointed out for improved definition of acceptable standards, evaluation of the optimum base (i.e. weight, fat-free mass, or lean body mass), documentation of the accepted data, and more awareness of the ways in which homeostatic mechanisms conserve body Mg. Although the database of the 1997 reference values (Table 2) has eliminated the poorer studies, a question remains as to the accuracy of many balance studies in terms of adherence to acceptable methodology.

Dietary Intake

Estimates of Mg intakes in NHANES III (1988-1991) indicated that children 2 to 11 years grouped by gender, age, and race/ethnicity had median intakes well above their RDA. Those ages 1 to 5 years in the lower fifth percentile took in approximately 90% of the RDA. On the other hand, males and females from 12 to more than 60 years, grouped by race and ethnicity, with the exception of non-Hispanic white males, had low median intakes in terms of the RDA. The Third Report on Nutrition Monitoring in the United States (1995) analyzed intake in relation to the

RDA for age and gender; it concluded that Mg presents a potential public health issue requiring further study. One reason given was that the medium intakes of Mg from food were lower than the RDAs in various population groups. Assessment of Mg status at various dietary Mg intakes has not been performed. It is therefore impossible to estimate what level of intake would place one at risk for a Mg-deficient-associated problem.

ASSESSING MG STATUS

Analytic Procedures

As Mg is mostly within cells or in bone, assessment of Mg status is most difficult. A number of laboratory techniques are used in clinical and research investigations (19).

Atomic absorption spectrophotometry (AAS) has been widely used to determine total Mg in many sources and still remains the reference method as it provides greatest accuracy and precision although a number of metallochromic indicators and dyes are commonly used in automated methods. Ion-selective electrodes (ISEs) can measure ionized Mg (70% of total Mg) in serum, plasma, and whole blood. However, calcium and lipophilic cations interfere with the determination of ionized Mg. Literature indicates that ISEs from various manufacturers differ in accuracy from each other and from AAS and may give misleading results in sera with low Mg concentrations. Also, in critically ill patients there is a poor correlation between total and ionized serum Mg levels (20).

Other techniques have been developed to assess intracellular Mg concentration, which includes nuclear magnetic resonance spectroscopy and fluorescent indicators (19). These methods are reserved as research tools. Mg isotopes have been used as biologic tracers to follow the absorption, distribution, and excretion of the Mg ion. The radioisotope ^{28}Mg has been used in human studies. Its value is limited by its radioactivity, its short half-life of 21.3 hours, and its short supply.

Assessment Tests

Total serum Mg is the only test available to clinicians to assess Mg status (21). There are a number of reports of normal serum/plasma levels associated with a variety of illnesses but with low values in various blood cells and other organs. Consequently, total serum/plasma Mg values in such situations may be considered unreliable indicators of depletion. The level of ionized Mg may be more relevant under certain circumstances than that of total Mg. As already discussed, it should be noted that there exist intermethod differences for ionized Mg and therefore reference ranges must exist for each analyzer and may not be comparable to a different manufacturer.

Erythrocytes and blood mononuclear cell Mg content have been measured in experimental human Mg deficiency and patient populations and suggest that these measurements are more accurate than the serum Mg in assessing Mg status. These are not commercially available and technical issues however appear to limit its use in assessing Mg status in any given individual.

Assessing urine Mg excretion may be of use. When there is a reduction in the amounts of Mg ingested, there is a fairly rapid reduction in urinary Mg excretion. Serum Mg

may still be within normal limits, but urine levels low. This would not indicate whether the Mg deficits were acute or chronic however. In situations in which renal Mg wasting occurs, the resulting hypomagnesemia is associated with excessive urinary Mg excretion ($>1\text{ mmol/day}$). Such a relationship would suggest renal tubular dysfunction as the cause of the hypomagnesemia.

The IV Mg retention test provides an estimate of the proportion of infused Mg that is retained over a given period. Persons retaining more than the percentage retained by Mg-replete individuals (e.g., 20–25%) are considered to have some body depletion. A suggested clinical protocol that has been tested in a relatively large number of hypomagnesemic patients, chronic alcoholics, and animal controls has been published (22). It is an invasive, time-consuming, nonstandardized, and expensive test, requiring hospitalization or other close supervision for the partial or full 24 hours after infusion, with careful urine collection for laboratory analysis.

RISK FACTORS/CAUSES OF DEFICIENCY

Prevalence

The many risk factors for Mg depletion (Table 3) suggest that this condition may not be a rare occurrence. Up to 11% of hospitalized patients having routine Mg determinations were hypomagnesemic (13). The true prevalence of hypomagnesemia is not known because this ion is not included in routine electrolyte testing in many clinics or hospitals. Similar high rates of depletion have been reported in studies of ICU patients.

Gastrointestinal Disorders

As already discussed, dietary Mg intake falls below the recommended intake in a large proportion of the population (12). Therefore, nutritional Mg deficiency can be observed and it contributes to Mg depletion when other conditions exist, which impair Mg balance. Gastrointestinal disorders (Table 3) may lead to Mg depletion in various ways (13). The Mg content of upper intestinal tract fluids is approximately 1 mEq/L. Vomiting and nasogastric suction therefore may contribute to Mg depletion. The Mg content of diarrheal fluids and fistulous drainage are much higher (up to 15 mEq/L), and consequently Mg depletion is common in acute and chronic diarrhea, regional enteritis, ulcerative colitis, and intestinal and biliary fistulas. Malabsorption syndromes may also result in Mg deficiency. Steatorrhea and resection or bypass of the small bowel, particularly the ileum, often results in intestinal Mg loss or malabsorption. Acute severe pancreatitis is associated with hypomagnesemia, which may be due to the clinical problem causing the pancreatitis, such as alcoholism, or to saponification of Mg in necrotic parapancreatic fat.

Recently, proton pump inhibitors have been reported to cause hypomagnesemia in some patients (23). The evidence suggests that it is due to intestinal Mg malabsorption. A primary defect in intestinal Mg absorption, which presents early in life with hypomagnesemia, hypocalcemia, and seizures, has been described as an autosomal recessive disorder linked to chromosome 9q22. This disorder appears to be caused by mutations in

Table 3 Causes of Mg Deficiency

1. Gastrointestinal disorders
 - a. Nutritional deficiency
 - b. Prolonged nasogastric suction/vomiting
 - c. Acute and chronic diarrhea
 - d. Intestinal and biliary fistulas
 - e. Malabsorption syndromes
 - f. Extensive bowel resection or bypass
 - g. Acute hemorrhagic pancreatitis
 - h. Primary intestinal hypomagnesemia (mutation of TRPM6 channel)
 - i. Proton-pump inhibitors
2. Renal loss
 - a. Chronic parenteral fluid therapy
 - b. Osmotic diuresis (glucose, urea, mannitol)
 - c. Hypercalcemia
 - d. Polyuric phase of acute renal failure, renal transplant, post renal-obstruction
 - e. Nondrug-associated tubulointerstitial nephropathy
 - f. Alcohol
 - g. Diuretics (furosemide, hydrochlorothiazide)
 - h. Epidermal growth factor blockers (cetuximab, panitumumab)
 - i. Renal tubular nephrotoxins (aminoglycosides, cisplatin, amphotericin B, pentamidine)
 - j. Calcineurin inhibitors (cyclosporin, tacrolimus)
 - k. Genetic mutations of Mg transport channels
 - l. Activating mutation of the calcium-sensing receptor
3. Endocrine and metabolic disorders
 - a. Diabetes mellitus (glycosuria-osmotic diuresis)
 - b. Phosphate depletion
 - c. Primary hyperparathyroidism
 - d. Hypoparathyroidism
 - e. Primary aldosteronism
 - f. Excessive lactation
4. Cutaneous loss
 - a. Sweat-athletics
 - b. Burns
5. Redistribution of Mg to bone/soft tissues
 - a. Hungry bone syndrome
 - b. Parenteral nutrition/refeeding syndrome

TRPM6, which expresses a protein involved with active intestinal Mg transport (14).

Renal Disorders

Excessive excretion of Mg into the urine may be the basis of Mg depletion (Table 3) (16,24). Renal Mg reabsorption is proportional to tubular fluid flow as well as to sodium and calcium excretion. Therefore, chronic parenteral fluid therapy, particularly with saline, and volume expansion states such as primary aldosteronism and hypercalcemic states, may result in Mg depletion. Hypercalcemia has been shown to decrease renal Mg reabsorption probably mediated by calcium binding to the calcium-sensing receptor in the thick ascending limb of Henle and decreasing transepithelial voltage. Osmotic diuresis due to glucosuria will result in urinary Mg wasting.

Hypermagnesuria also occurs during the polyuric phase of recovery from acute renal failure in a native kidney, during recovery from ischemic injury in a transplanted kidney, and in postobstructive diuresis. In such cases, it is likely that residual tubule reabsorptive defects persisting from the primary renal injury play as important a role as polyuria itself in inducing renal Mg^{2+} wasting (25). Renal Mg^{2+} wasting has occasionally been reported in patients with acute or chronic tubulointerstitial nephritis

not caused by nephrotoxic drugs, for example, in chronic pyelonephritis and acute renal allograft rejection (25). Alcohol ingestion may also cause renal Mg wasting and is one cause of the high prevalence of Mg deficiency in chronic alcoholics.

Many pharmaceutical drugs may cause renal Mg wasting and Mg depletion. The major site of renal Mg reabsorption is at the loop of Henle, therefore diuretics such as furosemide result in Mg wasting (26). Hypomagnesemia is common in patients receiving the epidermal growth factor (EGF) receptor blockers, cetuximab and panitumumab (27), which are monoclonal-blocking antibodies of the EGF receptor that are used in the treatment of metastatic colorectal cancer. Renal tubular nephrotoxins (aminoglycosides, amphotericin B, cisplatin, and pentamidine) have been shown to cause renal lesions that result in hypermagnesuria and hypomagnesemia (25,28–30). Similarly, calcineurin inhibitors (cyclosporine and tacrolimus) has been reported to result in renal Mg wasting in patients after organ transplantation due to a down-regulation of the distal tubule Mg channel, TRPM6 (31).

Several renal Mg-wasting disorders have been described, which may be genetic or sporadic (32). One form, which is autosomal recessive, results from mutations in the paracellin-1 gene on chromosome 3 (Claudin 16). This disorder is characterized by low-serum Mg as well as hypercalciuria and nephrocalcinosis. Another autosomal dominant form of isolated renal Mg wasting and hypomagnesemia has been linked to chromosome 11q23 and identified as a mutation on the Na^+, K^+ -ATPase γ -subunit of gene *FXYD2*. More recently, a mutation of the Mg channel, TRPM6 may result in Mg wasting. Gitelman's syndrome (familial hypokalemia-hypomagnesemia syndrome) is an autosomal recessive disorder due to a genetic defect of the thiazide-sensitive NaCl cotransporter gene on chromosome 16. Other undefined genetic defects also exist (32).

Diabetes Mellitus

Special consideration must be given to diabetes mellitus. It is the most common disorder associated with magnesium deficiency (33). It is generally thought that the mechanism for magnesium depletion in diabetics is due to renal magnesium wasting secondary to osmotic diuresis generated by hyperglycosuria. Dietary magnesium intake however falls below the RDA in diabetics therefore nutritional deprivation may be a factor. Magnesium deficiency has been reported to result in impaired insulin secretion as well as insulin resistance (34,35), which may contribute to hypertension (36). The mechanism is unclear but may be due to abnormal glucose metabolism as magnesium is a cofactor in several enzymes in this cycle. In addition, magnesium depletion may decrease tyrosine kinase activity at the insulin receptor and magnesium may influence insulin secretion by the β cell. Diabetics given magnesium therapy appear to have improved diabetes control. Two studies have reported that the incidence of type 2 diabetes is significantly greater in people on a lower magnesium diet (34,35). Genetic variants of TRPM6 and TRPM7 have been reported to increase the risk of type 2 diabetes in women when they are on a diet of less than 250 mg/day of Mg (37). Magnesium status should

therefore be assessed in patients with diabetes mellitus as a vicious cycle may occur: diabetes out of control leading to magnesium loss and the subsequent magnesium deficiency resulting in impaired insulin secretion and action and worsening diabetes control.

Other

Hypomagnesemia may accompany a number of other disorders (13). Phosphate depletion has been shown experimentally to result in urinary Mg wasting and hypomagnesemia. Hypomagnesemia may also accompany the "hungry bone" syndrome, a phase of rapid bone mineral accretion in subjects with hyperparathyroidism or hyperthyroidism following surgical treatment. Mg may also shift into soft tissue during the refeeding syndrome resulting in a fall in serum Mg (38,39). Mg loss may occur from the skin in sweat and in burn patients (40,41).

CLINICAL PRESENTATION OF MAGNESIUM DEFICIENCY

As Mg plays an essential role in a wide range of fundamental biologic reactions, it is not surprising that Mg deficiency may lead to serious clinical symptoms. Human subjects have been studied in the course of Mg deficiency induced by diets low in this element (13) and these observations, along with those in humans who have Mg deficiency due to secondary causes, identify the manifestations of this deficit. Symptoms and signs of deficiency are given in Table 4. Mg deficiency occurs in a number of

predisposing and complicating disease states. The clinical presentation of Mg deficiency in disease states may coexist or be masked by the signs and symptoms of the primary disorder.

Moderate-to-Severe Mg Deficiency

When Mg deficiency is recognized in the clinical setting, it is usually of moderate-to-severe depletion. Biochemical, neuromuscular, and cardiac complication are the most prevalent findings in the Mg-deficient patient.

Hypocalcemia

Calcium is the major regulator of parathyroid hormone (PTH) secretion. Mg however modulates PTH secretion via the Ca^{2+} -sensing receptor in a manner similar to calcium (42). Although acute changes in the extracellular Mg concentrations will influence PTH secretion qualitatively similar to calcium, Mg deficiency perturbs mineral homeostasis (42,43). Hypocalcemia is a prominent manifestation of Mg deficiency. Mg deficiency must become moderate to severe before symptomatic hypocalcemia develops. Mg therapy alone restores serum calcium concentrations to normal. Calcium and/or vitamin D therapy will not correct the hypocalcemia. One major cause for the hypocalcemia is impaired parathyroid gland function. The majority of patients with hypocalcemia due to Mg deficiency have low or inappropriately normal serum PTH levels. The administration of Mg will result in an immediate rise in the serum PTH level. The presence of normal or elevated serum concentrations of PTH in the face of hypocalcemia suggests that there may also be end-organ resistance to PTH action. Skeletal resistance to exogenous PTH in hypocalcemic Mg-deficient patients has been reported. Similarly, urinary excretion of cyclic AMP and/or phosphate in response to PTH in such patients has been observed (42,43).

The mechanism for impaired PTH secretion and action in Mg deficiency remains unclear. It has been suggested that there may be a defect in the second messenger systems in Mg depletion. Adenylate cyclase has been universally found to require Mg for cyclic AMP generation both as a component of the substrate (Mg-ATP) and as an obligatory activator of enzyme activity. PTH has also been shown to activate the phospholipase C second messenger system. Mg depletion could perturb this system via several mechanisms as an Mg^{2+} -dependent guanine nucleotide regulating protein is involved in activation of phospholipase C and Mg^{2+} has also been shown to be a noncompetitive inhibitor of IP₃-induced Ca^{2+} release (43).

Mg is also important in vitamin D metabolism and/or action (42,43). Patients with hypocalcemia and Mg deficiency have also been reported to be resistant to pharmacological doses of vitamin D, 1α hydroxy vitamin D and 1,25-dihydroxy-vitamin D. The exact nature of altered vitamin D metabolism and/or action in Mg deficiency is unclear. Serum concentrations of 1,25-dihydroxy-vitamin D have been found to be low or low normal in most hypocalcemic Mg-deficient patients. Because PTH is a major trophic for 1,25-dihydroxy vitamin D formation, the low serum PTH concentrations could explain the low 1,25-dihydroxy vitamin D levels suggesting that Mg deficiency in man impairs the ability of the kidney to

Table 4 Manifestations of Mg Depletion

I. Bone and mineral metabolism
a. Hypocalcemia
1. Impaired PTH secretion
2. Renal and skeletal resistance to PTH
3. Impaired formation and resistance to $1,25(\text{OH})_2$ -vitamin D
b. Osteoporosis
II. Neuromuscular
a. Positive Chvostek's and Trousseau's sign
b. Spontaneous carpal-pedal spasm
c. Seizures
d. Vertigo, ataxia, nystagmus, athetoid, and chorioform movements
e. Muscular weakness, tremor, fasciculation, and wasting
f. Psychiatric: depression, psychosis
III. Potassium homeostasis
a. Hypokalemia
1. Renal potassium wasting
2. Decreased intracellular potassium
IV. Cardiovascular
a. Cardiac arrhythmia
1. EKG: prolonged P-R interval and Q-T interval, U waves
2. Atrial tachycardia, premature contractions and fibrillation
3. Junctional arrhythmias
4. Ventricular premature contractions, tachycardia, fibrillation
5. Sensitivity to digitalis intoxication
6. Torsades de pointes
b. Myocardial ischemia/infarction (putative)
c. Hypertension
d. Atherosclerotic vascular disease (putative)
V. Other
a. Migraine
b. Asthma
c. Colon cancer

synthesize 1,25-dihydroxy-vitamin D. Mg is known to support the 25-hydroxy-1 α -hydroxylase *in vitro*.

Hypokalemia

A common feature of Mg depletion is hypokalemia (44,45). Experimental human Mg deficiency demonstrated a negative potassium balance resulting from increased urinary loss. During Mg depletion there is also loss of intracellular potassium. Attempts to replete the potassium deficit with potassium therapy alone are not successful without simultaneous Mg therapy. The reason for this disrupted potassium metabolism may be related to Mg dependence of the Na, K, ATPase. During Mg depletion, intracellular sodium and calcium rise, and Mg and potassium fall. Mg also appears to be important in regulation of potassium channels in cardiac cells that are characterized by inward rectification. This biochemical feature may be a contributing cause of the electrocardiographic findings and cardiac dysrhythmias discussed later.

Neuromuscular Manifestations

Neuromuscular hyperexcitability is a common presenting complaint of a patient with Mg deficiency (13). Latent tetany, as elicited by a positive Chvostek's and Trousseau's sign, or spontaneous carpal-pedal spasm may be present. Seizures may also occur. Although hypocalcemia contributes to the neurologic signs, Mg deficiency without hypocalcemia has been reported to result in neuromuscular hyperexcitability. Other signs occasionally seen include vertigo, ataxia, nystagmus and athetoid, and choreiform movements. Muscular tremor, fasciculation, wasting, and weakness may be present. Reversible psychiatric aberrations also have been reported.

There may be several mechanisms for these neuromuscular problems. Mg has been shown to stabilize the nerve axon. Lowering the serum Mg concentration decreases the threshold of axonal stimulation and increases nerve conduction velocity. Mg also has been shown to influence the release of neurotransmitters, such as glutamate, at the neuromuscular junction by competitively inhibiting the entry of calcium into the presynaptic nerve terminal. It is likely that a decrease of extracellular Mg would allow a greater influx of calcium into the presynaptic nerves and the subsequent release of a greater quantity of neurotransmitters, resulting in hyperresponsive neuromuscular activity.

Cardiovascular Manifestations: Dysrhythmias

Cardiac dysrhythmias are an important consequence of Mg deficiency. Electrocardiographic abnormalities of Mg deficiency in humans include prolonged P-R interval and Q-T interval. Intracellular potassium depletion and hypokalemia are complicating features of Mg deficiency and may contribute to these electrocardiographic abnormalities. Mg-deficient patients with cardiac dysrhythmias have been treated successfully by Mg administration (46). Supraventricular dysrhythmias including premature atrial complexes, atrial tachycardia, atrial fibrillation, and junctional arrhythmias have been described. Ventricular premature complexes, ventricular tachycardia, and ventricular fibrillation are more serious complications. Such

dysrhythmias may be resistant to usual therapy. As intracellular Mg depletion may be present despite a normal serum Mg concentration, Mg deficiency always must be considered as a potential factor in cardiac dysrhythmias.

Cardiovascular: Acute Myocardial Infarction

Acute myocardial infarction (AMI) is the leading cause of death in the United States. Mg deficiency may be a risk factor as it has been shown to play a role in systemic and coronary vascular tone (see later), cardiac dysrhythmias as mentioned earlier, and by inhibiting platelet aggregation. Over the past decade, debate arose over the clinical utility of adjunctive Mg therapy for AMI. Although several small controlled trials suggested that adjunctive Mg therapy reduced mortality from AMI by 50%, three major trials define our understanding regarding Mg therapy in AMI (47). LIMIT-2 was the first study involving large numbers of participants. Over a 6-year period, 2316 participants with suspected AMI were randomized to receive adjunctive Mg therapy or placebo. The Mg-treated group showed an approximately 25% lower mortality rate (7.8% vs. 10.3%; $P < 0.04$). The ISIS-4 study randomized over 58,000 participants over a 3-year period to examine the effects of captopril, nitrates, and Mg on AMI. Unlike LIMIT-2, the mortality rate in the Mg-treated group was not significantly different from the control group (7.64% vs. 7.24%). The conclusion was that Mg therapy was not indicated in suspected AMI. Despite the null result, some suggested that the ISIS-4 design masked the benefits of Mg therapy. Two major criticisms involved the timing of the Mg therapy and the severity of patient illness. ISIS-4 randomized participants up to 24 hours after presentation. The leading theory regarding the role of Mg therapy in AMI involves the prevention of ischemia-reperfusion injury. The recently published MAGIC Trial was designed to address the issues regarding ISIS-4 study design; namely, early intervention in higher risk patients would more likely show the benefit of Mg therapy (47). Over a 3-year period, 6213 participants were studied. The Mg-treated group mortality at 30 days was not significantly different from the placebo group mortality (15.3% vs. 15.2%). Unless there is a high suspicion of Mg deficiency, the overall evidence from clinical trials does not support the routine application of adjunctive Mg therapy in patients with AMI (48).

CHRONIC LATENT MAGNESIUM DEFICIENCY

Although the diets ordinarily consumed by healthy Americans fall below the RDA (12), they do not appear to lead to symptomatic Mg depletion. A number of clinical disorders however have been associated with a low-Mg diet. It has been suggested that more mild degrees of Mg deficiency present over time may contribute to disease states such as hypertension, coronary artery disease, preeclampsia, and osteoporosis.

Hypertension

A number of studies have demonstrated an inverse relationship between populations that have low dietary intake of Mg and blood pressure (7,49). Hypomagnesemia

and/or reduction of intracellular Mg have also been inversely correlated with blood pressure. Patients with essential hypertension were found to have reduced free Mg^{2+} concentrations in red blood cells. The Mg^{2+} levels were inversely related to both systolic and diastolic blood pressure. Intervention studies with Mg therapy in hypertension have led to conflicting results. Several studies have shown a positive blood-pressure lowering effect of Mg supplements whereas others have not. Other dietary factors may also play a role. A diet of fruits and vegetables, which increased Mg intake from 176 to 423 mg/day (along with an increase in potassium), significantly lowered blood pressure (50). The addition of nonfat dairy products that increased calcium intake as well further lowered blood pressure. The mechanism by which Mg deficiency may affect blood pressure is not clear but may involve decreased production of prostacyclin, increased production of thromboxane A₂, and enhanced vasoconstrictive effect of angiotensin II and norepinephrine. Recently, it has been suggested that vascular TRPM7 Mg channel may be altered in hypertension (7).

Atherosclerotic Vascular Disease

Another potential cardiovascular complication of Mg deficiency is the development of atheromatous disease (51). Lipid alterations have been reported in hypomagnesemic human subjects; however, they are often complicated by factors related to underlying lipoprotein abnormalities occurring in diabetes, coronary artery disease, myocardial infarction, and other diseases. Epidemiologic studies have related water hardness (calcium and Mg content) inversely to cardiovascular death rates. Platelet hyperactivity is a recognized risk factor in the development of cardiovascular diseases. Mg has been shown to inhibit platelet aggregation against a number of aggregation agents. Diabetic patients with Mg depletion have been shown to have increased platelet aggregation. Mg therapy in these subjects returned the response toward normal. The antiplatelet effect of Mg may be related to the finding that Mg inhibits the synthesis of thromboxane A₂ and 12-HETE, eicosanoids thought to be involved in platelet aggregation. Mg also inhibits the thrombin-induced Ca influx in platelets as well as stimulates synthesis of PGI₂, the potent antiaggregatory eicosanoid.

Preeclampsia and Eclampsia

Preeclampsia complicates 1 in 2000 pregnancies in developed countries and is responsible for over a 50,000 maternal deaths per year. Mg therapy has been used for decades in both preeclampsia and eclampsia and contributes to the very low mortality rate in developed countries (52). Despite decades of use, no large randomized trial examining the efficacy of Mg therapy had been performed until the MAGPIE Trial in 2002. This trial, which compared women with preeclampsia treated with MgSO₄ to nimodipine, a specific cerebral arterial vasodilator, showed a lower risk (0.8% vs. 2.6%) of eclampsia in the Mg therapy group (52). The Mg status of women with preeclampsia has been difficult to establish. No difference was found in the plasma Mg levels of women with preeclampsia and those of healthy pregnant women; however, in women

with preeclampsia there was a decreased RBC Mg level. Women with preeclampsia and women pre-term labor had no differences in ionized or total serum Mg levels. Although subtle deficits in total body Mg may contribute to hypertension during pregnancy, the role of Mg may relate more to its stabilizing neuronal and vascular effects rather than the correction of an electrolyte deficit. Mg therapy is clearly indicated for women with preeclampsia. It has been shown to decrease the incidence of eclampsia and likely to decrease overall mortality.

Osteoporosis

Dietary Mg deficiency in animals results in a decrease in growth of the skeleton (17,43). Osteoblastic bone formation has been found reduced. Markers of bone formation have been reduced, suggesting a decrease in osteoblastic function. An increase in the number and activity of osteoclasts in the Mg-deficient rats and mice has been reported. Bone from Mg-deficient rats has been described as brittle and fragile. Biomechanical testing has directly demonstrated skeletal fragility in both rats and pigs. In humans, epidemiologic studies have demonstrated a correlation between bone mass and dietary Mg intake in appendicular and axial skeleton (43). Few studies have been conducted assessing Mg status in patients with osteoporosis. Low serum and red blood cell Mg concentrations as well as high retention of parenterally administered Mg has suggested a Mg deficit, however these results are not consistent from one study to another. Similarly, whereas low skeletal Mg content has been observed in some studies, others have found normal or even high Mg content. The effect of dietary Mg supplementation on bone mass in patients with osteoporosis has not been extensively studied. The effect of Mg supplements on bone mass has generally led to an increase in bone mineral density, although study design limits useful information. Larger long-term placebo-controlled double-blind investigations are required.

There are several potential mechanisms that may account for a decrease in bone mass in Mg deficiency. Mg is mitogenic for bone cell growth that may directly result in a decrease in bone formation. Mg also affects crystal formation; a lack of Mg results in a larger, more perfect crystal, which may affect bone strength. Mg deficiency results in a fall in both serum PTH and 1,25(OH)₂D as discussed earlier. Because both hormones are trophic for bone, impaired secretion or skeletal resistance may result in osteoporosis. An increased release of inflammatory cytokines may result in activation of osteoclasts and increased bone resorption in rodents (17,43).

OTHER DISORDERS

Mg deficiency has been associated with migraine headache and Mg therapy has been reported to be effective in the treatment of migraine (53). Because Mg deficiency results in smooth muscle spasm, it has also been implicated in asthma and Mg therapy has been effective in asthma in some studies (54). Lastly, a high dietary Mg intake has been associated with reduced risk of colon cancer (55).

MANAGEMENT OF DEPLETION

Seizures, acute arrhythmias, and severe generalized spasticity require immediate IV infusion. One to two grams $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (8.2–16.4 mequiv Mg^{2+}) is usually infused over 5 to 10 minutes, followed by continuous infusion of 6 g over 24 hours or until the condition is controlled. Correction of electrolyte (especially potassium) and acid–base imbalances should accompany the Mg therapy. In addition, levels of serum Mg and other electrolytes should be determined at least twice daily in such patients.

Less severe manifestations (e.g., paresthesias with latent or active tetany) are likewise best treated by the IV route, again in conjunction with appropriate therapy for the underlying condition and with correction of other electrolyte and acid–base abnormalities. When renal function is good, 6 g (48 mequiv) of Mg sulfate may be given intravenously over 24 hours in saline or dextrose solutions, with other nutrients as required. This may be continued for 3 to 5 days until the signs and symptoms and/or electrolyte abnormalities are corrected. When the IV route cannot be used, IM injections can be given, although these are painful. This regimen is continued for 2 or more days, and the situation is then reassessed. The dosages given must always exceed the daily losses as indicated by serum levels and urinary excretion. The return to the normal or slightly higher range of serum Mg levels with any of these schedules is relatively rapid. However, repletion of Mg lost from bone and other tissues requires more prolonged Mg therapy.

When intestinal absorption is normal and renal Mg wasting is present, supplements should be added to the usual diet to tolerance (onset of diarrhea) to maintain normal serum levels. In some instances, oral Mg may not be sufficient and IM and/or IV Mg may be required. Those with continuing severe Mg and potassium losses in the urine (as in cisplatin nephrotoxicity or hereditary renal defects) may require long-term supplements by IV infusion via an indwelling central catheter for home administration.

When depletion is modest and persistent, initial efforts should be directed to increased intake of Mg-rich foods. When necessary and feasible, supplementary oral Mg may be taken. Three hundred to six hundred milligrams may be given in divided doses three to six times per day, with a full glass of water to prevent or minimize Mg-related diarrhea and to ensure solubilization. For the individual on enteral feeding, one of these salts may be dissolved in the formula. Improvement of existing steatorrhea by dietary or other medical means will decrease fecal Mg losses. Again, treatment of underlying disease and replacement of potassium deficits are essential.

CONCLUSIONS

In conclusion, Mg is a vital nutrient necessary for essential biologic processes. Despite its rigid homeostasis by the body, Mg deficiency is not uncommon due to numerous diseases, disorders, and medications, which impair the normal metabolism of magnesium. The relatively low dietary Mg intake compounds this problem in many patients. Indeed, even the reduction in suggested dietary

Mg may contribute to chronic disease states in otherwise healthy individuals. It is clear that education is necessary for the general population to increase awareness of the importance of Mg in maintaining health. In addition, further research efforts in both basic science and clinical science are needed to clarify the role of Mg deficiency in disease states.

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Melatonin

Amnon Brzezinski and Richard J. Wurtman

INTRODUCTION

Melatonin is a hormone, like the estrogens and testosterone: It is synthesized in the pineal gland and secreted into the blood and cerebrospinal fluid. It conveys signals to distant organs, principally the brain, which affect the synthesis of second messengers and, ultimately, sleep and circadian rhythms. However, unlike the estrogens and testosterone, melatonin is marketed in the United States as a dietary supplement, which implies that people normally obtain this compound from the diet and that melatonin pills simply supplement that which the diet provides. No food has ever been found to elevate plasma melatonin levels nor is there acceptable evidence that any food actually contains more than trace amounts of the hormone. This entry describes the history of our knowledge of melatonin; the hormone's synthesis, metabolism, and physiologic regulation; the factors that affect plasma melatonin levels; the known effects of endogenous and exogenous (oral) melatonin; and the present usage of melatonin and some synthetic analogs.

HISTORY OF MELATONIN

Few people would now doubt that the human pineal gland is an important structure, and that it transmits signals to the brain and other organs by secreting a unique hormone, melatonin. However, this consensus is only a few decades old. For most of the twentieth century, the pineal was generally dismissed as a "vestige"—a "third eye" in certain lower vertebrates, which, in humans, died and became calcified early in life. Tumors of the pineal gland were known sometimes to be associated with a reproductive disorder—precocious puberty, especially in boys—and some scientists attributed this phenomenon to the destruction of functioning pineal tissue. However, most concluded that the accelerated sexual maturation simply resulted from increased intracranial pressure or from the secretion of gonadotropins from tumor tissue.

The modern history of the pineal gland probably began with the discovery in 1917 (1) that extracts of cow pineals could lighten the skin of frogs. The physiologic significance of this relationship seemed obscure, inasmuch as bovine pineal extracts had no effect on pigmentation in bovines (or humans), and frog pineals lacked detectable skin-lightening ability. However, the finding did indicate that the pineal contained a compound with at least some biological activity, and it provided a way of

identifying the active compound, using assays based on the ability of purified extracts to aggregate the melanin granules in the frog's pigment cells. In 1958, Lerner et al. (2) discovered the compound's chemical structure to be 5-methoxy-*N*-acetyltryptamine and named it melatonin.

Around that time, scientists made four seemingly unrelated discoveries, which became coherent, like a partly completed crossword puzzle, once melatonin was identified. In chronologic sequence, these were (i) the demonstration, by Kitay and Altschule, that surgical removal of the rat's pineal accelerated the growth of the ovaries, whereas administration of bovine pineal extracts had the opposite effect (3); (ii) Fiske's observation that housing rats in a continuously lit environment led to a decrease in the weights of their pineals (4); (iii) Ariens-Kappers' discovery (5) that, though the pineal gland originates embryologically as part of the brain, it loses most or all of its CNS connections by birth, and instead receives its innervation from peripheral sympathetic nerves; and (iv) the demonstration that both pinealectomy and prolonged light exposure accelerate the growth of the rat's ovaries to an equal extent, and that both responses are blocked by administering pineal extracts (6). In 1963–1964, it was shown that melatonin is a true hormone in rats, that it is the gonad-inhibiting substance previously described in pineal extracts (7), and that its synthesis in the pineal gland is suppressed when rats are exposed continuously to light, the light acting not directly, as on a "third eye," but indirectly, via the animal's eyes and sympathetic nerves (8). [The chemical that mediates the sympathetic nervous signals was shown to be norepinephrine (9), which stimulates pineal beta-receptors and increases cyclic-AMP production (10).] The rates at which the rat's pineal synthesizes serotonin and melatonin were soon shown to vary with circadian rhythms, and the melatonin rhythm was ultimately found to be generated by intrinsic circadian signals emanating from the suprachiasmatic nucleus (SCN) of the brain (11), the phasing of which was controlled primarily by the light–dark cycle.

Finally, in 1975, it was shown that melatonin production in humans also exhibits a pronounced circadian rhythm (12), causing nocturnal plasma melatonin levels to be at least 10-fold higher than those observed in the daytime. Moreover, this rhythm was not simply a response to the environmental light–dark cycle, because if people were suddenly placed in an environment that was dark between 11 a.m. and 7 p.m. (instead of the usual 11 p.m. to 7 a.m.), it took their melatonin rhythms 5 to 7 days to re-entrain. The view thus became canonized that the

pineal is a “neuroendocrine transducer” (13) that tells all mammals when it is dark outside by raising plasma melatonin levels. The uses to which the body puts this information vary considerably among species: In diurnal, but not nocturnal, animals, melatonin promotes sleep onset and maintenance; in animals that breed seasonally, melatonin affects the choice of breeding season (i.e., spring or fall); and in those like humans and rats, which breed throughout the year, melatonin’s reproductive effects can be minimal.

Much subsequent pineal research has concerned the human brain’s responses to melatonin. The most compelling evidence now available supports two such uses that are discussed below: the involvement of nocturnal melatonin secretion in initiating and maintaining sleep, and control by the day/night melatonin rhythm of the timing of other 24-hour rhythms. It is melatonin’s effect on sleep that underlies most of its current use as a “dietary supplement.” Some additional possible benefits of melatonin supplementation have been proposed (e.g., as an antioxidant, or to slow aging, or to suppress cancer growth and hypertension). However, evidence supporting these effects is sparse.

Evidence is even more sparse that there is any rational basis for calling melatonin a “dietary supplement.” For melatonin to earn this appellation, it would have to be shown that at least some of the melatonin in human plasma derives from food sources, and that “supplementary” exogenous melatonin simply adds to what the foods provide. But as described later, there is no satisfactory evidence, on the basis of contemporary analytic techniques, that any actual foods contain more than trace amounts of melatonin—if that—and no evidence at all that eating any food elevates human plasma melatonin levels. Melatonin is a *hormone*, such as thyroxine and estrogens, and should be labeled and regulated as such. Only its extraordinary lack of overt toxicity apparently keeps the Food and Drug Administration (FDA) from insisting that it undergo such regulation.

MELATONIN SYNTHESIS AND METABOLISM: NEURAL AND PHOTIC CONTROL

Almost all the melatonin formed in mammals is synthesized within the pineal gland, starting with the uptake of the amino acid tryptophan from the plasma. Because the pineal lies outside the blood–brain barrier, this process—in contrast to tryptophan’s uptake into the brain—is not subject to competition from other circulating neutral amino acids and is not enhanced by carbohydrate consumption and insulin secretion. The tryptophan is first 5-hydroxylated (by the enzyme tryptophan hydroxylase) and then decarboxylated (by the enzyme aromatic L-amino acid decarboxylase) to form 5-hydroxytryptamine or serotonin (Fig. 1) (9).

During daylight hours, the serotonin in pinealocytes tends to be stored and is unavailable to enzymes (monoamine oxidase and the melatonin-forming enzymes) that would otherwise act on it. With the onset of darkness, postganglionic sympathetic outflow to the pineal increases and the consequent release of norepinephrine to pinealocytes causes stored serotonin to become accessible for intracellular metabolism. At the same time, the norepinephrine activates the enzymes [especially serotonin-*N*-acetyltransferase (SNAT), but also hydroxyindole-*O*-methyltransferase (HIOMT)] that convert serotonin to melatonin (Fig. 1) (9,11). Consequently, pineal melatonin levels rise manyfold. (Pineal levels of 5-methoxytryptophol, the corresponding deaminated and *O*-methylated metabolite of serotonin, also rise (14) even though formation of this compound is independent of SNAT.)

The melatonin then diffuses out of the pineal gland into the blood stream and cerebrospinal fluid (15), rapidly raising human plasma melatonin levels from approximately 2–10 to 100–200 pg/mL (12). Melatonin is highly lipid soluble, because both the ionizable groups in serotonin—the hydroxyl and the amine—have been blocked by its *O*-methylation and *N*-acetylation (Fig. 1).

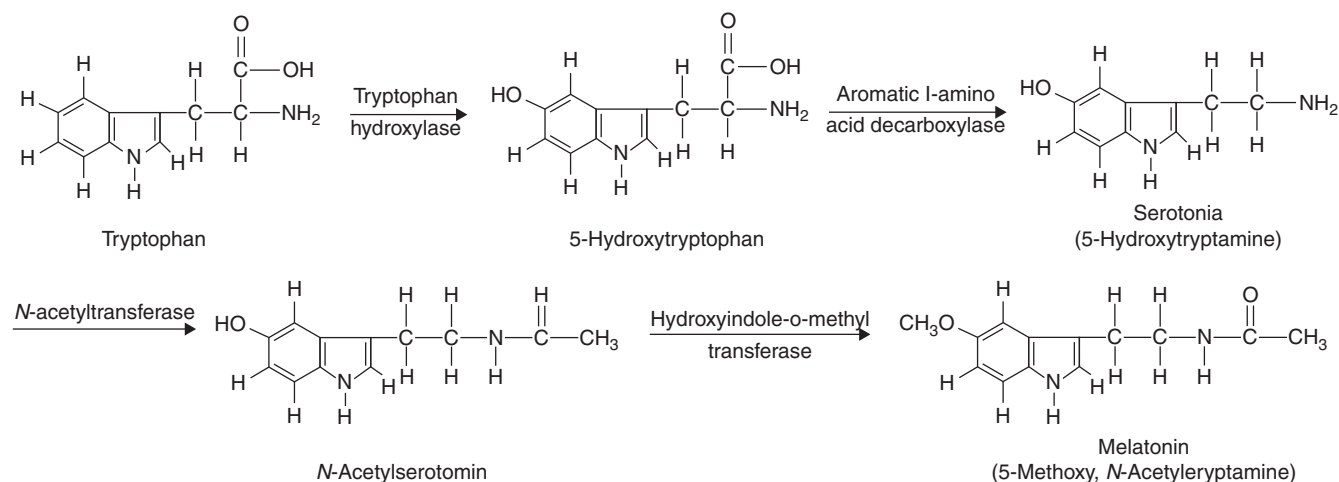


Figure 1 Metabolism of tryptophan to melatonin in the pineal gland. Source: From Ref. 91.

Thus, it diffuses freely across cell membranes into all tissues, and travels in the blood largely bound to albumin.

Most of the melatonin in the circulation is inactivated in the liver, where it is first oxidized to 6-OH-melatonin by a P450-dependent microsomal oxidase and then largely conjugated to sulfate or glucuronide before being excreted into the urine or feces (16). Approximately 2% to 3% is excreted unchanged into the urine or saliva, enabling measurements of urinary or salivary melatonin to be used as rough estimates of plasma melatonin levels. (Salivary melatonin apparently corresponds to the 25–30% of blood melatonin that is not bound to albumin.)

Studies using radioactively labeled melatonin of high specific activity have identified three probable melatonin receptors, two of which have been cloned by using human sources (17). These macromolecules are concentrated, respectively, within the suprachiasmatic nucleus of the hypothalamus, the pars tuberalis of the pituitary and cardiac blood vessels (mt_1), in the retina and hippocampus (MT_2), and in kidney, brain, and various peripheral organs (MT_3). Their affinities for melatonin are enhanced by several G-proteins. Activation of the mt_1 and MT_2 receptors by melatonin suppresses cAMP production. The MT_3 site shares 95% homology with a detoxifying enzyme, quinone reductase 2; its effects on specific signal transduction pathways await identification. Because of melatonin's unusual lipid solubility, its receptors could be located intracellularly, in contrast to the plasma membrane receptors characteristic of neurotransmitters; indeed, a nuclear-binding site has been identified. The mt_1 receptors in the SCN allow melatonin to inhibit the firing of SCN neurons during the night—an action that might contribute to melatonin's sleep-promoting effects. The SCN's MT_2 receptors apparently mediate melatonin's effects on the SCN's own circadian rhythms, as well as on other rhythms that this brain region controls.

In all species examined thus far, melatonin secretion manifests a characteristic circadian rhythm, causing plasma levels to be low during the daylight hours, ascend after the onset of darkness, peak in the middle of the night between 11 p.m. and 3 a.m., and then fall sharply before the time of light onset. (It is interesting that high nocturnal plasma melatonin levels characterize *both* diurnally active species, in which these levels promote sleep onset and maintenance, and nocturnally active ones, in which melatonin has no obvious relationship to sleep.) Although this rhythm is normally tightly entrained to the environmental light cycle, it does persist when people are placed for a few days in a dark room, and it does not immediately phase-shift when the light schedule is altered, indicating that it is not simply generated by the light-dark cycle but also by cyclic endogenous signals, probably from the SCN (11). These reach the pineal via a retinohypothalamic tract, the superior cervical ganglia, and postganglionic sympathetic fibers that re-enter the cranial cavity (5). In certain fish, birds, and reptiles, pineal glands also contain true photoreceptors, and denervated (or even cultured) glands can sustain circadian rhythms in melatonin synthesis that can be entrained by the light-dark cycle; in contrast, light has no known direct effects on melatonin synthesis in human or other mammalian pineals.

PLASMA MELATONIN LEVELS

Plasma melatonin normally reflects the amounts secreted by the pineal gland, the flux of melatonin in and out of tissues, melatonin's destruction in the liver, and its secretion into urine and saliva. Because melatonin is now also available as a dietary supplement, plasma levels can reflect consumption of the exogenous compound as well. Available evidence does not support the view that humans derive any plasma melatonin from foods. Several laboratories have described a compound in dietary fruits or vegetables [e.g., tomato (18,19)] that they concluded was melatonin. But in only one of these studies (19) was the identity of the melatonin unambiguously confirmed by gas chromatography-spectrometry (GCMS), and in that study, the melatonin concentrations determined by GCMS were very low (less than 20 ng/kg of fruit), and the "... concentrations ... indicated by RIA were 6–100-fold higher than ... by GCMS for the same extracts, suggesting ... contamination by an immunological interference ...". Of perhaps greater relevance, no investigator has ever presented evidence that feeding any amount of any food to humans can raise plasma melatonin levels.

Usually, the principal factor affecting plasma melatonin levels is its rate of secretion, which varies with the circadian rhythm described above and as a function of age (Fig. 2). Nocturnal melatonin levels are also affected by drugs that interfere with the transmission of neurotransmitter signals to pineal cells (like propranolol, a beta-blocking agent (20), those that inhibit melatonin's metabolism (like 8-methoxypsoralen (21), and a few drugs that lack clear links to melatonin's synthesis or metabolism (e.g., caffeine, ethanol (22), ibuprofen, and indomethacin, which decrease melatonin). Nocturnal melatonin secretion is also suppressed by exposure to environmental lighting (23), even by a relatively dim 100–200 lx, when pupils are dilated.

Melatonin secretion by the human pineal gland exhibits a pronounced age dependence (Fig. 2). Secretion is minimal in newborns, starts during the third or fourth months of life (coincident with the consolidation

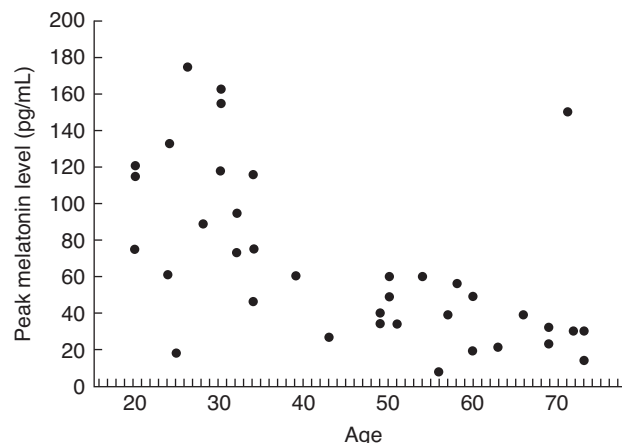


Figure 2 Nighttime peak serum melatonin levels in subjects of different ages (years). Source: From Ref. 92.

of sleeping at night (24)), increases rapidly at ages 1–3 year, and then declines slightly to a plateau that persists through early adulthood. Nocturnal melatonin secretion then starts a marked continuing decline in most people, with peak nocturnal levels in most 70-year-olds being only a quarter or less of what they are in young adults (25). This decline may reflect the progressive unexplained but ubiquitous calcification of the pineal gland and resulting loss of secretory tissue. Obviously, one strategy in using supplemental melatonin is to administer to older people doses that are just sufficient to compensate for this age-related decline.

The first person to examine the effects of exogenous melatonin was the scientist who discovered it, Aaron Lerner; he explored its actions (and possible toxicities) by giving himself 200 mg IV/day for 5 consecutive days. Lerner described feeling “relaxed.” Neither he nor the investigators who subsequently gave it (in doses of 10 mg to 6.6 g) to 96 other subjects prior to 1977 measured its effects on plasma melatonin levels. However because most of them administered doses in excess of 1 g, it can be assumed that massive increases in plasma melatonin ensued. When Waldhauser et al. (26) administered 80-mg doses to two male volunteers in 1987, plasma levels increased more than 1000-fold, and serum prolactin levels rose significantly—an effect not observed with physiologic melatonin doses.

In 1993, Dollins et al. examined the effects of 10, 20, 40, or 80 mg melatonin on various behavioral indices (auditory vigilance; self-reported fatigue, confusion, and sleepiness; reaction times), body temperature, and plasma melatonin levels. All the doses tested produced similar changes in the behavioral assays and in body temperature. And all raised plasma melatonin levels to at least 5000 pg/mL—well beyond the normal nocturnal range of 100 to 200 pg/mL (27). Hence, the study was repeated using much lower doses (0.1–10 mg orally) (28). The authors found that oral doses as low as 0.1 to 0.3 mg caused dose-related decreases in sleep latency and increases in sleep duration and self-reported sleepiness and fatigue, but without reducing body temperature or elevating plasma melatonin levels beyond their normal nocturnal range (Fig. 3). This suggested that nocturnal melatonin secretion—which produces plasma melatonin levels similar to those seen after the 0.3-mg dose—has a physiologic effect on sleep. It also identified the dosage range that investigators needed to use if they wanted to examine melatonin’s physiologic effects.

It should be noted that there is considerable person-to-person variability in the bioavailability of melatonin: In one study using single 80-mg doses, there were 25-fold variations in areas under the curve (AUCs) in the five subjects studied. In another, using 0.5 mg oral doses, peak plasma melatonin levels among four subjects varied from 480 to 9200 ng/L (29). Melatonin’s bioavailability was relatively poor—10% to 56%—which the authors attributed to person-to-person differences in first-pass hepatic extraction, perhaps reflecting such differences in hepatic function. Older subjects given a 0.3-mg oral dose of melatonin exhibit considerably greater increments in plasma melatonin levels, with correspondingly greater variability, than young adults receiving that dose.

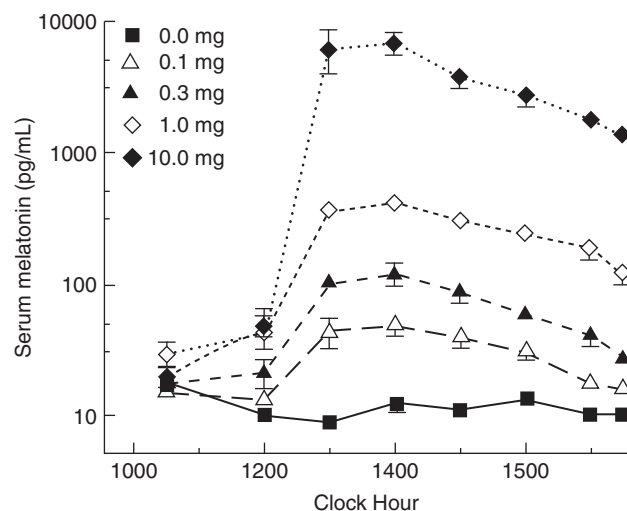


Figure 3 Mean serum melatonin profiles of 20 subjects sampled at intervals after ingesting 0.1, 0.3, 1.0, and 10 mg of melatonin or placebo at 11:45 a.m. Source: From Ref. 28.

These findings all suggest that although a 0.3-mg dose given to young subjects during the daytime, or to older insomniacs at night, can, on average, produce normal nocturnal plasma melatonin levels, some individuals may need a little more, or a lot less, melatonin to attain this effect. The pharmacokinetic properties of any oral dosage of melatonin can also vary depending on the lipid solubility of the inert ingredients that accompany it. A preparation containing corn oil plus 0.05 mg melatonin elevated plasma melatonin levels to as high a peak [from 4 to 118 pg/mL (30), though for a shorter period, as one containing 0.3 mg melatonin plus microcrystalline cellulose (15–105 pg/mL (28))].

EFFECTS OF MELATONIN

Because melatonin is available as a dietary supplement and is relatively nontoxic, physicians, researchers, and even consumers are able to administer or consume doses that elevate its plasma levels to hundreds or even thousands of times those ever occurring normally (26,31). Indeed, even the 1 to 10 mg doses most commonly marketed raise these levels to 3 to 60 times their normal peaks (Fig. 3) (28). Not surprisingly, such concentrations produce biological effects, for example, sleepiness, which might or might not also occur physiologically. Does the demonstration that a pharmacologic dose of melatonin produces such an effect indicate that the effect also occurs at normal nighttime plasma melatonin levels? Or, by extension, that a deficiency in melatonin (e.g., in older people) can contribute to a related disease process? Alas, no; enormous melatonin concentrations inhibit the aggregation of A-beta peptides to form amyloid *in vitro* (32); however, this no more means that the age-related decline in plasma melatonin causes Alzheimer’s disease than that poison ivy dermatitis—which can be treated with cortisone—is a sign of adrenocortical insufficiency.

What evidence must be added before one can propose that some effect of a melatonin megadose also occurs in response to secreted melatonin? First, that the effect occurs when plasma melatonin levels rise or fall within their normal range. Second, that administering melatonin in the daytime, in doses that increase plasma melatonin concentrations to—but not beyond—peak nighttime levels, also produces the effect. This type of study can sometimes be done *in vitro*. If melatonin were found to suppress β -amyloid aggregation at concentrations found nocturnally in plasmas of young people (up to approximately 1 nM), but not in concentrations more typical of many older people (less than 0.3 nM), this would indeed be suggestive.

By using these criteria, two probable physiologic effects have been associated with melatonin administration—the promotion of sleep onset and maintenance (28), and the phase-shifting of circadian rhythms, including the rhythm in melatonin itself (33). Both are produced by physiologic doses, that is, 0.1 to 0.3 mg for sleep and 0.5 mg for phase-shifting. Melatonin's actions on sleep include both a *direct* action (which decreases sleep latency, increases sleep efficiency, and increases total sleep time) and an *indirect* effect on the daily rhythm in the phasing of sleep onset.

Sleep

A 1997 review (34) on melatonin's hypnotic effects listed 24 papers, almost all of which described sedation, fatigue, decreased alertness, increased reaction time, shortened sleep latency (i.e., number of minutes needed to fall asleep), increased sleep efficiency (i.e., percentage of the total sleep period actually spent sleeping), and/or increased total sleep time. A recent (2005) meta-analysis (35) of all the 17 studies (36–43), involving 284 subjects, which satisfied inclusion criteria demonstrated a significant decrease in sleep latency and significant increases in sleep efficiency and total sleep duration. The inclusion criteria were that a study include at least six subjects, all adults, be randomized and double-blinded, involve placebo-controlled clinical trials, and use objective measures of sleep evaluation. Studies could use cross-over or parallel group designs; however, case reports were excluded. Statistical significance was obtained in spite of considerable variations among the studies in melatonin doses and routes of administration, the general health of the subjects, and the measures used to evaluate sleep.

The effects of exogenous melatonin on sleep have been examined under three types of experimental conditions in relation to the onset or offset of endogenous melatonin secretion.

In some studies, the hormone was administered during the daily light period, such that blood melatonin levels would be transiently elevated but would then return to baseline before the initiation of nocturnal melatonin secretion. Such experiments were used to demonstrate that melatonin decreases sleep latency at any time in the afternoon or evening, and that this effect is independent of an action on sleep rhythms (as no treatment can immediately shift the phase of a circadian rhythm by 8–10 hour).

In others, the hormone was given close enough to the onset of darkness for blood melatonin levels to still be elevated when nocturnal melatonin secretion started.

The period during which plasma melatonin levels were continuously elevated would thus be prolonged. Such experiments reflected the use of melatonin to decrease sleep latency and maintain continuous sleep in, for example, a shift worker or eastbound world traveler who needed to start sleeping earlier.

In yet others, the hormone was given at the end of the light period to older insomniacs with low nighttime plasma melatonin levels. The intent was to prolong the portion of the night during which their plasma melatonin concentrations would be in the same range as those of noninsomniac young adults.

In all these situations, oral melatonin decreased sleep latency and, when tested, increased sleep duration and sleep efficiency. A 0.3-mg dose was either as effective as, or more effective than (44), higher doses, particularly when the hormone was administered for several days. This dose had no effect on body temperature, affirming that, although pharmacologic doses can cause hypothermia, melatonin's ability to promote sleep is not mediated by such a change, as had been suggested. The hormone had no consistent effect on sleep architecture (e.g., REM time). Its effects differed from those of most hypnotic drugs, as after receiving melatonin, subjects could readily keep from falling asleep if they so chose and their cognitive abilities the next morning were unchanged or improved.

In a relatively large ($N = 30$) study (44) on people who were 50 years old or older and did or did not suffer from clinically significant insomnia (i.e., sleep efficiencies of 70–80% in the insomniacs vs. 92% in controls), melatonin was found to produce statistically and clinically significant improvements in sleep efficiency among insomniacs (Fig. 4). A 0.3-mg dose caused the greatest effect ($P < 0.0001$), particularly during the middle portion of the nocturnal sleep period (Fig. 5). No effects were noted in subjects without insomnia, or in latency to sleep onset (which is not abnormal in this population). Dose-related increases in plasma melatonin levels were observed (Fig. 6), the 0.3-mg dose causing peak levels in the range usually observed nocturnally among young adults. When subjects received a higher dose (3.0 mg) but not 0.3 mg, plasma melatonin levels remained significantly elevated during much of the following day, and the subjects exhibited hypothermia (Fig. 7).

Circadian Rhythms: Phase-Shifting and Jet Lag

The ability of exogenous melatonin to synchronize and shift the phases of various human circadian rhythms is generally accepted. As little as 0.5 mg of pure melatonin (33), or 0.05 mg of melatonin in corn oil (30) (which causes earlier peaks in plasma melatonin levels), advanced the onset of nocturnal melatonin secretion when administered at 5 p.m., (30) and larger doses caused greater phase advances. [The hormone was also able to shift the core body temperature rhythm. However, a statistically significant effect was found only after a dose that elevated plasma melatonin levels well beyond their normal range, i.e., to 1327 pg/mL (30).] As previously described, melatonin can also control the timing of sleep and sleepiness rhythms—an effect readily demonstrated among blind people with free-running melatonin and sleep rhythms (45) but also among sighted individuals.

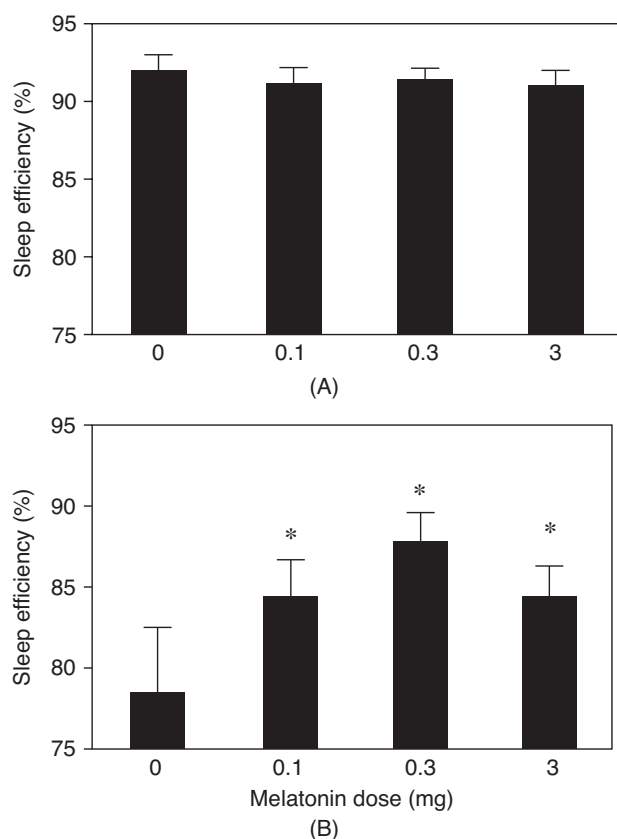


Figure 4 Sleep efficiency in subjects with normal sleep (A) and age-related insomnia (B) following melatonin or placebo treatment. * $P < 0.05$. Source: From Ref. 44.

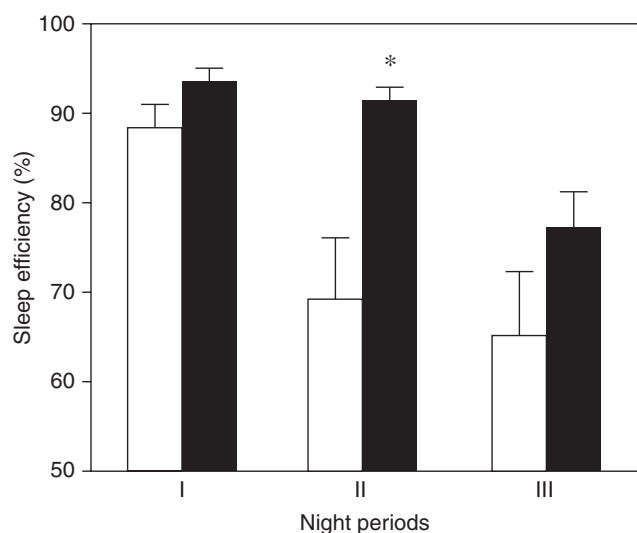


Figure 5 Sleep efficiency in insomniacs during three consecutive parts (I, II, and III) of the night, following placebo (light bar) or melatonin (0.3 mg, dark bar) treatment. * $P < 0.05$. Source: From Ref. 44.

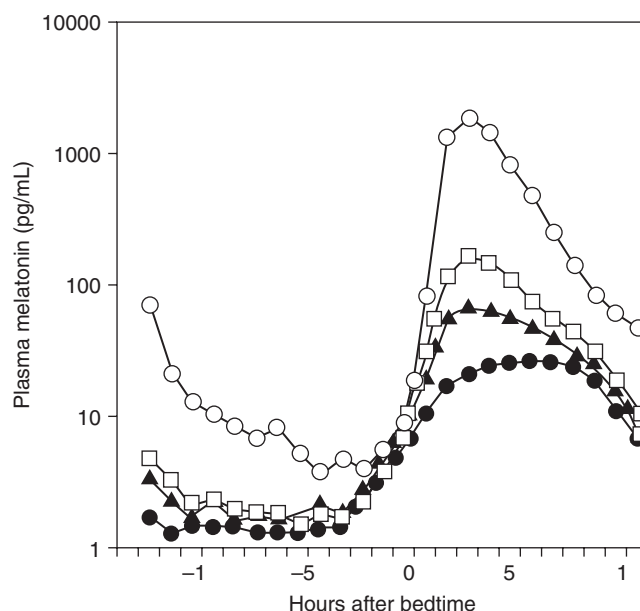


Figure 6 Mean group ($n = 30$) plasma melatonin profiles after melatonin or placebo treatment 30 minutes before bedtime. Circles, placebo; triangles, 0.1 mg; squares, 0.3 mg; diamonds, 3 mg. Source: From Ref. 44.

Melatonin's ability to phase-shift circadian rhythms underlies its common use to prevent or treat "jet lag"—particularly that which is associated with eastbound travel (possibly because the melatonin can be taken while the traveler is still awake). A 1999 review (46) cited nine placebo-controlled field studies on this use; in seven, subjective measures of sleep and alertness improved. Adequate data are not available on the relationship between the ability of a particular melatonin dose to treat jet lag

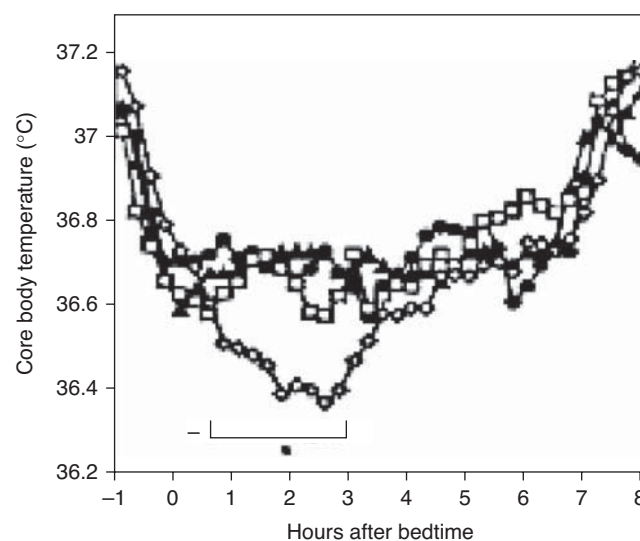


Figure 7 Core body temperature profiles following melatonin or placebo treatment. Circles, placebo; triangles, 0.1 mg; squares, 0.3 mg; diamonds, 3 mg. * $P < 0.05$. Source: From Ref. 44.

and to raise plasma melatonin levels. Some investigators recommend taking the melatonin at a specific time (e.g., at 2 a.m. in the traveler's new geographic environment); others simply propose "... a ... pre-flight early evening treatment before an eastbound flight, followed by treatment at bedtime for four days after arrival. ..." (46). Westbound, the traveler is advised to take the melatonin late in the evening, to sustain nocturnal plasma melatonin levels for as long into the night as possible.

Reproduction

Melatonin affects reproductive performance in a wide variety of species. In 1963, one of us (RJW) first reported that exogenous administration of melatonin reduces the weight of the ovaries of female rats (47). Since then, abundant evidence has been adduced that the pineal gland, acting via melatonin, affects reproductive performance in a broad range of animals (48). There is mounting evidence that the pattern of melatonin secretion, coupled to photoperiod, directly affects reproductive function. The major physiologic role of melatonin is to encode the daily light-dark cycle. The onset and offset of pineal melatonin secretion synchronize to dusk and dawn, respectively, and therefore the duration of the melatonin signal varies in proportion to the length of the night. In seasonal mammals, this variation in melatonin signal duration is used to synchronize neuroendocrine rhythms with the annual variation in day-length. In addition, fetal and newborn animals use the maternal melatonin signal to entrain endogenous circadian rhythms before direct photic information is available. The efficacy of exogenous melatonin in modifying particular reproductive functions has been found to vary markedly among species, depending on the age of the animal, the time at which melatonin is administered relative to the prevailing light-dark cycle, or phase of the estrus cycle (49).

In most, but not all, animals, melatonin has an antigonadotrophic effect. Species that exhibit major seasonal shifts in gonadal function also tend to exhibit the greatest responses to exogenous melatonin. Seasonal changes in the number of hours per day that melatonin is secreted mediate the temporal coupling of reproductive activity to seasonal changes in day-length. In animals with short life cycles or a duration of gestation and time to weaning of approximately 1 year, circadian rhythms can provide a sense of time of the year. Thus day-to-day changes in the time of the re-entraining stimulus of light may be associated with the tightly controlled seasonal onset of puberty and adult infertility/fertility cycles. For example, the hamster reproductive system is inhibited by short photoperiod, leading to testicular regression in males and to anestrus in females (48).

In rodents, puberty onset is altered by day-length so that long durations of darkness inhibit sexual maturation. The potent inhibition of GnRH-induced calcium signaling and gonadotropin secretion by melatonin provides an effective mechanism to protect premature initiation of pubertal changes that are dependent on plasma gonadotropin levels. The impact of short day-length is obvious in animals such as hamsters, which, outside the laboratory setting, live in higher latitudes and/or environments where food availability is highly seasonal. In

longer-lived species such as sheep, the time of puberty of animals born late in the season may even be delayed until the following year (48).

These observations stimulated a search for a role for the pineal gland and melatonin in **human reproduction**. The clinical experience has yielded inconclusive and sometimes conflicting results (50). The following is a brief review of currently available information about the effects of melatonin on human reproductive processes (e.g., puberty, ovulation, fertility).

Although humans are obviously not a seasonally breeding species, a seasonal distribution in human natural conception and birth rates has been reported by epidemiological studies in several geographical areas (51). Suppressed pituitary-ovarian activity (52) and reduction in the conception rate have been reported to occur during the dark winter near the Arctic Circle (53). These observations and the data from studies in mammals stimulated reproductive physiologists to search for a role for the pineal gland and melatonin in human sexual maturation (i.e. puberty) and reproduction (54,55).

Puberty results from withdrawal of the "gonadostat" mechanisms and from increased gonadotropin sensitivity to GnRH. It has been hypothesized that GnRH release may be modulated by a nonsteroid-mediated mechanism. Modifications of neuropeptides, neurotransmitters, and neurosteroids may underlie the onset of pubertal processes (56). Melatonin may be an important factor in the complex process that occurs in the awakening of hypothalamic-pituitary-ovarian axis. The origin of the hypothesis that the human pineal affects puberty dates back to 1898 when Heubner (57) described a 4.5-year-old boy who exhibited both precocious puberty and a nonparenchymal tumor that destroyed the pineal. Many other similar cases were later described, mostly in boys (58). There are data showing an association between endogenous melatonin levels and the onset of puberty. It was hypothesized that melatonin has an antigonadotrophic effect on sexual maturation and that the timing of the onset of puberty is related to the observed statistical reduction in circulating melatonin concentrations with pubescence (59). It remains to be clarified, using longitudinal studies, whether plasma melatonin levels do, in fact, progressively decline in individuals undergoing puberty.

Early studies reported conflicting observations on circulating melatonin levels during the normal menstrual cycle. More recent studies (60,61) failed to detect any fluctuations in melatonin secretion throughout the menstrual cycle. In fact, a remarkable consistency of the circadian pattern of melatonin secretion was observed, independent of the changes in plasma estrogen and progesterone levels. No significant effect on melatonin's rhythm was noted in response to varying endogenous (ovarian stimulation (62) or exogenous [oral contraceptives (63)] sex steroids. These observations suggest that in humans, unlike some other species (64), melatonin secretion is not significantly modulated by sex hormones.

The lack of effect of sex steroids on human melatonin secretion does not necessarily rule out a role for melatonin in human reproduction. In female rats, for example, which like humans are not seasonal breeders, large doses of exogenous melatonin completely inhibited ovulation and prevented the LH surge when administered during

the critical period of proestrus. There are indications that abnormally elevated endogenous melatonin levels and pharmacologic doses of melatonin have antigonadal effects in humans as well. Increased plasma concentrations of melatonin were repeatedly found in women suffering of functional ("hypothalamic") amenorrhea (60,65,66). Similar results were also reported for hypogonadotrophic men (67) and melatonin administration apparently alters semen quality in healthy men (68). Pharmacologic doses of exogenous melatonin, given to healthy young women (daily oral 300 mg for 4 months) altered ovarian activity and partially inhibited ovulation (69). A synergistic inhibitory effect on ovulation by a melatonin-progestin combination was reported by the same group.

Observations of elevated melatonin levels in both men and women with hypogonadism and/or infertility are consistent with a hypothesis that melatonin is antigonadal in humans. The significant increase in circulating melatonin levels in women with functional amenorrhea raised the possibility of a causal relationship between high melatonin concentrations and suppressed hypothalamic-pituitary-gonadal axis in humans. Acute elevations of melatonin occur in response to fasting and sustained exercise (70). Both of these events, if prolonged, may cause amenorrhea. However, the hypersecretion of melatonin may merely be coincidental and reflect adrenergic, dopaminergic, or opiodergic secretions that are characteristic of these conditions. Another possible explanation lies in the fact that melatonin stimulates the production of prolactin in humans (71). Hyperprolactinemia in turn is known to interfere with the ovulatory process in humans.

It has also been suggested that melatonin could exert an effect on human reproduction by directly modulating ovarian function (72) and spermatogenesis (68). Substantial amounts of melatonin are present in the ovarian follicular fluids of both stimulated (73) and spontaneous (74) menstrual cycles. The follicular concentrations of the hormone markedly exceeded those of serum samples obtained concurrently. Receptors to melatonin are highly concentrated in the human ovary (75) and have been observed on human granulosa cell membranes (76). Physiologic nighttime concentrations of melatonin-stimulated progesterone synthesis by human granulosa lutein cells in vitro (77) and increased the stimulatory effect of hCG on progesterone production by these cells (78). All these findings strongly suggest that melatonin may play a role in the intraovarian regulation of steroidogenesis and thus abnormally high concentrations of the hormone might interfere with normal ovarian function. Pharmacologic high doses of melatonin have been tried as a contraceptive agent but the preliminary efficacy results did not justify further development (69).

In conclusion, the data presented earlier demonstrate that the antigonadal effects of melatonin in humans are apparently much less significant than in some seasonally breeding mammalian species. This is not surprising as humans are clearly not "seasonally breeding" species. Currently, the balance of evidence from clinical studies suggests that the effect of melatonin on human reproductive processes such as ovulation and fertility is attenuated. Its role in the timing of the onset of puberty is substantial but it is difficult to differentiate its effect from the

complex interplay among neuropeptides, neurotransmitters, and neurosteroids, which occurs in the awakening of hypothalamic-pituitary-ovarian axis.

The genes that drive the circadian rhythm are emerging as central players in gene regulation throughout the organism, particularly for cell-cycle regulatory genes and the genes of apoptosis. The biological adaptation of humans probably includes development of some degree of photorefractoriness, which is correlated with a change in circadian expression of clock genes in the SCN (circadian pacemaker) and the pars tuberalis (PT, a melatonin target tissue) (79).

Melatonin may have some modulatory effects on human diseases that are related to the reproductive system. For example, lighting during the night of sufficient intensity apparently reduces circulating melatonin levels and resets the circadian pacemaker of the suprachiasmatic nuclei. This phenomenon has been related to increased risk of breast cancer (80) (perhaps by downregulating gonadal synthesis of steroids, by acting on receptor sites within the neuroendocrine reproductive axis, or by altering estrogen receptor function). Therefore, in the right circumstances, it is possible that by reinforcing and optimizing our temporal organization, melatonin may have substantial benefits for reproductive health.

Other Reported Effects

It has been suggested (81) that melatonin is a potent antioxidant, and that supplements of the hormone may protect against such age-related diseases as atherosclerosis, cancer, and Alzheimer's disease. None of these proposed uses has been tested in a controlled clinical trial and all remain controversial because of lack of confirmation, the enormousness of the melatonin concentrations or doses needed to produce the effect, the failure of the investigators to provide data on actual blood or tissue melatonin concentrations after treatment, and the lack of studies comparing melatonin's effects with those of known antioxidants such as vitamins C or E (31,82). It has usually been possible to demonstrate antioxidant or free radical scavenger effects in vitro; however, these have generally required melatonin concentrations 1000 to 100,000 times those ever occurring in vivo (31). Similarly, although high doses of melatonin (10–450 mg/kg body weight parenterally) have sometimes elicited antioxidant effects in experimental animals in vivo, neither their long-term safety nor their effects on the animals' blood melatonin levels have been characterized. In humans—if not in nocturnally active laboratory rodents—such megadoses might ultimately impair sleep or various circadian rhythms, perhaps by downregulating melatonin receptors.

Only one study (31) has described careful dose-response studies on the ability of melatonin to protect against autoxidation and compared melatonin, with known antioxidants. That study, by Duell et al. (31), examined the cell-mediated (by human macrophages) and cell-free (by copper sulfate) oxidation of low-density lipoproteins (LDL), a process believed to contribute to atherosclerosis. Melatonin did exhibit weak antioxidant activity, but only at 10,000- to 100,000-fold physiologic concentrations. In contrast, a vitamin E preparation

(alpha-tocopherol) was 50- to 100-fold more potent than melatonin and was efficacious at physiologic concentrations. Similarly, vitamin C (ascorbic acid) and tryptophan, melatonin's indolic circulating precursor (Fig. 1) were significantly more potent than melatonin and were active at physiologic concentrations.

Some investigators suggest—on the basis of small studies on laboratory rodents—that melatonin “maintains juvenile conditions” and is a “geroprotector.” There is no evidence that melatonin has any “antiaging” actions in humans.

In several small studies, melatonin was found to reduce blood pressure when given to normotensive men or women in daytime or the early evening, or to patients with essential hypertension. This possible effect should be explored further.

Recent studies of gene loci associated with elevated fasting plasma glucose concentrations, involving samples from more than 50,000 European subjects, report that variants in the gene encoding a melatonin receptor (1B; MTNR1B) are consistently associated with such elevations, and also with an increased risk of type 2 diabetes (83,84,85). As this receptor is known to be transcribed in human islets (25,86) and melatonin can inhibit insulin secretion (26,87), melatonin may have largely unexplored but important effects on metabolic homeostasis.

PRESENT USAGE OF MELATONIN

In the United States, the hormone melatonin is sold, without regulation by the FDA, as a dietary supplement. In most of the rest of the world, it is not sold at all, because it is regulated as a drug and no pharmaceutical company has presented an appropriate regulatory body with a successful new drug application (NDA) for its use. Some countries allow very low doses—less than 100 mg—to be sold without regulatory approval.

Why is melatonin not subject to FDA approval and oversight, whereas other hormones are subject to such regulation? This is a consequence of the way the Dietary Supplement Health and Education Act of 1994 (Public Law 103-147) has been implemented. That act exempts from FDA regulation a product that is “. . . intended to supplement the diet that . . . contains one or more of the following dietary ingredients . . .,” a list that includes “(D) an amino acid” (e.g., tryptophan) and “(F) . . . a metabolite . . . of any ingredient described in clause . . . (D)” (e.g., melatonin). Not exempted are products like L-dopa that have been “. . . approved as a new drug . . .” or “. . . authorized for investigation as a new drug . . .” Thyroxine, estrogens, and testosterone had also been approved as drugs prior to passage of the 1994 Dietary Supplement Act, whereas melatonin had not; thus, melatonin is treated as a dietary supplement, even though there is virtually no “dietary melatonin” for the “dietary supplement” to supplement.

What have been the consequences of melatonin not being regulated by the FDA? Apparently no deaths to date have been reported; if melatonin-related deaths had occurred, the 1994 Act would have allowed the FDA to investigate, and then perhaps to start regulating it. In fact, few serious side effects have been described. A 2001 ar-

ticle described a 35-year search (1966–2000) of reports on melatonin toxicity by using the Medline database. Nine articles were found to describe adverse effects of melatonin; in all cases, the doses administered were in the pharmacologic range (1–36 mg). Individual patients exhibited, autoimmune hepatitis, confusion, optic neuropathy, a psychotic episode, headache, or nystagmus. Four suffered fragmented sleep, four described seizures, and two exhibited skin eruptions. Obviously, no clear pattern of side effects emerges from this review.

In the absence of FDA regulation, companies are able to sell melatonin of uncertain purity, at dosages that are many times those needed for promoting sleep or shifting rhythms, or for restoring normal nocturnal plasma melatonin levels in older people. These dosages can elevate plasma melatonin to levels thousands of times greater than those that ever occur normally, and produce mild but not benign side effects like hypothermia and “hangovers.” Paradoxically, they also may, through receptor downregulation, exacerbate the insomnia that the consumer was trying to treat.

Several synthetic melatonin analogs have recently been approved in the United States and/or the European Union for treating insomnia [ramelteon (88) tasunekteib (89)] or depression [agomelatine (90)]. All require doses substantially higher than sleep-promoting doses of melatonin (44). Moreover, there is no compelling evidence that melatonin itself has antidepressant activity, so the possibility arises that the antidepressant effect of agomelatine results from a different mechanism, not involving melatonin receptors.

CONCLUSIONS

This entry describes melatonin, a hormone that is presently marketed as a dietary supplement. Melatonin is synthesized at night in the human pineal gland and released into the blood and cerebrospinal fluid. It acts on the brains of humans to promote sleep, and also affects the phasing of sleep and various other circadian rhythms. During the day, plasma melatonin levels are low; at night, they rise 10- to 100-fold or more in young adults, but by considerably less in older people—who often may have frequent nocturnal awakenings as a consequence. Very small oral doses of melatonin—approximately 0.3 mg or less—raise daytime plasma melatonin to night-time levels, thus making it easier for people to fall asleep in the afternoon or evening. Such doses can also help older people remain asleep during the night. Melatonin has also occasionally been claimed to confer other medical benefits—for example, preventing such age-related diseases as atherosclerosis, cancer, and Alzheimer's disease. The evidence in support of such claims is sparse.

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Milk Thistle

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INTRODUCTION

Milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae); also *Carduus marianus* L.] (Fig. 1) is a herb widely used in Europe for the treatment of liver and biliary disorders. Although milk thistle is the most commonly used name for the herb, other names include silymarin, holy thistle, St. Mary thistle, Mary thistle, Marian thistle, Mariendistel, and lady's thistle. The plant is indigenous to Europe but can be found in the western and southwestern United States. In ancient times, the leaves of milk thistle were used as part of the European diet. The medicinal properties of the herb reside in its seeds (Fig. 2). The primary active component, silymarin, is a potent antioxidant mixture composed of several related flavonolignans.

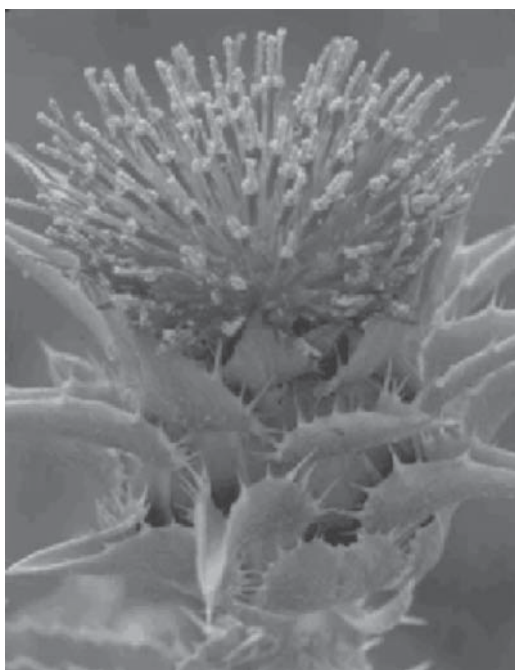


Figure 1 Milk thistle (*Silybum marianum*). Herbal supplements from this plant are most commonly made from organic extracts of the fruits/seeds present at the base of the spiny tuft.

HISTORY

Use in Historical Times

Milk thistle has been used medicinally for over 2000 years, primarily as a treatment for liver dysfunction. In ancient Greece, Dioscorides recommended the herb as a treatment for serpent bites (1). Subsequently, Pliny the Elder (A.D. 23–79) prescribed milk thistle for “carrying off bile” (1,2). In the Middle Ages, Culpepper reported it to be effective for relieving obstructions of the liver (1,2). In 1898, the eclectic physicians Felter and Lloyd stated that the herb was good for congestion of the liver, spleen, and kidneys (1,2).

Current Use

Native Americans use milk thistle to treat boils and other skin diseases. Homeopathic practitioners use preparations from the seeds in the treatment of jaundice, gallstones, peritonitis, hemorrhage, bronchitis, and varicose veins (1). Currently, the German Commission E recommends



Figure 2 Milk thistle seeds. The seeds of the thistle are relatively large (5–6 mm in length) and, when hulled, should contain 1% to 2% (w/w) silybins. The most common crude extract, silymarin, is off-white to yellow powder composed primarily of seven flavonolignans and the flavonoid taxifolin (dihydroquercetin). Selective extraction of silymarin yields silibinin, once thought to be a pure compound but now known to be a mixture of two silybins (see Table 1 for comparison).

its use for dyspeptic complaints, toxin-induced liver damage, and hepatic cirrhosis, and as a supportive therapy for chronic inflammatory liver conditions (3).

CHEMISTRY

Although the chemical composition of milk thistle seed extracts has been studied since the 1950s, a precise nomenclature for the biologically active constituents has been evasive until very recently. Historically, the terms “silymarin” and “silibinin,” or “silybinin,” have been used interchangeably (4) to denote the content of standardized milk thistle extracts. However, none of these terms refers to a single pure compound (Table 1).

Many of the primary active compounds in milk thistle extracts are classified as flavonolignans, each derived from the biosynthetic condensation of taxifolin, a flavonoid, and coniferyl alcohol, a precursor of lignins and lignans. The terms *silymarin*, *silymarin group* and *silymarin complex* have all been used to refer to the group of flavonolignans present in organic extracts of dried milk thistle seeds. The primary flavonolignan present in silymarin is silibinin, a 1:1 diastereomeric mixture of silybin A and silybin B (Fig. 3) (6).

Silymarin also contains several other flavonolignans, each with a formula weight of 482. Isosilybin is a 1:1 diastereomeric mixture of isosilybin A and isosilybin B, each of which differs from its corresponding silybin only in the interchange of substituents at the C-10 and C-11 positions (Fig. 3) (6). The other flavonoid compounds found in the seed of milk thistle are shown in Table 1, and these remaining structures have been known for quite some time. The resolution of these eight compounds was

Table 1 Compounds Present in Silymarin and Silibinin

Silibinin ^a	Silymarin ^b	
Silybin A	Silybin A	Silychristin
Silybin B	Silybin B	Isosilychristin
	Isosilybin A	Silydianin
	Isosilybin B	Taxifolin

^aApproximately a 1:1 mixture of silybin A and B.

^bThe major part (65–80%), of silymarin is a variable mixture of these eight compounds; 20% to 35% is accounted for by other polyphenolics and undefined compounds. Some milk thistle extracts are incorrectly labeled as standardized for 65% to 80% silymarin. With the exception of the small percentage of taxifolin, a more accurate term for these extracts would be 65% to 80% silibinin equivalents, because the other seven compounds share the same chemical formula. Milk thistle products, especially those used in clinical trials, should be analyzed for the composition of each of these compounds and manufacturers should be encouraged to provide these data to consumers for each lot (5).

accomplished in 2003 (6), and the stereochemical assignment of the silybins and isosilybins was confirmed thereafter (Fig. 4) (7).

The relative biological importance of each of these flavonolignans likely depends on the therapeutic indication. For example, in preclinical studies of human prostate cancer, silibinin is comparable in its growth-inhibitory and antitumor effects to silymarin (8). In contrast, silymarin is eightfold more potent than silibinin in scavenging free radicals in vitro (9). Consistent with this observation, silydianin and silychristin (present only in silymarin) are 2- to 10-fold more potent than silibinin (9). Therefore, future biological studies will be aided by the recent advances in milk thistle flavonolignan chemistry in determining whether different product formulations are better suited for specific indications.

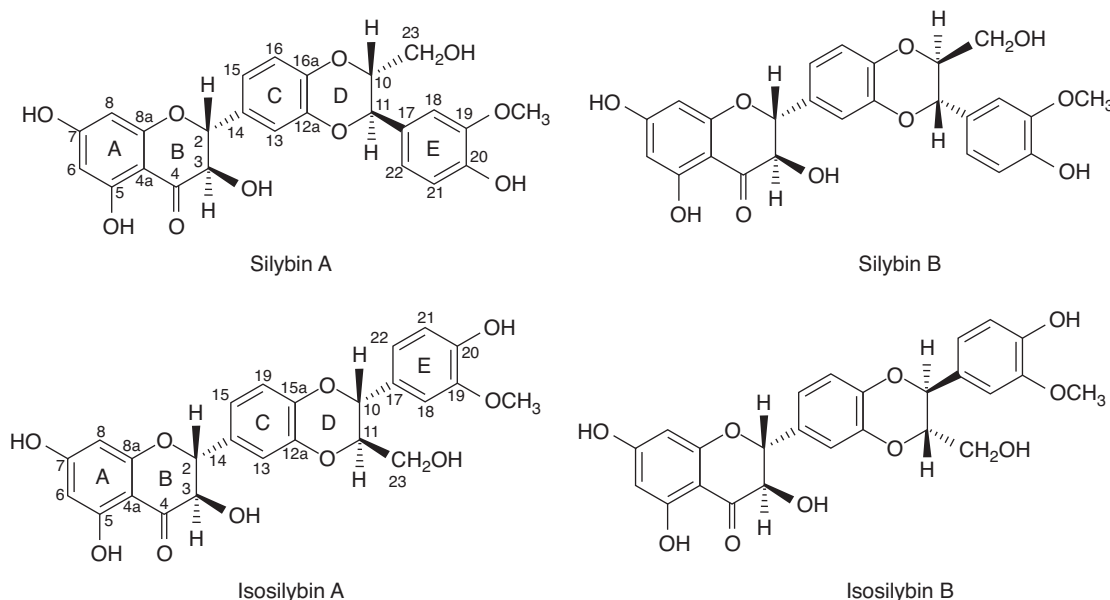


Figure 3 Structures of silybins and isosilybins. Silibinin is a 1:1 diastereomeric mixture of the two enantiomers silybin A and silybin B but contains no other flavonolignans. In contrast, silymarin is composed of the two silybins as well as varying concentrations of isosilybins A and B and other compounds. Source: From Ref. 6.

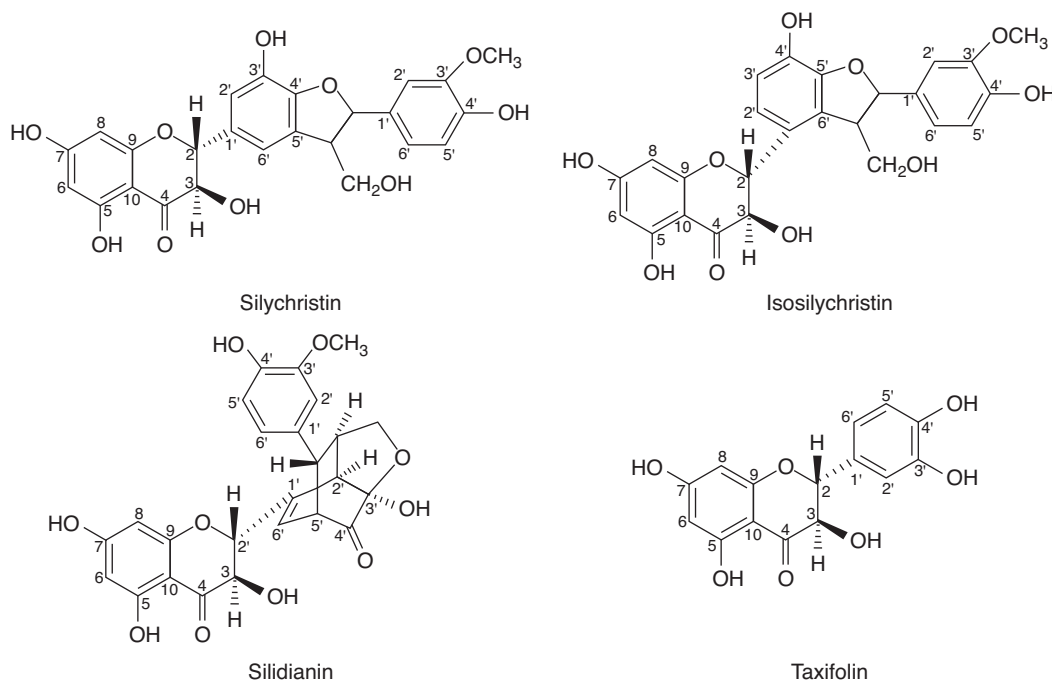


Figure 4 Structures of silychristin, isosilychristin, silydianin, and taxifolin. The three flavonolignans and the flavonoid, taxifolin, are present only in silymarin. Silychristin, isosilychristin, and silidianin share the same chemical formula and formula weight (482) as the silybins and isosilybins shown in Figure 3. Despite this similarity, silidianin is the most potent free-radical scavenger of the class. Source: From Ref. 6.

ABSORPTION AND TRANSPORT

Silymarin is not water soluble; therefore it is used primarily in capsule rather than in tea (aqueous decoction) form. To facilitate absorption, silibinin bound to a phosphatidylcholine complex is used in most human trials. The bioavailability of this complex is on average 4.6 times higher than that of free silibinin (10), and in patients post cholecystectomy, biliary bioavailability of silibinin-phosphatidylcholine was 4.2-fold greater than that of a similar dose of silymarin (11). Studies suggest that silibinin is absorbed directly by the portal pathway from the intestinal tract. It then undergoes extensive metabolism, as evidenced by the presence of sulfate and/or glucuronide conjugates in the blood (10,12). Once in the liver, silibinin may be packaged into lipid micelles and transported to extrahepatic tissues, a hypothesis supported by the observation of radiolabeled silibinin in micelles in increasing quantities according to their lipophilicity, with the highest concentrations in triglycerides and very-low-density lipoproteins (VLDL). Approximately 80% of silibinin is excreted in bile, with only 3% excreted in urine (13).

Pharmacokinetic studies in humans have found absorption to be rapid, with peak plasma levels occurring within 2 hours after a single dose and within 1 hour after multiple doses (10,12). The pharmacokinetics of single dose and multiple doses exhibit superimposable curves, although secondary peaks have been observed in multiple-dose studies (10,12). This may be due to extensive enterohepatic cycling (13). Following a sin-

gle dose of silibinin-phosphatidylcholine (80 mg silibinin equivalents), conjugated silybins are observed at 2.5-fold greater concentrations than free silybins, with a mean residence time of nearly 7 hours (14). Pharmacokinetic studies in humans have shown that steady-state levels are achieved within 4 days of dosing with the complex (10,12). The median peak plasma level of unconjugated flavonolignans (usually expressed as silibinin equivalents, with molar values relative to flavonolignan, which has a formula weight of 482.1) found in multiple human studies is 185 ng/mL, or 0.38 μ M (range 67–3787 ng/mL; 0.14–7.8 μ M), with high interindividual variability (10,12,15). However, the interpretation of existing pharmacokinetic studies is hampered by varying dosing regimens with numerous products, and only one study has attempted to examine the differential pharmacokinetics of individual flavonolignan isomers (16). Adverse effects have not correlated with higher plasma levels of free or free and conjugated silibinin (17). This is possibly due to its short half-life (approximately 2–6 hours). Human studies have found similar pharmacokinetic patterns in studies of patients with cirrhosis of the liver (18,19).

Studies have found that the silibinin-phosphatidylcholine form and silymarin are more concentrated in the bile relative to plasma. In nine cholecystectomy patients administered a single oral dose of either silibinin-phosphatidylcholine or silymarin (120 mg silibinin equivalents), peak biliary concentrations reached 116 and 29 μ g/mL, or 240 and 60 μ M, respectively (11). Mean residence time for both preparations was in excess of 10 hours (11).

ACTION AND PHARMACOLOGY

Milk Thistle as an Antioxidant

Silibinin demonstrates potent antioxidant effects in vitro and in vivo. As with other flavonolignans, this mixture has been found to be a free-radical scavenger, to mildly chelate metals, and to inhibit lipid peroxidation (20–23). Silibinin is an effective scavenger of OH and HOCl species, but has not exhibited any affinity toward H_2O_2 and O_2^- radicals (24). It inhibits lipid peroxidation induced by ADP/Fe_2^+ (20), Fe^{111} (23), *tert*-butyl hydroperoxide (25), and phenylhydrazine (26), as measured by effects on malondialdehyde (MDA) production. Silymarin has also been found to be effective in decreasing lipid peroxidation in human platelets in a dose-dependent manner (27). Despite their common chemical formulas, the flavonolignan isomers vary substantially in their in vitro potency for sequestering 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (9). Silydianin and silychristin are 2- to 10-fold more potent than the silybins or isosilybins, and this may account for the eightfold greater potency of silymarin compared with the silybins (9).

In vitro data suggest that silibinin affects enzymes involved with phase II detoxification through its effects on intracellular glutathione concentrations and glutathione-S-transferase enzymes. It stimulates enzyme transcription and the activity of glutathione-S-transferase enzymes and has been found to prevent a decrease in glutathione in the liver cells of rats treated with acetaminophen (28–30). This mechanism is agent specific, as treatment with buthionine sulfoximine, a glutathione-depleting agent, failed to prevent the depletion of intracellular glutathione in rat hepatocytes (21).

In addition, pretreatment with silymarin [Legalon® (10 $\mu\text{g}/\text{mL}$, 0.1 mg/mL , and 15 mg/mL)] had no effect on MDA production or glutathione or glutathione peroxidase activity in human erythrocytes exposed to doxorubicin or acetaminophen (31). The significance of these antioxidant effects in the intact organism is uncertain.

Effects on Liver Cells

By acting on protein synthesis, silymarin may accelerate liver regeneration and production of hepatocytes through its actions on DNA-dependent RNA polymerase I and 5.8S, 18S, and 28S ribosomal RNA (32,33). These hypotheses were later supported in one clinical trial that found improvements in liver histology in patients with acute and subacute liver disease (34). Silymarin has also been found to protect the liver from toxic substances, presumably through alteration of hepatocyte membrane permeability. In vitro studies demonstrate that silymarin exerts a protective effect in hepatocytes exposed to *tert*-butyl hydroperoxide, phenylhydrazine, acetaminophen, and carbon tetrachloride (23,25,26,35). This protective effect has been supported by human case reports as well (36). Silymarin may also affect phospholipid metabolism in the liver. Silibinin has been shown to inhibit alcohol-induced phospholipid synthesis (37), by decreasing the rate of glycerol incorporation into phospholipids (38). Investigations in rat liver Kupffer cells indicate that it inhibits leukotriene B_4 formation with an IC_{50} of 15 μM . This significant inhibition is also observed with as little as 5 μM , a concentration achievable in the liver (32). The relevance of this

effect in the prevention of fibrotic liver disease continues to be studied.

Silymarin has been found to inhibit signals that promote fibrosis of the liver tumor necrosis factor (TNF)- α and nuclear factor (NF)- κB involved in the development of cirrhosis (39,40). Silibinin inhibited intrahepatic activation of NF- κB and inhibited intrahepatic expression of TNF, interferon- γ , interleukin (IL)-4, IL-2, and inducible nitric oxide synthase (iNOS) in mice at doses of 25 mg/kg (41). IL-2 and -4 were expressed in mice fed 10 mg/kg of silymarin, but significant increases were not observed at doses of 50 or 250 mg/kg . Expression of TNF- α and proinflammatory cytokines (1 L-6, IL-1 β , iNOS) was stimulated in mice treated with 50 and 250 mg/kg of silymarin. The antioxidant effects of silymarin may also account for its ability to prevent or slow the progression of liver disease.

Antilipidemic Effects

Silymarin may be an effective hypocholesterolemic drug (Table 2) (42). Preliminary research suggests that silymarin on its own or in combination with other polyphenolic compounds found in milk thistle may inhibit absorption of lipids from the gastrointestinal tract, decrease synthesis of lipids in the liver, inhibit enzymes involved in lipid neogenesis, and prevent oxidation of low-density lipoprotein (LDL) vesicles.

Silibinin and silibinin-phosphatidylcholine complex do not prevent the accumulation of cholesterol in the liver (46,47). However, silymarin in combination with other milk thistle flavonoids decreased cholesterol absorption from the small intestine in rats fed a high-fat diet, through an action similar to bile-acid sequestrants. Taxifolin, a flavonoid compound found in milk thistle, has also been found to inhibit cholesterol absorption (48). This suggests that other components of the herb besides silibinin are responsible for its anticholesterolemic effects.

Silymarin inhibits key enzymes involved in cholesterol biosynthesis. Silibinin inhibits 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity in a dose-dependent manner in cell lines, but this has not been observed in rat liver microsomes (44). Silymarin decreases the concentration of cholesterol in VLDL (42,47,48), but its effect on plasma cholesterol levels is uncertain. An inverse relationship between plasma cholesterol levels and silymarin was seen in one study (46), but another study has not found an association (48,49).

The effects of silymarin on plasma triglycerides, VLDL, LDL, and high-density lipoproteins (HDL) has been evaluated. It lowers plasma VLDL (46–48) but has no effect on plasma LDL (46,48). Silymarin and silibinin increase HDL (46,48). The mechanisms through which silymarin may exert these effects is unknown, but its antioxidant properties may be responsible for inhibition of LDL oxidation (47).

Anticancer Effects

The effects of silymarin and silibinin have been investigated in various cancer models (Table 3).

The two mixtures have been evaluated for their ability to exert direct cytotoxic effects, mitigate the toxicity of certain anticancer agents, and enhance the efficacy of chemotherapeutic agents. These effects have been most

Table 2 Summary of Laboratory Studies: Antilipidemic Effects

Reference	Model	Treatment (concentration)	Results
(43)	Rat liver homogenates	Silibinin (7.5×10^{-6} mol/L)	Inhibition of precursors of cholesterol synthesis
(44)	Rats	Silibinin (100 mg/kg; 50 mg/kg)	↓ Biliary CHO and phospholipid concentration at higher dose only No effect on liver CHO Inhibition of HMG-CoA reductase activity
(45)	Rat liver microsomes	Silymarin and silibinin (100 mg/kg)	↓ Turnover of phospholipids only in vitro (findings not confirmed in vivo)
(37)	Rats	Silibinin (100 mg/kg IV)	Inhibition of EtOH-induced phosphosynthesis
(38)	Rat hepatocytes	Silibinin (1 or 0.1 mM)	↓ Incorporation of glycerol in TG synthesis ↓ TG production in the liver
	Rat liver removed after IP administration (liver homogenates)	Silibinin (7.5×10^{-6} to 7.5×10^{-4} mol/L)	Stimulation of phosphatidylcholine synthesis ↑ Choline phosphate cytidyltransferase
(46)	Hypercholesterolemic rats	Silymarin	↓ Liver and plasma CHO, VLDL, phospholipids ↑ HDL No effect on TG
		Silibinin	No effect
(47)	Rats	Silymarin	↓ Liver and Plasma VLDL ↑ HDL
		IdB 1016	Not effective
(48)	Rats	Silymarin	↓ Liver CHO, TG ↓ Plasma VLDL, TG ↓ Concentration of CHO in VLDL ↑ HDL
	Rabbits	Silymarin-phospholipid complex	No effect on plasma CHO and LDL
		Silymarin alone	↓ CHO in liver homogenates ↓ CHO in liver microsomes No significant effect

Abbreviations: CHO, cholesterol; EtOH, ethanol; HDL, high density lipoprotein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; IP, intraparenteral; IV, intravenous; TG, triglyceride; VLDL, very low density lipoproteins; ↑ increase; ↓ decrease.

extensively investigated in prostate cancer cell lines (DU 145, LNCaP, PC-3) (53–56,70) and a mouse skin cancer model (64,65,72–75). Other in vitro studies have investigated their properties in breast cancer (MDA-MB 468, MCF-7) (8,51,52) hepatic cancer (HepG2) (58,76), epidermoid cancer (A431) (58), colon cancer (Caco-2) (77), ovarian cancer (OVCA 433, A2780) (78), histiocytic lymphoma (U-937) (61), and leukemia (HL-60) (50,79) cells. Silymarin and silibinin have been investigated in animal tumor models of skin (64,65,72–74) tongue cancer (65,71–75) bladder cancer (69), and adenocarcinoma of the colon (62,63) and small intestine (62).

Silymarin and silibinin have activity against prostate cancer. They can inhibit growth factors and cell-to-cell signaling that stimulate cell growth (53,55,56,70), promote cell cycle arrest in G₁ (54,55), and inhibit antiapoptotic activity (57). Silymarin (75 µg/ mL of medium) inhibits epidermal growth factor B1 and subsequent signaling processes leading to growth inhibition of DU 145 cells (54). In LNCaP cells, the G₁ arrest caused by silibinin appears to be mediated by an increase in complex formation between the retinoblastoma gene product, Rb, and members of the E2F transcription factor family. Administration of silibinin in the diet to nude mice significantly lowered tumor volume and wet tumor weight (70). These effects on human prostate cancer xenografts correlated with plasma levels of 14 to 27 µM. Importantly, the investigators monitored food consumption and found that mice ingested 1.8 to 3.5 mg silibinin/day. By using typical allometric scaling to a 70-kg human, this correlates to a daily silibinin dose of 650 to 1300 mg. Although this is higher than

the dose recommended for hepatic protection, the low toxicity of silibinin and silymarin should make it possible to increase the dose to achieve a therapeutically relevant anticancer concentration of the flavonolignans.

Silymarin and silibinin have also been extensively investigated in the SENCAR mouse nonmelanoma skin cancer model (64–67,73–75,80). Silymarin treatment significantly reduced tumor incidence, multiplicity, and volume in cells treated with ultraviolet (UV) B to induce tumor promotion, but not in cells treated with UVB to induce tumor initiation (65). Silymarin (6 mg dose in 0.2 mL of acetone) inhibits TNF-α mRNA in the mouse epidermis, possibly by inhibiting tumor promotion (68).

Silymarin and silibinin have chemopreventive effects in breast (MDA-MB-468) and cervical (A431) cancer cell lines (8). In male F344 rats with azoxymethane-induced colon cancer, supplementation with silymarin (100, 500, and 1000 ppm in the diet) resulted in a reduction of colon tumorigenesis and a decrease in multiplicity of tumor growth (63). Pretreatment of human promyelocytic leukemia (HL-60) cells with silibinin resulted in inhibition of cell growth and differentiation. Silymarin interferes with cell-to-cell signaling in breast cancer cell lines (MDA-MB-468) (52), histiocytic lymphoma (U-937) (61), and hepatoma cell lines (76). It may also have a chemopreventive role in tongue and bladder carcinogenesis (69,71).

Silymarin has also been investigated as a possible adjunctive agent in mitigating some of the toxicity associated with chemotherapeutic agents. Silibinin and silychristin exerted a protective effect on monkey kidney cells and rats exposed to vincristine or cisplatin chemotherapy

Table 3 Summary of Laboratory Studies: Anticancer Effects

Cancer type	Main findings/target of action
Cell lines	
Leukemia (50) (HL-60)	Inhibited cell proliferation; induced cell differentiation
Breast (51,52) (MCF-7, MDA-MB-468)	Inhibited VEGF (25, 50, 100 μ g/mL) Exerted antiangiogenic properties by inhibiting VEGF Inhibited cell growth, cell proliferation, and CDK expression
Prostate (43,45,51, 53–57) (DU145, PC-3, LNCaP)	Inhibited VEGF Inhibits erbB1; induced CDKIs; induced cell cycle arrest Stimulated IGFBP-3 Inhibited intracellular PSA and cell growth Inhibited EGF, erbB1, ERK1/2 Inhibited NF- κ B, p65, p50; Increased I κ B α levels; sensitized cells of TNF α Inhibited Rb phosphorylation; induced G ₁ arrest Exerted antiangiogenic properties by inhibiting VEGF secretion
Hepatic (58) (HepG2)	Inhibited binding and expression of NF- κ B Decreased APAP-induced toxicity
Other	
Testicular (59) (H12.1, 577LM, 1777NR Cl-A)	No interference in antitumor effects in cisplatin- and ifosfamide-treated cells
Gynecologic (60) (A2780, OVCA 433)	Enhanced efficacy of cisplatin; no stimulation of tumor growth by IdB 1016
Histiocytic lymphoma (61) (U-937)	Inhibited transcription of NF- κ B. Inhibited phosphorylation and degradation of I κ B α no effect on AP-1
Animal models	
Colon (62,63) (male F344 rats; Sprague-Dawley rats)	Reduced incidence and multiplicity of chemical-induced colon cancer of (dietary silymarin: 100, 500, 1000 ppm in diet) Decrease frequency of adenocarcinoma (silymarin flavonolignans; 0.10%)
Skin (64–68) (SENCAR mice)	Inhibited activity and mRNA of ODC Inhibited mRNA of TNA α Inhibited tumor promotion; no effect on tumor initiation Inhibited ligand binding of EGFR, cell cycle arrest, DNA synthesis Inhibited lipid peroxidation and proinflammatory cytokines; prevented depletion of antioxidant enzymes Inhibited MAPK/ERK 1/2, stimulated SAPK/JNK 1/2 and p38 MAPK
Other	
Bladder (69) (male ICR mice)	Inhibited initiation and proliferation of tumor cells
Prostate (DU145) (70) (athymic male nude mice)	Inhibited tumor volume; induced IGFBP-3
Tongue (71) (F344 rats)	Inhibited tumor initiation and promotion

Abbreviations: APAP, acetaminophen; CDKI, cyclin-dependent kinase inhibitor; EGF, epidermal growth factor; EGFR and erbB1, epidermal growth factor receptor; IGFBP-3, insulin-like growth factor binding protein 3; I κ B α , inhibitory subunit of NF- κ B; MAPK/ERK1/2, mitogen-activated protein kinase/extracellular signal-regulated protein kinase; NF- κ B, nuclear factor kappa B; ODC, ornithine deoxycarboxylase; PSA, prostate specific antigen; Rb, retinoblastoma; SAPK/JNK1/2, stress-activated protein kinase/jun NH₂-terminal kinase; TNF- α , tumor necrosis factor α ; VEGF, vascular endothelial growth factor.

(59,81,82). In germ cell tumors, silibinin did not interfere with the antineoplastic effects of cisplatin or ifosfamide. It potentiated the cytotoxic effect of cisplatin and doxorubicin in breast cancer and ovarian cell lines (60,78). The flavonolignan mixture may increase the chemosensitivity of DU145 prostate cancer cells resistant to chemotherapy (57). The clinical significance of these investigations in humans as well as the effective dose, timing, and duration of treatment with silymarin in humans with cancer needs further investigation.

INDICATIONS AND USAGE

Liver Disorders

Although research has been conducted in humans with silymarin and a variety of diseases of the liver, its mechanisms of action are largely unknown. Some human studies suggest that silymarin may be more effective in the earlier stages of liver disease (34). This may be explained by its ability to prevent toxins from entering the hepatocyte, thereby preventing initial damage to the cell (Table 4). The studies investigating silymarin in humans with liver disease are described later.

Cirrhosis

Three double-blind, randomized, controlled trials using similar dosing regimens have investigated milk thistle in the treatment of alcohol-induced cirrhosis. The studies have reported mixed results (Table 4). Ferenci et al. followed patients with alcohol- and non-alcohol-induced cirrhosis for 2 years and found increased survival in patients supplemented with 420 mg/day of silymarin. However, no effects on measurements of liver function were found. A later study using a similar dosing regimen and duration found that silymarin had no effect on survival (87). In a smaller study, patients with alcohol-induced cirrhosis were supplemented with 150 mg/day of silymarin. Significant increases in glutathione levels and decreased malondialdehyde levels were observed (88). No significant effects on measurements of liver function tests were found.

Alcoholic or Virus-Induced Hepatitis

Five studies have investigated the effect of silymarin in the treatment or prevention of progression of alcohol- or virus-induced hepatitis (17,83–86). Variations in the form and dosage of silymarin make comparisons difficult. In the only double-blind placebo-controlled trial, 59 subjects were randomly assigned to Legalon or placebo (89). Substantial increases in aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin were noted, but *P* values were not reported.

Subacute or Acute Liver Disease

Two double-blind, randomized, controlled trials and one observational study have investigated silymarin in the treatment of patients with liver disease (34,89,90). In a large observational study (*n* = 2637), subjects with liver disease of various etiologies and severity were supplemented for 8 weeks with a mean Legalon dose of 267 + 103.6 mg. Considerable decreases in ALT, AST, and γ -glutamyl transferase (GGT) and a decrease in the

Table 4 Summary of Human Studies

Reference	Type of study/sample size	Type of disease	Formulation/dosage	Duration of study	Results
<i>Hepatitis</i>					
(17)	Phase II randomized open trial/60	Viral or alcoholic hepatitis	IdB 1016/80–120 mg b.i.d./t.i.d	2 weeks	↓ALT, GGT
(83)	Controlled, randomized trial/52	Viral hepatitis B	Silymarin/210 mg	5 weeks	No significant findings
(84)	Double-blind trial/59	Acute viral hepatitis	Legalon/70 mg	21–28 days	Improvements in bilirubin, AST, ALT, (significance NR)
(85)	DBRCT/45	Chronic hepatitis	Silymarin/	NR	“Recuperation” was faster among patients with “normal” evolution of disease
(86)	DBRCT/77	Acute viral hepatitis	Legalon/420 mg		
<i>Cirrhosis</i>					
	DBRCT/170	Cirrhosis	Silymarin/140 mg t.i.d	2 yr	↑Survival
(87)	DBRCT/200	Alcohol-induced cirrhosis	Silymarin/150 mg t.i.d	2 yr	No significant findings
(88)	DBRCT/60	Alcohol-induced cirrhosis	Silymarin (MZ-80)/150 mg t.i.d	6 mo	Significant increases in erythrocyte glutathione ↓Platelet MDA values No significant differences in liver function tests
<i>Other liver diseases</i>					
(34)	DBRCT/106	Acute and subacute liver disease	Legalon/420 mg	4 weeks	↓LFTs Improved histology
(89)	Observational study/2637	Toxic liver pathology (unspecified)	Silymarin/267 mg (±103 mg)	8 weeks	↓ALT, AST, GGT (significance NR) ↓Number of patients with enlarged liver (significance NR)
(90)	DBRCT/66	Liver damage (unspecified etiology)	NR	28 days	↓AST ($P < 0.1$) ALT ($P < 0.05$), GGT ($P < 0.05$)
<i>Primary biliary cirrhosis</i>					
(91)	Noncontrolled, open trial/27	Primary biliary cirrhosis	Silymarin/140 mg t.i.d	1 yr	No significant findings
<i>Lipid</i>					
(92)	Nonrandomized/14	Hyperlipoproteinemia type II	Legalon/420 mg	3 mo	↓CHO, HDL, Apo AI/AII No effect on TG, CHO

Abbreviations: ALT, alanine aminotransferase; Apo A1, apolipoprotein A1; Apo AII, apolipoprotein AII; AST, aspartate transaminase; CHO, cholesterol; GGT, γ -glutamyl transpeptidase; HDL, high density lipoprotein; LFT, liver function test; MDA, malondialdehyde; NR, not reported; TG, triglycerides; ↑ increase; ↓ decrease.

number of patients with hepatomegaly were noted, but *P* values were not reported. In the two double-blind, placebo-controlled trials, significant decreases in ALT, AST, and GGT were observed.

Primary Biliary Cirrhosis

In a nonrandomized pilot study ($n = 27$), silymarin (140 mg three times daily) was administered to patients with primary biliary cirrhosis nonresponsive to standard medical care (91). No noteworthy changes were observed.

Lipidemia

One study suggests that silymarin may be a potential therapeutic agent in the prevention of atherosclerosis (92). Oxidation of low-density lipoprotein (LDL) particles plays an important role in the development of atherosclerosis. Silymarin may reduce atherosclerosis through its effects on apolipoproteins A-I and A-II. Apolipoproteins reside on the surface of HDL and activate lecithin/cholesterol acyltransferase (LCAT), thereby clearing cholesterol from extrahepatic tissues. Apolipoprotein A-I facilitates uptake of cholesterol into cells. A small trial of 12 weeks' silymarin supplementation in 14 adults with Type-II hyperlipidemia resulted in significant reduction in apolipoprotein A-I levels, thereby showing that silymarin is not beneficial in this condition. Significant decreases in apolipoprotein A-II lev-

els were also observed. However, the role of apolipoprotein A-II in atherosclerosis is not well defined (93). As apolipoprotein A-II is inversely associated with insulin resistance and plasma triglycerides, silymarin may be useful in both atherosclerosis and diabetes. Further study is needed.

Cancer

Silymarin may have a therapeutic role in the treatment of certain malignancies. However, no clinical trials have reported the safety or efficacy of silymarin in combination with cancer treatment. Two case studies have reported the use of silymarin in humans with cancer in combination with cancer therapy. Silymarin was used as an adjunctive treatment in a 34-year-old woman with elevated liver enzyme content undergoing chemotherapy. A daily dose of 800 mg was associated with reductions in AST and ALT and thereby enabled the patient to receive the prescribed chemotherapy (94). Spontaneous regression of hepatocellular carcinoma has been reported in a 52-year-old man taking silymarin (95).

Other

Amanita phalloides is a mushroom that, upon ingestion, causes amatoxin (*a*-amanitin) poisoning. This toxin damages both liver and kidney by irreversibly binding to RNA

polymerase II. This leads to hepatic failure and often results in death. IV administration of silymarin is the only treatment (36). Histochemical investigations in rat hepatocytes demonstrate that silymarin stabilizes cell membranes (96). Silibinin may also inhibit absorption of the toxin from the gut through its extensive enterohepatic cycling (96). The effectiveness of silibinin depends on the dose and timing of administration after amatoxin exposure (96).

Silymarin has been administered to pregnant women with cholestasis without any teratogenic effects on the fetus (97). Milk thistle has also been used as a lactagogue. Herbalists have also used milk thistle for the treatment of psoriasis. No clinical studies have been reported for these indications.

AVAILABLE DOSAGES/FORMS

Capsules and extracts of milk thistle are usually quantified as 65% to 80% silibinin or silibinin equivalents, with the remaining 20% to 35% consisting of less-defined polyphenolic compounds and fatty acids. Most of the clinical trials have used capsules standardized to silibinin content (Table 5).

However, the composition of the capsules can vary. The relatively straightforward selective precipitation of the silybins from milk thistle extracts has led to the widespread marketing of silibinin as the purified active principle component of silymarin. Analysis of a representative lot of Legalon (sold in Germany as Legalon and imported into the United States as Thisilyn[®]) revealed 66.1% flavonolignans in the proportions of 30.1% silybin, 9.1% isosilybin, 14.9% silychristin, and 12% silydianin (4,9). Another preparation, Siliphos[®] or Silipide[®] (1dB 1016), is a silibinin preparation sold by Indena S.p.A. and is a patented mixture of 33% silibinin and 66% soy lecithin (phosphatidylcholine). An IV preparation of milk thistle extract is sold in Europe as silibinin hemisuccinate in aqueous solution to be given for acute *A. phalloides* poisoning (20 mg/kg total silibinin per day given in four 5 mg/kg infusions of 2 hours each). Silibinin (S-0417) as sold by Sigma-Aldrich (St. Louis, MO) is the most commonly used source of milk thistle extract for preclinical

studies and comprises nearly identical proportions of silybin A and silybin B.

An average dose of 200 to 400 mg/day in divided doses has been used in most of the studies investigating silymarin for hepatic disorders and antilipidemic effects. Teas made from the crushed seed are used for mild gastrointestinal disorders (98); however, due to its lipophilic properties, only a small percentage of silymarin is found in aqueous solution (2,98). A list of formulations and suppliers is described in Table 5.

CONTRAINDICATIONS/ADVERSE REACTIONS

Few side effects are reported when silymarin and silibinin are used within the recommended dose ranges (3,98). Rare cases of a mild laxative effect have been described. One human dose escalation study reported nausea, heartburn, and dyspepsia in subjects treated with 160 mg/day, dyspepsia in patients treated with 240 mg/day, and postprandial nausea and meteorism in patients treated with 360 mg/day. None of these side effects were dose related. At doses greater than 1500 mg/day, mild allergic reactions have been reported. Episodes of sweating and gastrointestinal distress have been associated with the use of milk thistle (99). The symptoms resolved upon discontinuation of the supplement, but it is unknown whether these effects were due to milk thistle or contamination of the capsule.

DRUG INTERACTIONS

Interactions between milk thistle (silymarin) and medications or other herbal remedies are largely unknown. Silymarin inhibits CYP3A4 and UDP-glucuronyltransferase UGT1A6/9 in cultured human hepatocytes, and silibinin inhibits CYP2C9 and some activities of CYP3A4 in isolated human liver microsomes (100,101). However, the concentration at which inhibition is observed is high (100–500 μ M in hepatocyte studies) and not achievable with oral intake of silymarin (102). However, the biliary concentrations of flavonolignans can be greater than plasma levels by an order of magnitude or more: Single 120 mg doses of silymarin or silibinin–phosphatidylcholine result in

Table 5 Formulations and Suppliers of Silymarin and Silibinin

Brand name	Formulation ^a	Manufacturer
Legalon MZ-80	One tablet contains 35, 70, or 140 mg of silymarin. Standardized to silymarin content	Madaus A.G., Ostmerheimer Strasse 198, Cologne, Germany
Thisilyn, Thisilyn Pro TM	One capsule contains 175 mg of 80% silymarin (140 mg silymarin)	Manufactured in Germany by Madaus A.G. for US distribution by Nature's Way Products, Inc, Springville, Utah, [also doing business as Murdock Madaus Schwabe Professional Products (MMS Pro), Inc, 10 Mountain Springs Parkway, Springville, Utah 84663]
1dB 1016	One capsule contains 150 mg of a 1:2 ratio of silibinin complexed with soy-derived phosphatidylcholine	Indena S.p.A. Viale Ortles 12, Milan 20139, Italy
Silipide [®] Siliphos [®]		

Note: The regulatory status of herbal medicine varies between countries. For more information on the regulatory status of herbal therapies in selected countries, refer to *Legal Status of Traditional Medicine and Complementary/Alternative Medicine: A Worldwide Review*; WHO/EDM/TRM/2001.2: WHO, Geneva, 2001; 189 p. (ISBN 92-4-154548-8; Swiss Fr. 35).

^aThe information on formulations was supplied by the manufacturers of the product and has not been subject to confirmation by an outside agency.

maximum mean biliary concentrations of 60 and 240 μM , respectively (11). The observation that patients with HIV often use milk thistle to prevent or manage hepatitis or protect the liver from hepatotoxic drugs led to clinical trials investigating the potential for interactions with the HIV protease inhibitor indinavir and CYP3A4. Two independent trials of coadministration of milk thistle and indinavir within the recommended dosages in healthy individuals found no interference with indinavir pharmacokinetics (103,104). These findings are also consistent with the observation that a 2-day exposure of isolated human hepatocytes to 10 μM silymarin has no effect on CYP3A4 gene expression (105).

Theoretically, its antioxidant and free-radical scavenging properties suggest that silymarin may interact with any free-radical-generating medication, such as the anthracycline chemotherapy agent doxorubicin. This has not yet been investigated in human or laboratory studies.

OVERDOSAGE

No reports of overdosage have been documented. Silymarin has been well tolerated in high doses. IV administration of silibinin (20–50 mg/kg body weight) in the treatment of humans with *A. phalloides* poisoning resulted in no adverse effects. No toxicity has been observed in rats and mice when it is given in doses as high as 5000 mg/kg. Rats and dogs have received silymarin at doses of 50 to 2500 mg/kg for a 12-month period. Investigations including postmortem analyses showed no evidence of toxicity. The Merck Index lists no LD_{50} value in any species for silibinin or silymarin.

REGULATORY STATUS

Milk thistle is classified in the United States as a dietary supplement under the Dietary Supplement Health and Education Act (DSHEA) 1994 and cannot be marketed for the treatment of any disease. In Germany, the Commission E approves the internal use of crude milk thistle fruit preparations for dyspeptic complaints. Formulations consisting of an extract of 70% to 80% silymarin are also approved for toxic liver damage and for supportive treatment in chronic inflammatory liver disease and hepatic cirrhosis.

The U.S. Pharmacopoeia and National Formulary (USP-NF) monographs dictate that milk thistle seeds (with pappus removed) used for extraction contain not less than 2% (w/w) silymarin, expressed as silybin, using a USP spectrophotometric assay method and botanical identification confirmed by thin-layer chromatography and macroscopic and microscopic examinations (USP 24-NF 19, 1999). German pharmacopeial-grade milk thistle should also contain not less than 1.5% (*Deutsches Arzneibuch/DAB*, 1997).

CONCLUSIONS

Milk thistle is a herbal plant that has a long history of use in the treatment of a variety of illnesses. Laboratory and

clinical research suggests that silymarin, a complex of active components from milk thistle, may be a possible agent in the prevention or treatment of cancer, atherosclerosis, hepatitis, and cirrhosis. The use of milk thistle for these indications has been further reviewed elsewhere (1–3,97). The low-toxicity profile of silymarin makes it an attractive agent for further studies. Future investigations are needed to determine the effective dose, duration, and formulation so that standardized recommendations can be developed.

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Niacin

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ABBREVIATIONS

ADPR/P, adenosine diphosphate ribose/phosphate; ART, mono-ADP-ribosyltransferase; NAAD/NAADP, nicotinic acid adenine dinucleotide/nicotinic acid adenine dinucleotide phosphate; NE, niacin equivalents; NMNAT1–3/NaMNAT, nicotinamide/nicotinic acid mononucleotide adenosine 5'-triphosphate adenylyltransferase; Nrk, nicotinamide riboside kinase; PARP, poly(adenosine diphosphate ribose) polymerase; PBEF, pre-B; colony-enhancing factor; PRPP, phosphoribosyl pyrophosphate.

INTRODUCTION

Niacin, also designated vitamin B₃, is found mostly in meat, grains, milk, and eggs. In the United States, “niacin” means nicotinic acid, and the amide form, nicotinamide, is called niacinamide. Elsewhere, “niacin” denotes nicotinic acid and/or nicotinamide. Deficiency of this vitamin causes pellagra, a disease characterized by dermatitis, diarrhea, and dementia that is endemic today in parts of India and China, and may result in death in severe cases. As a precursor of pyridine nucleotides [nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP)], niacin participates in the function of numerous enzymatic pathways, which are critical for normal cell metabolism, involving, for example, redox reactions and those that consume NAD. Discovery of the antihyperlipidemic properties of pharmacological doses of nicotinic acid and of the importance of NAD metabolism for the maintenance of genome stability has renewed interest in this vitamin in developed countries.

GENERAL DESCRIPTION

The structures of nicotinic acid, nicotinamide, and nicotinamide riboside are shown in Figure 1. They consist of a pyridine ring substituted in position 3 with a carboxylic group in nicotinic acid and with a carboxamide group in nicotinamide. Niacin was initially studied because of its association with pellagra, a nutritional deficiency disease, symptoms of which are dermatitis, diarrhea, and dementia, with death as the eventual outcome. Pellagra was first documented by Casal as “mal de la rosa” in 1735, but was linked to niacin deficiency only about two centuries later, during an epidemic in the southern United States (reviewed in Ref. 1). The disease was initially thought to be of infectious origin, until Goldberg and Tanner observed

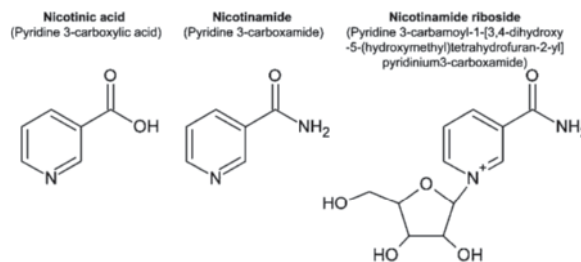


Figure 1 Structure of nicotinic acid, nicotinamide, and nicotinamide riboside.

its association with poor nutrition, inadequate meat and milk intake, and use of corn as the principal constituent of the diet. In 1922, they suggested that pellagra was an amino acid deficiency. Five years later, it was demonstrated that nicotinic acid cured pellagra and in 1949, that tryptophan reversed the symptoms. In 1961, Goldsmith quantified the conversion of tryptophan to nicotinic acid by monitoring such nicotinic acid metabolites as *N*¹-methyl-5-carboxamide-2-pyridone. Elucidation of the biochemical pathway for conversion of tryptophan to nicotinic acid mononucleotide (Fig. 2) took more than 10 years—from 1950, when Knox and Mehler showed that the first step in the biodegradation of tryptophan to *N*-formylkynurenine was catalyzed by tryptophan pyrrolase, to 1963, when work by Nishizuka and Hayaishi revealed that quinolinic acid reacts with phosphoribosyl pyrophosphate (PRPP) to form nicotinic acid mononucleotide, a reaction catalyzed by the enzyme quinolinic acid phosphoribosyltransferase.

ACTIONS AND PHARMACOLOGY

The major dietary sources of niacin are meats, poultry, and fish, followed by dairy and grain products (2). Pre-formed niacin exists in foods as nicotinamide, nicotinic acid, nicotinamide riboside, or the pyridine nucleotide coenzymes, NAD and NADP (Fig. 3). Nicotinamide riboside, a recently discovered salvageable precursor of NAD, is particularly abundant in cow milk (3). *L*-Tryptophan, the *in vivo* precursor of nicotinamide (Fig. 2), also contributes to the total niacin-equivalent (NE) content of foods and should be taken into account when calculating the vitamin intake. Eggs and milk, for instance, with their high tryptophan content, are a significant source of NE. Niacin

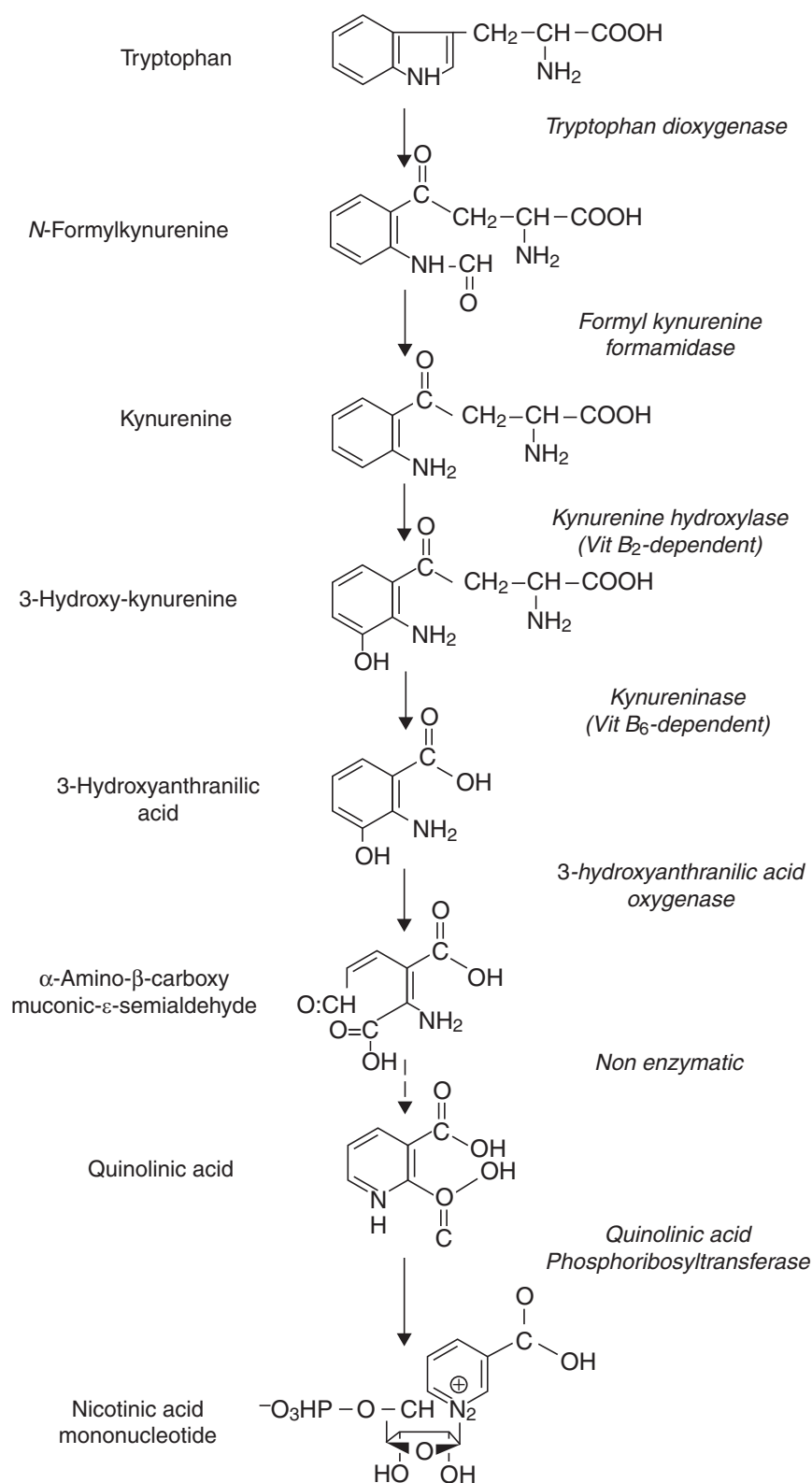


Figure 2 Conversion of tryptophan to nicotinic acid mononucleotide; “de novo” synthesis pathway.

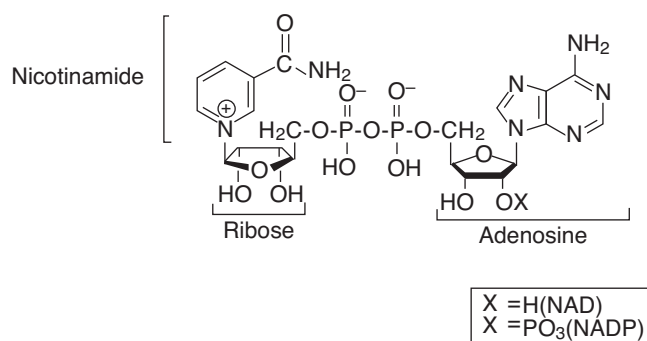


Figure 3 Structure of the pyridine coenzymes.

intake is, therefore, generally expressed in NE. It is estimated that 60 g of the amino acid is converted to 1 g of the vitamin, with a variation of approximately 30% (standard deviation) among individuals. The efficiency of tryptophan conversion to nicotinic acid depends on nutritional history and hormonal factors (4). Quantitatively, tryptophan is primarily used for protein biosynthesis, even in conditions of niacin deficiency.

Estimation of dietary niacin content should also consider another factor: bioavailability. Indeed, in certain cereal grains, such as corn, niacin is largely present as niacytin, a polysaccharide/glycopeptide/polypeptide-bound form, most of which is unavailable for intestinal absorption. In maize, for instance, 70% of niacin is in a biologically unavailable form (5). However, niacin availability can be improved by specific processes such as the alkali treatment of corn used in the preparation of tortillas (4). Otherwise, absorption of nicotinic acid and its amide by the gastric and intestinal mucosa is very efficient, proceeding via sodium ion-dependent facilitated diffusion at low concentrations, and passive diffusion at high concentrations. In the gut, NAD and NADP are degraded by glycohydrolase and pyrophosphatase activities into nicotinamide and nicotinamide ribonucleotide, respectively, which are bioavailable sources of the vitamin.

Tissues absorb free nicotinic acid as well as nicotinic acid bound to proteins. Metabolic trapping, in which nicotinic acid and nicotinamide are converted to NAD, accounts for retention of these vitamins (4). Nicotinic acid and, to a lesser extent, nicotinamide are lipid-soluble molecules, and adipose tissue is responsible for the rapid clearance of nicotinic acid after an IV dose. In addition, receptor-mediated uptake has been reported for nicotinamide (4). A transporter for nicotinamide riboside has been identified in yeast (6). In liver, nicotinic acid and nicotinamide can be converted to NAD and NADP or metabolized for clearance. Nicotinic acid is eliminated as a glycine conjugate, nicotinuric acid, whereas the main metabolites of nicotinamide are *N*¹-methylnicotinamide and its oxidized products, 2- and 4-pyridones (7). Nicotinic acid and nicotinamide metabolites are then excreted in urine; quantification of this excretion is useful in evaluating niacin nutritional status.

Nicotinic acid, nicotinamide, nicotinamide riboside, and tryptophan are precursors of NAD and NADP (Fig. 4).

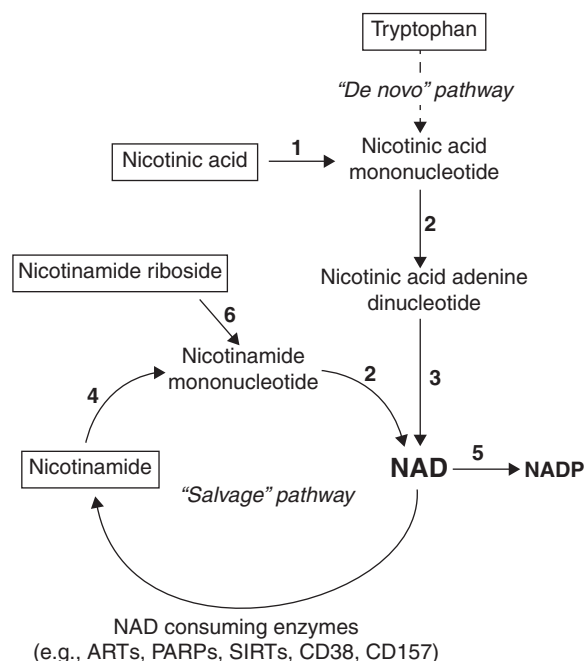


Figure 4 General pathways of NAD metabolism. In liver, NAD may be synthesized from dietary nicotinic acid, nicotinamide, nicotinamide riboside, and tryptophan ("de novo" pathway) or may be readily absorbed from foods. NAD may also be synthesized through the "salvage pathway," which is fueled by the nicotinamide resulting from the activity of NAD-consuming enzymes. *Abbreviations:* PARPs, poly(adenosine diphosphate ribose) polymerases; ARTs, mono-ADP-ribosyltransferases; SIRT, Sir2-like protein deacetylase; NAD, nicotinamide adenine dinucleotide. 1: Nicotinic acid phosphoribosyltransferase; 2: (nicotinamide/nicotinic acid) mononucleotide adenosine-5'-triphosphate adenyltransferase (NMNAT/NaMNAT); 3: NAD synthase; 4: nicotinamide phosphoribosyltransferase (or PBEF); 5: NAD kinase; 6: nicotinamide riboside kinases (Nrks).

These nucleotides can be synthesized "de novo," using tryptophan from the diet to generate nicotinic acid mononucleotide (Fig. 1) or through the "salvage pathway" (Fig. 4), using nicotinic acid, nicotinamide, and nicotinamide riboside absorbed from nutrients, or through nicotinamide recycled from signaling reactions that involve NAD catabolism [for review see Ref. (8)]. Tryptophan metabolism, initiated by tryptophan-2,3-dioxygenase, a tryptophan-inducible enzyme, occurs primarily in liver. Because quinolinic acid phosphoribosyltransferase activity in mammals was detected only in liver and kidney, other tissues rely mostly on an exogenous supply of nicotinic acid/nicotinamide/nicotinamide riboside for NAD biosynthesis, hence their role as essential nutrients. The rate-limiting step of the salvage pathway is catalyzed by a nicotinamide phosphoribosyltransferase, also known as pre-B colony-enhancing factor (PBEF), an inflammatory cytokine (9). The last step of NAD synthesis is catalyzed by nicotinamide/nicotinic acid mononucleotide adenyltransferases (NMNAT1–3, in humans) (10–12), which use both nicotinamide mononucleotide and nicotinic acid mononucleotide as targets for the adenyl-transfer reaction. In yeast, genes of the "de novo" synthesis pathway are silenced by an NAD-dependent histone deacetylase, which functions as a sensor of levels in

nuclear NAD pools (13). NADP is formed directly from NAD by phosphorylation catalyzed by a specific kinase present in most tissues except skeletal muscle (14).

These pyridine nucleotides are involved in numerous reactions, ranging from energy metabolism to cell signaling. As coenzymes, they are required in most of the metabolic redox processes of the cell, in which dehydrogenases use NAD/P(H) as coenzymes to oxidize or reduce substrates. NADP dehydrogenases are preferentially involved in anabolic reactions (e.g., synthesis of fatty acids and cholesterol) (15). In contrast, NAD is used in catabolic reactions to transfer the potential free energy stored in macronutrients such as carbohydrates, lipids, and proteins to NADH, which is then used to form ATP, the primary energy currency of the cell.

Besides its well-known role in energy transduction, NAD is also substrate for four other classes of enzymes, mono-ADP-ribosyltransferases (ARTs), poly(adenosine diphosphate ribose) polymerases (PARPs), ADP-ribosylcyclases (e.g., CD38, CD157), and Sir2-like protein deacetylases (SIRT1). ARTs and PARPs catalyze the activation of the β -N-glycosylic bond of NAD and transfer of the ADP-ribose moiety to acceptor proteins or another ADP-ribose in the case of PARPs. Many of the ARTs also demonstrate NAD glycohydrolase activities, in which water is the ADP-ribose acceptor, as do ADP-ribosylcyclases. The latter, in addition, catalyze the formation of potent calcium-mobilizing second messengers, cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide (NAAD) from NAD, as well as cyclic ADP-ribose phosphate (cADPRP), and nicotinic acid adenine dinucleotide phosphate (NAADP) from NADP. In contrast, most SIRT1s use NAD as an acceptor of the acetyl group that is removed from proteins, thereby generating 2'-O-acetyl-ADP-ribose although SIRT4 was recently identified as a mitochondrial ART (16).

ARTs transfer a single ADPR moiety per specific acceptor amino acid (e.g., arginine, cysteine, asparagine, histidine). In general, the purpose of this covalent modification is to alter the biological activity of the acceptor protein (reviewed in Ref. 17). The known vertebrate ARTs (ART1–7) are secreted or glycosylphosphatidylinositol-anchored proteins. Their enzymatic activities have been implicated in the regulation of diverse cell processes including myocyte differentiation and modulation of immune cell functions (e.g., T-lymphocyte cytotoxicity and neutrophil chemotaxis) (17). The well-established modulatory properties of mono-ADP-ribosylation on intracellular targets (e.g., G proteins, chaperone proteins, cytoskeleton, Golgi components) suggest that additional ARTs are yet to be identified.

The first described PARPs were recognized for their ability to synthesize highly negatively charged ADP-ribose polymers on themselves and/or target proteins, thereby affecting protein folding and, hence, protein function (for review, see Ref. 18,19). However more recently, other proteins carrying the PARP signature domain have been shown to transfer one single ADPR moiety similarly to ARTs (20). This posttranslational modification is transient as the polymers or monomers are rapidly metabolized by three enzymes, poly-ADP-ribose glycohydrolase, pyrophosphatases, and lyase. Poly-ADP-ribosylation is involved in the regulation of many vital cellular events,

for example, DNA replication and repair, chromatin structure, transcription, apoptosis, and regulation of telomere length (18,19). In general, members of the PARP family are nuclear DNA-binding proteins that catalyze the polymerization and branching of ADPR chains on target proteins. The most extensively studied member, PARP1, is markedly activated at sites of single-strand DNA breaks. Overactivation of PARP1 after extensive DNA damage leads to rapid depletion of NAD and ATP and ultimately cell death. Because niacin contributes to maintaining the NAD levels for PARP, niacin deficiency could compromise DNA repair and increase the risk of cancer. Alternatively, the inhibitory effect of niacin on PARP1 activity should also be taken into account; an excess of niacin could also impair DNA repair. However, there is a growing body of experimental and epidemiological evidence for a relationship between niacin status and genomic stability (18,19). Several PARP1 inhibitors are in clinical trial as potential antitumor drugs (21–24).

The third group of enzymes, the NAD-glycohydrolase/ADP-ribosylcyclases, includes CD38 and CD157 in mammals (25). These membrane proteins use NAD and NADP to generate signaling molecules, cADPR and cADPRP, by cyclization and NAADP by transglycosidation. These pyridine derivatives have critical signaling functions in the mobilization of intracellular calcium stores via modulation of the Ca^{2+} -releasing channel ryanodine receptors. Thanks to its very strong NADase activity, CD38 may also control NAD levels in cells and thus NAD-dependent physiologic processes (25). As shown by targeted gene inactivation in mice, CD38 is required for appropriate cell-dependent antibody and innate immune responses to bacterial pathogens (26), whereas CD157 participates in the regulation of the humoral T-cell-independent immune and mucosal thymus-dependent responses (27). Both cyclases have been implicated in the development of autoimmune disorders, although their role(s) is (are) not well established. CD38 has been proposed as a mediator of glucose-induced insulin secretion from pancreatic β -cells via the increase of intracellular Ca^{2+} concentration and may be involved in the pathogenesis of autoimmune diabetes (28). It has been postulated that upregulation of CD157 expression in some patients with rheumatoid arthritis may contribute to the development of this autoimmune disease (26).

Sir2-like proteins are related to the yeast silent information regulator (Sir2), an enzyme required for lifespan expansion in conditions of nutrient scarcity in many organisms (29,30). In humans, SIRT1, the most closely related to yeast Sir2, deacetylates p53, thereby inhibiting apoptosis in response to DNA damage. In mice, absence of SIRT1 leads to p53 hyperacetylation, impaired development, a shortened lifespan, and sterility. Thus, in several species, SIRT1 and homologs appear to regulate diverse pathways that have one common feature, that is, their impact on aging (31). Because nicotinamide is a potent inhibitor of SIRT activity, it has been proposed to serve as a physiologic regulator, whose level would be controlled by the rate of its conversion to nicotinic acid through the NAD⁺ salvage pathway, and/or to N-methyl nicotinamide, by the excretion pathway. Whether a decrease in nicotinamide, or an increase in NAD levels, is responsible

for the increased activity of Sir2 during caloric restriction is still debated (29,32).

Thus, NAD-consuming enzymes, by their activities, link the nutritional and metabolic status of the cell to the regulation of essential cell functions, such as gene silencing, maintenance of genome integrity, and innate immunity. Many of these reactions yield nicotinamide in addition to other molecules, thus fueling the NAD "salvage pathway" for NAD resynthesis.

INDICATIONS AND USAGE

Supplementation to Achieve Recommended Intake Levels

The recommended dietary allowance (RDA), as defined in the report of the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Niacin (5), is summarized in Table 1. These values were established according to the doses required to prevent pellagra (11.3–13.3 mg of NE/day). The tryptophan content of a reasonable dietary protein intake is itself likely to provide at least 13.2 mg/day NE (based on a 2000 kcal/day diet). There are no relevant data concerning niacin requirements in pregnancy and lactation. Thus, requirements were estimated based on an average increase in energy expenditure of 300 kcal/day during pregnancy and an average daily secretion of 1.4 mg of NE during lactation (5). As numerous NAD-dependent enzymes (e.g., PARPs, SIRT5) can affect genomic stability, insufficient nicotinic acid intake is likely to increase the risk of cancer and other diseases attributable to increased DNA damage (33). It has been suggested that maintenance of adequate NAD levels would, in the long term, prevent or retard the multistage

process of carcinogenesis and age-related diseases. Several lines of evidence in yeast and in rodents support this hypothesis (34). Furthermore, studies in rats suggest that niacin supplementation could decrease the risk for development of chemotherapy-related malignancies in cancer patients with compromised nutritional status (35,36). According to preliminary experimental data linking niacin status and genomic stability, the doses required to prevent pellagra would not be sufficient to promote genomic stability (37).

Niacin nutriture has been assessed in several ways by a variety of methods (for review, see Ref 38). Dowex-1 formate chromatography is used to separate pyridine nucleotides and N^1 -methylnicotinamide. Measurement of the latter and its 2-pyridone derivative in urine is most commonly used. Excretion of N^1 -methylnicotinamide below 0.8 mg/day indicates niacin deficiency (38). A ratio of N^1 -methyl-5-carboxamide-2-pyridone to N^1 -methylnicotinamide of 1.3 to 2.0 is considered normal; in niacin deficiency, it is less than 1.0. Niacin status can also be assessed by measuring its physiologically active forms, NAD(H)/NADP(H). A method devised by Lowry et al. in 1961, and modified by Slater and Sawyer in 1962 and Nisselbaum and Green in 1969, uses appropriate dehydrogenases specific for either NAD or NADP and thiazolyl blue, which, when reduced by NADH and NADPH, forms purple formazan in an amount proportional to the concentration of the coenzymes (oxidized and reduced). This assay is used to measure pyridine nucleotides in tissue and blood. Assays of NAD/NADP in erythrocytes and cultured cells suggest that the intracellular NAD level may reflect niacin status, whereas NADP levels do not. Measurement of NAD/NADP content and tryptophan level in erythrocytes has been proposed to evaluate niacin deficiency (4).

Table 1 Recommended Dietary Allowances of Niacin^a

Age (yr)	RDA (mg) ^b
Infants	
0–0.5	2 ^c
0.5–1.0	4 ^c
Children	
1–3	6 ^d
4–8	8 ^d
Males	
9–13	12 ^d
14–18	16 ^d
19 and above	12
Females	
9–13	12 ^d
14–18	14 ^d
19 and above	14
Pregnancy	
14–50	17
Lactation	
14–50	18

^aRDA, 1998 United States Food and Nutrition Board of the Institute of Medicine.

^bOne milligram niacin = 60 mg tryptophan = niacin equivalent (NE).

^cFor infants, given values correspond to the adequate intake (AI) level, which is based on the observed mean intake of preformed niacin by infants fed with human milk.

^dNo data being available for these ranges of age, RDAs were estimated by extrapolation from adult values.

Treatment of Niacin Deficiency

In humans, the combination of inadequate intakes of tryptophan and niacin leads to pellagra. This name was given by Frapolli in 1771, from the Italian words "pelle" for "skin" and "agra" for "rough" to describe the roughened, sunburned-like appearance of the skin of niacin-deficient patients exposed to sunlight. Other symptoms include diarrhea and neuropathy (39). In its most acute form, deficiency can lead to death. Maize-based diets predispose to pellagra because of the limited bioavailability of the niacin contained in this grain and its low tryptophan level. Niacin bioavailability can be improved, however, by alkaline treatment. In Central America, where corn used for the preparation of tortillas is first soaked in lime solution, the incidence of pellagra is very low, despite the corn-based staple diet (4). Today, this disease seems to be endemic mostly in parts of India, China, and Africa (39). Advanced stages of pellagra can be cured with nicotinamide in IM doses of 50 to 100 mg three times a day for 3 to 4 days, followed by similar quantities orally, supplemented with 100 g of proteins daily.

Other factors such as alcoholism or AIDS may promote the appearance of pellagra (reviewed in Ref 40). Vitamins B6 and B2 (riboflavin) are coenzymes required for the efficient conversion of tryptophan to niacin (Fig. 2). Hence, an inadequate intake of these vitamins is likely to

predispose to pellagra. An excess of leucine also impairs tryptophan bioconversion by competing for transport and by inhibiting kynureninase, resulting in decreased NAD formation.

There are several reports of a higher incidence of pellagra in women than in men. This difference may have several causes, including cultural factors that determine food intake, metabolic stresses due to repeated pregnancies, and lactation. In addition, estrogen metabolites can inhibit kynureninase and kynurenine hydroxylase activity. When the intake of preformed niacin and tryptophan is low, inhibitory effects of estrogens on tryptophan bioconversion could contribute to a greater susceptibility of women to pellagra.

Inborn disorders of tryptophan metabolism can cause nondietary pellagra (reviewed in Ref 41). In Hartnup's syndrome (an autosomal recessive disorder), decreased absorption and/or increased excretion of tryptophan lead to inadequate conversion of this essential amino acid to niacin. The symptoms of niacin deficiency can be alleviated by large doses of the vitamin (40–250 mg/day).

Treatment of Hyperlipidemia

In pharmacological doses (2–6 g/day), nicotinic acid, but not nicotinamide, significantly reduces atherosclerotic cardiovascular disease and mortality (42). The benefits of nicotinic acid treatment are due to its antihyperlipidemic effects at high doses. It decreases the levels of plasma low-density lipoproteins (LDLs), very low-density lipoproteins (VLDLs), and triglycerides (TGs), and increases the high-density lipoproteins (HDLs), thus reducing the LDL/HDL ratio. The mechanism of action of nicotinic acid on lipoprotein metabolism has not been completely elucidated (reviewed in Ref 43). Available data suggest that nicotinic acid decreases the formation of LDL and VLDL by inhibiting the lipolysis of TG in adipose tissue and TG synthesis in liver. In adipose tissue, the antilipolytic effect is mediated by niacin activation of a recently characterized Gi/o protein-coupled high-affinity receptor that inhibits cAMP-stimulated lipolysis (44,45). On the other hand, nicotinic acid promotes the synthesis of HDL by preventing the catabolism of a major protein component of HDL apolipoprotein A-I (apoA-I), but not of cholesterol esters from HDL. It has been proposed that an increase in the amount of apoA-I available for HDL synthesis would augment reverse cholesterol transport, facilitating the removal of excess cholesterol from peripheral tissues and thereby lowering the risk of atherosclerotic cardiovascular disease.

When nicotinic acid monotherapy does not lower the blood cholesterol level sufficiently, it is administered in combination with other lipid-lowering drugs that act through different mechanisms (e.g., bile-acid-binding resins, statins). This strategy has proved successful in several clinical trials (46).

Prevention of Oxidant-Induced Cell Injury in Pathological Conditions

At high doses (up to 3.5 g/day), nicotinamide is protective against cell death and inhibits the production of inflammatory mediators in animal and in "in vitro" models of oxidant-induced cell injury. In addition to these effects,

which are consistent with PARP1 inhibition, nicotinamide exhibits PARP1-independent actions (19) that may be attributable to its inhibition of other signaling pathways (e.g., SIRT6) and its function as a precursor of pyridine nucleotides (46). Nicotinamide has been proposed as a possible means of increasing the survival of pancreatic β -cells after diagnosis of Type I diabetes (insulin-dependent diabetes mellitus, IDDM), or to prevent onset of the disease in high-risk individuals (reviewed in Ref 19). This latter notion was not confirmed, however, by the recently published European Nicotinamide Diabetes Intervention Trial (ENDIT), a large-scale evaluation of nicotinamide benefits in first-degree relatives of Type I diabetic patients (47).

Adverse Effect of Drugs on Niacin Status

Isoniazid, which is commonly used to treat tuberculosis, causes vitamin B6 depletion and hence may lower the efficiency of the "de novo" synthesis pathway that converts tryptophan into nicotinic acid, thereby predisposing to pellagra (4).

CONTRAINDICATIONS

Because of its potential side effects, antihyperlipidemic treatment with nicotinic acid is contraindicated in patients with active peptic ulcer or frequent gout attacks. Until recently, those with Type II diabetes mellitus were also considered at risk, but new clinical data seem to indicate that nicotinic acid can be used safely to treat diabetic dyslipidemia (48).

PRECAUTIONS AND ADVERSE REACTIONS

Prostaglandin-mediated flushing is the major specific side effect experienced by users of pharmacological doses of nicotinic acid in the initial days of treatment. Symptoms can be reduced by ingestion of the drug with food and/or by gradually increasing the dose. Tolerance develops with continued use in most patients. Flushing has been documented in patients using immediate-release nicotinic acid (IR-nicotinic acid) and sustained-release forms, as well as by some subjects on extended-release nicotinic acid (ER-nicotinic acid). However, in general, the extended-release formulation achieves the efficacy of the immediate-release form with a reduced incidence of flushing and without the hepatic problems caused by slow-release nicotinic acid (49). Other reported adverse effects of nicotinic acid treatment include pruritis, nausea, gastrointestinal upset, hypotension, tachycardia, and elevated serum blood glucose and uric acid levels. Because of potential hepatic toxicity, liver enzymes (aminotransferases and/or alkaline phosphatase) should be monitored before the initiation of therapy, 6 weeks after initiation and/or any change of dose, and two or three times a year thereafter. If liver enzymes exceed three times the upper limit of normal, treatment should be discontinued. To avoid liver toxicity, it is recommended that the starting dose should not exceed 250 to 300 mg/day with monthly increments not greater than 250 to 300 mg/day until a maximum of 3 g/day is reached for IR-nicotinic acid and 1.5 to 2 g/day for the sustained-release form.

Nicotinic acid may cause insulin resistance, which requires compensatory insulin secretion, and, in patients with dysfunctional pancreatic β -cells, it may trigger hyperglycemia. Those with diabetes mellitus, therefore, require special monitoring during niacin treatment (19). No adverse effects of the pharmacological doses of nicotinamide used during the ENDIT study were reported (47). However, there has been concern that saturation of the nicotinamide excretion pathway may divert methylation equivalents required for anabolic pathways to nicotinamide methylation and lead to growth retardation in children. Furthermore, as a strong inhibitor of SIRT6, nicotinamide might interfere with cell survival (34). Thus, more data are needed on the long-term effects of therapeutic doses of nicotinamide.

OVERDOSAGE

No adverse events of the consumption of naturally occurring niacin in food have been reported. Side effects have been widely recognized in patients treated for hyperlipidemia with high doses (3–9 g/day) of pharmaceutical preparations of nicotinic acid for periods of months to years (48). Symptoms of nausea and vomiting and signs of liver toxicity with intake of more than 3000 mg/day of nicotinamide or 1500 mg/day of nicotinic acid have been reported. Most frequently, patients develop jaundice and increased levels of serum transaminases. In the most severe cases, liver dysfunction and fulminant hepatitis can result (49).

CONCLUSIONS

Meat, cereals, eggs, and milk are the main sources of vitamin B3, the general term to designate niacin (nicotinic acid and nicotinamide) and NE (tryptophan). Deficiency, which may be caused by poor dietary intake or inherited disorders (e.g., Hartnup's syndrome), results in pellagra. The diversity of pellagra symptoms is representative of the wide spectrum of pathways that require adequate niacin intake to function. The molecular mechanisms by which insufficient niacin uptake causes these symptoms are poorly understood. Some may reflect primarily the role of the vitamin as a precursor of NAD and NADP, others to its requirement, as coenzyme or substrate in many enzymatic reactions. Niacin nutritional status may have consequences for cellular functions as diverse as immunity, genomic stability, and energy supply. In addition to its role as a niacin source, nicotinic acid is well established, and widely employed therapeutically, because of its efficacy as an antihyperlipidemic agent. Risks of hepatotoxicity and other side effects have decreased with the development of new niacin formulations that improve drug delivery and are better tolerated by patients. The recent characterization of different types of nicotinic acid receptors may help in the development of more specific agonists with fewer side effects. Nicotinamide is the niacin source of choice to treat pellagra. Other pharmacological applications of nicotinamide have produced mixed results. It did not seem effective in preventing the development of autoimmune diabetes or for protection against

oxidant-induced cell death. This may be due to the roles of nicotinamide as both an NAD precursor and an inhibitor of several relevant enzymes (e.g., SIRT6, PARPs). Recent studies suggest that, by sustaining adequate NAD levels, pharmacological doses of niacin could contribute to the prevention or delay of age-related diseases in healthy individuals and protect cancer patients against secondary effects of anticancer therapy. However, because of their multiple functions, more studies are necessary to evaluate the benefits and consequences of pharmacological doses of nicotinamide and nicotinic acid.

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Noni

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INTRODUCTION

Morinda citrifolia L. (Rubiaceae) (noni) is an important medicinal plant of South Asian origin. All parts of this species have been used traditionally in regions such as South and Southeast Asia, Polynesia, Northeastern Australia, and the Caribbean for a wide range of ailments, primarily topical diseases. The fruits, and more rarely the leaves, are currently being marketed in the United States as a botanical dietary supplement for several chronic ailments and to promote overall general health. Several limited in vitro and in vivo studies in mice have suggested that noni may help treat or prevent cancer, pain, and cardiovascular disease. These studies are limited in scope and only a few pure compounds from noni have been tested for biological activity, including against targets related to cancer and inflammation. The biological activity primarily ascribed to noni in books and other promotional information written for a general audience is based on a hypothesis developed by Ralph M. Heinicke. This idea, centered on the mythical compound “xeronine,” has not been scientifically validated. A phase I clinical trial on a freeze-dried noni fruit extract, sponsored by the National Center for Complementary and Alternative Medicine (NCCAM), National Institutes of Health, Bethesda, MD, was conducted in Hawaii in cancer patients. There is an active scientific literature on aspects of the phytochemistry, biological activity testing, and potential safety of the constituents of the various plant parts of *M. citrifolia*, with such reports found as peer-reviewed research articles, meeting abstracts, and patents.

Background

M. citrifolia L. (Rubiaceae) has a host of common names, including ach, awl tree, canary wood, doleur, gardenia hedionda, gogu atogi, great morinda, hog apple, Indian mulberry, kura, lada, mengkoedoe, mengkudu, morinda, mulberry, nen, nhàu, nino, and noni (Fig. 1) (1,2).

Botanical Description

M. citrifolia L. (noni) is a small evergreen tree or shrub, 3 to 10 m in height, which grows throughout the Pacific and other regions. Noni is known to be very adaptive and can easily colonize new islands and terrains, as it grows in a wide range of soil types and in both wet and dry areas. The leaves of noni are opposite, pinnately veined, glossy, elliptic to elliptic-ovate in shape, and are 20 to 45 cm long and 7 to 25 cm wide. The flowers are perfect with five-lobed, small, white corollas. The fruits are syncarpous, yellowish white, fleshy, and 5 to 10 cm long and 3 to 4 cm in diameter at maturity. The lumpy fruits are soft with



Figure 1 Drawing of *Morinda citrifolia* L. (calibration bar = 1 cm). Source: From Singh YN, Ikahihifo T, Panuve M, et al. Folk medicine in Tonga. A study on the use of herbal medicines for the obstetric and gynaecological conditions and disorders. *J Ethnopharmacol* 1984; 12:305-529, with permission from Elsevier.

a waxy, semitranslucent skin, and the surface is covered with four- to six-sided outlines with a central “eye.” The ripe fruit is reported to vary in scent, with some trees containing nonscented to slightly scented fruits to other trees with a strong, unpleasant butyric acid odor (2). The seeds include an air chamber, which makes them buoyant, and can remain viable after months of floating in water. This attribute led to the hypothesis that noni was spread among the Polynesian islands by floating from island to island (1,3,4). There are two recognized varieties of noni, namely, *M. citrifolia* var. *citrifolia* and *M. citrifolia* var. *bracteata* and one cultivar, *M. citrifolia* cultivar *Potteri*. Of these, *M. citrifolia* var. *citrifolia* is the most commonly found and has the greatest importance economically (3,4).

Noni is currently cultivated for commercial use in the Pacific islands such as Tahiti and Hawaii and in Australia. The fruits are harvested year-round and can be picked just prior to fully ripening to be shipped or can be gathered when ripe for local processing (1–4).

Ethnomedical Use of Noni

The leaves and fruits are eaten as food in times of famine, although it has been suggested in several botanical reports on noni that some cultures have ingested this plant as a regular food (1,2). Medicinally, various parts of *M. citrifolia* are used both externally and internally, alone or in combination with other plants. Externally, the fruits and leaves have been used to treat boils, pain and inflammation, ringworm, scabies, and wounds (5–7). In Fiji, the leaves and flowers are heated and the juice squeezed into ulcers, and the leaves are then used as a bandage (6). Internally, the different parts of the noni plant have been used to stimulate the appetite in patients with wasting diseases and also as an antiemetic (8). The juice of the fruit is also applied to aching teeth or sore gums and to treat halitosis. A concoction of the root with coconut is taken internally for scabies and skin eruptions and to treat intestinal infections (6). In Vietnam, the root-bark is used to treat hypertension and a decoction made from the leaves is used for diarrhea and fever. The fruit is also used to treat dysentery, neuralgia, and a number of other common ailments (9). Also, noni fruits have been used in combination with kava (*Piper methysticum* Forst. f.) (Piperaceae) to treat tuberculosis (1).

Current Use of Noni in the United States

The use of noni fruit in the United States as a botanical dietary supplement is becoming more widespread, and noni products are widely available in health food stores, pharmacies, chain grocery stores specializing in natural foods, and on the Internet. The current rise in popularity of noni may be due to the increase in publicity as a general “cure-all” or panacea for chronic conditions. This is exemplified by a number of books devoted entirely to the subject of noni, which provide anecdotal evidence of the use of noni to alleviate problems such as cancer, chronic pain, depression, drug addiction, hypertension, and obesity (10,11). Cancer patients, in particular, are using noni as an alternative method to treat or to complement their anti-cancer drug regimens. A small number of scientific studies (summarized later), along with ethnobotanical use, has suggested that noni juice may help alleviate symptoms and/or increase efficacy of cancer chemotherapy. The disparity between traditional ethnobotanical uses and currently marketed uses of noni has recently been expounded upon in a review by McClatchey (2). Briefly, his ethnobotanical research in Fiji, Hawaii, Rotuma, and Soma found that expert healers primarily use the leaves, followed by the young green fruit and the root bark and inner stem for topical ailments. The ripe fruit, which is the predominant formulation used in the United States is used in these same areas as home remedies by nonhealers.

Chemistry and Preparation of Products

Chemistry

Investigation of the chemical constituents of this valuable medicinal plant began over 150 years ago. In

1848, Anderson crystallized a compound described as light yellow needles of faintly bitter taste, which was named morindin, from the root bark of noni (12). A few other structurally similar compounds (anthraquinone glycosides) were also obtained by extraction and crystallization from other *Morinda* species (12,13), and all of these isolates were also named “morindin.” The structure of morindin isolated from the root bark of noni was determined to be 1,5-dihydroxy-2-methyl-6- β -primverosyloxyathraquinone on the basis of the chemical conversion evidence and analysis of its physical and spectroscopic data. In addition to anthraquinones, other major compounds isolated from this plant are flavonol glycosides (14), iridoid glycosides (14–16), lignans (17,18), lipid glycosides (19,20), and triterpenoids (21–23). So far, over 130 compounds have been isolated from different anatomical parts of noni and the structures of a few selected components are given in Figure 2. It is noteworthy that most of the iridoid glycosides isolated from noni are oxygenated at C-10 (Fig. 2). Also, an alkaloid (21), fatty acids (23,24), monoterpenoids (24), and steroids (22,23) have also been isolated and characterized. Recently, the composition of an ethanol-insoluble polysaccharide constituent has been determined for a noni fruit sample obtained from Vietnam (25).

Despite these phytochemical studies, the precursor of a compound called “xeronine” has been touted to be the major biologically active constituent of noni in books and other promotional materials oriented toward the lay public. This stems from a 1985 paper by Ralph Heinicke entitled “The Pharmacologically Active Ingredient of Noni,” where he proposed a hypothesis as to how noni is able to help cure a wide range of diseases (26). Briefly, he stated that “xeronine” is required by all cells and that a deficiency can lead to a number of ailments that can be prevented or cured by noni supplementation. However, Heinicke’s paper, along with all subsequent documentation, lack any supportive evidence for the presence, structure, or biological requirement of “xeronine” and, therefore, his theory is questionable at best (2).

Preparation of Products

Although noni fruit, and occasionally the leaves, is sold as tablets and in the form of herbal teas, it is encountered most commonly as a juice derived from the fruits. These noni fruit juices are frequently prepared by diluting the dried powdered fruit with other juices such as grape juice to increase palatability. A few noni products are available, which claim to be standardized to a given percentage of polysaccharides. This is presumed to stem from the studies demonstrating the potential anticancer activity of the polysaccharide-rich partition (summarized later). The main delineation between the currently marketed noni juice products in the United States is the plant source, with most either of Tahitian or Hawaiian origin. Two recent reports have pointed to various marker molecules that may be used in the quality control of commercial noni juice and/or capsule products by using chromatographic methods, including substances of the anthraquinone, coumarin, fatty acid glucoside, flavonoid, and iridoid classes (27,28).

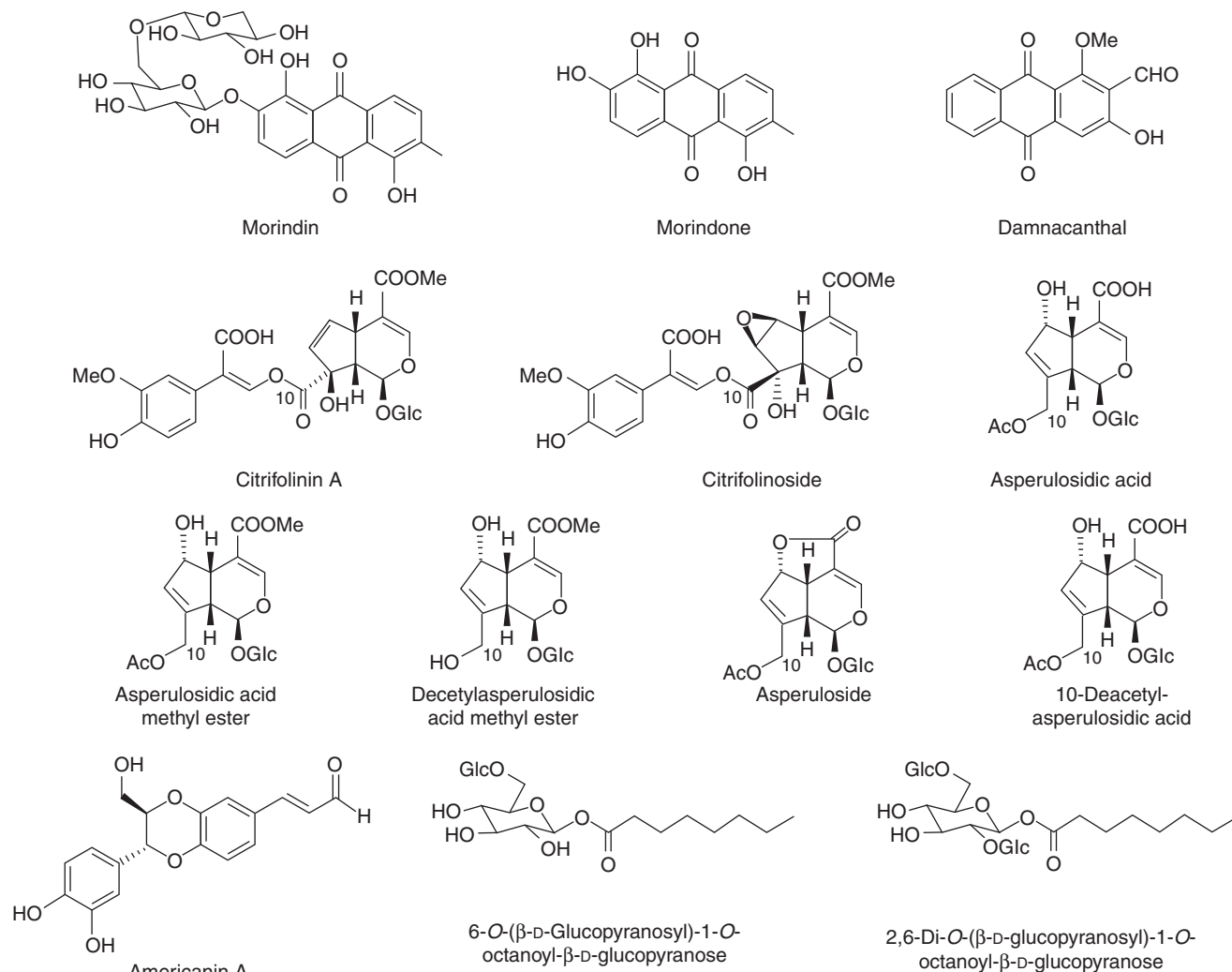


Figure 2 The structures of selected constituents of *M. citrifolia* (noni).

Stability

No information is available.

Preclinical Studies

Cancer

A number of in vitro bioassay evaluations have been performed on isolated compounds from noni, which suggest possible cancer preventative or therapeutic activities. For example, several compounds have demonstrated inhibition of activator protein-1 (AP-1) in different cell systems. AP-1 is a transcription factor involved in the tumor promotion stage of carcinogenesis and its inhibition may be an important mechanism in a variety of cancers at this stage. The iridoids, citrifolinin A and citrifolinoside, isolated from the leaves of noni prevented ultraviolet B (UV-B)-induced AP-1 activity in cell culture. UV-B irradiation acts as both an initiator and a tumor promoter in the development of skin cancers (29,30). Two other compounds, asperulosidic acid and the glycoside 6-O-(β-D-glucopyranosyl)-1-O-octanoyl-β-

D-glucopyranose, isolated from noni fruits, suppressed 12-O-tetradecanoylphorbol-13-acetate (TPA)- and epidermal growth factor-induced AP-1 transactivation in JB6 mouse epidermal cells (31). Furthermore, an anthraquinone isolated from noni roots, damnacanthal, is a potent tyrosine kinase inhibitor. Although damnacanthal has been shown to be an inhibitor of specific tyrosine kinases (32), the activity disappeared in one study when the authors switched from a cell-free to a whole-cell system (33). Damnacanthal and its structural analog, morindone, have demonstrated strong topoisomerase-II inhibition in a cell-free test system (34). Damnacanthal was also found to induce normal morphology in cells expressing the *ras* oncogene, termed K-ras^{ts}-NRK cells, but not in cells expressing the *src* oncogene (35).

Several recent reports have correlated constituents of *M. citrifolia* with cancer chemoprevention. The anthraquinone, 2-methoxy-1,3,6-trihydroxyanthraquinone, a trace component of noni fruits, was demonstrated as a very potent inducer of quinone reductase (QR), a phase-II-metabolizing enzyme considered protective at the

initiation stage of carcinogenesis. Furthermore, this compound was not found to be cytotoxic for the Hepa 1c1c7 murine hepatoma host cells (36). Four structurally related anthraquinones from noni roots were also found to be QR-inducing agents (37). Several constituents (anthraquinones, saccharide fatty acid esters, the iridoid glycoside, asperulosidic acid, and the flavonoid glycoside, rutin) of noni fruits were reported as inhibitors of the activation of Epstein-Barr virus early antigen induced by TPA (12-*O*-tetradecanoylphorbol 13-acetate) (38). However, none of these *in vitro* results have been followed up by a relevant *in vivo* bioassay germane to cancer chemoprevention, such as a two-stage carcinogenesis inhibition test.

Angiogenesis Inhibition

In order for solid tumors to grow, they must be able to develop new blood vessels through a process termed angiogenesis. Without angiogenesis a tumor would cease growing due to metabolic deprivation. Therefore, there has been an interest in finding antiangiogenic agents that can prevent the development of tumors or target their recently formed vasculature. In an *in vitro* angiogenesis model using placental vein explants, noni juice at a concentration of 5% in the growth media was able to effectively suppress angiogenic initiation. In a similar experiment where the placental explants were allowed to grow for 7 days and then treated with 10% noni juice, there was an observed inhibition of new growth and a breakdown in the recently developed vasculature. The same experiments were carried out using human breast cancer explants where new blood vessel development was also suppressed with media supplemented with noni juice. No positive controls appear to have been used for these studies (39).

Immunostimulation

Several studies performed at the University of Hawaii have demonstrated potential anticancer activity of the polysaccharide-rich, ethanol-insoluble precipitate (EtOH-ppt) of noni fruits. Initially, the juice from the noni fruits significantly prolonged the lifespan of mice injected IP with Lewis lung carcinoma cells. Further studies to compare the EtOH-soluble precipitate and EtOH-ppt demonstrated the life-prolonging activity to be in the EtOH-ppt. This antitumor activity was abrogated by concurrent administration of the immunosuppressants, 2-chloroadenosine or cyclosporin, indicating that the activity may be due to an immune-stimulating effect of the EtOH-ppt (40). Furthermore, there was an increase in survival by the concurrent administration of the EtOH-ppt with the chemotherapeutic agents vincristine, 5-fluorouracil, cisplatin, and adriamycin when compared with each of these agents alone, suggesting a potential therapeutic complementary treatment or synergistic effect of noni (41). In a more recent study, this same research group performed similar studies using a sarcoma 180 tumor system in mice that is particularly responsive to immune system modulation. The EtOH-ppt, obtained from noni grown in both Hawaii and Tahiti, was similarly injected and demonstrated an increase in survival rate of mice, particularly when given prior to tumor cell inoculation. The number of mice surviving up to 40 to 50 days

without signs of tumor growth was increased by 45% and 53% when given prophylactic treatment with noni from Hawaii and Tahiti, respectively. This increase in survival in both the prophylactic and the therapeutic treatments with EtOH-ppt was inhibited using 2-chloroadenosine, anti-asialo GM1 antibody, and cyclosporin. These are specific inhibitors of macrophages, natural killer cells, and T-cells, respectively, which further support the theory of immune system involvement. Furthermore, similar increases in the survival of test mice were seen when the EtOH-ppt was administered along with certain anticancer agents, but not all (41). It should be emphasized that these studies, while promising, involve the IP injection of EtOH-ppt physically on top of the IP cancer cells, and no studies appear to have been published where the EtOH-ppt was administered orally with analogous efficacy. Recently, it has been shown that noni fruit juice and fruit juice concentrates (dose 1.5 mg/mL) from Tahiti activated cannabinoid 2 (CB₂) and inhibited cannabinoid 1 (CB₁) receptors, in a concentration-dependent manner. Coupled with this observation, in the same study administration of the fruit juice to mice for 16 days (*ad libitum*) decreased the production of interleukin-2 and increased that of interferon- γ cytokines, suggesting a modulation of the immune system (42). The agents responsible for these effects were not structurally defined.

Cardiovascular Disease

A methanol-soluble extract of noni leaf was tested for its ability to prevent oxidation of low-density lipoprotein (LDL) and upregulation of LDL receptors (LDLr) to determine if noni can prevent or slow down the processes involved in cardiovascular disease. The oxidation of LDL, referred to colloquially as "bad cholesterol," is considered a risk factor for atherosclerosis. The upregulation of LDLr in liver cells is believed to decrease one's risk for atherosclerosis by decreasing the overall LDL levels in the blood stream. By using these two *in vitro* bioassays, of 12 plants tested, noni was one of the two that did not demonstrate inhibition of LDL oxidation, yet it was one of the four that caused a significant increase in LDLr in liver cells. In fact, the noni extract was more effective than the positive control, green tea, at upregulating LDLr (43). In another study, noni fruit extract inhibited copper-induced LDL oxidation. Bioassay-guided fractionation of the ethyl acetate partition led to the isolation of six lignans, four of which, 3,3'-bisdemethylpinoresinol, americanol A, morindolin, and isoprincepin, demonstrated potent activity similar to or stronger than the positive control, butylated hydroxytoluene (17). In a recent report on noni produced in Okinawa, Japan, *ad libitum* intake of 10% noni juice given in the diet to mice for 7 days resulted in a protective effect on neuronal damage after ischemic stress, whereas a lower dose of 3% noni juice in the diet was not effective in this regard (44).

Antioxidant Activity

In a study funded by Morinda Inc. (Orem, Utah, USA), their product, Tahitian Noni Juice (TNJ), demonstrated antioxidant activity in both lipid hydroperoxide and tetrazolium nitroblue assays. TNJ exhibited a dose-dependent antioxidant activity that was compared with vitamin C, pycnogenol, and grape seed powder using the current

recommended daily allowance or manufacturer recommendations for testing concentrations. TNJ had a greater free-radical scavenging activity than the positive controls, but because varying doses were used, a direct comparison is difficult. Animal studies using 10% TNJ in the drinking water of female SD mice and male C57 BL-6 mice were performed to determine if TNJ can prevent 7,12-dimethylbenzanthracene (DMBA)-DNA adduction formation. After 7 days of administering TNJ, intragastric administration of the carcinogen, DMBA, was done and the animals were sacrificed after 24 hours. In both animal models, TNJ was able to decrease the amount of DMBA-DNA adducts in the heart, lung, liver, and kidney compared with negative controls (45). In a further study, using the leaf, root, and fruit methanol extracts and ethyl acetate partitions, antioxidant activity was measured using the ferric thiocyanate method and thiobarbituric acid test. The root methanol extract had comparable antioxidant activity to the positive controls, α -tocopherol and butylated hydroxytoluene. The ethyl acetate partitions of all parts of the plant tested were similar to the positive controls (46). In another study to determine possible antioxidant constituents, diphenylpicrylhydrazyl and peroxyxynitrite free-radical scavenging assays were used to test compounds isolated from a powdered noni fruit extract. Of the 19 compounds isolated from the *n*-butanol-soluble partition, the neolignan, americanin A, was found to exhibit potent free-radical scavenging activity in these bioassays (18).

Anti-inflammatory and Analgesic Activity

The ability of ethanol extracts of the bark and leaves, the fresh fruit juice, and the fruit powder of noni to inhibit cyclooxygenase I (COX-1) was determined using an in vitro bioassay that measured the arachidonic acid metabolites, PGE₂ and PGD₂. Using 3.4 mg/mL as test concentration, the fruit powder had a "high" inhibition of COX-1 whereas the leaf extract had a "moderate" inhibition, as defined by the authors. The IC₅₀ value of the noni fruit powder was 163.3 μ g/mL and the positive controls, aspirin and indomethacin, had IC₅₀ values of 241.15 and 1.19 μ g/mL, respectively (47). It has been suggested that TNJ has a higher selectivity for COX-2 than for COX-1, with a COX-2/COX-1 IC₅₀ ratio of 0.76, with celecoxib (CelebrexTM), indomethacin, and aspirin having a ratio of 0.34, 40, and 119, respectively (48). Three lignans and two common flavonoids (quercetin and kaempferol) isolated from noni fruits collected in Tahiti were found to exhibit inhibitory effects (IC₅₀ values <10 μ M) on two enzymes implicated in the inflammatory response, namely, 5-lipoxygenase (5-LO) and 15-lipoxygenase (15-LO) (49). Several saccharide fatty acid esters from the fruits of noni cultivated in Japan demonstrated inhibitory activities when tested in a mouse ear inflammation assay. These compounds exhibited IC₅₀ values in the range of 0.46 to 0.79 mg/ear, when skin irritation was generated using the phorbol ester, TPA (12-*O*-tetradecanoylphorbol 13-acetate; 1 μ g/ear) (38).

To determine potential analgesic activity, an aqueous extract of noni roots was given by IP administration to 9-week-old male Swiss mice prior to evaluation in writhing and hotplate tests (50). Acetic acid (1.2%) was injected for the writhing test and contortions and stretchings were counted over a 30-minute period. For both the writhing and the hotplate tests, morphine sulfate was used as the

positive control and each mouse was used as its own negative control. Larger doses of noni decreased the number of contortions in the writhing test (800–1600 mg/kg) and increased the reaction time in the hotplate test (400–800 mg/kg). The morphine antagonist, naloxone, reversed these effects. In further studies to examine the locomotor activity of the mice, it was found that noni did decrease locomotor activity. The authors of this study suggested that the observed sedative activity, in addition to the other findings, was supportive of a central analgesic activity of noni (51).

Antitubercular Activity

Both ripe noni fruit and leaves have demonstrated in vitro activity against *Mycobacterium tuberculosis*. Isolation work revealed several active compounds, including scopoletin and a mixture of the two ketosteroids, stigmasta-4-en-3-one and stigmasta-4,22-dien-3-one (23,52).

Safety, Toxicity, and Adverse Effects

There are several studies involving the administration of noni to laboratory animals with no perceived toxicities. However, one clinical report of a possible adverse event related to noni juice from a patient with chronic renal insufficiency who demonstrated elevated potassium levels despite claims of compliance with a low potassium diet. The patient was reported to be taking a "shot glass of noni juice before each meal." The authors surmised that the noni juice may be responsible for the hyperkalemia and tested its potassium content. They found the potassium level of a specific noni juice product to be 56.3 mequiv/L, which is similar to that of orange and tomato juice. The authors concluded that because the recommended dose is 1 to 3 oz of juice per day, noni juice does not pose a great threat to those on a potassium-restricted diet unless they exceed this amount. Unfortunately, the authors were unable to confirm whether the patient was compliant with the manufacturer's recommended dosage of noni juice or what product was used (53). There have been further reports of cases of hepatotoxicity associated with the use of noni juice (54–56). Accordingly, a follow-up toxicology was carried out and no evidence of genotoxicity was shown in primary rat hepatocytes and 4AIIIE rat hepatoma cells, although a slight mutagenic effect against *Salmonella typhimurium* strain TA1537 was observed and attributed to the presence of flavonoids (57). As one of the clinical reports suggested that the hepatotoxicity of noni juice might be due to the presence of anthraquinones (55), an analytical study was conducted and indicated the absence of such compounds in noni juice by high-performance liquid chromatography, with a limit of sensitivity of <1 ppm (57).

In addition to these reports, noni juice has been found to inhibit angiotensin-I-converting enzyme (ACE) (58). Because ACE inhibitors cause a decrease in potassium secretion, this activity, in addition to the potassium content, may be a concern for patients on potassium-restricted diets. Noni has not been clinically evaluated for safety in pregnant or lactating women. It has been reported that the ingestion of a large amount of noni fruits can cause an abortion and the root bark has been used as an abortifacient in the Pacific island of Futuna. This activity has not been confirmed experimentally (59).

Clinical Studies

There is an ongoing phase I clinical trial that began in November 2001 at the Cancer Research Center of Hawaii, in patients with clinically diagnosed cancer for which there is no standard treatment available. In this phase I clinical trial, freeze-dried noni fruit extract in 500 mg capsules is being given orally with several major goals, including determining the maximum tolerated dose, defining toxicities, determining any antitumor and/or symptom control, and identifying marker compounds for bioavailability and pharmacokinetic studies. This study is being funded by the NCCAM, National Institutes of Health, Bethesda, MD. More information on this study can be found online (60).

Regulatory Status Worldwide

Noni juice was approved as a "novel food" in the European Union in 2003 (61). Noni juice is widely available in retail outlets in the United States as a botanical dietary supplement.

CONCLUSIONS

Noni is regarded as one of the most important medicinal plants of Polynesia and is growing in popularity in the United States for a wide range of diseases including cancer, cardiovascular disease, and arthritis. The ability to cure or prevent most diseases of aging attributed to noni in promotional material is based on a compound called "xeronine." To date, there are no scientific data to confirm the presence of its precursor in noni fruits or the requirement of this compound in the body. A number of studies have demonstrated the potential beneficial activities of noni to treat or help prevent multiple diseases. However, these studies are limited in scope but warrant further investigation.

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Omega-3 Fatty Acids

William S. Harris

BIOCHEMISTRY AND FUNCTIONS

Long-chain, highly unsaturated fatty acids (HUFA) of the omega-3 (ω -3 or n -3) family include eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA), which are 20- and 22-carbon molecules, respectively, with five double bonds (Δ 5, -8, -11, -14, -17). Docosahexaenoic acid (DHA) has 22-carbon molecules and six double bonds (Δ 5, -8, -11, -14, -17, -20). All fatty acids (FAs) in the omega-3 family are characterized by having their first double bond at the third position counting from the terminal (ω or n th) methyl group in the molecule (Fig. 1). As with the omega-6 family, omega-3 FA cannot be synthesized *de novo* by mammals, and must be obtained from the diet. The extent to which EPA and/or DHA *per se* are essential nutrients is currently unknown.

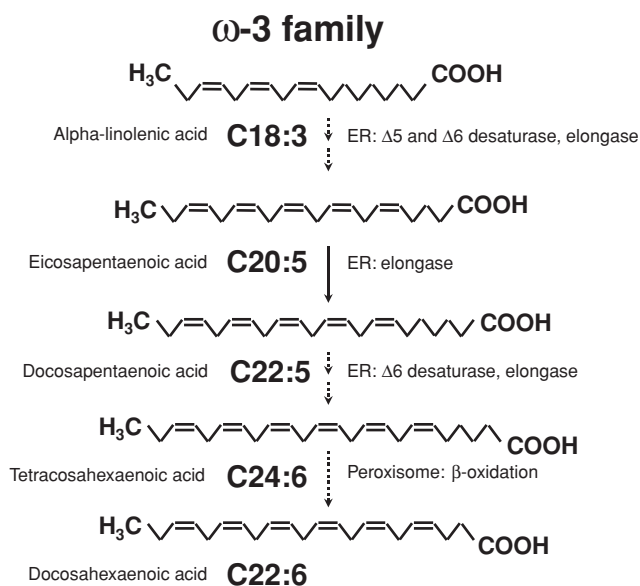


Figure 1 The principal FAs in the omega-3 family are alpha-linolenic acid (C18:3), which is derived from plant oils such as flaxseed, soybean and canola, and EPA (C20:5) and DHA (C22:6), which are usually consumed in fish and fish oils. There is quite limited conversion of α -linolenic acid to EPA and DHA. EPA feeding does not raise DHA blood levels, however, DHA feeding will produce a small increase in EPA levels. Whether this is from retroconversion or some other mechanism is not clear. *Abbreviation:* ER, endoplasmic reticulum.

Synthesis of EPA and DHA

EPA and DHA are natural constituents of the lipids of most marine and some fresh-water animals. These FAs are originally synthesized by microalgae at the base of the marine food chain (1). They move up the chain via phytoplankton, zooplankton, and small fish to larger fish and marine mammals (Fig. 2).

The mammalian biosynthesis of the long-chain omega-3 FAs from the parent omega-3 FA, α -linolenic acid (ALA), requires a series of elongations and desaturations (Δ -6 and Δ -5) and is believed to require processing in two organelles (the endoplasmic reticulum and peroxisomes) (2) (Fig. 1). Feeding EPA raises DPA but not DHA plasma levels, whereas feeding DHA has a minor EPA-raising effect (3). Whether this reflects true retroconversion or some other process is yet to be determined.

Efficiency of Conversion of ALA to EPA and DHA

In adult humans, the only currently known role for ALA is to serve as a precursor of DHA, but conversion is exceedingly slow (4). Reported rates vary from a low of less than 1% to 3–6% in young men up to 9% for young women; conversion of up to 15% have been reported, depending on the methodologic approach. Most of the dietary ALA (approximately 75%) is shunted to β -oxidation making it unavailable for conversion to the longer chain FAs.

Tissue Distribution of EPA and DHA

These FAs are found predominantly esterified in membrane phospholipids of tissues, circulating cells, and plasma lipoproteins. The distribution within plasma differs by lipid class, with phospholipids carrying the most omega-3 FAs in individuals consuming typically low omega-3 amounts from their diets. With omega-3 supplementation, EPA and DHA rise markedly in all lipid classes, the only anomaly being that DHA is relatively poorly transferred from phospholipids to cholesteryl esters by lecithin–cholesteryl ester acyltransferase (5). As EPA and DHA appear to exert their beneficial effects as components of membranes (6), the erythrocyte membrane has become a common tissue for assessing omega-3 status (7). In Western cultures where fish intake is low, EPA and DHA typically constitute approximately 3% to 5% of total RBC phospholipid FAs (8). However, in Japan, where EPA and DHA intake is approximately 5- to 10-fold higher, RBC EPA+DHA is approximately twice as high (9). Certain tissues are particularly rich in DHA and contain little EPA. These include the brain, spermatozoa, and rod outer segments of the retinal epithelium (10).

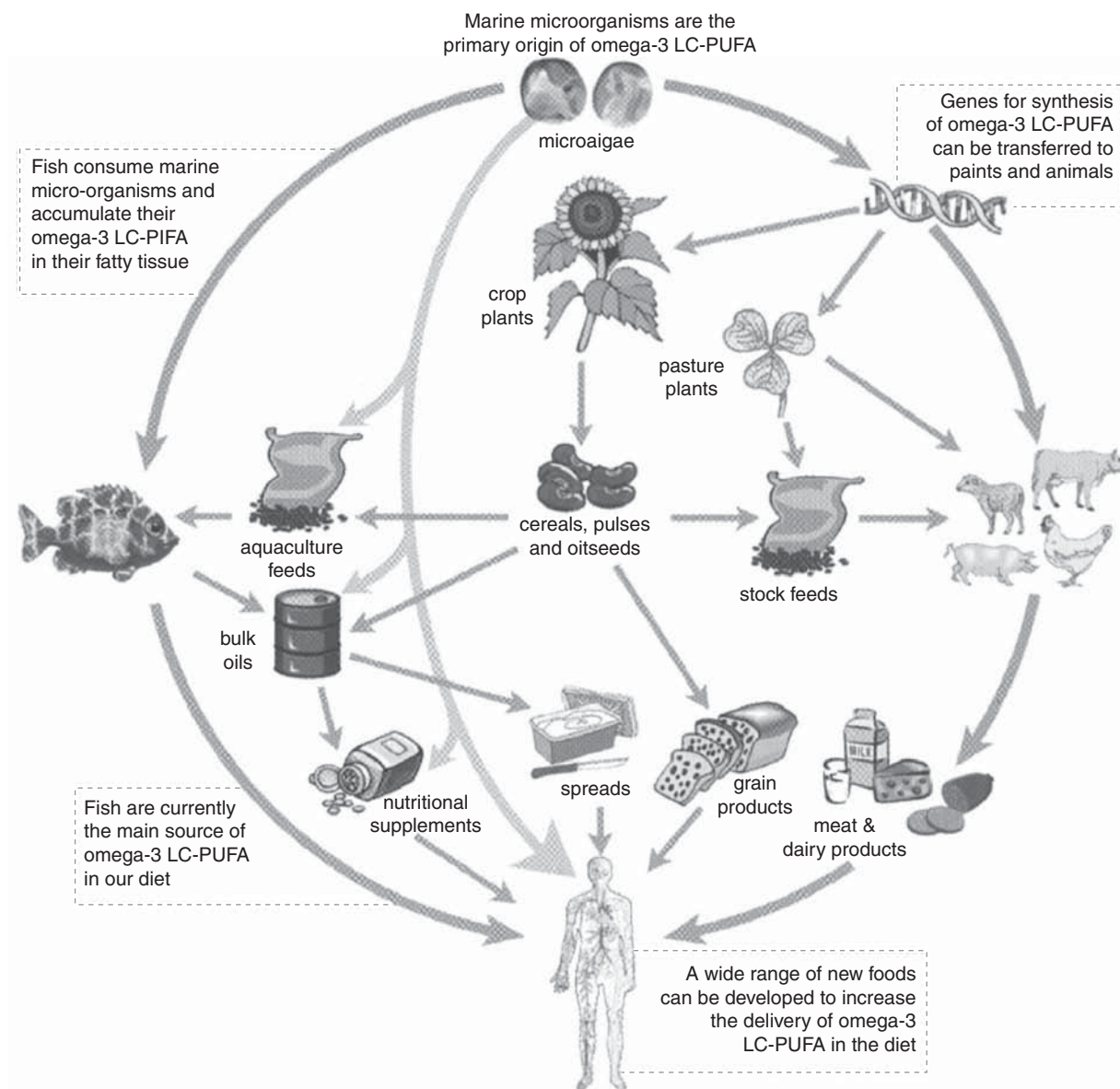


Figure 2 Present and future sources of EPA and DHA. The original source of the long-chain omega-3 FAs is marine microorganisms (algae, fungi, bacteria). These form the base of the marine omega-3 food chain resulting in fish (and fish oils) being enriched in EPA and DHA. Presently, DHA is (and likely EPA will be in the future) isolated from these organisms as a source for human nutrition. In addition, genes from these organisms can be transferred to certain plants (29) and even animals (30), enabling them to produce EPA and/or DHA.

Conversion of EPA and DHA to Eicosanoids and Docosanoids

In tissues undergoing physiological stresses, phospholipase A₂ becomes activated by a G-protein-mediated pathway, liberating the HUFA at position 2 of membrane phospholipids. Once cleaved, these FAs are available for conversion into eicosanoids (20-carbon moieties) and docosanoids (22-carbon moieties). The principal 20-carbon tri-, tetra- and pentaenoic HUFA are dihomo- γ -linolenic acid (DGLA; C20:3 *n*-6), arachidonic acid (AA; C20:4 *n*-6) and EPA. These three HUFA substrates give rise to three classes of eicosanoids: DGLA gives rise to the 1-series prostaglandins and the 3-series leukotrienes; AA to the

2- and 4-series leukotrienes, and EPA to the 3-series and 5-series leukotrienes. Beyond the eicosanoids, there are now a bewildering variety of other molecular species produced from EPA and DHA including thromboxanes; prostacyclins; lipoxins; resolvins and protectins (11); metabolites of P-450 enzymes (12); and anandamides and 2-acylglycerols (13) (Fig. 3).

With regard to biochemical potency, the eicosanoids produced from AA are generally more potent mediators of inflammation, vasoconstriction, and platelet aggregation than those made from EPA (14) or DGLA (15). There are, however, important anti-inflammatory and inflammation-resolving metabolites of AA as well, making it impossible

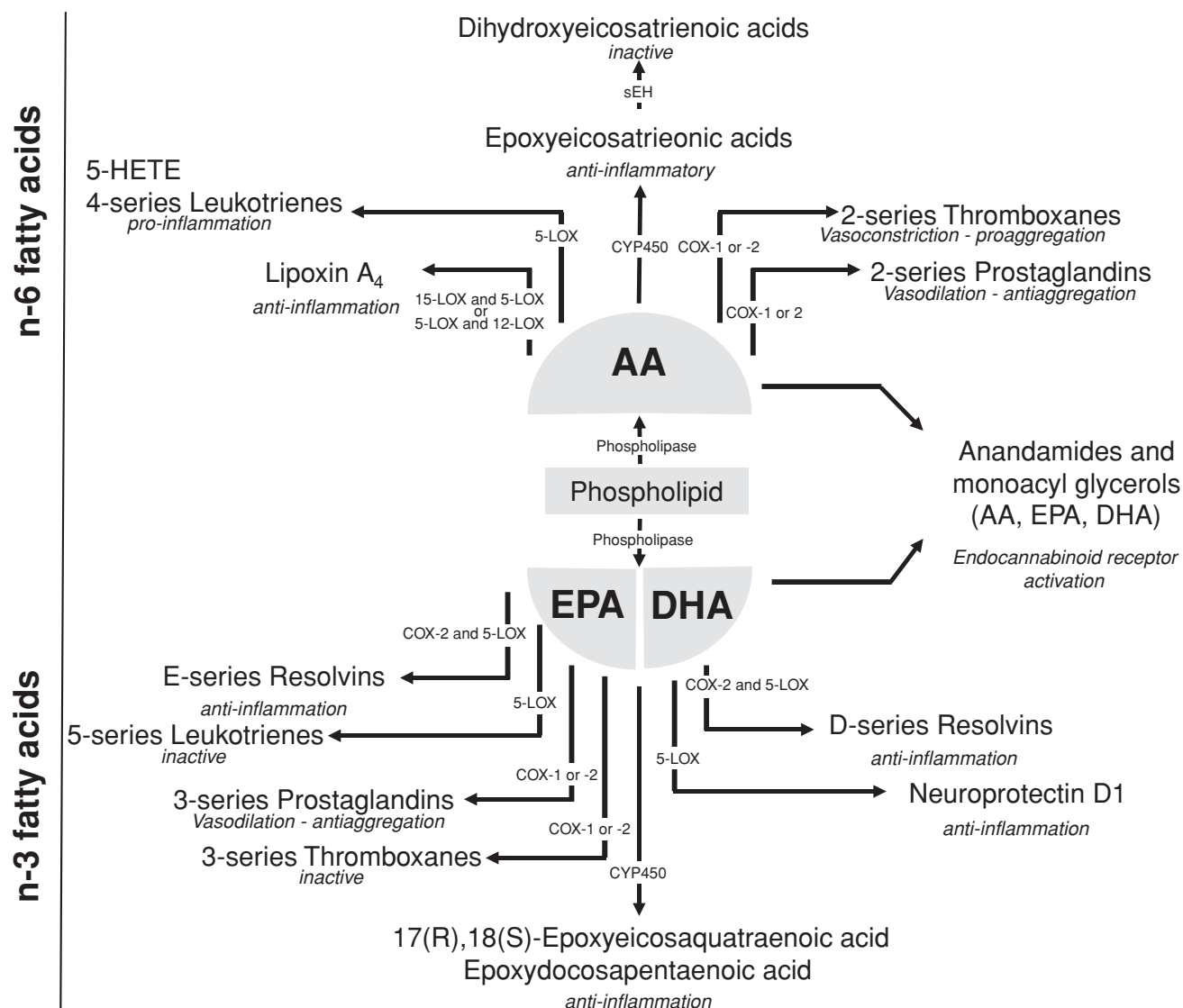


Figure 3 The eicosanoid and docosanoid metabolites of EPA and DHA contrasted with the metabolites of arachidonic acid (AA). Source: Courtesy of B. de Roos.

to characterize AA (and omega-6 FAs in general) as “proinflammatory” (16). AA is the predominant HUFA in tissue membranes in Western cultures where intakes of EPA and DHA are very low. The relative proportions of these precursor molecules present in membranes determine, to some degree, which eicosanoids are ultimately produced. Accordingly, a higher EPA and DHA content in membrane HUFA has the potential to diminish the biochemical and, thus, physiological responses to stress.

Membrane Ion Channels

The presence of omega-3 FAs, especially DHA, in biological membranes appears to play an important role in homeostasis (17). As an example, these FAs appear to alter the activity of certain ion channels. Original observations in experimental myocardial infarction (MI)

from Lands’ laboratory (18), as well as later studies by McLennan et al. (19) in rats and monkeys suggested that omega-3 FAs may have a direct protective effect on the heart itself independent of effects on serum lipids and platelets. In a dog model of ventricular tachyarrhythmia (20), infused omega-3 FAs reduced the number of potentially fatal arrhythmias. This led to investigations in isolated cardiac myocytes that revealed that EPA and DHA could prolong the refractory state of these cells by interaction with fast-acting sodium channels and L-type calcium channels (21). As noted later, the evidence for an antiarrhythmic effect of the relatively low doses of EPA+DHA associated with reduced risk for coronary heart disease (CHD) death (approximately 500–1000 mg/day) is relatively sparse. A study in 10 patients undergoing cardiac electrophysiological testing reported beneficial effects of an infused, omega-3 FA-rich emulsion on the susceptibility of the myocardium to dysrhythmias

(22), and the threshold for induced tachycardia was increased by omega-3 FA supplementation (23). Tissue levels of EPA+DHA achieved by supplementation with 1 g/day increase from approximately 1.7% to 2.9% of total cardiac FA (24). It is unknown whether this level of tissue enrichment with EPA+DHA produces the same biophysical effects on membrane function than the higher levels produced in experimental settings in cells, animals, or humans.

FOOD SOURCES

Fish are the primary source of the omega-3 FAs. The content per serving can vary markedly depending on the species of fish (Table 1). Higher fat fish such as salmon, mackerel, albacore tuna, herring, and sardines are excellent sources of EPA and DHA, whereas low fat fish such as bass, perch, tilapia, and cod are poor sources. Commercially available dietary supplements providing EPA and DHA are made with refined fish oils obtained from the high-fat species (and menhaden and anchovy). Fish oils are also derived from fish liver; indeed, for centuries cod liver oil was (and continues to be) the most widely used fish oil in the world.

Recognizing that fish contain omega-3 FA only because their food contains them, and that harvesting fish to obtain these FA cannot be sustained forever, interest has turned to microorganisms, the ultimate sources of these FA in the marine food chain (Fig. 2). A variety of species of algae, fungi, and bacteria have been discovered which, under specific environmental conditions, synthesize either DHA (27) or EPA (28). Omega-3 FAs derived from microbial sources are free of the "fishy" flavors characteristic of most fish oil supplements and are uncontaminated by any lipid-soluble pollutants (as are the refined fish oils). At this point, cost issues have largely prohibited their wide-scale use in supplements, but increased demand may bring more "single-cell oils" to the market. Additional future (but nonsupplemental) sources of long-chain omega-3 FAs may be through oils derived from genetically modified plants (Fig. 2). Soybeans transfected with a Δ -15 desaturase can synthesize stearidonic acid (SDA; C18:4 *n*-3) from ALA (which is the Δ -6 desaturase product of ALA metabolism in mammals), and SDA is more readily converted to EPA than is ALA (31). SDA-enriched soybean oil could thus provide an essentially inexhaustible, nonmarine source of omega-3 FAs for food fortification programs.

SAFETY OF OMEGA-3 FATTY ACIDS

Omega-3 FAs have been a part of the human diet for thousands of years with no known toxicity. The apparent safety of these FAs has been underscored by a 1997 U.S. Food and Drug Administration (FDA) ruling given in response to a request that menhaden oil (a widely available fish oil) be granted generally recognized as safe (GRAS) status. SDA-enriched soybean oil was granted GRAS status in 2009. The FDA ruled that intakes of up to 3 g/day of marine omega-3 FA should be considered GRAS for inclusion in the diet, and up to 2 g/day from dietary

Table 1 Approximate Levels of EPA + DHA in Fish and Fish Oils, and the Amounts Required to Provide Approximately 1 g/day of EPA + DHA

Fish	EPA+DHA gm/3 oz serving (edible portion)	Oz/day required to provide \approx 1 g of EPA+DHA per day
Tuna		
Light, canned in water, drained	0.26	12
White, canned in water, drained	0.73	4
Fresh	0.24–1.28	2.5–12
Sardines	0.98–1.70	2–3
Salmon		
Sockeye or Pink	1.05	3
Chinook	1.48	2
Coho, farmed	1.09	3
Coho, wild	0.91	3
Atlantic, farmed	1.09–1.83	1.5–2.5
Atlantic, wild	0.9–1.56	2–3.5
Mackerel	0.34–1.57	2–8.5
Herring		
Pacific	1.81	1.5
Atlantic	1.71	2
Trout, rainbow		
Farmed	0.98	3
Wild	0.84	3.5
Cod		
Atlantic	0.13	23
Pacific	0.24	12.5
Catfish		
Farmed	0.15	20
Wild	0.2	15
Flounder/Sole	0.42	7
Oyster		
Pacific	1.17	2.5
Eastern	0.47	6.5
Lobster	0.07–0.41	7.5–42.5
Crab, Alaskan King	0.35	8.5
Shrimp, mixed species	0.27	11
Clam	0.24	12.5
Scallop	0.17	17.5
Capsules	Omega-3 fatty acids g oil/day	g/g of oil
Cod liver oil ^a	0.19	5
Standard fish body oil	0.30	3
Omega-3 FA concentrate	0.50	2
Highly concentrated omega-3 FA	0.7–0.9	1

Note: Values derived from literature (25,26) and the USDA Nutrient Data Laboratory (<http://www.nal.usda.gov/fnic/foodcomp/>). Fish intakes (oz/day) are only estimates because EPA+DHA content can vary markedly with season, diet, age, and storage/preparation methods.

^aThis amount of cod liver oil would provide the Recommended Dietary Allowance of vitamin A and twice that of vitamin D.

supplements is recommended (acknowledging that the background diet may contain up to 1 g/day). Thus, the safety of omega-3 FA up to this level of intake is not in question. However, the extensive (tablespoon amounts) use of fish *liver* oils (cod, shark) is ill advised, owing to the high levels of fat soluble vitamins (A and D) present in these oils. Concerns about the presence of mercury in supplements and organochlorines are largely unfounded (32,33). Although the safety of omega-3 FA is apparently not an issue, there are side effects of supplementation. Perhaps the most common (seen in approximately 50% of subjects) is a "fishy" belch occurring soon after the

Table 2 Agency for Healthcare Research and Quality (AHRQ) Evidence-Based Reviews on Omega-3 Fatty Acids

Topic	Link
Omega-3 fatty acids and asthma (34)	http://www.ahrq.gov/clinic/tp/o3asthmtpt.htm
Omega-3 fatty acids and cancer (35)	http://www.ahrq.gov/clinic/tp/o3cantpt.htm
Omega-3 fatty acids and cardiovascular disease	
Serum risk factors and intermediate markers (36)	http://www.ahrq.gov/clinic/tp/o3cardrisktp.htm
CVD outcomes (37)	http://www.ahrq.gov/clinic/tp/o3cardtp.htm
Arrhythmia in animal models (38)	http://www.ahrq.gov/clinic/tp/o3arrtp.htm
Omega-3 fatty acids and child and maternal health	http://www.ahrq.gov/clinic/tp/o3mchtp.htm
Omega-3 fatty acids and cognitive function with aging, dementia, and neurological disease (39)	http://www.ahrq.gov/clinic/tp/o3cogntp.htm
Omega-3 fatty acids and eye health (40–42)	http://www.ahrq.gov/clinic/tp/o3eyetp.htm
Omega-3 fatty acids and lipids and glycemic control in type II diabetes and the metabolic syndrome and on inflammatory bowel disease, rheumatoid arthritis, renal disease, systemic lupus erythematosus, and osteoporosis (43)	http://www.ahrq.gov/clinic/tp/o3lipidtp.htm
Omega-3 fatty acids and mental health	http://www.ahrq.gov/clinic/tp/o3menttp.htm
Omega-3 fatty acids and organ transplantation (44)	http://www.ahrq.gov/clinic/tp/o3organtpt.htm

capsules are taken. This can be minimized by bed-time or meal-time consumption of supplements.

POTENTIAL HEALTH BENEFITS OF OMEGA-3 FATTY ACIDS

In early 2004, the first of a series of NIH-funded evidence-based reviews on omega-3 FAs and a wide variety of disease conditions was released by the Agency for Healthcare Research and Quality. These reviews examined the existing medical literature in several disease categories. All reports have now been issued and links to the reports as well as references to published summaries are listed in Table 2. As the effects of omega-3 FAs are most well established in cardiovascular disease, this evidence is summarized here.

Coronary Heart Disease

The first three reviews published all focused on the effects of omega-3 FA on cardiovascular disease (CVD): (i) clinical endpoints, (ii) risk factors, and (iii) possible arrhythmogenic mechanisms. The first concluded that the evidence for CVD benefit from consumption of fish or fish oil supplements was strong. The second found a consistent beneficial effect of EPA+DHA on serum triglyceride levels, but beyond that the benefits of omega-3 FA on CVD risk are not well explained by their effects on cardiovascular risk factors. The third concluded that EPA and/or DHA “might” have anti-arrhythmic effects but that the data were not conclusive. Some of the effects on membrane function and ion channel activity were already discussed and have recently been reviewed (45) as has the evidence for effects on serum lipids (46). The strongest support for a CHD benefit from increased omega-3 FA intake comes not from effects on surrogate risk markers but from studies on event rates themselves. These are summarized below and in the 2002 Scientific Statement from the American Heart Association (47).

Epidemiological Studies

Data indicating that omega-3 FA may protect against CHD began to accumulate in the 1970s when Danish investigators found that acute MI rates were significantly lower in Greenland Inuits than in age- and sex-matched Danes (48). On further investigation, a strong link between the

omega-3 FAs in their diets and their apparent protection from heart attacks began to emerge (49). In studies of other fish-eating populations such as the Japanese, omega-3 FA intakes were also associated with lower rates of CHD (50). A meta-analysis of 25 studies reporting the relations between blood or tissue omega-3 levels and risk for CHD events found significant inverse relations for DHA and for EPA+DHA and risk (51). The two perhaps most influential studies linking low blood omega-3 FA levels specifically to risk for sudden cardiac death were those from Siscovick et al. (52) and Albert et al. (53) (Fig. 4).

Randomized Clinical Trials

The strongest evidence for omega-3 FA benefit in CHD has come from large, randomized, controlled clinical trials with “hard” CHD endpoints such as total mortality, fatal and nonfatal stroke, and/or MI. These have been

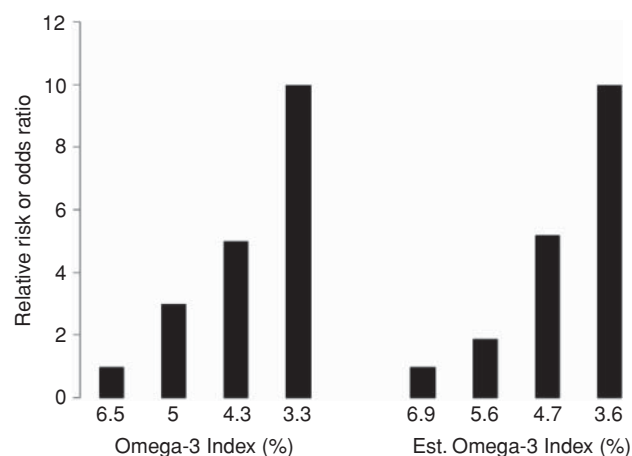


Figure 4 Relationship between the estimated omega-3 index (RBC EPA+DHA) and relative risk (RR) for sudden cardiac death (right), and the omega-3 index and odds ratios (OR) for primary cardiac arrest (left). The latter were derived from Siscovick, et al. (52) in a population-based case control study, and the former from Albert et al. (53) in a case-control study prospectively nested in the Physicians' Health Study. The estimated omega-3 index values were calculated from the whole blood EPA+DPA+DHA as described by Harris and von Schacky (24). * $P < 0.05$ versus quartile 1. Source: From Ref. 54.

performed with both recommendations to consume oily fish (55,56) and with omega-3 capsules (57–59). A major review in 2006 concluded that the evidence was strong for a benefit of omega-3 FAs for reducing risk for death from CHD; moderate for ischemic stroke; equivocal for nonfatal events, recurrent ventricular tachyarrhythmias, atherosclerosis progression, and restenosis postangioplasty; and limited for atrial fibrillation and congestive heart failure (60). When restricted to supplementation studies alone, significant effects on cardiac mortality were observed, but not on total mortality. This is partly because the largest study included (JELIS, the Japan EPA Lipid Intervention Study, described later) was conducted in a population in which cardiac death was among the lowest in the world (presumably owing to an average EPA and DHA intake of approximately 1 g/day), and there was little room for improvement (59).

JELIS

JELIS was the single largest omega-3 treatment trial and was conducted with EPA alone. It included 18,645 hypercholesterolemic patients who were all placed on statins, and then randomly assigned to receive either 1800 mg/day of EPA ethyl esters (Mochida, Japan) or usual care for 4.6 years (59). The primary endpoint was any major adverse coronary event (MACE), including sudden cardiac death, fatal and nonfatal MI, and other nonfatal events including unstable angina pectoris, angioplasty, stenting, or coronary artery bypass grafting. Both primary and secondary prevention patients were included. In the overall analysis, allocation to EPA was associated with a 19% ($P = 0.01$) reduction in major coronary events (Fig. 5). EPA treatment was also associated with a significant 24% reduction in the incidence of unstable angina and a 19% decrease in nonfatal coronary events. This treatment also produced nonsignificant reductions of 21%, 25%, and 14% in fatal MI, nonfatal MI, and revascularizations, respectively.

A secondary prevention subanalysis of JELIS focused on the effects of EPA in the approximately 3400

statin-treated patients with established CAD for which 5-year follow-up was available, the overall MACE rates were significantly lower in the EPA group (8.7% vs. 10.7%, $P = 0.017$), and among the 1050 patients with prior MI, EPA reduced MACE rates by 27% (15.0% vs. 20.1%, $P = 0.033$) (61). In the latter category the number needed to treat was only 19. Thus, in this highest of risk groups, EPA proved to be highly effective, even in combination with statin therapy and a high background fish consumption.

Stroke was an endpoint of particular interest for two reasons: Japanese stroke rates are higher than CHD rates, and there has been some concern that EPA (because of its mild antiplatelet effects) might actually increase risk for hemorrhagic strokes. In another subanalysis, the effects of EPA on the secondary prevention of stroke were examined (62). Recurrent stroke rates were reduced from 10.5% to 6.8% ($P < 0.05$). Thus, EPA was not only safe (vis-à-vis stroke risk), but it also actually reduced risk for this major endpoint in those with a previous history of stroke.

The vast majority of patients in JELIS had no history of CHD ($n = 14,981$). In a subanalysis of these primary prevention patients, MACE rates (regardless of other risk factors) were 71% higher ($P = 0.014$) in those with both low high density lipoprotein cholesterol (HDL-C) and high triglycerides (TG) (63). In this subgroup, EPA treatment reduced MACE risk by 53% ($P = 0.043$). Thus, a metabolic-syndrome-like profile increased risk for MACE in these hypercholesterolemic patients, and in those with this pattern, even while taking statins, EPA was very effective in reducing risk. EPA produced no clinically meaningful changes in lipids, glycemic measures or blood pressure, but the cardioprotective effects seen in the normoglycemic patients was also observed in those with impaired fasting glucose levels (64).

Hypertriglyceridemia

There are multiple clinical trials that show that pharmacologic doses of omega-3 FAs can have a significant effect on elevated triglycerides. The magnitude of the effect is

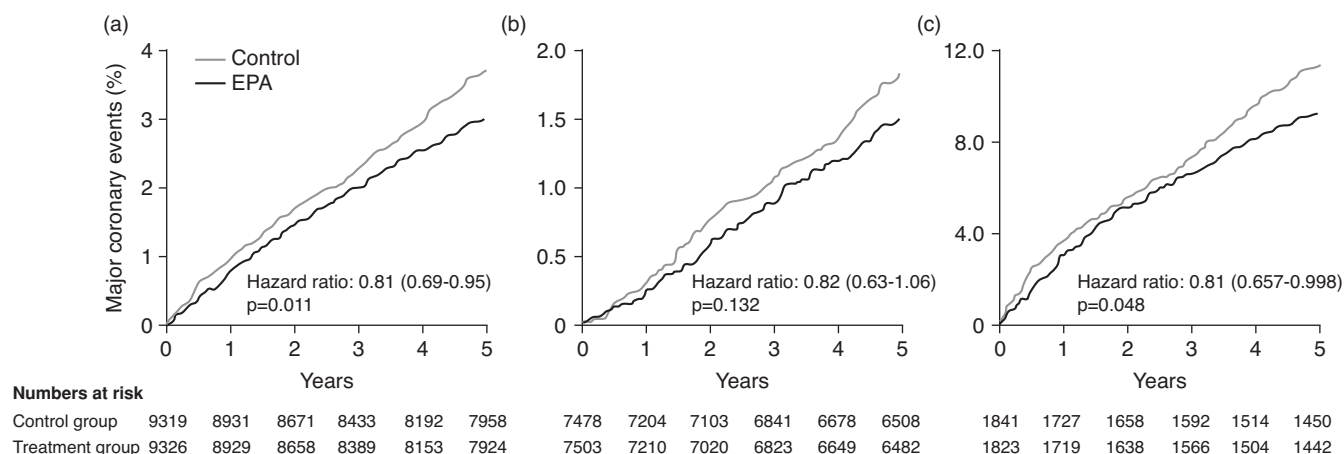


Figure 5 The Japan EPA Lipid Intervention Study (JELIS). The effects of EPA versus control in hypercholesterolemic patients taking statins on major coronary events in the entire cohort (A), the primary prevention cohort (B), and the secondary prevention cohort (C). Source: From Ref 59.

dependent on the baseline triglyceride level and there appears to be a dose response effect above 2 g/day (46). In general, triglycerides can be lowered 20% to 50% but may require doses of up to 4 g of EPA+DHA. The prescription omega-3 product (Lovaza in the United States, Omacor elsewhere; Pronova Biocare, Oslo) is approved for treatment of triglycerides >500 mg/dL at a dose of 4 g/day per day. In Europe, it is also approved at 1 g/day for secondary prevention in post-MI patients. The complete mechanism of action of omega-3 fish oil is unknown but appears to involve the activation of PPAR receptors, decreasing the incorporation of triglycerides in VLDL particles and perhaps increases in beta oxidation of FAs (65,66). Omega-3 FAs can be combined with fibrates, niacin and/or statins (67) for additive effects. The adverse effect profile of fish oil is very limited and its use is generally very safe.

Blood Omega-3 FA as a CHD Risk Factor

Given the evidence summarized above, blood biomarkers of EPA+DHA are independently associated with increased risk of death from CHD. This suggests that they might serve as a new risk marker, if not a risk factor (68). We have proposed that the content of (i.e., percent of total FAs as) EPA+DHA in RBC membranes (the "Omega-3 Index") be considered such a risk factor (24,54). Based on clinical studies in our laboratory and a review of the literature, we proposed that an Omega-3 Index of 8% to 10% was associated with the greatest cardioprotection, whereas an Index of less than 4% was associated with the least. This target was recently supported by reports that the average omega-3 index in older Japanese subjects (where cardiovascular disease is rare) is in the 8% to 10% range (9). Although further work will be needed to thoroughly validate and refine such a marker, the Omega-3 Index may represent a novel, physiologically relevant, easily modified, independent, and graded risk factor for death from CHD that could have significant clinical utility.

Neurocognitive Health

There are data supporting the relationship between fish intake during pregnancy and reduced incidence of postpartum depression in England (69) but a similar study from Holland did not confirm the findings (70). In the former study, children born of women underconsuming fish during pregnancy showed some evidence of developmental delay (71), an important finding if confirmed. Later in life, the possible relationship between low intakes of omega-3 FAs and hostility has been suggested (72,73). Finally, there is currently great interest in the possibility that a chronic low omega-3 FA status may increase risk for hyperactivity, depression, and perhaps even dementia (74). Randomized trials to test the effects of omega-3 FA supplements in all of these conditions are urgently needed.

RECOMMENDED INTAKES

In the United States, there are currently no specific, federally endorsed dietary recommendations for the long-chain omega-3 FAs. In 2002, the National Academy's Institute of Medicine recommended intakes of ALA of up to 1.2% of

energy on the basis of median intakes of healthy individuals in the United States (75). Although the report noted that up to 10% of the ALA target could be supplied as EPA and DHA (approximately 300 mg), it did not make a specific recommendation per se for these FA. The US Food and Drug Administration, however, considers an intake of 3 g of EPA and DHA (combined) to be safe, but recommends that no more than 2 g be consumed as supplements. A variety of expert panels from around the world have recommended intakes of 200 to 800 mg/day of EPA+DHA (76). The American Heart Association currently (47) recommends that for patients with known CHD, an intake of approximately 1 g of EPA+DHA appears to be prudent, and for those without known CHD, two (preferably oily) fish meals per week. This would translate into an intake of approximately 500 mg/day of EPA+DHA, an amount that has been associated with the lowest rates of death from CHD observed in major epidemiological trials conducted in the United States (52,77–80). In pregnant women, the focus is specifically on DHA, and the current recommendation of a consensus group in Europe is 200 mg/day (81). There are currently no official guidelines for intakes in children and adolescents, but another consensus panel recently recommended that infant formulas contain between 0.2% and 0.5% of total fat as DHA, and that EPA not exceed DHA (82).

Regulatory Status

At present, there is a qualified health claim for omega-3 FAs that may be included on the labels of dietary supplements or conventional foods: "Supportive but not conclusive research shows that consumption of EPA and DHA omega-3 FAs may reduce the risk of coronary heart disease. One serving of [name of food] provides [x] grams of EPA and DHA omega-3 FAs." There is, however, no Dietary Reference Intake for EPA and DHA. As noted earlier, there is a pharmaceutical omega-3 preparation approved by the FDA as an adjunct to diet for the treatment of very high triglyceride levels. Recommended doses of this agent are 4 g/day.

CONCLUSION

There is compelling evidence that increased intakes of omega-3 FAs may contribute to the prevention of CHD, and tantalizing evidence supporting benefit in variety of other diseases. Although dietary (oily fish) sources should continue to be the first-line approach to increasing intakes of omega-3 FA, increasing concerns about the safety of the fish supply will undoubtedly lead many to consider using supplements that carry a lower risk for contamination. Because an apparent cardioprotective intake is approximately 1 g/day and most capsules contain at least 300 mg of EPA+DHA per 1-g capsule, only 3 g of fish oil concentrate may be needed to confer benefit. There is no evidence to date that such an intake would have any adverse health effects, nor interact negatively with any known drugs. Consequently, interest in and use of omega-3 FAs for health promotion is likely to continue to grow.

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Omega-6 Fatty Acids

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INTRODUCTION

The term “essential fatty acids” (EFAs) applies to vitamin-like fatty acids required in the diet and necessary for animal and human health (1). The two types of EFAs, omega-6 (ω -6) and omega-3 (ω -3), have similar biochemical properties that can lead them to compete with each other in many metabolic and physiologic processes. Both types of EFAs originate from plants and photosynthetic algae, and both occur throughout the human food chain. The best-known ω -6 EFAs are linoleic acid (LA; 9Z,12Z-octadecadienoic acid) and arachidonic acid (AA; 5Z,8Z,11Z,14Z-eicosatetraenoic acid). α -Linolenic acid (ALA; 9Z,12Z,15Z-octadecatrienoic acid) and eicosapentaenoic acid (EPA; 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid) are the corresponding ω -3 homologs. The intake of ω -6 EFAs in the United States averages approximately 7% of food energy (7%), which is eight times the average intake of ω -3 EFAs. Dietary supply and metabolic competition affect the proportions of ω -3 and ω -6 EFAs that accumulate in tissues (2). Chain length and the number and position of double bonds in fatty acids determine their ability to interact with metabolic enzymes and other protein targets. Questions about the metabolic specificity for EFAs are confounded by the presence of multiple related enzymes and receptors with overlapping selectivities. A few proteins discriminate appreciably between ω -6 and ω -3 structures with potentially important physiologic consequences. For example, linoleic acid and AA and their ω -3 counterparts are precursors for the formation by cells of potent hormone-like products called eicosanoids. Cyclooxygenases involved in prostaglandin (PG) biosynthesis and certain PG receptors show selective actions with ω -6 versus ω -3 structures. Although dietary requirements for dietary ω -6 EFAs can be attributed to beneficial ω -6 eicosanoid actions, excessive ω -6 eicosanoid production is associated with undesired immune-inflammatory events, tumor cell proliferation, and thrombosis. Some scientists hypothesize that significant decreases in inflammatory and cardiovascular diseases would occur were the ratio of ω -6 EFA to ω -3 EFA in foods decreased to values closer to 1, which would lower the availability of ω -6 eicosanoid precursors in tissues and consequently decrease formation of ω -6 eicosanoids.

STRUCTURES AND SOURCES OF EFAs

Vertebrates form saturated fatty acids such as palmitic (hexadecanoic) and stearic (octadecanoic) acids through

combined actions of acetyl CoA carboxylase and the fatty acid synthetase multienzyme complex that give sequential addition of two carbons to the carboxyl end of the fatty acid carbon chain (3). Vertebrates also form the monounsaturated fatty acids, ω -7 palmitoleic (9Z-hexadecenoic acid) and ω -9 oleic acid (9Z-octadecenoic acid), by oxidation of saturated fatty acids with molecular oxygen catalyzed by the Δ 9 desaturase (4,5). Monounsaturated fatty acids can be converted to polyunsaturated fatty acids (PUFAs) with more than one double bond and to highly unsaturated fatty acids (HUFAs) with 20 or more carbon atoms and three or more double bonds by other desaturases acting at different positions along the carbon chain to form methylene-interrupted cis double bonds ($-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$) characteristic of biological systems (4,5).

Figure 1 shows how ω -6 LA, an 18-carbon PUFA containing two double bonds, is converted to various HUFAs with 20 or more carbon atoms and three or more double bonds, such as ω -6 AA. Formation of ω -6 AA from ω -6 LA involves sequential actions of an acyl-CoA synthetase, Δ 6 desaturase, two-carbon chain elongation, Δ 5 desaturase, and an acyl-CoA hydrolase. A unique, comprehensive study of the conversion of ω -6 LA to various elongated and desaturated HUFA showed that both ω -6 LA and its ω -3 analog, ALA, have very similar dynamics of distribution and turnover in 25 different tissues, with liver being the major locus for HUFA formation (6). The many ω -6 HUFA intermediates in Figure 1 must be formed to the same extent as their subsequent products, but selective transfers that are poorly understood (see later) cause only a few HUFAs to accumulate appreciably in various tissue lipids. Over the course of 600 hours, 18% of the initial ω -6 LA was accumulated unchanged in various tissues (especially adipose triacylglycerols), 79% was catabolized and 2.6% accumulated in tissue lipids as ω -6 HUFA esters [especially muscle phospholipids; (6)]. The “pulse-chase” design confirmed an earlier report of similar precursor-product dynamics for both ω -6 and ω -3 types of HUFAs, which compete with each other in the elongation-desaturation events (7).

Vertebrates do not have Δ 12 or Δ 15 desaturases (4,5) to form EFAs with double bonds at 6 (ω -6) and 3 (ω -3) carbons from the methyl end of the chain. However, various plants have different amounts of Δ 12 and Δ 15 desaturases, and they synthesize different amounts of the 18-carbon fatty acids, ω -6 LA and ω -3 ALA (4). Diatoms, which are photosynthetic phytoplankton, some fungi and moss, and the worm *Caenorhabditis elegans* can form both LA and ALA and also elongate and desaturate these PUFAs to form HUFAs (4,5,8). For example, up to 30% of

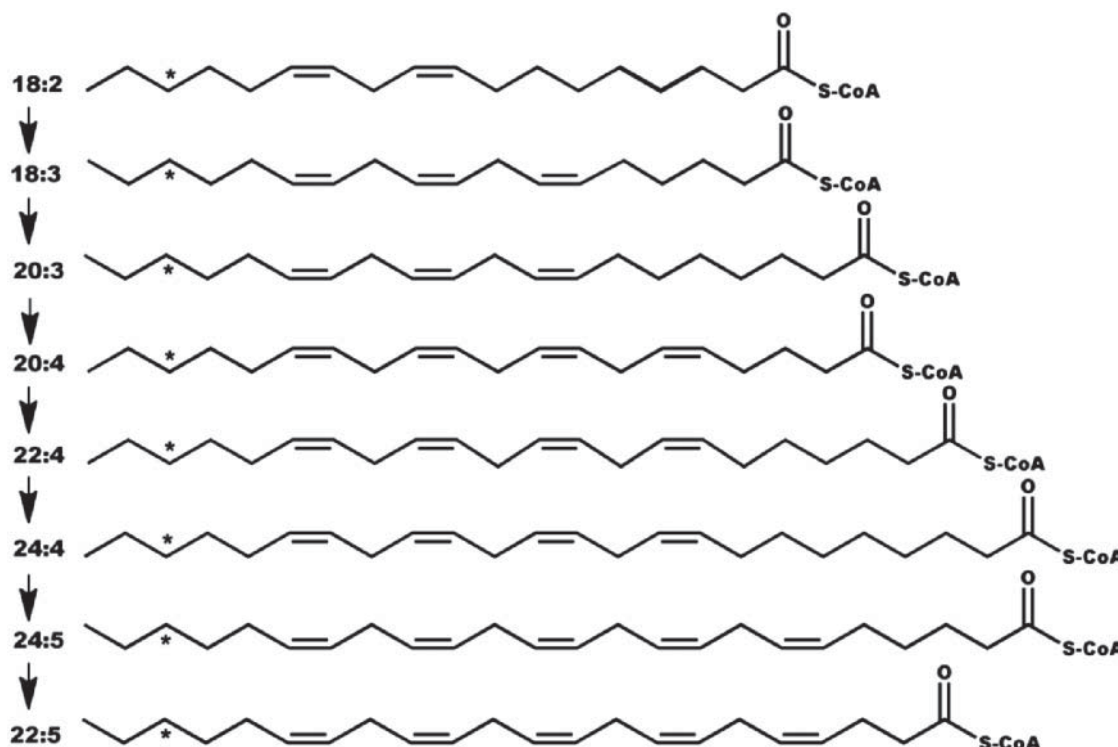


Figure 1 Structures and biosynthetic relationships of ω -6 highly unsaturated fatty acids (HUFA) formed from ω -6 LA by the elongation-desaturation pathway. The asterisk indicates the location of the ω -3 double bond in the competing ω -3 homolog that affects the proportions of various HUFAs available to tissues.

the fatty acids in diatoms can be the ω -3 HUFA, EPA. Diatoms provide a rich supply of ω -3 HUFA to the maritime food chain—an abundance, which is not found in land-based foods. The relative availability of ω -6 EFA in typical American foods has many important consequences.

Seed oils

A major source of ω -3 and ω -6 fatty acids for humans is plant-based oils, which are mixtures of triacylglycerols. Their relative proportions of saturated (mostly palmitic acid (hexadecanoic acid), monounsaturated (mostly oleic acid), and polyunsaturated ω -6 LA and ω -3 ALA vary widely [Fig. 2; (9)]. Corn oil (53% LA) is a common food oil in the United States, used in cooking and baking and in salads. Similarly, soybean oil (51% LA) has abundant ω -6 EFA, and its modest content (6.8%) of ω -3 ALA is the major source of ω -3 EFA for Americans. High percentages of LA are also in safflower (74%) and sunflower (66%) oils, although some commercial high-oleic acid variants of these oils have only 14% and 3.6% LA, respectively. Olive oil is also high in oleic acid (76%), and it has only 9.8% LA and 0.8% ALA. In contrast to most food oils with more ω -6 than ω -3, the high percentage of ω -3 ALA (53%) in flaxseed oil is several-fold more than that of ω -6 LA (13%).

Meats

Meats consumed in the United States (except seafood) contain relatively high proportions of the ω -6 LA and AA and low proportions of the ω -3 ALA, EPA, and DHA (4Z,7Z,10Z,13Z, 16Z,19Z-docosahexaenoic acid). This is

primarily because most farm animals are raised on diets containing LA-rich corn and soybeans, and part of the LA is accumulated as AA. For example, a 100 g (3 oz) serving of broiled pork loin has 508 mg of LA, 20 mg of ALA, and 59 mg of AA; a similar serving of beef has 488, 189, and 34 mg, respectively; and a similar serving of lamb has 310, 140, and 30 mg, respectively. All have very small amounts of ω -3 HUFAs.

Seafood

Freshwater and saltwater fish contain relatively high levels of the 20- and 22-carbon ω -3 HUFAs with lower levels of ω -6 fatty acids. A 100 g serving of broiled sockeye salmon has 113 mg of LA, 62 mg of ALA, 30 mg of AA, 530 mg of EPA, and 700 mg of DHA. However, farm-raised fish contain higher levels of ω -6 fatty acids (373 mg of LA, 76 mg of ALA, 94 mg of AA, 408 mg of EPA, and 871 mg of DHA) because of diets selected by farmers. Readers can learn more from the US Department of Agriculture web-accessed nutrient database (10) and the National Institutes of Health web page with downloadable software describing the amounts of essential PUFAs and HUFAs in over 9000 different food servings (11).

PATHOLOGIES ASSOCIATED WITH ω -6 EFA DEFICIENCIES

Studies in the late 1920s and early 1930s showed that rats fed chow diets that had been extracted with organic

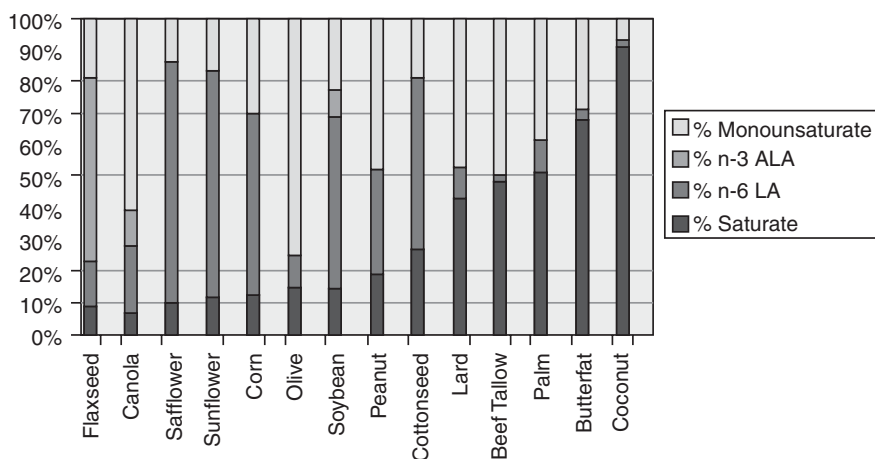


Figure 2 The percentages of saturated fatty acids, monounsaturated fatty acids, LA (*n*-6), and ALA (*n*-3) comprising the triglycerides (oil fraction) of various seeds, lard, and beef.

solvents to eliminate fats had a syndrome manifested by (a) reproductive difficulties, especially with parturition; (b) scaly skin associated with excessive water loss; and (c) a general failure to grow and thrive (12). This syndrome is called EFA deficiency. An accompanying accumulation of ω -9 HUFA in tissues was recognized later (13), consistent with a deficit of competing ω -3 and ω -6 HUFA CoA esters. Although most studies on EFA deficiencies were done with rodents, similar evidence occurred for humans fed artificial infant diets (14) or parenteral diets lacking EFAs (15). Dermal signs of deficiency were absent when dietary ω -6 EFA was more than 0.3% of daily food energy (13,14) or when tissue ω -9 HUFA formed from ω -9 oleic acid was less than the ω -6 HUFA formed from ω -6 linoleic acid (15,16). Both LA (12,17) and AA (17) were effective against some aspects of deficiency. However, reports have described the ω -3 fatty acid ALA as less effective (12), ineffective (17), or equally effective (13). Thomassen (18) obtained consistent differences in measured ω -3 and ω -6 efficacies by using a water-limited diet.

In the case of impaired parturition seen in EFA deficiency, an ω -6 prostanoid metabolite of AA—probably PG $F_{2\alpha}$ (PGF $_{2\alpha}$)—is apparently not produced in sufficient quantity. Indeed, descriptions of difficulties in parturition in EFA-deficient rats (17) and in cyclooxygenase-1 knockout mice (19) are strikingly similar, and animals lacking cytosolic phospholipase $A_{2\alpha}$ (20) or the PGF receptor, FP, (21) also exhibit a similar parturition phenotype. As discussed in more detail later, PG endoperoxide H synthases (known generically as cyclooxygenases) catalyze the conversion of ω -6 AA to oxygenated metabolites called prostanoids, one of which is PGF $_{2\alpha}$ (22).

The growth retardation and failure-to-thrive abnormalities characteristic of EFA deficiency may also reflect a deficit of oxygenated metabolites of AA involved in regulating hypothalamic/pituitary hormone secretions (23,24). ω -3 ALA, presumably because it is converted to EPA and its oxygenated metabolites, can also support body growth (12,13).

Interestingly, the scaly skin syndrome of EFA deficiency that is ameliorated by LA may not require AA or its oxygenated metabolites. Felinae have low levels of the Δ 6 desaturase needed to convert LA to longer chain HUFAs (Fig. 1), but dietary LA resolves the scaly skin problems of EFA-deficient cats (25). LA (or AA) may function in skin to form *O*-acylated sphingolipids such as 1-*O*-linoleoyl-ceramide (2) or hydroxy fatty acids such as 13-hydroxy-9*Z*,13*E*-octadecadienoic acid (13-HODE) (27). However, AA also resolves this abnormality, so homologous ceramide or hydroxyl fatty acid metabolites of AA (instead of LA) may also function to relieve the scaly skin problem.

Conversely, there is a distinct ω -3 fatty acid deficiency, most likely a deficit of ω -3 DHA in neurons, which results in various neurological deficits. This deficiency is not compensated by accumulated ω -6 DPA (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-docosapentaenoic acid) (1,28). The ω -3 fatty acids are treated as a subject elsewhere in this encyclopedia.

ACCUMULATION OF ω -6 EFAs IN DIFFERENT TYPES OF LIPIDS

ω -6 AA and LA do not accumulate to a significant extent as nonesterified 'free' fatty acids in vivo. Most acylated versions have the carboxyl group bonded to an oxygen, sulfur, or nitrogen atom including (a) arachidonoyl- and linoleoyl-CoA esters (i.e. thioesters); (b) triacylglycerol esters; (c) arachidonoyl and linoleoyl esters esterified at the *sn*2 position of glycerophospholipids (i.e. phosphatidylcholine, phosphatidylserine, phosphatidylinositol, or phosphatidylethanolamine) that comprise cell membranes; (d) arachidonoyl ethanolamide (anandamide); and (e) 1-*O*-acyl sphingolipids; (f) phospholipid degradation products retaining arachidonoyl and linoleoyl chains esterified at the *sn*2 position of

metabolic or signaling intermediates such as (i) phosphatidic acid formed from phosphatidyl inositol derivatives by the action of phospholipase D; (ii) 1,2-diacylglycerol formed by the action of phospholipase C; (iii) 2-arachidonylglycerol formed by sequential actions of phospholipase C and diglyceride lipase; and (iv) 2-acylglycerophospholipids formed via phospholipase A₁.

The percent of ω -6 LA in food calories has a linear affect on the weight percent of LA stored in adipose fat (23,29). With 25% of body mass as fat and approximately 15% of that as LA, an "average" 70-kg American male maintains a supply of 2625 g of ω -6 LA that is steadily mobilized and mixes with newly ingested fatty acids as ω -6 LA is replenished daily. ω -6 AA is only 1% of plasma triacylglycerols and nonesterified fatty acids (23,30); however, continued release from the corresponding 175 g supply in adipose may have more physiologic impact than is currently acknowledged. The plasma free acids released from adipose tissue or generated by hydrolysis of circulating triacylglycerols and phospholipids have a very short 1-minute half-life and seem to enter cells nonselectively. However, once inside the cell, the relatively low *K_m* of long-chain acyl-CoA synthetases for HUFAs such as AA may help to sequester HUFAs into cellular lipids (31). Because of the overlapping specificities of the five known acyl-CoA synthetases, it is difficult to test the hypothesis that acylation of HUFAs by these synthetases concentrates these fatty acids in cells (32).

ACCUMULATION OF ω -6 EFAs IN MEMBRANE PHOSPHOLIPIDS

The relative proportion of ω -6 fatty acids in tissue HUFA esters responds in a predictable manner to dietary intake (2,23) most likely reflecting competitions during the elongation-desaturation processes in liver (6,7). Acyl chains of HUFA accumulate at the *sn*2 position of 1,2-diacylglycerophospholipids during *de novo* synthesis or lipid "retailoring" (Fig. 3). During *de novo* biosynthesis of glycerophospholipid from glycerophosphate, an unsaturated acyl group (most often oleate or linoleate) from an acyl-CoA esterifies 1-acylglycerophosphate (LPA; lysophosphatidic acid). Exclusion of the ω -6 AA from the *de novo* pathway is evident from *in vivo* studies, but the apparent specificity *in vitro* depends on incubation conditions (33); for example, acylation of the *sn*2 hydroxyl group is less selective when LPA levels are elevated. The altered apparent specificity when increased nutrient intake elevates LPA abundance likely involves multiple acyltransferases (33).

Extensive studies of detailed acyl-chain structure differences have shown acyltransferase activity to have surprising specificities not predicted by general biophysical concepts (2). Thus, careful interpretations with extensive controls will be needed to describe the factors that affect accumulation of ω -6 HUFA among the multiple-membrane phospholipids (Fig. 3). Progress is now being

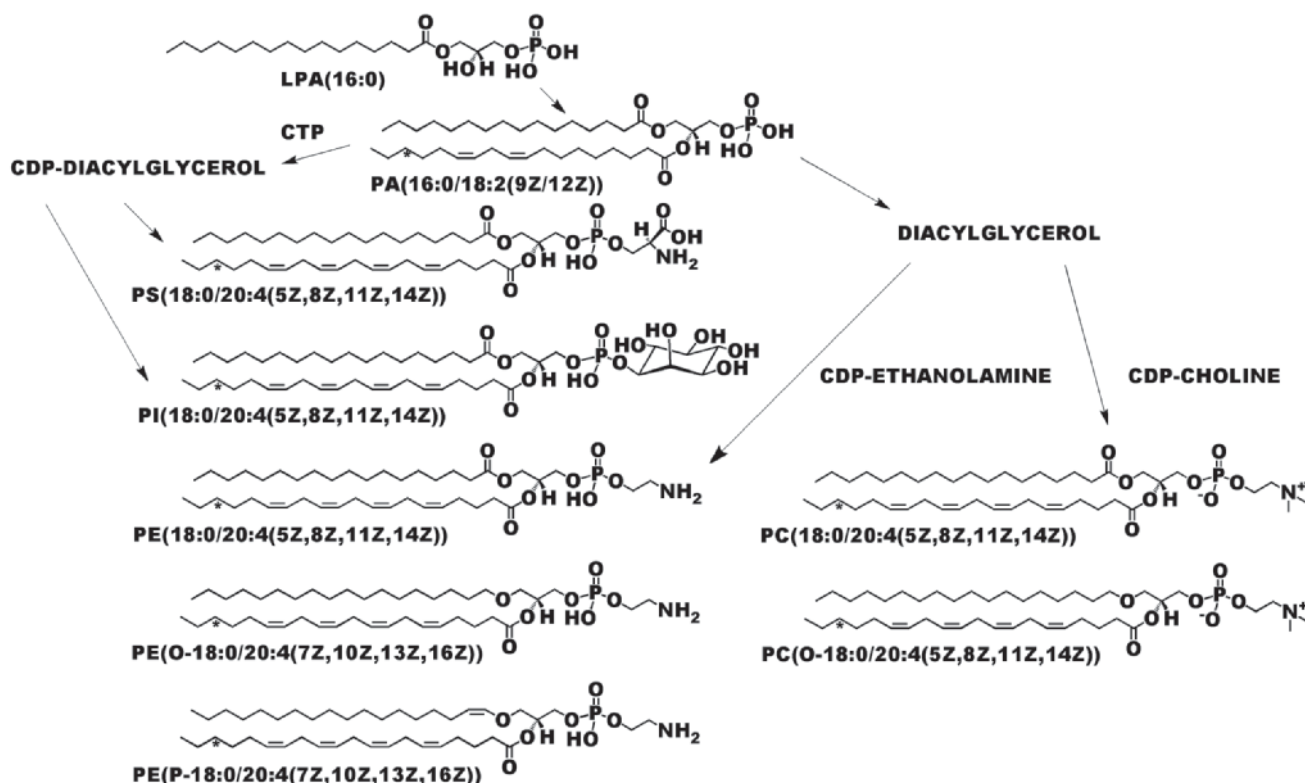


Figure 3 Phospholipids formed by *de novo* glycerolipid biosynthesis and by retailoring processes. HUFAs tend to be excluded when acylating 1-acyl-GP (LPA) during normal physiological conditions. They appear in diacyl, *O*-alkenyl, and *O*-alkyl analogs of PC, PE, PI, and PS after retailoring events involving hydrolysis of the acyl chain and re-esterification with an acyl chain coming from a HUFA-CoA. The asterisk indicates the location of the ω -3 double bond when corresponding ω -3 HUFA homolog is esterified.

made by cloning and expressing individual acyltransferases (34) and measuring multiple products formed in membrane-associated conditions with mixtures of available competing substrates (35). Six cloned candidates for mammalian LPA acyltransferase (LPAAT) include ones having high or low preferences for AA (34). This leaves uncertain whether LPAAT1 or LPAAT2 is the important transferase excluding ω -6 AA from the de novo pathway. Some acyltransferases act on multiple acceptors with multiple acyl-CoA donors (34), but further study may yet identify currently unknown enzymes with high specificity for acyl chains or phospholipid head groups. As cloned enzymes become available, studies with iRNA and knockout mutants will inform us of the importance of each catalyst in managing the proportion of ω -6 fatty acids in HUFAs of membrane phospholipids.

A unique study of the acyltransferase preferences used a dual substrate choice design with eight acyl-CoAs as donors and six lysophospholipids as acceptors with four different sets of cells expressing human membrane-bound O-acyltransferases [MBOAT; (35)]. After subtracting background activity, results with the dual substrates showed a strong preference of MBOAT-1 for esterifying oleate to phosphatidyl serine, MBOAT-2 for esterifying oleate to phosphatidic acid and phosphatidylethanolamine, and MBOAT-7 for selectively esterifying AA to phosphatidylinositol (35). Although quite selective for inositol lipids, the latter enzyme esterifies both EPA and AA (36). Phosphatidylinositol in wild type *C. elegans* has mostly ω -3 EPA rather than the ω -6 AA, which is so abundant in reports of human and rodent lipids. This fact emphasizes again the importance of making available many diverse substrates when designating acyltransferase specificity (2). Although plasma phospholipids of humans tend to have approximately 15% of their fatty acids as HUFAs, the average% ω -6 in the HUFA for different ethnic groups ranges from 32% to 87% (2). This reflects food choices rather than enzyme selectivity.

The bulk of the HUFAs in mammalian phospholipids seem to be introduced during "retailoring" by either acyl-CoA acyltransferases or CoA-independent transacylases (31,37). The major membrane glycerophospholipids in eukaryotes are phosphatidylethanolamine and phosphatidylcholine with smaller amounts of phosphatidylserine and phosphatidylinositol [Fig. 3; (37,38)]. All four classes occur as diacyl derivatives with the fatty acids at the *sn*1 position being approximately 60% saturated and 40% monounsaturated acyl groups and those at the *sn*2 position of the glycerol being unsaturated, especially HUFAs. Some ethanolamine- and choline-containing phospholipids also contain ether lipids having 1-O-alkyl or 1-O-alkenyl groups at the *sn*1 position and unsaturated fatty acids, mainly HUFAs, at the *sn*2 position [Fig. 3; (37)].

Hydrolytic release of ω -6 HUFA from the *sn*2 position of membrane phospholipids is catalyzed by three types of phospholipase A₂ including secretory PLA₂ (sPLA₂), cytosolic PLA₂ (cPLA₂) and Ca²⁺-independent PLA₂ (iPLA₂) (39). Each type has different subcellular locations, Ca²⁺ interactions and acyl chain preferences, but all PLA₂s cleave the major types of phospholipids (Fig. 3). The sPLA₂ enzymes are 14-kDa proteins that require Ca²⁺ and cleave most common acyl chain structures in

most phospholipids with no clear preference (40). cPLA₂ is an 85-kDa enzyme sensitive to Ca²⁺ as it acts on all the major types of phospholipid. It has a preference for acyl chains with more double bonds (HUFA > PUFA > monounsaturated acids) (41,42); however, cPLA₂ hydrolyzes DHA only slowly, likely because of steric hindrance by the nearby Δ 4 double bond as shown decades earlier for pancreatic lipase. Nonetheless, phospholipids with ω -3 DHA bind well to the enzyme and interfere with enzymatic release of the alternate substrate, ω -6 AA (41). DHA and AA are likely released from phospholipids of astrocytes by Ca²⁺-independent iPLA₂ and Ca²⁺-sensitive cPLA₂, respectively (43). There are different variants of iPLA₂ but the splice variants of the Group VI enzymes range in molecular mass from 85 to 88 kDa (39). The known iPLA₂ variants are regulated by cellular levels of cAMP and cAMP-activated kinases in ways that differ from cPLA₂ (39).

TRANSFORMATION OF ω -6 EFAs INTO EICOSANOIDS

Free AA can be transformed to three types of active hormone-like compounds collectively known as eicosanoids including prostanoids (PGs), leukotrienes (LTs), epoxides (EETs), and related hydroxyl fatty acids (22). Also, LA can be converted to some 18-carbon oxygenated products by the same synthetic enzymes as eicosanoids. AA may exert some of its actions as a free acid. For example, it will activate NADPH oxidase (44) and serve as an activating ligand for peroxisomal proliferator-activated receptors [PPARs; (45), activate ion channels such as a two-pore domain K_p channel (44), and stimulate apoptosis (47)]. [ω -Hydroxylated epoxy fatty acids and certain prostanoids may also activate PPARs (48).] However, selectivity or specificity claimed for various actions of nonesterified PUFAs and HUFAs is often modest. As noted earlier, ω -6 AA synthesized from LA is currently the most abundant ω -6 HUFA in tissue phospholipids, and some attributions of AA-selective events (such as cPLA₂-catalyzed cleavage from phospholipids) result from the availability of AA rather than its selective interaction with proteins (49).

PGs, leukotrienes, and epoxides are made by many cells and tissues, where they act locally before being rapidly inactivated by further metabolism. Hence, their biology is difficult to study. However, because ω -6 eicosanoid overproduction occurs in important pathologies, potent enzyme inhibitors and receptor antagonists have been developed for many therapeutic applications. The relatively recent availability of specific knockout mice for most of the various biosynthetic enzymes and prostanoid receptors, plus the use of specific enzyme inhibitors, and specific receptor agonists and antagonists has greatly enhanced our understanding of eicosanoid physiology and pathology (22).

Biosynthesis and Actions of (PGs)

The structures and biosynthetic interrelationships of the most important prostanoids are shown in Figure 4. Letters after PG and TX (thromboxane) denote the nature and location of the oxygen-containing substituents present in the cyclopentane ring. Prostanoids with a subscript "2" are

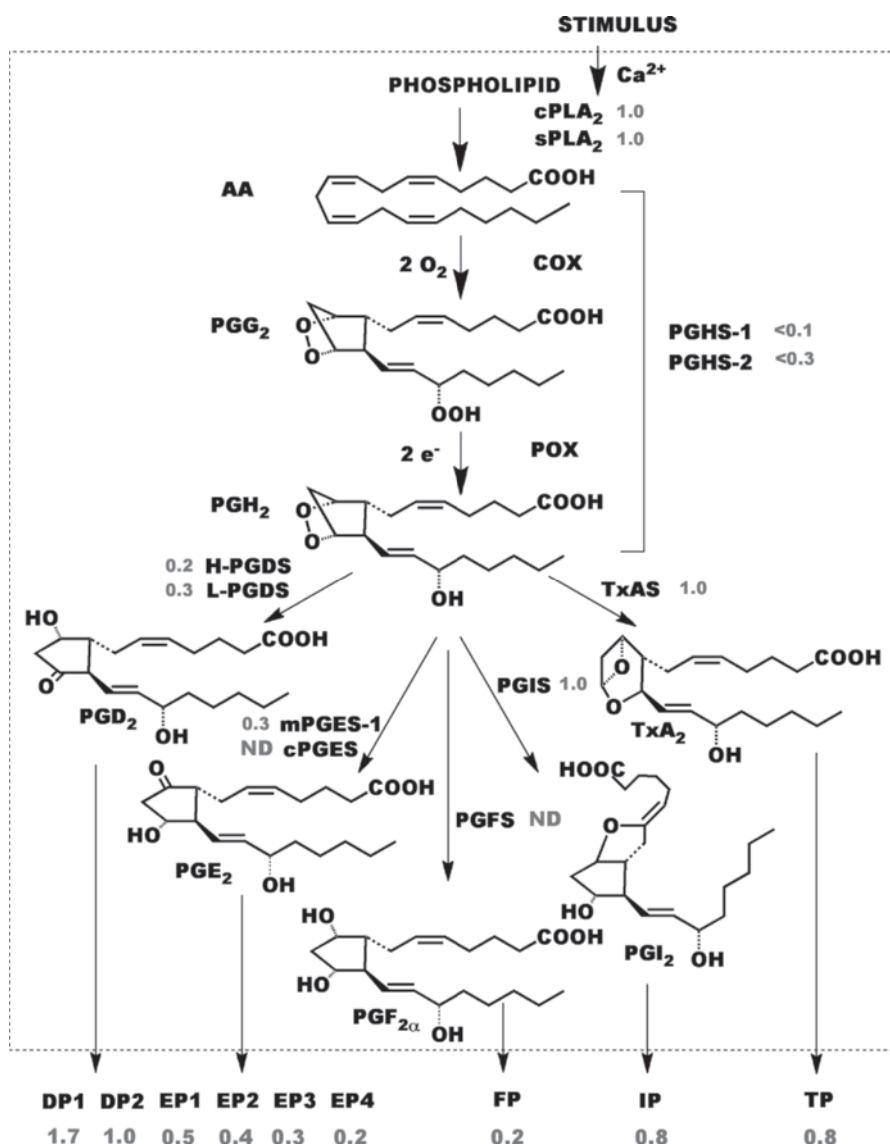


Figure 4 Pathways for the synthesis and actions of prostanoids. Selective events in the biosynthesis and action of prostaglandins. *Abbreviations:* PG, prostaglandin; PGHS, prostaglandin H synthase; COX, cyclooxygenase; POX, peroxidase; DP, receptor for PGD; EP, receptor for PGE; IP, receptor for PGI; TP, receptor for TxA; PGDS, PGD synthase; PGES, PGE synthase, PGFS, PGF synthase(s); TxAS, TxA synthase; PGIS, PGI synthase. Numbers shown in gray adjacent to various enzymes and receptors are the ratio of activity with ω -3 EPA versus ω -6 AA or the corresponding products formed from these precursors.

formed from ω -6 AA and those with subscript "3" are from ω -3 EPA. The PGs are synthesized and released rapidly by cells in response to certain hormones and physical stimuli (22). The dynamics of stimulus-induced prostanoid formation and action involves four distinct stages (Fig. 4). Each has counteracting forces that can cause modest differences in the forward rates to amplify into important physiological differences:

1. Mobilization of free AA from membrane phospholipids (22) through the activation of Ca²⁺-dependent cPLA₂ and sPLA₂. This is augmented by phosphorylation of cPLA₂ by various kinases and counteracted by the actions of acyl-CoA synthetases that rapidly mediate re-esterification of HUFAs. Figure 4 notes similar rates of hydrolysis for ω -6 and ω -3 structures by cPLA₂ and sPLA₂.
2. Conversion of AA to PG endoperoxide H₂ (PGH₂) by a PG endoperoxide H synthase (PGHS; also known as cyclooxygenase or COX). The oxygenation reaction can be counteracted by removal of obligatory hydroperoxide activators (50) or changes in the levels of nonesterified, nonsubstrate fatty acids (51). PGHS activity is also diminished by "suicide" inactivation of the enzyme (52).
3. Conversion of PGH₂ to one of the major types of active prostanoids by a specific synthase (e.g., TxA synthase). Formation of active prostanoid is counteracted by rapid inactivation by a 15-hydroxy dehydrogenase (53) and other metabolic reactions (22). Frequently, specific prostanoids are formed by specific cell types; for

example, TXA₂ is almost exclusively made by platelets, whereas PGI₂ is synthesized by vascular endothelial cells.

4. Binding of active prostanoids to specific G-protein-linked receptors (54,55). Activated receptors interact with downstream effectors such as adenylate cyclase and phospholipase C to modulate the formation and action of second messengers such as cAMP, Ca²⁺, and diacylglycerol to affect physiologic processes. The efficacy of prostanoids formed from ω -6 versus ω -3 fatty acids (i.e., AA vs. EPA) is an understudied area. A unique, comprehensive report (56) compares the relative specificities of AA versus EPA and their metabolites with enzymes and receptors of the PG pathways. Products derived from ω -6 AA tend to be more active on prostanoid receptors than those from ω -3 EPA (56).

Two isoforms of PGHSs are known as PGHS-1 and -2 or COX-1 and -2 (57). The enzymes are encoded by separate genes. In general, PGHS-1 is expressed constitutively, and its ω -6 products are essential in parturition, platelet aggregation, and crypt stem cell survival (20,58). In contrast, PGHS-2 is essential for ovulation, implantation, resolution of inflammation, perinatal kidney development, ductus arteriosus remodeling, and ulcer healing (58). PGHS-2 is induced in response to growth factors, cytokines, and inflammatory stimuli, and thus it can be suppressed by steroidal anti-inflammatory glucocorticoids-like dexamethasone (22,59). Figure 4 notes much lower rates of formation for ω -3 than ω -6 structures with both PGHS-1 and PGHS-2. Both enzymes are inhibited by nonsteroidal anti-inflammatory drugs such as aspirin, ibuprofen, and naprosyn. Examples of COX-2-specific inhibitors are celecoxib and rofecoxib. PGHS-2 can convert 2-arachidonoyl-glycerol to 2-PGH₂-glycerol efficiently, and this intermediate can be converted to 2-prostanoyl-glycerol esters (with the exception of TXA₂) (57). 2-Arachidonoyl-glycerol has hormonal actions on cannabinoid receptors, but the importance of 2-prostanoyl-glycerol derivatives from PGHS-2 action is unknown.

The substrate specificities of PGHS-1 and -2 have been examined in detail (56,60). AA is the most efficient substrate for both PGHS-1 and -2. EPA is a particularly poor one for PGHS-1 and DHA is inactive. Indeed, EPA and DHA can inhibit AA oxygenation by PGHS-1 (61,62). EPA is a substrate for PGHS-2, and DHA can also be oxygenated by PGHS-2. However, neither of these fatty acids inhibit AA oxygenation by PGHS-2 as effectively as PGHS-1 (41). LA can be converted to 9- or 13-HODE by PGHS-1 and -2 at about one-fourth the efficiency of AA. The degree to which oxygenation of LA by PGHS occurs and has biological importance is not known. 13-HODE formed via a 15-LO has been implicated as an effector of cell growth (63).

Formation of active prostanoids from PGH₂ (and PGH₃) is catalyzed by two different PGD synthases, at least three PGE synthases, two forms of PGF synthase, a PGI synthase, and a TXA synthase (22). Not all cells express all of these synthases, making prostanoid formation somewhat cell specific. Transcellular formation occurs when the PGH₂ formed by platelets diffuses to endothelial cells where PGI₂ can be formed. The active eicosanoids rapidly disappear, making a fast rate of formation neces-

sary to provide sufficient active ligand bound to nearby cellular receptors. When synthase rates are less than inactivation rates, little signaling occurs even though appreciable hormone may be formed.

Nine distinct prostanoid receptors are G-protein-linked receptors (54,55)—two for PGD, four for PGE, and one each for PGF, PGI, and TXA. Figure 4 notes a fivefold greater action of ω -6 PGF_{2 α} compared with ω -3 PGF_{3 α} at the FP receptor (56). This may provide more effective parturition and more intense dysmenorrhea. The greater action of ω -6 than ω -3 forms of PGE at the four EP receptors (Fig. 4) may have important consequences. In addition, the greater action of ω -3 PGD₃ than ω -6 PGD₂ at the DP receptor may enhance observed antiplatelet actions of ω -3 HUFA (56).

Biosynthesis and Actions of Leukotrienes (LTs)

The biosynthetic pathway for the formation of LTs is shown in Figure 5 (22). Like prostanoids, LTs have four stages in their formation and action when they are formed in response to cellular stimuli that mobilize HUFA from phospholipids by activating cPLA₂. The LTs are produced from ω -6 AA (or its ω -3 analog) by the action of 5-lipoxygenase (5-LO), which both forms 5-hydroperoxy-6,8,11,14- (*E,Z,Z,Z*)-eicosatetraenoic acid (5-HPETE) and then dehydrates this product to form LTA₄. The enzyme acts at the nuclear membrane and requires several cofactors for activity, including Ca²⁺, ATP, hydroperoxides, and phosphatidylcholine (22). The activity is regulated by a suicide inactivation mechanism, making continued

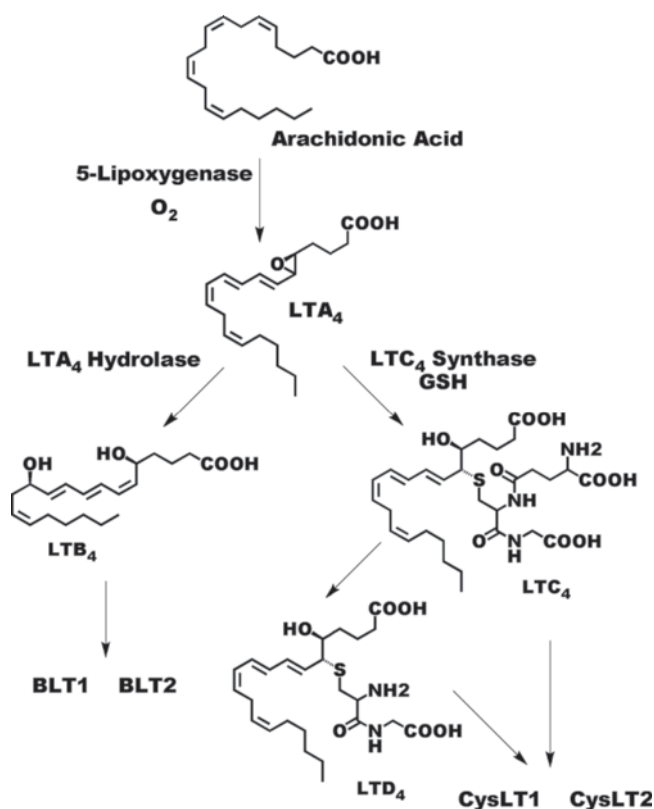


Figure 5 Pathways for the biosynthesis of leukotrienes.

synthesis of the enzyme important for sustained formation of active hormone. During the search for pharmacological antagonists of LT biosynthesis, a protein called 5-lipoxygenase-activating protein was discovered. This protein is important for the efficient production of LTs by cells, but it is not clear how it functions. It may serve as a protein that transfers HUFA to 5-LO (22). A methylene-interrupted cis double bond system is the major determinant in the action of various lipoxygenases toward C-18 and C-20 fatty acids (64). Most lipoxygenases use the HUFA, AA, in preference to the PUFA LA, although some attributions of selective actions with AA may be explained by a high availability of AA under many experimental conditions (49). This is notably the case in 5-lipoxygenase action during LT formation when little ω -3 EPA is provided to the oxygenase.

Two different types of potent leukotriene hormones are formed from LTA₄. LTA hydrolase hydrolyses LTA₄ to produce LTB₄ whereas LTC synthase conjugates glutathione to carbon-6 of LTA₄ to create the peptidoleukotriene called LTC₄. Cellular locations for forming LTB and LTC from LTA can be limited and cell specific (22). For example, human neutrophils produce LTB₄, whereas mast cells and eosinophils form LTC₄. Unequivocal evidence for transcellular formation *in vivo* showed transfer of more than half the total LTA₄ produced moved efficiently between cells (65). LTB₄ is a very potent chemotactic and chemokinetic agent for human neutrophils. LTC₄ constricts bronchial smooth muscle and mediates leakage of vascular fluid during edema. Metabolism and inactivation of leukotrienes is rapid, making their action evanescent without a continued availability of LTA₄. The peptidoleukotriene, LTC₄, can rapidly form LTD₄, which also activates receptors. However, peptidase cleavage to LTE₄ gives a less active leukotriene that is excreted in the urine as the *N*-acetyl derivative.

The biological activities of both LTB₄ and LTC₄ are mediated by specific G-protein-coupled receptors (22,66). Two LTB receptors, BLT1 and BLT2, mediate chemotactic effects, and three receptors, including CysLT1 and CysLT2, mediate cysteinyl leukotriene actions (22). Even before BLT receptors were recognized, the much greater chemotactic proinflammatory action of the ω -6 LTB₄ over the ω -3 LTB₅ (67) indicated that the receptors discriminate between ω -6 and ω -3 structures with important consequences. LTC₄ receptor antagonists (e.g., montelukast, pranlukast, and zafirlukast) and a 5-LO inhibitor zileuton are commercially available to treat asthma. There are lipoxygenases other than 5-LO, including 8-, 12- and 15-lipoxygenases that introduce oxygen at different positions

in the AA chain (68). Certain of these lipoxygenases (e.g., 15-LO) will use LA as a substrate such as in the formation of 13-HODE, which, as already noted, may be able to ameliorate the scaly skin of EFA-deficient animals (27).

Biosynthesis and Actions of Related Oxygenated Acids—P450 Hydroxylase and Epoxygenase Pathways

AA can be hydroxylated by many different cytochrome P450 isoforms (CYP1 A, CYP2B, CYP2 C, CYP2D, CYP2G, CYP2 J, CYP2 N, CYP4 A, CYP), leading to epoxyeicosatrienoic acids [EETs; Fig. 6; (22,69)]. Some epoxides have potent roles as an endothelium-derived hyperpolarizing factor regulating renal vascular tone and fluid/electrolyte transport (22). The epoxide availability can be counteracted by enzymatic hydrolysis to dihydroxy acids, conjugation with glutathione, activation to acyl-CoA esters followed by esterification into lipids or β -oxidation. Because of desirable antihypertensive actions of certain EETs, researchers are exploring epoxy hydrolase inhibitors to increase EET availability at its receptors.

The AA esterified at the *sn*2 position of any phospholipids can also be oxidized nonenzymatically to yield a complex racemic mixture of esterified "isoprostanes," which are then mobilized presumably through the actions of phospholipase A₂ (70). Isoprostanes are formed in abundance, particularly under conditions where tissue-free radical damage occurs and some isoprostanes have potent biological activities (71).

PATHOLOGIES ASSOCIATED WITH ω -6 EFA ACTIONS

Although an absence of ω -6 LA or ω -6 AA in the diet can cause EFA deficiency, intakes as low as 0.3% of daily calories prevent it. Current diets in the United States, Europe, and highly developed countries in the Far East (e.g., Japan) contain levels of LA 10- to 20-fold greater. The proportions of ω -6 in tissue HUFAs show a correspondence with mortality from cardiovascular deaths in various human populations (2). One ω -6 eicosanoid—TXA₂—is an important causal mediator in cardiovascular mortality (72,73). There are correlations showing that COX-2 is overexpressed in colon cancers (74), that knock out of microsomal PGE synthase-1 in mice diminishes tumor development and that nonsteroidal anti-inflammatory drugs may decrease mortality from colon cancer. This suggests that overproduction of AA-derived eicosanoids that could

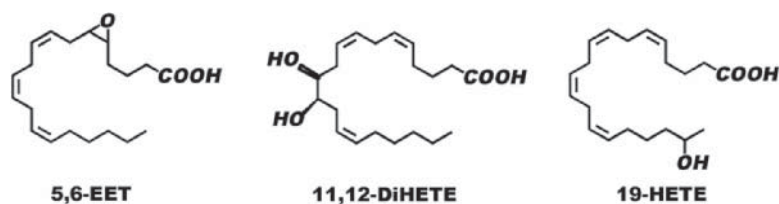


Figure 6 Representative eicosanoids formed via various cytochrome P450s. Abbreviation: EET, epoxy eicosatetraenoic acid.

result from excessive intake of ω -6 fatty acids can lead to serious health consequences.

Actions of ω -6 eicosanoids continue to be intensively studied by pharmaceutical researchers looking for new therapies to treat ω -6 eicosanoid-mediated pathologies. The intensity of interest in developing agents to decrease excessive actions of ω -6 metabolites in the "arachidonate cascade" is reflected in the fact that 38,360 articles retrieved by the term "arachidonic" in a recent PubMed search contained 21,420 articles retrieved by the combined terms "arachidonic" and "drug", and 1579 of those were reviews. The present review offers readers insight to some of the established evidence about selective and nonselective competitions that occur during the metabolism of ω -6 and ω -3 molecules. These competitions accompany daily personal food choices that have profound influence on physiologic states and clinical status. Knowing the established basic biochemistry and physiology of ω -6 fatty acids will allow readers to interpret more fully the relevance of data gathered and discussed in less-detailed epidemiologic and clinical studies in which intakes of ω -6 and ω -3 fats have been altered.

CONCLUSIONS

The major ω -6 EFAs in human tissues are LA and AA. Relatively small amounts of LA are required in the diets of humans for growth, health, and reproduction. The basis for the requirement is partially explained by tissue AA being converted to ω -6 eicosanoids essential for reproduction and other important physiologic actions. However, overproduction of ω -6 derived eicosanoids can amplify cellular responses to pathological levels. This may be prevented by decreasing the upstream dietary intake of ω -6 LA and increasing the dietary intake of ω -3 EFAs such as EPA and DHA.

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Pancreatic Enzymes

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INTRODUCTION

Pancreatic enzymes are critical for the normal physiological digestion of fats, proteins, and carbohydrates. Many additional conditions and molecules throughout the digestive system complement and assist in the digestion of essential nutrients. As occurs often in diseases involving the pancreas such as cystic fibrosis (CF), deficiencies of pancreatic enzymes can result in significant malabsorption and nutritional deficiencies.

This chapter briefly outlines the normal functions of the pancreas and pancreatic enzymes. Enzymes that are primary components of pancreatic supplements are defined and the function of each is described. We then consider conditions in which the use of pancreatic enzyme supplements is recommended. We review also clinical studies in which pancreatic enzyme supplements were used to improve digestion or as a supplement to augment the natural age-related decrease in pancreatic exocrine output and explore the uses of pancreatic enzymes in cases of disease, for example, cystic fibrosis for which pancreatic enzyme supplementation may be beneficial. The chapter concludes with a summary of recommendations for pancreatic enzyme supplementation.

THE PANCREAS

The pancreas has both endocrine and exocrine functions. The exocrine pancreas secretes enzymes crucial to digestion. Cells responsible for exocrine functions comprise the largest part of the pancreas. Clusters of acinar cells constitute the primary functional unit and release between 6 and 20 g of digestive enzymes and zymogens each day in approximately 2.5 L of fluid. These products are secreted into the duodenum of the small intestine via the pancreatic ducts, in which they are mixed with sodium bicarbonate secretions produced by pancreatic ductal cells. Pancreatic enzymes reduce complex nutrients into simple molecules that can be absorbed by the small intestine; sodium bicarbonate secretions neutralize the acidic chyme as it moves from the stomach to the duodenum (1).

PANCREATIC ENZYMES AND FUNCTIONS

Amylase, lipase, and protease are three main categories of pancreatic enzymes. Each type serves a specific digestive function.

Amylase, which is also produced in less quantity by salivary glands, acts on carbohydrates. Pancreatic

α -amylase cleaves internal α -1,4-glucose linkages, yielding glucose and dextrins. It cannot cleave terminal glucose residues or α -1,6-glucose linkages. The dextrins produced by α -amylase digestion are further digested into glucose by intestinal brush border enzymes.

Lipase hydrolyzes fats into fatty acids and glycerol. The pancreatic lipases are responsible for the majority of fat digestion, with minor contributions from lingual and gastric lipases. Pancreatic triglyceride lipase (PTL) is a carboxyl esterase, catalyzing the hydrolytic cleavage of acylglycerides into glycerol and free fatty acids. The pancreas secretes excess PTL, meaning that a large decrease in PTL secretion must occur before signs of fat malabsorption are apparent. Many digestion products present in the duodenum have inhibitory effects on PTL activity. There are also molecules that complement PTL, such as colipase, which is a coenzyme required to maintain PTL activity. Colipase, which stabilizes the active conformation of PTL, has itself no enzymatic activity. It is secreted as a procolipase, which is active after cleavage of its N-terminus by trypsin. Other major pancreatic contributors to lipid digestion include phospholipase A2, which catalyzes hydrolysis of the acyl ester bond in phospholipids and carboxyl ester lipase, which hydrolyzes triglycerides, cholesterol esters, phospholipids, lysophospholipids, ceramides, vitamin esters, and galactolipids.

Three important serine proteases are produced by the pancreas: trypsin, chymotrypsin, and elastase. Trypsin is the most abundant of the pancreatic digestive enzymes. It hydrolyzes peptide bonds at the C-termini of arginine and lysine residues, except those in a proline linkage. Trypsin is secreted from the pancreas as a zymogen trypsinogen, which is activated by cleavage of its activation peptide catalyzed by enterokinase, a duodenal enzyme. Autoactivation of trypsin can also be mediated by calcium and pH, specifically pH between 7.5 and 8.5. Trypsin is inactivated through autolysis, which is inhibited by calcium. Another contributor to pancreatic enzyme-catalyzed digestion is chymotrypsin, which cleaves peptide bonds at the C-terminus of an aromatic amino acid (i.e., tyrosine, phenylalanine, tryptophan). The zymogen chymotrypsinogen is secreted from the pancreas and then activated through autolysis. Elastase also hydrolyzes peptides at the C-terminus of amino acids alanine, glycine, and serine. It is secreted as proelastase and is both activated and inactivated by trypsin (2).

Several additional molecules that are present in pancreatic secretions contribute to nutrient digestion and absorption, including proteases, such as carboxypeptidase, nucleases, and enzyme cofactors (1).

NORMAL DIGESTION AND PANCREATIC FUNCTION

Digestion serves to extract necessary nutrients from ingested complex food sources such as proteins, carbohydrates, and fats. Protein digestion begins within the stomach, where acid and proteases hydrolyze proteins into peptides. This continues within the small intestine via pancreatic proteases and intestinal brush border proteases. Carbohydrates and starches are substrates for salivary amylase within the mouth and further digested by pancreatic amylase and intestinal brush border oligosaccharidases within the small intestine. Fat digestion begins in the stomach and is catalyzed by the lingual and gastric lipases, yielding glycerol and long-chain fatty acids. Gastric hydrolysis accounts for only about 10% of lipid digestion. Products of intragastric lipolysis are transferred to the duodenum for additional hydrolysis by pancreatic lipases, a process requiring the neutralization of acidic chyme by pancreatic bicarbonate secretions to facilitate the pH-determined functioning of pancreatic lipases. Products of completely digested lipids are absorbed by the intestinal mucosa, after which the fatty acids are reincorporated into triglycerides to be packaged as chylomicrons for delivery to the bloodstream via lymphatic vessels (3).

Regulation of exocrine pancreatic secretions is influenced significantly by nutrients acting in the distal bowel. Digestive products, especially free fatty acids, stimulate release of the hormone cholecystokinin from the small intestine. This in turn stimulates the pancreas to release its enzymatic secretions. In normal, healthy individuals, the pancreas releases between 10 and 20 times the amount of prandial enzyme required for digestion (4).

When considering activities of pancreatic enzymes, it is important to assess the stability of enzymatic activity during intestinal transit. Amylase is the most stable of all pancreatic enzymes as a majority of that released reaches the terminal ileum in its active state. Proteases are less resistant to degradation, and only 20% to 30% of protease activity is retained. Lipases are the most sensitive to inactivation during intestinal transit. Although the presence of lipid substrates does enhance lipase stability, only a small fraction of released lipase is active on reaching the terminal ileum (4).

The coexistence of numerous digestive processes that overlap pancreatic enzyme activities allows maintenance of protein and carbohydrate digestion even in the face of severe pancreatic insufficiency. Fat digestion is not, however, appreciably augmented by nonpancreatic mechanisms, thus critical fat malabsorption can be associated with pancreatic insufficiency.

ENZYME DEFICIENCIES

Pancreatic insufficiency (PI) is caused by a significant deficit in pancreatic enzyme output and differs widely in severity. Symptoms of PI usually manifest after a 90% reduction in pancreatic enzyme output. Although protein and starch digestion are relatively easily corrected by pancreatic enzyme supplementation, fat malabsorption is more difficult to treat effectively because of the instability of lipase molecules (3).

Deficiency in the production of any of the pancreatic enzymes can impair gastrointestinal (GI) function. Decreased lipase production and subsequent fat malabsorption is associated with steatorrhea, or fatty stool. A secondary consequence of pancreatic insufficiency is an inadequate uptake of fat-soluble vitamins, notably vitamins A, D, E, and K. Fat malabsorption occurs earlier in pancreatic insufficiency than does malabsorption of other nutrients for several reasons: fat digestion is not adequately compensated by other digestive mechanisms; declines in the synthesis and secretion of pancreatic lipases are more rapid than they are for other pancreatic enzymes and are readily inactivated in an environment of high pH caused by the lack of pancreatic bicarbonate secretions; and lipases are more sensitive than other digestive enzymes to protease degradation (5). Protein malabsorption can also occur, with evidence of creatorrhea or protein in the stool. Both protein and carbohydrate malabsorption are usually seen only late in the course of severe PI, and sometimes not at all, because of supplementation of digestive function by intragastric proteolysis or intestinal brush-border peptidases and salivary amylase plus intestinal oligosaccharidases for starches (4). As a consequence of impaired enzyme output in PI, the site of maximal absorption within the small intestine becomes more distal (3). Further reduced nutrient digestion and absorption increases gastric and intestinal motility (6). Impaired protein digestion has been linked to food allergies (7), and proteolytic enzymes are necessary for preservation of a healthy intestinal microbial flora (8).

PANCREATIC ENZYME REPLACEMENT THERAPY

Pancreatic enzyme replacement therapy (PERT) is intended to correct insufficient pancreatic enzyme levels in the proximal small intestine through the ingestion of exogenous enzymes. Delivery of exogenous pancreatic enzymes with each meal can restore enzymatic activity to the duodenum thereby reducing many of the symptoms associated with pancreatic insufficiency. To accomplish this, PERT needs to address effectively the need for exogenous enzymes to survive the acidic and proteolytic environment of the stomach and to be in a form that will mix and efficiently enter the duodenum simultaneously with food. A third requirement is the temporal and geographical localization of enzymes with substrates to ensure an optimal absorption of necessary nutrients.

Two types of enzyme formulations are generally used: unprotected, conventional preparations and acid-resistant enteric-coated preparations. Because gastric acids and proteases can cause significant inactivation of lipases, effective doses of conventional preparations will be large. Protected preparations contain pancreatin, which is enteric-coated as protection from enzymatic action in the stomach, but allowing release in environments of pH > 5, such as the duodenum, which facilitates its administration in doses lower than those of uncoated preparations (9).

Protected preparations and brands differ in methods of delivery. Enzyme packaging is very important to achieve adequate mixing with food and transit from stomach to duodenum. Two predominant forms of

enteric-coated pancreatin preparations are tablets and microspheres, which have proven superior to tablets with regard to the three aforementioned criteria. Enteric-coated particles >2 mm in diameter cannot be adequately emptied from the stomach and thus, lead to increased gastric retention (5) so that enzymes and food are not simultaneously traversing the digestive system. In a direct comparison, delivery of lipase via microspheres was significantly superior to that by tablets for increasing body weight and reducing abdominal pain as well as fecal fat excretion. Through their ability to enter the duodenum simultaneously with food (10), microspheres appeared to prevent the problem of pyloric retention.

Timing of ingestion of enzymes supplements is critical. Patients should swallow supplemental capsules just after beginning a meal, during the meal, and immediately after. This allows for protection of supplemental enzymes from gastric acid and proteases and ensures optimal mixing with food (11). If with standard dosages symptoms of steatorrhea persist, dosages should be increased (9). Impaired bicarbonate secretion in patients with PI and the resulting low duodenal pH can hinder enzyme release from enteric-coated particles. Administration of medication to reduce gastric acidity, such as famotidine, an H₂-receptor antagonist, or omeprazole, a proton pump inhibitor, has been shown to reduce fecal fat content, especially when given in combination with high doses of pancreatin (12,13). When supplemental enzymes were used in combination with antacids, both duodenal pH and enzyme activities were increased. Patients with the lowest responses to PERT experienced the greatest benefits of a regimen of antacids during meals (14). Increasing gastric pH because of antacid treatment also increased the efficacy of gastric lipase, which has its highest enzymatic activity at pH 5.4 (15).

PANCREATIC INSUFFICIENCY IN CYSTIC FIBROSIS

CF is highly associated with PI. Approximately 95% of patients with cystic fibrosis will develop steatorrhea by adulthood due to PI (16). CF is an autosomal recessive disorder caused by a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), which functions as a cyclic AMP-regulated chloride channel, and is expressed on the luminal surface of pancreatic ductal cells. Normal function and water secretion involves a chloride/bicarbonate anion exchange, which is disrupted by the malfunctioning chloride channel, resulting in pancreatic secretions that contain lower amounts of bicarbonate, lower pH, and lower volumes (13). This results in hyperconcentrated, viscous pancreatic secretions that obstruct pancreatic ducts and lead to the destruction of acinar cells with pancreatic fibrosis (17).

CF patients with different genetic defects have different probabilities of developing pancreatic symptoms. A $\Delta F508$ mutation, which is almost always associated with PI, is present in the majority of Caucasian CF patients (18). Pancreatic dysfunction and its severity are highly correlated with mutations in the CFTR gene, regardless of clinical manifestations of CF (19).

Early diagnosis of CF is important for children to ensure proper growth and development to allow for recog-

nition of the need for and the prompt initiation of enzyme replacement therapy. The majority of CF patients suffering from PI present with symptoms within the first year of life, although in some patients PI may develop with age. Symptoms in the latter group are usually first recognized as acute pancreatitis following ingestion of high-fat meals (20).

Maintenance of proper nutrition through modified diet and pancreatic enzyme replacement therapy is of primary importance as it both enables normal development and improves respiratory function (21). Children within normal weight ranges have a higher FEV₁ and better survival rates than those who are underweight (22). It is recommended that children with CF have 110% to 200% of the energy intake of healthy children of similar age, sex, and size to compensate for malabsorption. Diets high in fat and protein are also recommended and have been shown to maximize fat digestion and absorption (23).

SUPPLEMENTATION OF NORMAL DIGESTION

Although pancreatic enzyme supplements are traditionally prescribed for individuals with pancreatic dysfunction, supplements may also improve digestion for those with normal pancreatic function. Studies have shown that pancreatic secretions change in response to diet, specifically depending on its composition (24). This feedback mechanism, however, begins to alter pancreatic enzyme secretions only after a sustained change in diet. For more immediate effects on digestion, the use of pancreatic enzyme supplementation has proven useful.

Microencapsulated pancreatic enzymes were shown to reduce postprandial symptoms in normal individuals. In a study analyzing the use of pancreatic enzyme supplementation in normal digestive function, it was found that healthy volunteers experienced a significant reduction in abdominal symptoms associated with indigestion after eating a high-calorie, high-fat meal. Healthy volunteers reported reductions in bloating, gas, and a feeling of fullness (25). Pancreatic enzyme supplements have also been recommended for idiopathic digestive disorders with symptoms of excessive gas and bloating. It is thought that exogenous enzymes can break down undigested food material that is responsible for abdominal discomfort after meals, although more research is needed to define the exact mechanism of action and the utility of pancreatic enzyme supplementation for treatment of common GI symptoms (26).

AGING

Earlier studies showed a significant decrease in pancreatic output of bicarbonate and digestive enzymes with increasing age (27,28). Also, pancreatic enzyme output was less in the elderly than in the younger individuals (29). With age physiological changes in pancreatic structure and function are seen that affect the entrance of pancreatic secretions to the duodenum. These include increased caliber of the main pancreatic duct, vascular calcification, fibrosis, accumulation of fat in the pancreas, defective protein synthesis, and decreased pancreatic cell mass (30). In

a study of pancreatic secretions during stimulation by IV infusion of secretin and cerulein in healthy volunteers, pancreatic bicarbonate and enzyme output were significantly lower in the elderly than in the younger groups (31). Given the importance of pancreatic function in nutrient absorption, malnutrition in the elderly could potentially be prevented through pancreatic enzyme supplementation. Treatment of aged endotoxemic rats with pancreatic enzymes improved their nutritional status and recovery from infection (30). This evidence supports the possibility that use of pancreatic enzymes to supplement normal pancreatic function is beneficial for digestive health, as malabsorption and nutritional deficiencies due to impaired digestion tend to increase in frequency with age.

FOOD ALLERGIES

When malabsorption of nutrients occurs due to pancreatic insufficiency, complex proteins and carbohydrates remain undigested. With an accumulation of undigested materials, proteins begin to leak into the circulation where they may be received by components of the immune system as foreign and subjected to immunological attack. In food allergies, the body treats innocuous substances as if they were harmful, triggering a hypersensitive immune response (7). It is hypothesized that malabsorption can contribute to food allergies by allowing certain undigested food materials to pass into the circulation and elicit an immune response. Symptoms associated with food allergies are typically gastrointestinal, and include diarrhea, abdominal pain, and dyspepsia. Treatment with pancreatic enzymes has been shown to reduce the clinical symptoms of food allergies and also limit the allergen-induced inflammation (in studies with patients with confirmed food allergies). Pancreatic enzymes cannot only reduce GI discomfort, but also prevent allergen activation of the immune system, potentially through degradation of the allergen (32).

ALTERNATIVE CANCER THERAPIES

Pancreatic enzyme supplementation is used in alternative cancer therapies. The Gonzalez therapy arose from the similar Gerson Therapy, which also involved pancreatic enzyme supplementation (33); it is based on the belief that toxins from environmental and processed food sources collect in tissues and gradually lead to imbalances in the autonomic nervous system, weaken immunity, and lead to cell damage that ultimately gives rise to cancer. Proponents of the Gonzalez therapy believe that removing these toxins will help in combating cancers. To detoxify the body, a regimen of orally ingested pancreatic enzymes, an organic diet, nutritional supplements, and coffee enemas are prescribed. Pancreatic enzymes are thought to play a role in detoxification by helping eliminate abnormal cells, toxins, and waste material, and help repair cell damage. Therefore, pancreatic enzyme supplements are the main anticancer component of the Gonzalez therapy (34). A case study was conducted by the originators of the therapy in which 11 patients diagnosed with

inoperable stage II to IV pancreatic adenocarcinoma underwent the Gonzalez therapy. This group reported a one-year survival of 81%, a two-year survival of 45%, and a three-year survival of 36%. This exceeded the National Cancer Data Base Report on Pancreatic Cancer from 1995, in which the overall one and two-year survival rates were 25% and 10%, respectively (35). These findings led to a National Cancer Institute-funded nonrandomized study comparing the Gonzalez therapy to gemcitabine-based chemotherapy among pancreatic cancer patients. Patients who chose the chemotherapy lived 14 months, compared to those who chose the Gonzalez therapy, who survived 4.3 months (36).

Pancreatic enzymes are also used in the Wobe-Mugos E treatment, which is a mixture of calf thymus extracts and enzymes from papaya plants, cow pancreas, and pig pancreas (37). This treatment has been shown to reduce the side effects of radiation, and is thought to function by eliminating toxic metabolic and inflammatory substances, as well as disintegrating microthrombi to improve microcirculation (36).

NEW TREATMENT APPROACHES

As an alternative to conventional and enteric-coated pancreatic enzyme supplements for patients with PI, a new pancreatic enzyme replacement product, TheraCLEC-Total is being developed. The formulation substitutes bacterial lipase and fungal protease and amylase for the porcine enzymes currently used in pancreatic enzyme supplementation (38). Notable in this new formulation is bacterial lipase, which has been shown to be more resistant than porcine lipases to acid and protease degradation. In dogs, bacterial lipases proved more effective for correction of steatorrhea and required only 1/75 the amount of porcine lipase usually used (240 mg bacterial lipase vs. 18 g porcine lipase) (39). To provide additional stability, lipases and proteases in TheraCLEC-Total are crystallized, and lipases are cross-linked. The crystallization and cross-linking of lipase also provides greater protection and maximizes lipolytic activity, thereby enabling the use of lower doses, which should aid in reducing the risks of fibrosing colonopathy seen with high doses of supplemental pancreatic enzymes in CF patients (38).

STANDARD DOSAGE

To improve digestion and decrease malabsorption, enzyme activity must be delivered to the duodenum with each meal. Mean lipase activity in the duodenal chyme must be between 40 and 60 units/mL, which requires a timely intraduodenal delivery of 25,000 to 40,000 units of lipase per meal. Dosages can be increased if additional lipolytic action is needed, but should not exceed 75,000 units of lipase per meal. If the need exceeds this amount, alternatives should be considered. Recommended doses for infants are 400 to 800 units of lipase/g of dietary fat (18). For children and young adults, recommended dosage is 500 to 2000 units of lipase/kg/meal, or 500 to 4000 units of lipase/g of fat ingested. Dosages more than 2500 units

of lipase/kg/meal or 10,000 units of lipase/kg/day are not recommended (3).

CONTRAINDICATIONS AND OVERDOSING

Ingestion of very large doses of pancreatin has been associated with a dose-dependent risk of fibrosing colonopathy in cystic fibrosis patients. Generally, dosages in excess of 75,000 units of lipase per meal are not recommended (5). In children younger than 12 years, dosages in excess of 6000 units of lipase/kg/bw/meal have been associated with colonic strictures (3). Diminished folate absorption has also been seen with the use of exogenous pancreatic extracts, and therefore should be monitored during the use of pancreatic enzyme supplements (40).

FDA REGULATION

The FDA has recently required that all pancreatic enzyme products gain FDA approval because of variations in activity and release rate due to different formulations, dosages, and manufacturing methods (41). Creon (pancrelipase) has thus far been the only delayed-release pancreatic enzyme product to be approved; it was approved in May 2009. All unapproved pancreatic enzyme products can remain on the market until the approval deadline, April 28, 2010, but must gain FDA approval to be sold after the deadline (42).

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Pantothenic Acid

Lawrence Sweetman

INTRODUCTION

Pantothenic acid is a water-soluble B vitamin (vitamin B₅) that is not synthesized by animals but is widely available in the diet. Pantothenic acid is metabolized to two important cofactors for enzymes: coenzyme A (CoA) and acyl carrier protein (ACP). Both cofactors contain a sulfhydryl group (-SH), which reacts with carboxylic acids to form thioesters. ACP has a central role in the synthesis of fatty acids. CoA forms thioesters with a very wide range of metabolic intermediates and has been estimated to be a cofactor for about 4% of all known enzymes (1). It is also involved with fatty acid synthesis but has broader functions in fatty acid oxidation, ketone body metabolism, oxidative metabolism of pyruvate via pyruvate dehydrogenase and the citric acid cycle, and in the metabolism of a wide variety of organic acids, including those in catabolic pathways of amino acid metabolism.

MICROBIAL SYNTHESIS

Micro-organisms synthesize pantoic acid (pantoate) from α -ketoisovaleric acid, the keto acid derived from the amino acid valine (1). A hydroxymethyl group is attached to α -ketoisovaleric acid, and the keto group is reduced to a hydroxy group to form pantoic acid. Beta-alanine produced by the decarboxylation of the amino acid aspartate is condensed with pantoic acid to form pantothenic acid (pantothenate) (Fig. 1). This synthesis does not occur in humans or in other animals, which must obtain pantothenic acid from the diet. Pantothenic acid is quite widely distributed in foods, giving rise to its name from the Greek word pan- (also panto-) meaning all or every. Liver, meats, milk, whole grain cereals, and legumes are good sources. It is contained in foods in various bound forms, including CoA and CoA esters, ACP, and as a glucoside in tomatoes.

COFACTOR SYNTHESIS

Coenzyme A Synthesis

Within cells, pantothenic acid is metabolized to CoA in the cytosol (Fig. 2). The initial reaction is phosphorylation of the hydroxyl group of the pantoic acid portion of pantothenic acid with ATP, catalyzed by pantothenate kinase, to form 4'-phosphopantothenate. This is the rate-limiting step for synthesis of CoA, and regulation of pantothenate kinase activity is the primary control of the rate of CoA synthesis. The activity of pantothenate kinase is strongly

inhibited by acetyl CoA and malonyl CoA and activated by free CoA (3). It is also inhibited by the intermediates 4'-phosphopantothenate and dephospho-CoA as well as CoA in other studies (4). Carnitine protects from the inhibition by CoA and acyl CoA by competing with them for their binding to pantothenate kinase. It has been shown that palmitoylcarnitine can prevent and reverse the strong inhibition of the human mitochondrial pantothenate kinase 2 isoform, and enable the synthesis of CoA, which is needed for mitochondrial beta oxidation of palmitate and other long-chain fatty acids (5). The human genes for the last four enzymes of CoA synthesis have been cloned, expressed, purified, and reconstituted *in vitro* to synthesize CoA (6). 4'-Phosphopantothenate is the substrate for 4'-phosphopantothenoylcysteine synthetase, which couples ATP hydrolysis with the formation of an amide bond between the carboxyl of 4'-phosphopantothenate and the amino group of the sulfur amino acid, cysteine. The product, 4'-phosphopantothenoylcysteine, is decarboxylated by 4'-phosphopantothenoylcysteine decarboxylase to form 4'-phosphopantetheine. A bifunctional protein with adenylyltransferase activity adds the 5'-AMP group of ATP to the 4'-phospho group of 4'-phosphopantetheine to form dephospho-CoA, and a kinase activity catalyzes the final step in the synthesis of CoA, the phosphorylation of the 3'-hydroxyl of dephospho-CoA utilizing ATP. The adenylyltransferase may be a secondary point of control of the biosynthesis of CoA. All the enzymes in the CoA biosynthetic pathway are present in the cytosol, but the last two enzymes can also be found in mitochondria. Notable features of the structure of CoA are the 3'-phospho-AMP moiety linked with the pantoate portion, and the reactive sulfhydryl group at the end of the long flexible chain derived from β -alanine and cysteine. CoA is often abbreviated as CoASH to illustrate this reactive sulfhydryl group, while thioesters of organic acids with CoASH are often referred to as acyl-SCoA.

Coenzyme A Subcellular Location

The majority of the CoA in cells is found within the mitochondria, with about 75% of liver CoA in mitochondria and 95% of heart CoA in mitochondria. This is consistent with the mitochondria being the major cellular organelle involved in fatty acid oxidation and in the final oxidative steps in the catabolism of all fuels; CoA plays a major role in these processes. Because the mitochondria represent only a small fraction of the cellular volume, the concentration of CoA here (2.2 mM) is 40 to 150 times that in the cytosol (0.015–0.05 mM). This large difference is

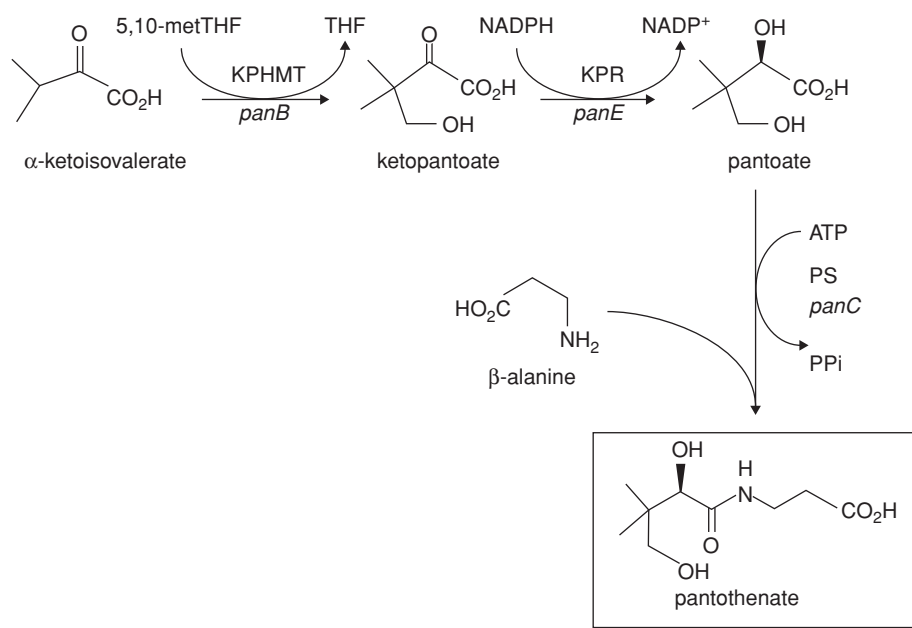


Figure 1 Pantothenic acid structure and biosynthetic pathway in micro-organisms. Source: From Ref. 2.

maintained by the transport of the negatively charged CoA into the mitochondria, which is driven by the membrane electrical gradient (8). CoA is also involved in the oxidation of very-long-chain fatty acids in peroxisomes, but little is known about how it enters these organelles. In the cytoplasm, CoA is also utilized for the synthesis of the ACP domain of the fatty acid synthase enzyme, which catalyzes fatty acid synthesis.

Acyl Carrier Protein Synthesis

There are several ACPs known in yeast and bacteria, but the ACP domain of fatty acid synthase is the most important and best studied. Fatty acid synthase is the only mammalian enzyme complex containing the ACP domain that has been well characterized. It is a single, homodimeric, multifunctional protein with seven enzymatic activities required for fatty acid synthesis (9). The synthase is synthesized with the ACP domain as an enzymatically inactive apoprotein lacking the prosthetic group. But after covalent attachment of the phosphopantetheine group, it becomes the enzymatically active holo-acyl carrier protein (holo-ACP) (10). This reaction, catalyzed by 4'-phosphopantetheinyl transferase, utilizes CoA to form a phosphoester bond between the 4'-phosphopantetheine portion of CoA and a specific serine residue of the ACP, with the release of the 3',5'-ADP moiety of CoA (Fig. 3). Note that as in CoA, the reactive sulphhydryl group of ACP is at the end of the long chain derived from β -alanine and cysteine.

COFACTOR DEGRADATION

The intermediates in the degradation of CoA are the reverse of those in the synthesis but involve different

enzymes. CoA does not appear to be degraded in the mitochondria, but in the lysosomes, the 3'-phosphate group is removed by nonspecific phosphatases to form dephospho-CoA. This is degraded to 4'-phosphopantetheine and 5'-AMP by a nucleotide pyrophosphatase located in the plasma membrane fraction. CoA is also degraded by this enzyme but at a much lower rate and with a much higher K_m . Surprisingly, acyl CoAs are also readily degraded to 4'-phosphopantetheine. There is an ACP hydrolase that releases 4'-phosphopantetheine from holo-ACP to reform apo-ACP. Interestingly, the combined action of the ACP hydrolase and synthetase results in the rapid turnover of the 4'-phosphopantetheine of ACP with a half-life measured in hours compared to that of the fatty acid synthase, which is measured in days in rat tissues (11). Whether 4'-phosphopantetheine is derived from the degradation of CoA or from the turnover of ACP, the phosphate is removed by phosphatases to give pantetheine. This is hydrolyzed to pantothenic acid and cysteamine by pantetheinase, which is found in both the microsomal and lysosomal fractions of rat liver and kidney. The pantothenic acid can be excreted or used for resynthesis of CoA. The cysteamine is oxidized to hypotaurine and further oxidized to taurine, which may be excreted in the urine.

METABOLIC ROLE

CoA has many functions in metabolism including its role in the formation of ACP. Both CoA and ACP are used to form thioesters with carboxylic acid groups of fatty acids and other compounds. Much of the metabolism of fatty acids and certain amino acid derivatives, as well as a number of amphibolic steps in metabolism, occurs

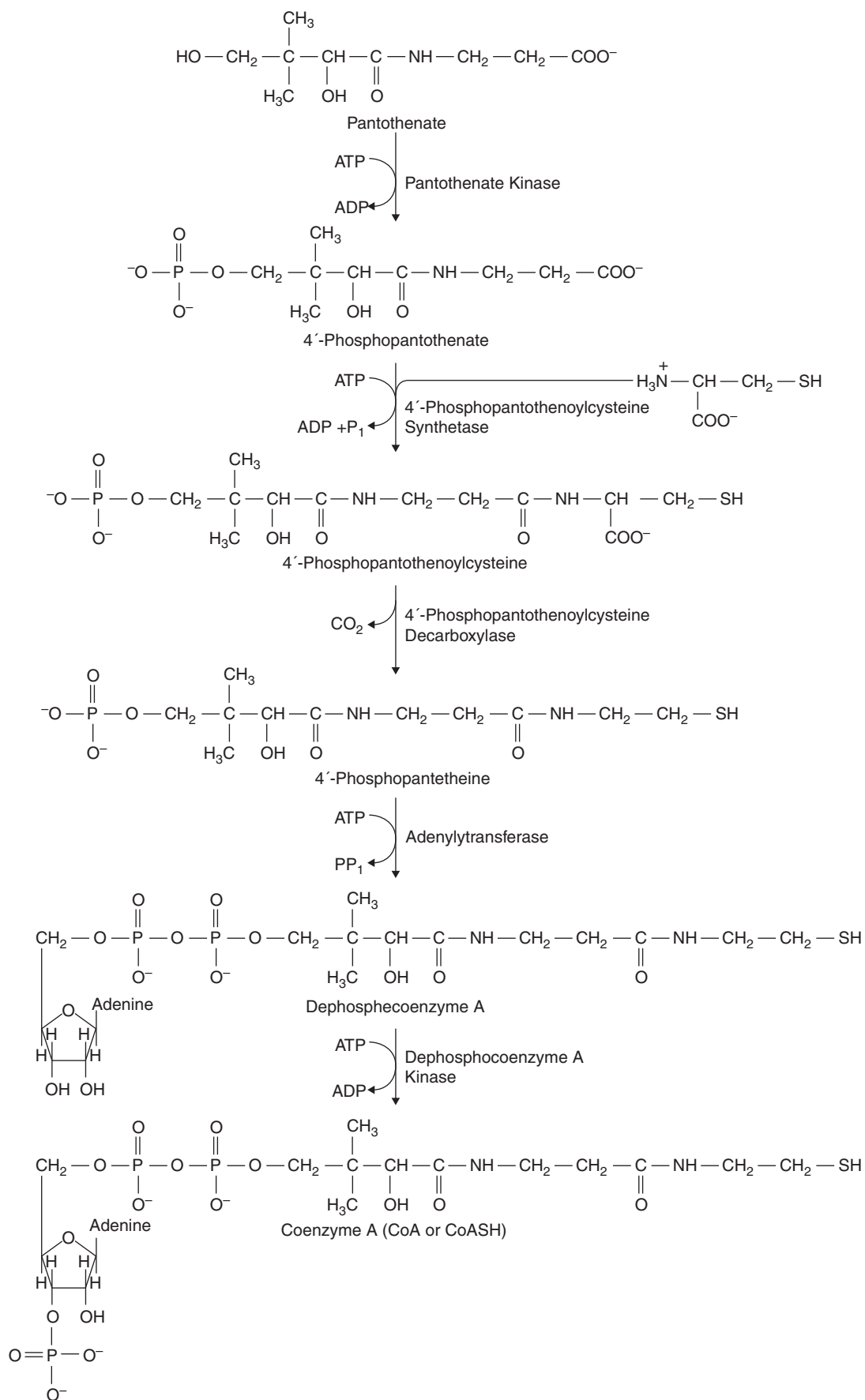


Figure 2 CoA synthesis and structure: CoA is synthesized from pantothenic acid, the amino acid cysteine, and ATP in mammalian cells. Source: From Ref. 7.

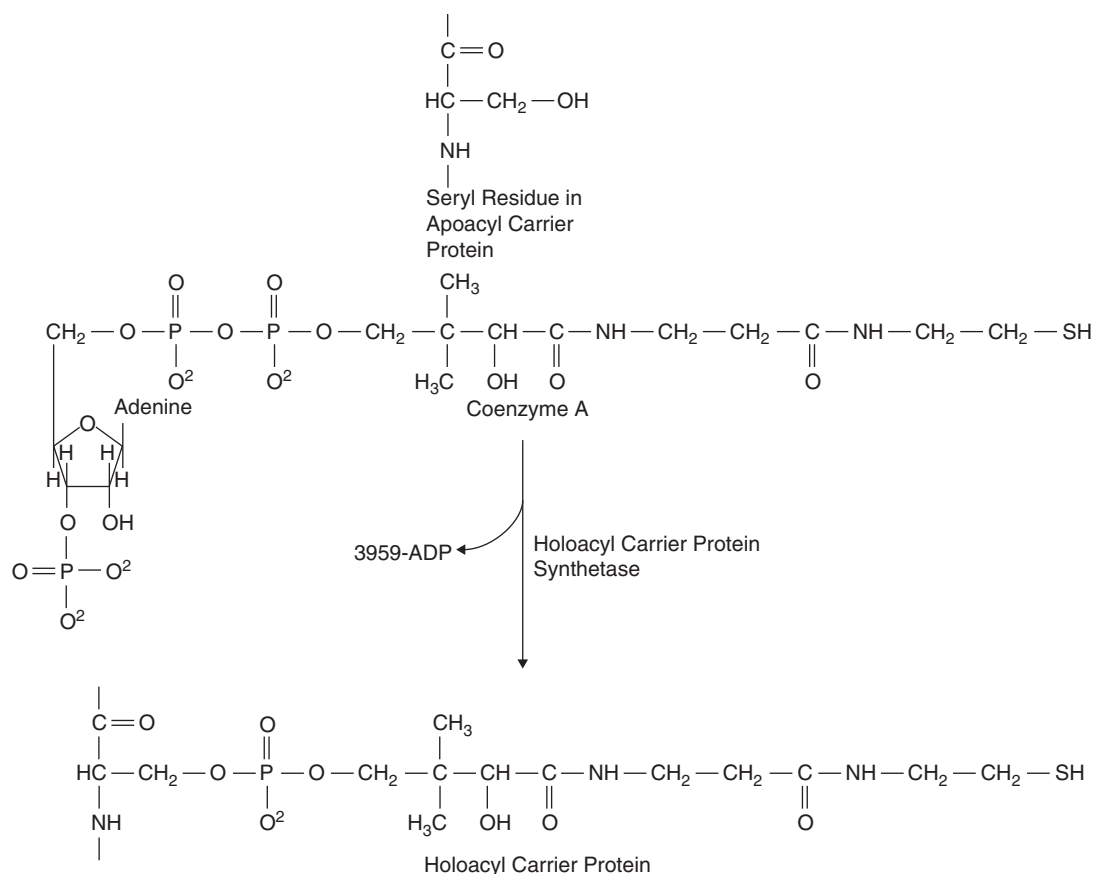


Figure 3 Acyl carrier protein synthesis: CoA is cleaved to form 3',5'-ADP and attach 4'-phosphopantetheine as a phosphate ester of the hydroxyl of a serine residue in apo-ACP, a component of fatty acid synthetase. Source: From Ref. 7.

using CoA thioester substrates and producing CoA thioester products.

Acyl Carrier Protein and Coenzyme A in Fatty Acid Synthesis

Both CoA and ACP are essential for the synthesis of fatty acids in the cytosol. Acetyl CoA, the substrate for fatty acid synthesis, is generated from citrate and CoA by citrate lyase in the cytosol. The citrate is transported out of the mitochondria where it was formed in the tricarboxylic acid cycle from acetyl CoA produced by the oxidation of pyruvate. Acetyl transacylase transfers the acetyl group from acetyl CoA to the pantetheine sulfhydryl of ACP, releasing free CoA in the process. These two carbons from acetyl CoA form the methyl end of the fatty acid that will be synthesized. A biotin-containing enzyme, acetyl CoA carboxylase, utilizes bicarbonate and ATP to convert acetyl CoA to malonyl CoA. Fatty acid synthetase utilizes this malonyl CoA to sequentially add two carbon units to the acetyl or acyl ACP, with the liberation of the third carbon of malonyl CoA as CO₂. This process results in the synthesis of even-numbered fatty acids of 16 or 18 carbons. When the synthesis of a fatty acid is complete, a thioesterase hydrolyzes the ACP-fatty acid thioester, releasing the fatty acid and regenerating the ACP sulfhydryl. The rate of fatty acid synthesis is primarily regulated by

the concentration of malonyl CoA, which is determined by regulation of the activity of acetyl CoA carboxylase.

Coenzyme A in Oxidative Decarboxylation

A key role for CoA in fuel metabolism is its function in α -keto acid dehydrogenase complexes that catalyze the oxidative decarboxylation of keto acids. In the metabolism of carbohydrates, the end product of the glycolytic pathway for glucose is the simple three-carbon α -keto acid, pyruvate. In order for pyruvate to be completely oxidized via the tricarboxylic acid cycle and oxidative phosphorylation, it is oxidatively decarboxylated to acetyl CoA [with release of CO₂ and reduction of nicotinamide adenine dinucleotide (NAD)] by the pyruvate dehydrogenase complex. This complex reaction involves five coenzymes (four of them derived from vitamins): thiamine pyrophosphate, NAD, flavine adenine dinucleotide, lipoate, and CoA. In the decarboxylation of pyruvate, the two-carbon aldehyde unit is attached to thiamine pyrophosphate, oxidized, transferred to a lipoyl enzyme, and then to CoA to form acetyl CoA. Acetyl CoA is a central compound in metabolism, having several catabolic as well as anabolic fates. The CoA is eventually released as free CoA as further metabolism of acetyl CoA progresses. Two other enzyme complexes catalyze the oxidative decarboxylation of keto acids with the formation of acyl CoA products.

The α -ketoglutarate dehydrogenase in the tricarboxylic acid cycle converts α -ketoglutarate to succinyl CoA. The CoA is released from succinyl CoA in the next step of the tricarboxylic acid cycle. The branched-chain α -keto acid dehydrogenase complex, again in a series of reactions analogous to those of pyruvate dehydrogenase, catalyzes the first committed step in the catabolic pathway for the branched-chain amino acids. The α -keto acids from transamination of valine, isoleucine, and leucine are oxidatively decarboxylated to form branched-chain acyl CoA products with one less carbon in the chain. These are metabolized in a number of different steps as CoA esters and ultimately yield simple acyl CoA products, such as acetyl CoA and propionyl CoA, which enter general metabolism.

Coenzyme A in Fatty Acid β -Oxidation

CoA plays a major role in the β -oxidation of fatty acids in the mitochondria, which may result in the complete degradation of fatty acids to acetyl CoA that can be further oxidized in the tricarboxylic acid cycle. Most of the fatty acids consumed in dietary triglycerides (fat or oils) or obtained from adipose tissue stores have chains of 16 or 18 carbons. These long-chain fatty acids require a carrier system for their transport from the cytosol into the mitochondria. In the cytosol, the free long-chain fatty acids are activated to CoA thioesters by acyl CoA synthetases that couple ATP hydrolysis with thioester formation. These fatty acyl CoAs are transesterified to carnitine to form "energy-equivalent" acyl carnitines, which can be transported across the mitochondrial inner membrane. On the outer mitochondrial membrane, the enzyme carnitine palmitoyl transferase I (CPT I) converts the fatty acyl CoA to acyl carnitine and free CoA. A carnitine/acyl carnitine translocase moves the acyl carnitines into the mitochondria and free carnitine out of the mitochondria. Carnitine palmitoyl transferase II (CPT II) on the inner mitochondrial membrane regenerates fatty acyl CoA in the mitochondria, freeing up carnitine for transport out of the mitochondria. In the β -oxidation, two-carbon segments of the fatty acyl CoA are sequentially removed as acetyl CoA. The series of reactions for each cycle are dehydrogenation to the unsaturated acyl CoA, hydration to 3-hydroxyacyl CoA, dehydrogenation to the 3-ketoacyl CoA, and thiolytic cleavage by CoA to release acetyl CoA and a fatty acyl CoA with two less carbons. There are multiple dehydrogenases with overlapping chain length specificities that favor acyl CoAs with very long, long, medium, or short chains. Reducing equivalents generated in the various dehydrogenation steps are funneled into the electron transport chain. Although most tissues other than the brain can use fatty acids as fuel, cardiac muscle and skeletal muscle are especially dependent on fatty acid oxidation for energy. The rate of fatty acid oxidation is controlled by the rate of transport of fatty acids into the mitochondria. The rate of transport is controlled largely by the activity of CPT I, which is strongly inhibited by malonyl CoA. When fatty acid synthesis is increased by insulin activation of acetyl CoA carboxylase to produce more malonyl CoA as substrate for fatty acid synthetase, the increased malonyl CoA inhibits CPT I, decreasing fatty acid transport into the mitochondria, and thus preventing

the reoxidation of newly synthesized fatty acids. Increased glucagon enhances fatty acid β -oxidation indirectly by inhibiting acetyl CoA carboxylase, decreasing the synthesis of malonyl CoA and fatty acids, and reducing malonyl CoA inhibition of CPT I so that fatty acids enter the mitochondria for β -oxidation.

Coenzyme A in Ketone Body Metabolism

Ketone bodies are an important source of fuel derived from fat metabolism when glucose is limiting as in starvation. Acetoacetate and its reduction product, 3-hydroxybutyrate, were called ketone bodies because some acetoacetate is spontaneously decarboxylated to acetone, a ketone. Ketone bodies are synthesized in the liver from acetoacetyl CoA and acetyl CoA produced via β -oxidation of fatty acids. Acetoacetyl CoA is condensed with acetyl CoA to form 3-hydroxy-3-methylglutaryl CoA and free CoA by mitochondrial 3-hydroxy-3-methylglutaryl CoA (HMG CoA) synthetase. This is then cleaved by HMG CoA lyase to form free acetoacetate and acetyl CoA. The net result of this cycle is the conversion of acetoacetyl CoA to acetoacetate and free CoA, but there is no enzyme that directly catalyzes this hydrolysis. Acetoacetate and 3-hydroxybutyrate are interconverted by 3-hydroxybutyrate dehydrogenase with NAD and NADH, with 3-hydroxybutyrate being the major form. Acetoacetate and 3-hydroxybutyrate are released from the liver into the blood and are then taken up by other tissues that are able to use them as fuels. In the extrahepatic tissues, the acetoacetate is converted to a CoA ester using succinyl CoA as the CoA donor. The acetoacetyl CoA can then be metabolized to acetyl CoA (last step of β -oxidation) and further oxidized by the tricarboxylic acid cycle and oxidative phosphorylation. The brain, which cannot utilize fatty acids for energy, can use the ketone bodies produced from fatty acids by the liver.

Coenzyme A in Organic Acid Metabolism

CoA is also involved in the mitochondrial metabolism of a large number of other carboxylic acids as CoA thioesters. The catabolism of many amino acids involves the removal of the amino group, leaving a carboxyl group that can be esterified to CoA for further metabolism. As described earlier, the branched-chain α -keto acids derived from valine, isoleucine, and leucine are oxidatively decarboxylated to form acyl CoA derivatives. Leucine is catabolized to HMG CoA, which is cleaved to acetoacetate (a ketone body) and acetyl CoA by the lyase involved in ketone body synthesis. Valine and isoleucine are metabolized via pathways involving acyl CoAs to form propionyl CoA and propionyl CoA plus acetyl CoA. The amino acids threonine and methionine are also metabolized to propionyl CoA. The propionyl CoA is converted to succinyl CoA and enters the tricarboxylic acid cycle. The amino acids lysine, hydroxylysine, and tryptophan are catabolized to acetoacetyl CoA. In addition to catabolic pathways, acyl CoAs are involved in many synthetic reactions. The CoA ester of 3-hydroxy-3-methylglutamate (HMG CoA), formed in the cytosol, is the starting material for the synthesis of isoprenoids, cholesterol, and steroids. Acetyl CoA is a substrate for the acetylation of amino and hydroxyl groups of many compounds. Another role for CoA is the

detoxification of drugs and other exogenous compounds. An example is the conversion of aspirin to a CoA ester, then transfer to the amino group of glycine to form salicylurate for excretion.

Carnitine interrelations

The esters of CoA and carnitine have very similar energy contents. They are maintained in equilibrium by carnitine acyl CoA transferases. The carnitine palmitoyl CoA transferases and their role in transporting long-chain fatty acids into mitochondria for fatty acid β -oxidation have already been described. In addition, carnitine acetyl CoA transferase catalyzes the interconversion of a number of short-chain carnitine esters and CoA thioesters. Additional transferases act on medium-chain length acids. Free carnitine and carnitine esters act as a buffer to maintain free CoA and acyl CoA levels. If acyl CoAs accumulate as that occurring in inherited disorders of fatty acid oxidation or metabolism of some organic acids, free CoA could be depleted below the levels needed for its essential roles in metabolism. The conversion of some acyl CoAs to acyl carnitines frees up CoA and maintains a more normal ratio of free-to-esterified CoA. In addition, acyl CoAs are inhibitors of a number of enzymes, and decreasing their concentration by converting them to acyl carnitines reduces this inhibition. The acyl carnitines can also be translocated out of the mitochondria, enter the blood circulation, and be excreted by the kidneys as a means of removing accumulated esters of CoA that may be toxic. A side effect of this is that in inherited disorders, in which acyl carnitines are excreted in large amounts, carnitine itself may become depleted in tissues and this, in turn, will decrease the transport of fatty acids into the mitochondria.

PHYSIOLOGY

Absorption

CoA and ACP from the diet are enzymatically degraded in the intestine to release free pantothenic acid (12). CoA, dephospho-CoA, and phosphopantetheine are not absorbed by the intestine and must be digested to pantothenic acid before absorption. Uptake of pantothenic acid is mediated by a saturable Na^+ -dependent transporter utilizing the Na^+ electrochemical gradient for active transport with the highest rate of transport in the jejunum (13). This multivitamin transporter, which also transports biotin and lipoic acid, has been cloned from human intestinal cells (14). Pantetheine is also absorbed by the intestine but is hydrolyzed to pantothenic acid in the intestinal cells. The absorbed pantothenic acid is transported by the blood, primarily as bound forms in red blood cells. How this is made available to tissues is unclear, and it may be that the low concentration of free pantothenic acid in plasma (0.06–0.08 mg/L as compared with 1.0–1.8 mg/L in whole blood) is the form taken up by tissues (4).

Transport

Pantothenic acid (pantothenate) is transported into mammalian cells by the saturable Na^+ -dependent multivitamin transporter, which also transports biotin and lipoic

acid (15). The transport across the blood–brain barrier is also saturable but does not appear to be Na^+ -dependent (4).

Excretion

In the kidney tubules, pantothenic acid is largely reabsorbed at physiological concentrations by a Na^+ -dependent process (4). At higher concentrations, there is tubular secretion of pantothenic acid (excretion of a higher concentration in the urine than is present in the plasma). As a result, there is a positive correlation between dietary intake of pantothenic acid and its excretion in the urine. There are no known catabolites of pantothenic acid; only pantothenic acid is excreted in urine.

DIETARY SOURCES

Pantothenic acid is widely distributed in plant and animal sources, existing both free and bound as ACP and CoA. Total pantothenic acid in foods is determined by hydrolysis of the bound forms to free pantothenic acid and quantitation of the released pantothenic acid by microbiological growth assays, radioimmune assays, or more recently, stable isotope dilution mass spectrometric assays (16). There is considerable loss of pantothenic acid in highly processed foods (4). The average dietary intake of pantothenic acid in the composite Canadian diet is about 5 to 6 mg/day, with somewhat lower intake in the elderly and young children (17). Another study of mixed total diet composites of young adults in the United States found a mean pantothenic acid intake of $5.88 \text{ mg} \pm 0.50$ standard deviation (18). There is limited information about the bioavailability of pantothenic acid (19). From studies of dietary intake and urinary excretion, it is estimated that only about 50% of dietary pantothenic acid is available.

RECOMMENDED INTAKES

No recommended daily allowance has been determined for pantothenic acid. Ingestion of 1.7 to 7 mg/day, depending on age, is considered adequate dietary reference intake (Table 1). Pantothenic acid is included in most multivitamin supplements, generally in the amount of 10 mg.

Table 1 Adequate Intakes of Pantothenic Acid

Group	Amount (mg/day)
Infants	
0–6 mo	1.7
7–12 mo	1.8
Children	
1–3 yr	2
4–8 yr	3
9–13 yr	4
Males	
> 13 yr	5
Females	
> 13 yr	5
Pregnancy	6
Lactation	7

Source: From Ref. 20.

DEFICIENCY

Because of the wide distribution of pantothenic acid in foods, no spontaneous deficiency has been reported. Deficiency has been induced in a small number of human volunteers with a pantothenic acid-free diet. There were no clinical symptoms at nine weeks, even though urinary excretion of pantothenic acid had decreased by 75%, but the volunteers appeared listless and complained of fatigue (21). Others fed a diet deficient in pantothenic acid together with an antagonist (ω -methylpantothenic acid) to block pantothenic acid utilization developed headaches, fatigue, a sensation of weakness and numbness, and burning sensations in hands and feet (22,23). Additional symptoms included personality changes, sleep disturbances, impaired motor coordination, and gastrointestinal disturbances. All symptoms were reversed by stopping the antagonist and giving pantothenic acid.

SUPPLEMENTATION

The effect of supplementation with very high levels of pantothenic acid and thiamin derivatives on physiology and performance of trained cyclists was compared to placebo in a randomized double-blind study (24). There was no difference in any of the physiological parameters or in time trials. Pantothenic acid together with ascorbic acid may improve wound healing, giving more solid and resistant scars, by affecting the trace metal concentrations (25). Exposure of rats to gamma radiation lowers liver CoA, glutathione, cholesterol, and phospholipid levels and causes lipid peroxidation. Administration of pantothenol, which is readily converted to pantothenic acid, prevented these effects, presumably by maintaining CoA levels (26).

Patients with fatty liver and hypertriglyceridemia treated with large amounts of pantothenic acid show decreased fat in liver and the viscera, but increased subcutaneous fat (27).

In the dietary supplement marketplace, there are many more claims for a wide range of beneficial health effects of very large doses of 500 to 1000 mg of pantothenic acid. These amounts are hundreds of times the adequate daily intake, considered to be about 5 mg/day for adults. No data on toxicity of pantothenic acid in humans at these or higher doses have been reported, and only minor gastrointestinal effects occur at even higher doses. Studies in rats showed that the extremely high dose of 3% calcium pantothenic acid by weight in the diet for 29 days caused enlargement of the testes, diarrhea, hair damage, reduced food intake, and reduced weight gain (28). There were no ill effects from calcium pantothenate at 1% by weight of the diet for 29 days, or the control (normal) diet with calcium pantothenate at 0.0016% by weight of the diet. Although there appears to be no risk of toxicity with gram quantities of pantothenic acid, there is very little evidence to support the health claims for clinical benefits of pantothenic acid. The broad health claims include increased energy and athletic ability, a cure for acne, decreased symptoms in arthritis, increased immunity, prevention of hair loss and graying, anti-aging, activation of the adrenal glands, synthesis of the neurotransmitter acetylcholine, lowering cholesterol and triglyceride levels, and improved wound

healing. The claims are often based on a single or a very few old studies with a small number of subjects, and well-controlled double-blind clinical studies with a larger number of subjects have not been done to validate the claims.

INHERITED DISORDER

An inherited disorder, pantothenate kinase-associated neurodegeneration (previously called Hallervorden-Spatz syndrome), has been shown to be due to mutations in a pantothenate kinase gene, *PANK2* (29). This disorder is an autosomal recessive neurodegenerative disorder with iron accumulation in the basal ganglia of the brain, onset in childhood, and a progressive course with early death. There are four different *PANK* genes in humans, with different expressions in different tissues. *PANK1* is most expressed in heart, liver, and kidney; *PANK3* in liver; *PANK4* in muscle; and *PANK2*, the mitochondrial isoform, in most tissues, including basal ganglia. A mutation in *PANK2*, which resulted in low activity of pantothenate kinase in those tissues, where it is the major expressed pantothenate kinase, would be expected to affect CoA levels since this enzyme is rate limiting for the synthesis of CoA. In a large study of patients with *PANK*, all those with the classic syndrome showing early onset with rapid progression had mutations in *PANK2*, most often resulting in protein truncation (30). Only about a third of patients with atypical disease (often with prominent speech-related and psychiatric symptoms) had *PANK2* mutations, and these generally caused an amino acid change. Some of these patients with residual activity of pantothenate kinase may benefit from treatment with large doses of pantothenic acid. The classic and atypical disease due to *PANK2* abnormalities is now generally referred to as pantothenate kinase-associated neurodegeneration. A recent review compares pantothenate kinase-associated neurodegeneration with other inherited disorders that cause neurodegeneration with brain iron accumulation (31). A common mutation of the *PANK2* gene accounts for 25% of the alleles in affected individuals. A mouse model lacking *PANK2* does not show the neurological phenotype of *PANK* but does show growth retardation, azoospermia, and retinal degeneration. When fed a pantothenic acid-deficient diet, these mice died suddenly without discernable neurological problems (32). Wild type mice on the pantothenic acid-deficient diet developed azoospermia and a movement disorder, which were reversible on restoring use of pantothenic acid.

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Pau d'Arco

Memory P. F. Elvin-Lewis and Walter H. Lewis

INTRODUCTION

Pau d'arco and lapachol are the Portuguese and Spanish names used to identify about 26 species of shrubs and trees of the genus *Tabebuia* (Bignoniaceae). These species are indigenous to the American tropics from Mexico to southern South America, with the majority of species found in Brazil and neighboring states. They possess numerous bioactive compounds, with core activity in the naphthoquinones, particularly lapachol and α - and β -lapachones. Other classes of compounds include the anthraquinones, flavonoids, iridoids, lignans, and terpenoids, all less well known or active than the more prevalent naphthoquinones. The stem bark and trunk or heartwood of *T. impetiginosa* (Mart. ex DC.) Standl. (synonym *T. avellanedae* Lor. ex Griseb.), sometimes referred to as the Ipe Roxo tree, *T. rosea* (Bertol.) DC., and *T. serratifolia* (Vahl) Nichols. are the materials and species most commonly used in the preparation of botanicals and traditional and herbal medicines, and for research and clinical purposes. The inner bark of *T. impetiginosa* and possibly other related species are the source of taheebo, a phytomedicine also referred to as ipê-cavatã, ipê-comum, ipê-reto, ipê-rosa, ipê-roxo-damata, lapacho negro, pau d'arco-roxo, peúva or piúva.

Historical uses of pau d'arco species are most commonly reported for use as a tonic, for the treatment of syphilis, fevers, malaria, cutaneous infections, backache, toothache, and stomach and bladder disorders. With a research impetus starting in the 1960s in Brazil, which led to preliminary clinical claims of efficacy for treating cancers, fresh interest in the significance of pau d'arco and its bioactive components has arisen, both regarding basic and clinical research among the general public.

BACKGROUND

Widely used historically and currently in South America to treat a variety of infections and diseases, pau d'arco became known in North America and Europe in the early 1980s. Acceptance of this botanical has been rapid, however, for the bark or wood prepared as an infusion or decoction is ingested regularly by at least one million people (1). According to Information Resources Inc. (IRI), in 2009 pau d'arco was ranked 46th in single herb supplement sales in the United States with dollar sales amounting to \$35,636. This was an increase of 11.68% over the 2008 sales (C. Cavaliere, written communication, 2010). However, in Canada the Federal Health Protection Branch has banned



Figure 1 Flowering branch of *Tabebuia impetiginosa*, a 30-m tree common in tropical South America. Source: Courtesy of Al Gentry, Missouri Botanical Garden.

its use until distributors prove their products are safe and effective.

One of the most important sources of pau d'arco inner bark and heartwood is *T. impetiginosa* (Fig. 1), a large tree up to 30 m tall, with deep pink to purple flowers, found from Mexico and Central America to tropical South America, south to northern Argentina, and Bolivia. Significant also are the large trees *T. rosea*, with pink to purple flowers, found from Mexico to northern South America, and *T. serratifolia* (Fig. 2), having yellow flowers, which occurs from Colombia to the Guianas south to Brazil and Bolivia. These species and a majority of the remaining 23 species of *Tabebuia* tested contain lapachol or related compounds, each with varying concentrations in their inner stem bark, heartwood, and leaves.

For medicinal purposes, indigenous people prefer the inner bark, although the heartwood is considered more potent. Leaves and flowers are less frequently used, and the roots rarely so. Usually these phytomedicines are used as infusions or decoctions. Reports of traditional medicinal uses of *Tabebuia* species can be found on herbarium labels and in the literature, and they provide useful anecdotal evidence of prior use. Pau d'arco is most commonly reported for treatment of syphilis, fevers, malaria, cutaneous infections, tooth and back pain, and bladder and stomach disorders. Some examples are:

- *T. impetiginosa* to treat impetigo in Brazil around 1843 (2).



Figure 2 Flowering branch of *Tabebuia serratifolia*, a 30-m tree common in Amazonian Brazil. Source: Courtesy of Al Gentry, Missouri Botanical Garden.

- *T. rosea* bark drunk to treat malaria (Steyermark 51372, F); bark to treat rabies in Guatemala (Ruano 425, US); decoction of flowers, leaves, and roots taken internally and also applied externally for treating snakebites in Costa Rica; bark decoction prepared as a remedy for fevers, colds, and headaches (3); bark infusion or decoction as a gargle in Colombia to treat throat ailments and fevers, and as an astringent (4).
- *T. serratifolia* bark as a medicinal for the stomach among the Panaré of Venezuela (Boom and Grillo 6209, MO).

Other examples of pau d'arco's early ethnopharmacological uses are cited in current reviews of *T. impetiginosa* (5) and lapachol (6).

Sometimes such information is difficult to separate from neo-Western herbalism (7) when those practicing domestic medicine adopt new uses reported in popular press releases on the basis of limited research. This is particularly evident in reports involving anticancer uses conducted in Brazil since 1960; no primary herbarium material or literature reference of traditional antineoplastic use could be found prior to this date. Thus, the citation of Schunke 14259 (MO) in 1998 reporting that bark infusions of *T. impetiginosa* are drunk by Peruvian natives to cure diabetes, malignant tumors, leukemia, other cancers, anemia, and Parkinson's disease, is undoubtedly an instance of recent incorporation into indigenous pharmacopeia. Use of *T. rosea* bark by the Maya of Mexico against cancer can likewise be traced to the year 1985 (8). Similarly, the utilization of *T. serratifolia* bark in Colombia as an anticancer infusion or decoction can be attributed to Brazilian research of the 1960s and 1970s (4).

All species known as pau d'arco should not be considered equivalent in toxicity or efficacy. The purple-flowered species are considered less toxic than the yellow-flowered ones, and the former are preferred. However, the bark and heartwood are routinely collected and used without regard to taxonomic identification, chemical composition, or biological activity.

CHEMISTRY

Of the 26 species of *Tabebuia* known as pau d'arco, the secondary metabolic chemistry of about half has been well documented. Most contain naphthoquinone derivatives, but only *T. impetiginosa* also contains anthraquinones. In addition, some species possess flavonoids, iridoids, lignans, triterpenes, and other classes of compounds.

The naphthoquinones (Fig. 3) are the most prevalent class of compounds in *Tabebuia*. While a few species lack lapachol (2), it is commonly found in the three most important species, *T. impetiginosa*, *T. rosea*, and *T. serratifolia*. The derivatives prenylnaphthoquinone (1) and lapachol methyl ether (3) are also found in *T. impetiginosa*. Other prenylnaphthoquinones found in these species include dehydro- α -lapachone (4), dihydro- α -lapachone (5), and β -lapachone (LAPA, ARQ 501) (6). In addition, the furanonaphthoquinone derivatives 7–18 are common in *T. impetiginosa*. The majority of these (2, 4–7, 10–12, and 17 and 18) have been found in the inner bark (9). The anthraquinone-naphthaquinol dimer, tabeuin 22, has also been reported for this species (10). In addition to lapachol, 4, 7, 11, and 14 (11) have been isolated from the root, bark, and heartwood of *T. rosea*, along with tabeuin 22 (12), dehydrotectol 19, and the tecomaquinones 20, 21, 23, and 24 from its heartwood (Fig. 4) (13). *T. serratifolia* also contains the constituents 2, 4, 5, 11–13, and 15, obtained from extracts from the trunk's heartwood (14). Studies on

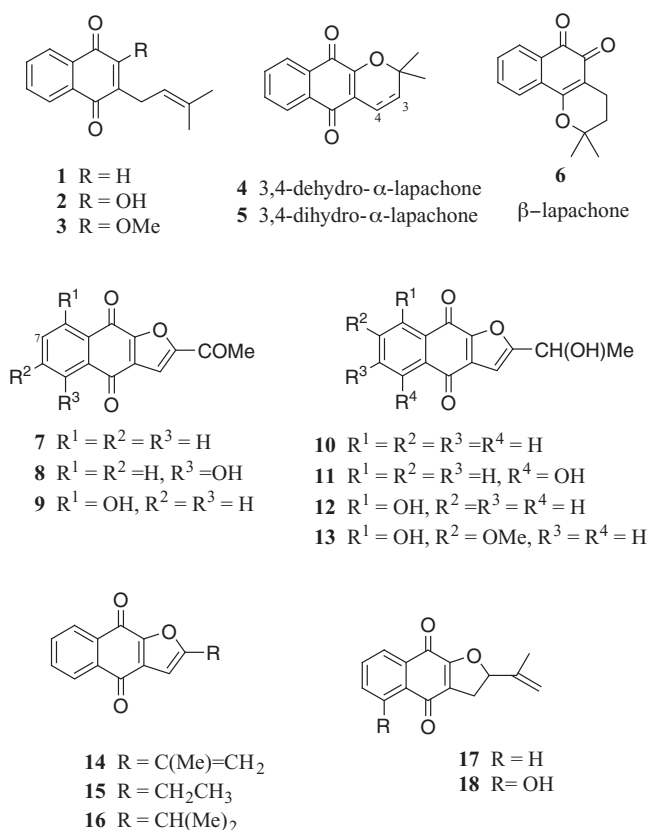


Figure 3 Naphthaquinones from *Tabebuia* spp.

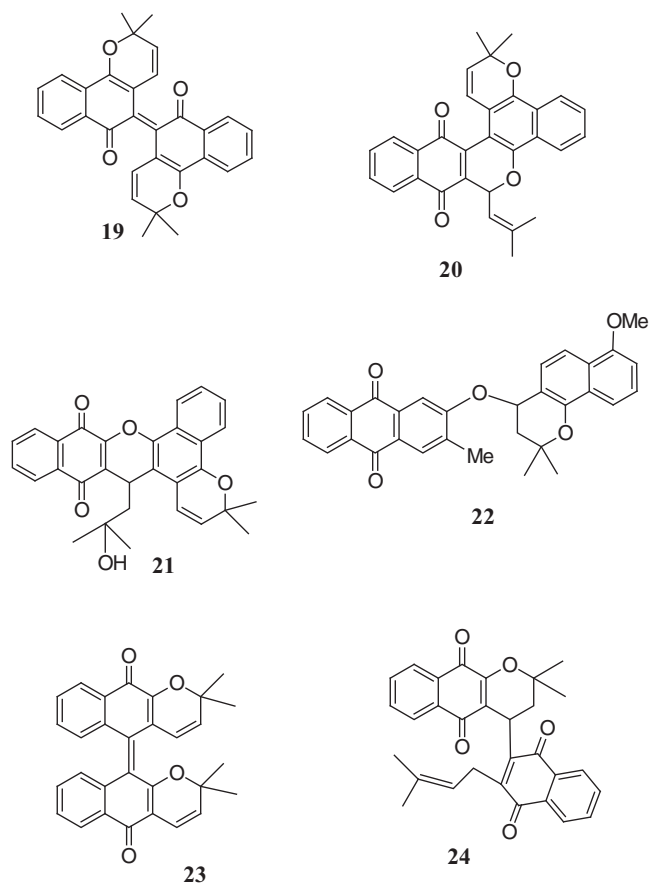


Figure 4 Dimeric naphthaquinones from *Tabebuia* spp.

pau d'arco's compounds and synthetic derivatives are also discussed in other current reviews (5,6).

Initially pharmacological research focused on lapachol; however, this is only one of the N-factors (naphthaquinones) responsible for pharmacological activities of pau d' arco (15). Lapachol is obtained in gram quantities from these species ranging from 2% to 7% from the heartwood (16), a level confirmed by Linardi (17) who obtained 3.2% of the compound from petroleum and methanol extracts of finely powdered wood of *T. impetiginosa*. It was also obtained as a minor constituent from nonaqueous extracts of its inner bark (9), but not detectable in aqueous extracts. In an early publication (1914), *T. serratifolia* wood was reported to have 7.64% lapachol (18). Lapachol and β -lapachone are readily synthesized from 2-hydroxy-1,4-naphthaquinone in high yields (19).

A number of current mechanistic studies and patents have also cited the use of the naturally occurring o-naphthaquinone derivative of lapachol, namely 3, 4-Dihydro-2,2-dimethyl-2H-naphtho(1,2-b)pyran-5, 6-dione or β -lapachone, which is also referred to as LAPA, and ARQ 501. Because of its potential pharmaceutical value as an anticancer and anti-infective agent (20) addressing sourcing issues, physiochemistry and pharmacotech characterization has identified suitable purifying and storage processes in addition to the isolation

of a contaminant, its isomer α -lapachone (21). The compound has also been synthesized (22).

Mechanisms by which synthetic and biosynthetic pathways have been utilized to produce these compounds are known. Although these techniques are important in verifying these and other related structures, the abundance of the natural product as a metabolite in many tropical trees species negates this approach as an alternate source unless such species become threatened by overexploitation (6).

BOTANICAL PRODUCTS, USES, AND ADVERSE REACTIONS

Products

Pau d'arco is available as capsules, tablets, skin salves, extracts, and tea bags. Products with lapacho as a component are sold under such names as Advance Defense System Tablets, Brazilian Herbal Tea, Candistroy, Cat's Claw Defense Complex, Cellguard Coq 10 Nac, Healthgard with Echinacea, Immuno-Nourish, Ipe Roxo, Lapacho, Pau d'Arco, Pau d'Arco Inner Bark, Taheebo, and many others.

Powdered inner bark and/or heartwood are often prepared in the United States as decoctions, with one cup taken two to eight times per day. A decoction is prepared by boiling one teaspoon of powdered bark/heartwood for each cup of water for five to ten minutes (23). In other examples two to three teaspoons of inner bark are simmered in 500 mL of water for 15 minutes and taken three times per day (24). In a more specific example using 460 mg capsules of inner bark/heartwood orally, 1 to 2 capsules are ingested at meals with water twice daily, or 3 to 4 capsules 3 times daily for not more than 7 days, depending on use.

In a study of 15 commercial products of pau d'arco obtained in Canada, naphthaquinones were detected in all samples except two, although no naphthaquinones were found in the three concentrates examined. Lapachol was detected in only two of these products. However, in two Brazilian products studied, one wood and the other a concentrate, both contained lapachol and related compounds (25).

Antioxidant Use

Lapacho or *T. impetiginosa* bark extracts are used to stabilize compositions that contain oxygen-labile active agents particularly for cosmetic use (26), to whiten skin with limited irritation (27), and in an antioxidant Japanese carbonated drink known as Purple Ipe (28).

Antifungal Use

As an example of herbal medicinal use, pau d'arco tea is drunk or applied vaginally as a douche to treat *Candida*, or an extract-soaked tampon is used to treat this and similar infections, often with associated inflammations (29,30). Such extracts are also part of a patented nail varnish formulation for the treatment of human onychomycosis and paronychia (31).

Toxicity, Side Effects, and Interactions

Drinking extracts of yellow-flowered *T. umbellata* or *T. pedicellata* caused abnormal swellings similar to burns and skin pustules to form. Only weak teas made from 1 part bark to 10 parts water should be drunk (1). Pau d'arco should be avoided when pregnant, breast-feeding, and taking anticoagulants and by persons having severe liver disease, von Willebrand's disease, or thrombocytopenia. Its use may cause anemia, nausea, pinkish urine, unusual or excessive bleeding or vomiting, and diarrhea (5,32,33).

Allergic Reactions and Irritation

Exposure to wood dusts may cause skin and mucosal symptoms associated with allergic dermatitis. In the timber trade, allergic reactions to pau d'arco sawdust are common (1). Both lapachol and deoxylapachol are considered allergens (34).

PRECLINICAL STUDIES

In Vitro Studies

Antibacterial Activity

Both gram-positive and -negative microorganisms are affected by certain naphthoquinones through the generation of superoxide anions and hydrogen peroxide (35), by the uncoupling of oxidative phosphorylation, and through electron transfer inhibition (36). Of relevance to traditional uses to treat skin and gastrointestinal infections are inhibitory activities of lapachol, β -lapachone, α -xyloidone, and related compounds against *Staphylococcus aureus* (36), including those with methicillin resistance (37), and *Salmonella* (29,30). Susceptibility of other organisms has also been demonstrated (38).

Using the disc technique (100 μ g/disc), lapachol's effects on intestinal bacteria can vary with greater sensitivity being demonstrated for *Clostridium paraputrificum*, and less so for *C. perfringens* and *Escherichia coli*. Unaffected, even at high concentrations of 1000 mg/disc were *Bifidobacterium adolescentis*, *B. bifidum*, *B. infantis*, and *Lactobacillus acidophilus*, and *L. casei*. When the chemical components of the dried inner bark of *T. impetiginosa* were tested against *Helicobacter pylori* by disc diffusion and minimum inhibitory concentration (MIC) bioassays it was found that 2-(hydroxymethyl) anthroquinone exhibited strong activity at 0.01 mg/disc, whereas 2-carboxylic acid, lapachol and the control metronidazole only exhibited moderate effects at 0.1 mg/disc (39). Overall these compounds were more effective than metronidazole but less effective than amoxicillin and tetracycline. The basis for lapachol and its derivatives' antimicrobial activities are related to the presence of a methyl group in the C-2 position of the 1,4-naphthoquinone (40). Additional studies with an analog of lapachol, "furanonaphthoquinone" show that significant antibiotic effects on five strains of *H. pylori* can occur at MIC levels of 0.1 μ g/mL. Also, when furanonaphthoquinone was combined with several antibiotics such as ampicillin, cefaciator, levofloxacin, minocycline, and vancomycin their MIC values against *H. pylori* were reduced two- to eightfold (41).

Anticancer Activity

The use of pau d'arco in Brazilian remedies to treat cancers has led to the identification of pau d'arco's active components, the production of some derivatives, partial elucidation of their mechanisms of action, and the conduct of preclinical and clinical evaluations in the treatment of carcinomas and leukemias (42,43).

Current in vitro studies have provided a better understanding of the antiproliferative and immunosuppressive effects observed originally (44). For example, in an attempt to identify a selective estrogen receptor for breast cancer therapy that did not elicit adverse effects, human breast carcinoma ER+ MCF-7 cells were treated with aqueous extracts of pau d'arco (taheboo). This preparation elicited time-dependant growth inhibition of the S phase and the initiation of apoptosis associated with a downregulation of the cell cycle regulation and estrogen responsive genes and an upregulation of apoptosis specific and xenobiotic metabolic specific genes (45).

The naphthoquinones inhibit enzymes critical to cellular DNA replication, and other cellular functions, resulting in cell death. Their effects occur at macromolecular levels and result in the selective killing of certain cancer cell lines (colon, lung, prostate, breast, ovary) in unique ways that suggest that lapachol derivatives have the potential for use in therapies for specific cancer types, for example, breast and prostate (46). Some also have increased cytotoxicity (47) and others are effective in drug-resistant cell lines (48).

Also, in lower doses, the naphthoquinones are effective radiosensitizers. They act by specifically and synergistically enhancing the cytotoxic effects of DNA-damaging agents and the effects of X-rays following prolonged drug exposures. For example, β -lapachone may elicit DNA conformational changes, which can inhibit potentially lethal damage repair or during this process, enhance lethality by converting single- into double-stranded DNA breaks (49). The mechanism by which β -lapachone radiosensitizes cancer cells with elevated NQO1 levels is related to its ability to inhibit error-prone nonhomologous end joining needed in the repair of DNA double-stranded breaks (50).

Among these the isomer of lapachol, β -lapachone appears to be the most promising and has replaced lapachol in the US National Cancer Institute's (NCI) anticancer chemotherapeutic studies (5). This is because this molecule and other potent β -cycled-pyran-1, 2-naphthoquinones [0.1 β ¹/₄M < IC₅₀ < 0.6 β ¹/₄M] produce hydrogen bond interactions that are able to affect hydrophobic areas of a receptor more completely than those of the moderately active 1,4-naphthoquinone derivatives (51). These molecules have currently been studied extensively to understand the molecular basis for their anticancer activities and their potential uses in cancer chemotherapy.

While not affecting the regulatory systems of normal cells, β -lapachone can affect unchecked and persistent expression patterns of unscheduled checkpoint molecules present in regulatory-defective precancer and cancer cells (52). It exerts its antineoplastic activity by inducing either apoptotic or necrotic cell death within the range of 1 to 10 μ M (IC₅₀). Cytotoxicity has been elicited in a wide variety of transformed cell lines including those derived

from patients with promyelocytic leukemia (53), prostate (54), malignant glioma (55,56), hepatoma (57), colon (58), breast (59), ovarian (60), pancreatic (50,61–65), retinoblastoma (66), and multiple myeloma cell lines (67), including drug-resistant lines (48).

The mechanisms by which anticancer activities are elicited are complex. Shortly after exposure to β -lapachone, malignant cells rapidly release cytochrome c. This activity causes a decrease in mitochondrial transmembrane potential ($\Delta\psi$), which is followed by the activation of caspase-3 in apoptotic cell death but not in necrotic cell death (68). Studies indicate that its antitumor effect occurs indirectly by inducing p53-independent apoptosis and the arrest of cell cycles through the altered activities of cell cycle control regulatory proteins such as the downregulation of retinoblastoma protein (pRB), a transcriptional repressor target at transcription factor E2F-1 and the induction of the expression of cyclin dependent kinase inhibitor 1A (CDKN1A or p21). During the cell cycle, G1/S-phase transition requires both E2F-1 and p21, and several studies indicate that β -lapachone affects the E2F1 checkpoint pathway and induces cell death in cancer cells from a variety of tissues without affecting normal cells from these tissues. Also, cytotoxicity occurs through the induction of reactive oxygen species in vivo (69). In addition, apoptosis elicited by β -lapachone may also be due to the loss of reduced NADH or NAD(P)H. This is because NAD(P)H:quinone oxidoreductase (NQO1) causes a reduction of β -lapachone causing "futile cycling" between the quinone and hydroquinone forms with a concomitant loss of these reduced enzyme cofactors (70). Its potential use as a chemopreventive agent for liver cancer is also associated with its ability to induce apoptosis in hepatocarcinoma HepG2 cells. The mechanisms involved relate to the formation of apoptotic bodies and DNA fragmentation. These activities are linked to the downregulation of antiapoptotic Bcl-2 and Bcl-X (L) and upregulation of proapoptotic Bax expression with the proteolytic activation of caspase-3 and -9 and degradation of poly (ADP-ribose) polymerase (PARP) protein without affecting either the Fas/FasL system or the inhibitor of apoptosis family proteins (71).

β -lapachone inhibits cell viability and migration of human hepatocarcinoma cell lines, HepG2, and Hep3 in a dose-dependant manner. Western blot analysis indicates that at an early point in time, amplified levels of protein, as well as mRNA expression of early growth response gene-1 (Egr-1) and thrombospondin-1 (TSP-1) occurs and then decreases in a time-dependent manner. Also, in the matrigel invasion assay a decrease in invasive ability takes place indicating a downregulation of Snail and upregulation of E-cadherin expression. The viability of HepG2 cells is affected through the induction of apoptosis and the formation of apoptotic bodies and DNA fragmentation. This activity is associated with a proteolytic activation of caspase-3 and -9 and degradation of PARP protein, downregulation of antiapoptotic Bcl-2 and Bcl-XL and upregulation of proapoptotic Bax expression. However, the family of inhibitor proteins and the FAS/FasL system are not affected. These studies indicate that this compound is potentially a chemopreventive agent for liver cancer (71,72). Similar results have been observed using the T24 line of bladder cancer cells (73), cultured human prostate carci-

noma DU145 cells (74), human colon cancer cells (HCT-116 (75), SW480, SW620, and DLD1), (58) and breast cancer cells. (59,76).

The role of the ubiquitous enzyme, NAD(P)H:quinone oxidoreductase-1 (NQO1) is to detoxify cells affected by xenobiotics containing quinone moieties by catalyzing a two-electron reduction in quinones, using NADH or NADPH as the electron donor. Unlike other reductases, NQO1 produces a highly stable intermediate in the presence of β -lapachone treatment. In certain tumors of the breast, cancer, and lung NQO1 is overexpressed (77) and treatment with this compound causes cell death due to the catalytic action of NQO1 by eliciting futile oxidoreductions of β -lapachone leading to reactive oxygen species' generation, a novel caspase independent of p53, DNA breaks, β -H2AX foci formation, and hyperactivation of poly(ADP-ribose) polymerase-1 (5,48).

Unlike normal cells, the vulnerability of cancer cells to topoisomerase inhibition is linked to their ability to overcome certain restrictive mechanisms enabling them to continually divide. Initial studies on the effects of β -lapachone indicated that its mode of action on irradiated cells differs from camptothecin, which causes chromosome damage and strand breaks (43). It was proposed that enhanced cytotoxicity was due to inhibition of topoisomerase I activity and the modification and inhibition of lesion repair (49,54,78,79). β -lapachone blocks the binding of human DNA topoisomerase I to DNA (80,81), and by preventing DNA repair sensitizes cells to DNA-damaging agents including radiation (82,83). By interacting directly with these enzymes β -lapachone prevents catalysis and blocks the formation of a cleavable complex (80) or with the complex itself, causing religation of DNA breaks and dissociation of the enzyme from DNA (84). However, inhibition of either topoisomerase I or topoisomerase II (81,84) activities is no longer considered the primary way in which cell lethality is induced by β -lapachone but rather that NAD(P)H:quinone oxidoreductase activity is the principal determinant of β -lapachone cytotoxicity (43,70).

Prevention of neovascularization is an important factor in inhibiting tumor growth. Studies on the effect of β -lapachone on endothelial cell death, indicate that intracellular cGMP levels and the mitochondria membrane potential (MMP) are decreased, and calpain and caspases are activated. Addition of nitric oxide (NO), which is an important factor in mediating vascular cell growth and migration, downregulates the β -lapachone-induced cGMP depletion and protects the cells from apoptosis by blocking the MMP decrease and increases of calcium. The fact that antiangiogenic effects are not affected in this process suggests that β -lapachone may have a potential as an antiangiogenic drug (85).

The apoptotic synergistic action of β -lapachone and taxol on cancer cells is based upon their ability to affect cell arrest at conflicting checkpoint signals. By exploiting these cell death "collisions" this combination has the potential of treating cancers such as multiple myeloma (43). It is recommended that the combination of a G1 or S phase drug, such as a β -lapachone should be given first, with a G2/M drug, such as a taxane derivative added either simultaneously or after β -lapachone. In this way β -lapachone causes cell-cycle delays in late G1 and S phase and taxol arrests cells at G2/M, resulting in multiple checkpoint delays

before committing to apoptosis (60). Additional studies with human retinoblastoma Y79 cells combining β -lapachone with paclitaxel suggest that the basis for this synergistic apoptotic action is related to phospho-Akt lowering of inhibitor apoptosis proteins and by activation of Bid and caspases-3 and -6 with lamin B and PARP breakdown. Phospho-Akt is a serine/threonine protein kinase, which acts as a powerful promoter of cell survival as it antagonizes and inactivates various components of the apoptotic cascade, and PARP is a protein involved in a number of cellular processes involving mainly DNA repair and programmed cell death (86).

Antifungal Activity

Pau d'arco extracts and their components lapachol and β -lapachone are active against more than one known infection of the skin (ringworm) and nails (36), and pathogenic yeasts such as *Candida* and *Cryptococcus neoformans* (36). Activities of these naphthoquinones differ in that lapachol activity is comparable to that of ketoconazole, while β -lapachone is superior (36). In vitro tests using lapachol show it is an effective antifungal agent against the human yeast *Pityrosporum ovale*, which causes the chronic skin infection called Tinea versicolor in addition to the wood rotting basidiomycete, *Gloeophyllum trabeum* (87). *Tabebuia caraiba* extracts are also effective against the dermatophyte, *Trichophyton rubrum* (88). In a U.S. patent application 2003-674145, 2005-049207, *T. impetiginosa* is cited for use against cancers at antifungal doses (89). Modest antifungal activity has also been associated with synthesized naphthoquinones based on the naphtho[2,3-b]furan-4,9-dione skeleton such as (-)-5-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione and its positional isomer, (-)-8-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione (90). Lapachol's significant antifungal activities against *Candida albicans*, *C. tropicalis*, *C. elegans*, and *Cryptococcus neoformans* is considered because of its interaction with their cellular membranes (91).

Anti-inflammatory activity

Studies on the affects of various solvent fractions of pau d'arco (taheebo) on washed rabbit platelets and cultured rat aortic vascular smooth muscle cells (VSMCs) suggest that platelet aggregation induced by collagen and arachidonic acid is inhibited in a dose dependant way by n-Hexane, chloroform, and ethyl acetate fractions. Of these, the chloroform fractions were the most active by significantly suppressing arachidonic acid liberation induced by collagen in [(3)H]AA-labeled rabbit platelets, effectively inhibiting cell proliferation and DNA synthesis induced by platelet derived growth factor (PDGF)-BB, in addition to inhibiting the levels of phosphorylated extracellular signal regulated kinase (ERK1/2) mitogen-activated protein kinase stimulated by PDGF-BB, in the same concentration range that inhibits VSMC proliferation and DNA synthesis (92). Aqueous and ethanolic extracts of pau d'arco (taheebo) studied for their anti-inflammatory potentials indicated that the aqueous extract possessed the ability to negatively modulate macrophage-mediated inflammatory responses by suppressing prostaglandin E(2) production. This activity was evident by treating LPS-stimulated RAW264.7 macrophages where significant suppression of

NO production and blockage of COX-2 and inducible NO synthase (iNOS) occurred as did the phosphorylation of extracellular signal-related kinase (ERK) and U0126, a selective ERK inhibitor. In vivo blockage of COX-2 was also evident when mouse ear edema was diminished in arachidonic acid treated mice given 100 mg/kg of pau d'arco (taheebo), but not those whose edema was evoked by croton oil, an activator of lipoxygenase (93). Using the carrageen inflammatory paw model in rats, lapachol at doses of 100 and 500 mg/kg was found to significantly inhibit the production of edema and abscess formation (20,94).

A number of studies indicate that β -lapachone (LAPA) has the potential of being a useful anti-inflammatory agent. It is not only able to inhibit the expression of NO and iNOS in alveolar macrophages but when studied in lipopolysaccharide-stimulated BV2 microglial cells it inhibits NO and the fever inducing, prostaglandin, dinoprostone (PGE2). A blockage at transcriptional levels is suggested by inhibition of iNOS and COX-2. In addition, the expression of mRNA and proteins of proinflammatory cytokines, such as interleukin IL1B, IL-6, and tumor necrosis factor (TNF-alpha) are affected in a dose-dependant manner. β -lapachone further elicits its anti-inflammatory activity by suppressing the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) by blocking IkappaBalpha degradation and downregulating the ERK, p38 mitogen-activated protein kinase and the Akt pathway associated with cellular survival (95). Furthermore as a topoisomerase inhibitor, β -lapachone has the potential of being useful in the treatment of inflammatory disorders (96) and as antiarthritic agents (97). Likewise, two lapachol derivatives 3-iodo- α -lapachone (I) and 3-iodo- β -lapachone (II) are considered useful as probable immunomodulatory, antimicrobial and anti-inflammatory agents (98).

Antileishmania Activity

β -lapachone and its derivatives have also been synthesized and tested as antiparasitic agents. A number of β -lapachone derivatives obtained via the Prins reaction of lapachols possess Leishmanicidal activities (99). Also 3-allyl- β -lapachone can elicit negative affects on the developmental cycle of *Trypanasoma cruzi* by inhibiting the growth and functions of epimastigotes as well as decreasing or causing the total disappearance of trypomastigotes from mouse-infected blood (100). Additional studies on the inhibition of epimastigote growth and trypomastigote viability with lapachol, α - and β -lapachone derivatives indicate the substitution of the benzene ring by a pyridine moiety enhances these activities (101).

Antimalarial Activity

Early antimalarial studies claimed that lapachol was almost as active as quinine (102). However, when tested, its activity against *Plasmodium falciparum* proved disappointing (103), although several analogs of lapachol show enhanced bioreactivity (104,105). In addition several benzo[a]phenazines synthesized from 1, 2-naphthoquinone, lapachol, and β -lapachone proved effective against *P. falciparum*. (106) as have the dihydroxyfuranonaphthoquinones, 5 and 8-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-diones isolated from *T. incana* bark infusions (107).

The antimalarial activity of lapachol and other naphthoquinones is linked to their ability to interfere with electron transport and thus to inhibit the respiratory chain (43,108). A study with *Plasmodium knowlesi* treated with 100 mg/mL of lapachol indicated that absorption of oxygen was inhibited by 74% and succinate oxidase by 26%. This inhibition is possibly related to lapachol's ability to inhibit the interaction between cytochromes b and c. (6,109).

Anti-Giardia Activity

Giardia lamblia is a diplomonad protozoan that parasitizes the small intestine of vertebrates and is responsible for millions of people acquiring intestinal infections and diarrhea. In vitro studies with β -lapachone indicate it can elicit apoptosis by cell shrinkage, chromatin condensation, membrane blebbing, and vacuolization. Drug treatment also can alter lipid rafts, co-localized with regions containing membrane blebbing. By inducing encystation it may also affect mechanisms of parasite resistance (110).

Antimetastatic Activity

Alterations in the protein profile and inhibition of cellular invasiveness was demonstrated in a HeLa cell assay using lapachol at a nontoxic concentration of 400 μ g/mL (corresponding to 10^{12} molecules of the drug/cell). These activities correspond to a significant antimetastatic potential of this compound (111).

Antioxidant Activity

The antioxidant activity of volatile constituents of the dried inner bark of *T. impetiginosa* is comparable to that of the antioxidants α -tocopherol and butylated hydroxytoluene (112).

Antioxidant activities have also been identified in a number of *T. heptaphylla* bark compounds (113).

Antipsoriatic Activity

Evaluations of several naturally occurring pau d'arco (lapacho) compounds from the inner bark of *T. impetiginosa* and a number of synthetic analogs on the growth of the human keratinocyte cell line HaCaT indicate that β -lapachone is comparable to the antipsoriatic drug anthralin with an IC_{50} value of 0.7 μ M. Other active constituents of lapacho inhibited keratinocyte growth, with IC_{50} values in the range of 0.5 to 3.0 μ M with the most potent synthetic analog 2-acetyl-8-hydroxynaphtho[2,3-*b*]furan-4,9-dione eliciting an IC_{50} value of 0.35 μ M. Like anthralin, treatment of HaCaT cells with these potent lapacho compounds caused damage to the plasma membrane (9). Pharmaceutical compositions containing several synthetic lapacho derivatives have been proposed for the treatment of cell proliferative disorders including psoriasis, cancer, or precancerous conditions (114).

Antiviral Activity

Studies indicate that lapachol, β -lapachone, and certain of their derivatives possess broad-spectrum antiviral capacities in vitro. Of those DNA viruses affected, lapachol inhibits replication of members of the herpesvirus group including HHV1, 2 (115,116), and HHV4 (Epstein Barr Virus or EBV) (117). Inhibitory effects against representative RNA viruses, (118) including HIV-1 (119), and HIV

reverse transcriptase (120) have been demonstrated as has its ability to inhibit reverse transcriptases from myeloblastosis virus and Rauscher murine leukemia virus in addition to eukaryotic DNA polymerase- α (121). The ability of β -lapachone to inhibit regulatory proteins, including Tat, which affects the viral switch from latency to active replication, is the subject of several current patents (119). Acute and chronically infected HIV-1 infected cells treated with β -lapachone are unable to produce P 24 since the compound is a potent inhibitor of 1 LTR-directed gene expression of HIV-1 (119). A polyherbal invention containing *Tabebuia* combines a redox active polyphenol, an oxidizing agent and a redox-active transition metal ion and is claimed to be antimicrobial and antiviral (HIV) (122).

Antitrypanosomal Activity

Chagas' disease, or American trypanosomiasis, is a devastating disease in South America, and transmission through blood transfusions is a serious concern. In vitro and in vivo inhibitory effects on *Trypanosoma cruzi* of lapachol, β -lapachone, and several 1,2-naphthoquinone derivatives (100,123) have led to the development of oxazolic, imidazolic (124), and phenazine derivatives (125), which have the potential to replace crystal violet as blood sterilants. Activity against *T. cruzii* is associated with a three bond-distance from nitrogen to the imidazole ring, of the semi-synthetic pyran[b-4,3]naphtho[1,2-d]imidazoles from β -lapachone (126). The production of oxidation radicals leading to cytotoxic activities seen with Trypanosomatids *Crithidia fasciculata* and *Leptomonas seymouri* treated with β -lapachone and structurally related lipophilic o-naphthoquinones and mansonones are also related to their tricyclic structures, including the presence of a naphthalene ring, a 1,2b or 1,8bc pyran ring, and two ortho-carbonyl groups (127).

Neurodegenerative Effects

Lapachol and other naphthoquinones modulate the Tau aggregation of proteins, and thus are assumed to effect the treatment or prophylaxis of neurodegenerative diseases and/or clinical dementias, such as Alzheimer's disease (128).

Snakebites

Tabebuia rosea bark demonstrated 100% neutralization against the minimum hemorrhagic dose of *Bothrops atrox* venom in vitro. This correlates with traditional healers' statements from northwestern Colombia that *T. rosea* has antihemorrhagic properties (129).

Spasmolytic Activity.

When tested in the guinea-pig ileum model, Lapachol, α -lapachone and β -lapachone (or LAPA) can elicit nonselective spasmolytic activities. β -lapachone was also found to exert this effect by blocking voltage-gated calcium channels (L-type Cav channels) (130).

In Vivo Studies

Anticancer Activity (rodents)

Crude extracts and lapachol were tested in implanted rodents against Walker 256 carcinosarcoma. Lapachol showed highly significant antitumor activity especially when administered orally, with relatively little effect on

host body weight (131). In this model, a 92% reduction in tumor growth occurred, and lapachol's tetra-acetyl-glucoside derivative increased the life span by 80% in mice with lymphocytic leukemia P-388 (17). Treatment with β -lapachol following exposure to irradiation optimized the effects of delayed tumor growth in mice with RKO-induced (colorectal cancer cells) tumors (132). In human ovarian and prostate tumor prexenografted mouse models, a synergistic cytotoxic effect was demonstrated using taxol and β -lapachone (60).

Antileishmania (hampsters)

Lapachol and its derivatives can generate oxygen free radicals and through the induction of nitric oxide synthetase by IFN- γ , NO. Together they are considered capable of killing intracellular amastigotes in murine macrophages, with NO playing the major role in killing *Leishmania brasiliensis*. However, when hampsters were given oral doses of lapachol of 300 mg/kg/day for 42 days the development of LVb-induced lesions was not prevented. The rationale for this lack of clinical success was considered due to leishmanicidal activity being lost in macrophages, the formation of inactive metabolites or the inability to achieve sufficient plasma levels of the drug (133).

Antimalarial (mice)

Several benzo[a]phenazines synthesized from 1,2-naphthoquinone, lapachol, β -lapachone containing polar (-Br, -I) and ionizable (-SO₃H, -OH) groups were compared with the activities of lapachol and β -lapachone in the *Plasmodium berghei* mouse model. The best candidate was 3-sulfonic acid- β -lapachone-derived phenazine which elicited 98% inhibition of parasitemia in long term treatment (7 doses) subcutaneously whereas the phenazine from 3-bromo- β -lapachone was inactive. Those compounds with antimalarial activities are considered as potential prototypes for use against chloroquine resistant strains (106).

Molluscicidal Activity

In tropical South America schistosomiasis is caused by *Schistosoma mansoni*, with other species known elsewhere. This fluke is endemic to parts of Brazil, Suriname, and Venezuela but it is also prevalent in parts of the Caribbean, and tropical areas of Asia and Africa. Control of the disease is based upon eliminating freshwater snails such as *Biomphalaria*, which can serve as its intermediate host and primary reservoir. They release cercariae into lakes, ponds, and other water sources, which can penetrate the skin of those wading or swimming. In humans they become schistosomulae which develop into adult worms that reside in the mesenteric or rectal veins shedding eggs, which circulate in the liver and are shed in the stools. In water these eggs develop into miracidia that infect the snails (134).

In various parts of the world wherever schistosomiasis is prevalent snail-eradication programs for waterways can vary and are dependant upon the availability and costs of suitable methods. For this reason, much research has gone into identifying local plant species and their compounds that possess molluscicidal activity (135), which can be successfully and practically applied, for example, the Ethiopian Gopo berry (*Phytolacca dodecandra*) (136).

Species within the Bignoniaceae including *Tabebuia aurea* have been investigated for this purpose (137). Earlier research with lapachol (124,138) and a number of its derivatives (138,139) indicated that these compounds exhibited important molluscicidal activities against *Biomphalaria glabrata*, with potassium salts of lapachol and iso-lapachol eliciting significant molluscicidal activity against the adult snail (LC₉₀ < 7 ppm) and snail egg masses (LC₉₀ < 3 ppm) (140). An additional study with a number of 2-hydroxy-3-alkyl-1,4-naphthoquinones indicated that those easily reduced were the most active whereas low-to-moderate activity was elicited by new amino derivatives of lapachol and another which was partially hydrogenated (141). The basis for this lethal activity is because lapachol is bioactivated by P450 reductase into reactive species through redox cycling and the eventual generation of superoxide anion radicals, which cause DNA scissions to occur (140).

Antischistosomiasis Activity (mice and rats)

South American schistosomiasis is asymptomatic at first, except for a rash or itchy skin, and may not present with constitutional symptoms of fever, chills, cough, and muscle aches until one to two months after infection. Children who are repeatedly infected may be subclinically symptomatic with symptoms such as mild anemia, malnutrition, and learning difficulties. As the disease progresses into its chronic state it can cause granulomatous reactions and fibrosis, resulting in colonic polyposis with diarrhea, and portal and pulmonary hypertension. Only rarely is the brain and spinal cord involved causing seizures, paralysis, and spinal cord inflammation. Mortality rates are generally low (142).

Preclinical studies with lapachol and other naphthoquinones address preventing cercariae infection. Oral administration of lapachol protected mice from topical infection with *Schistosoma mansoni* cercariae and also significantly reduced the trematode burden in infected mice (143). Topical application of other naphthoquinones also prevented cercariae penetration with highest activities found when lapachol and its 0-alkyl and 9-acetyl derivatives or β -lapachone were used (144).

Cancer Chemopreventive Agents (mice)

1,4-Furanonaphthoquinones with an OH group on the dihydrofuran-ring, such as avicequinone-A and avicenol-A, showed the highest bioreactivities in the EBV early activation model and in a chemoprovocative tumor-inducing mouse model (145).

β -lapachone and several of its derivatives can prolong the lives of mice with Raucher leukemia (146). Several derivatives and analogs of β -lapachone are cited in a number of patents with a variety of substituent's at the 3-position as well as in place of the methyl groups attached at the 2-position (147-149), with substituents at the 2-, 3-, and 4-positions and 2-, 3-, 8-, and/or 9-positions (146), and substituents at the 2-, 8-, and 9-positions (150). Another publication describes sulfur-containing hetero-rings in the " α " and " β " positions of lapachone (151).

Antitumorogenic Activity (rats)

Studies using an ethnolic extract of the bark of *T. impetiginosa* (*T. avellaneda*) in rats with chemically induced

acute and chronic ulcerations indicate that its gastroprotective capacity involves the maintenance of protective factors such as mucus and prostaglandin, as well as reducing total gastric acidity (152).

Toxicity in vivo (rodents, dogs, monkeys)

Lapachol from the bark of *T. ochracea* was tested during rat fetogenesis from days 17 to 20 of pregnancy. While lapachol was not toxic to rat mothers, it was fetotoxic, leading to fetal intrauterine growth retardation of pups compared to untreated controls ($P < 0.01$). There was also significant weight reduction ($P < 0.01$) in lungs, livers, and kidneys of treated pups. Putative effects in women cannot be ignored (153,154).

In oral toxicity tests using lapachol, rodents, dogs, and monkeys developed moderate-to-severe anemia during the first two weeks of treatment, but recovery was evident at four weeks of treatment. Monkeys receiving up to 0.25 g/kg/day completed the treatment and recovery periods, with those receiving higher doses developing infrequent emesis, anorexia, pallor of mucous membranes, and periods of diarrhea. Dogs and rats were able to tolerate much larger doses of lapachol than either monkeys or mice. A gender difference was also evident in mice: males tolerated less lapachol than females (155).

The naphthoquinones, β -lapachone, 3-hydroxy β -N lapachone, and α -lapachone may have promise as topical antibiotics used to treat methicillin-resistant *Staphylococcus* infections since they do not elicit dermal toxicity on the skin of healthy rabbits (156).

CLINICAL TRIALS

Anticancer Efficacy

Promising anticancer reports for the use of pau d'arco in humans were widely disseminated in South America in the popular press and the medical literature in the 1960s (29,30). Even as late as 1993, analysis of data from trials evaluating whole plant extracts of pau d'arco versus their bioactive compounds indicated that potency is diminished as plant extracts are fractionated into their component parts. These studies implied that the totality of bioactive compounds including lapachol and other naphthoquinones in crude extracts act in a synergistic manner to elicit positive clinical effects (42). In Brazil pau d'arco extracts are used to treat cancers and leukemias. While not meeting the rigorous standards of current clinical evaluations, claims of efficacy have been made in a number of Brazilian and Argentinian studies (5). These types of assertions likely contribute to its use as an herbal remedy for every kind of medical complaint including cancer. Anecdotal reports of the efficacy of crude extracts continue to be lauded in the lay literature and internet fueling their value for these purposes (15).

Lapachol was the first component of pau d'arco to be evaluated in a phase I clinical trial by the U.S. National Cancer Institutes (NCI). Patients with nonleukemic tumors or chronic myelocytic leukemia were given lapachol at dose ranges of 250 to 3750 mg daily for five days and up to 3000 mg daily for 21 days. The trial was terminated because at critical plasma levels of 30 mg/mL, toxicity occurred causing nausea, vomiting, and reversible prolongation of prothrombin times due to its anti-vitamin K action.

According to NCI this study was disappointing and did not fulfill the criteria for further development (157).

Because lapachol targets vitamin K-dependent reactions, such as the reversible activation of ligand for the Axl receptor tyrosine kinase, this compound, like warfarin, may have value in cases where Axl is overexpressed. Consequently, lapachol use may be possible for the treatment of small cell carcinoma, metastatic colon cancer, and adenocarcinomas of the colon (158).

β -lapachone is considered a broad spectrum anticancer agent, which has promise as an anticancer agent, alone or in combination with radiation therapy. However, before these applications can be realized there is a need to overcome the low solubility of β -lapachone in common solvent systems needed for topical and parenteral administration such as enhancing its solubility in aqueous solution by providing either water- or oil-based solubilizing carrier molecules (159,160).

Under development by Arquile Inc. and Roche is the fully synthetic β -lapachone called ARQ-501, which activates E2F1-mediated checkpoint pathways leading to selective apoptosis of cancer cells. A number of phase trials have already been completed. These include two Phase 1 trials in subjects with cancer (NCT0075933), in combination with Docetaxel in patients with cancer (NCT00099190), and a Phase 1/11 trial involving an extension study for patients previously treated with ARQ 501 (NCT00622063). Also finished are three, Phase 11 trials with ARQ 501 in combination with Gemcitabine in subjects with pancreatic cancer (NCT00102700), with hydroxypropyl- β -cyclodextrin for safety and efficacy in adult patients with leiomyosarcoma (NCT00310518), and in patients with squamous cell carcinoma of the head and neck (NCT00358930) (161). An additional Phase 1 trial involving an exploratory biomarker study of ARQ 501 in patients with advanced solid tumors (NCT00524524) is ongoing and is no longer recruiting participants (162).

Of relevance, studies on one of its metabolites (glycosylsulfate conjugate (m/z 241) with ARQ 501) found in the plasma of treated (nu/nu) mice, rats, and human subjects suggest that this is the first time glycosyl conjugates have been found in mammals (22).

While none have been clinically evaluated, several derivatives show promise for the treatment of prostate cancer particularly β -lapachone (i.e., R and R1, both being hydrogen), allyl- β -lapachone, particularly 3-allyl- β -lapachone (i.e. R being allyl and R1 being hydrogen), and 3-bromo- β -lapachone (i.e. R being bromo and R1 being hydrogen). 3-allyl- β -lapachone is considered less toxic (163,164). β -lapachone is also proposed for use with kinetin riboside and glucocorticosteroid to inhibit cyclin D in cancers such as multiple myeloma, non-Hodgkins lymphoma, breast cancer, and other cancers dependent on cyclin D (165). Also, for future clinical assessment in the treatment of breast, non-small cell lung, pancreatic, colon, and prostate cancers are a number of naphthoquinone prodrugs with the ability to convert beta-lapachone with cancers having elevated NAD(P)H:quinone oxidoreductase 1 levels (64).

Anticervicitis/Antivaginitis

Twenty Brazilian patients suffering from cervicitis and cervicovaginitis caused by *Trichomonas vaginalis* or *Candida*

albicans were evaluated for the effectiveness of daily changes of tampons soaked with extracts of *T. impetiginosa* heartwood. After 5 to 29 days, successful treatment was considered complete, with re-epithelialization of inflamed areas. No patient reported adverse side effects (166).

REGULATORY STATUS

The availability of pau d'arco varies from country to country. In Germany its use is limited to some registered herbal medicines used as general stimulants but not for serious medications (M. Heinrich, written communication, 2010). It is not listed in either *The Complete German Commission E Monographs* (1998) or the *Herbal Medicine Supplement* (2000), which provides the standards for use of botanicals and phytomedicines in Germany (167). In the United Kingdom it is not registered or licensed, and in Canada, classified as a "new drug" its sale is prohibited throughout the country. In Brazil, lapachol is cited as being commercially available for antitumor therapy (168). There are no specific regulations for the sale of pau d'arco in the United States. It is "generally regarded as safe" (GRAS) by the Federal Drug Administration and is available in a variety of formulations defined as dietary supplements.

It is probable that much of the material from South America is poorly identified, adulterated, or harvested incorrectly. Many shipments may represent other genera, other *Tabebuia* species, or mixtures of several plants. There is little guarantee of quality control at the source and thus a product labeled as pau d'arco bark may be incorrect (5,169).

CONSERVATION

Since it became popular as a medicinal in the 1960s, many populations of *Tabebuia* species have been destroyed indiscriminately. Timber use is also impacting the availability of *T. angustatus* and *T. heterophylla* in the West Indies, *T. heptaphylla* and *T. rosea* wherever native, and *T. billbergii* and *T. chrysanthia* in Ecuador (1). A program to conserve these and other members of the Bignoniaceae is sorely needed throughout the Neotropics.

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Phosphorus

John J. B. Anderson and Sanford C. Garner

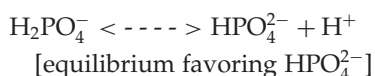
COMMON AND SCIENTIFIC NAME

Phosphorus is the name of the element (number 32 of the periodic table), but phosphorus does not exist in biological tissues or foods as such because of its chemical reactivity; rather it exists almost exclusively as phosphate anions. Most of these anions are inorganic, but some are derived from pre-existing organic molecules that contain phosphate groups (see general description).

In the biological sciences, the term phosphate is used rather than phosphorus.

GENERAL DESCRIPTION

The two major anionic forms are HPO_4^{2-} (metaphosphate) and H_2PO_4^- (orthophosphate), which are interconvertible by the addition or removal of a hydrogen ion. A third form, PO_4^{3-} , exists but it is quite rare in biological tissues: it is the anion of phosphoric acid (H_3PO_4). In human body fluids (pH 7.4), the usual ratio is 4 HPO_4^{2-} ions to 1 H_2PO_4^- ion. The structures of the two major biological phosphate anions and their relationship at equilibrium are as follows:



Phosphorus (P), primarily in the form of phosphates, is found in three major dietary sources: (i) foods containing natural phosphates; (ii) foods containing phosphate additives; and (iii) supplements containing phosphates. Although some amount of phosphorus is present in all foods, foods high in protein are typically also high in phosphorus. Milk, eggs, meat, poultry, and fish contain the highest amounts of phosphorus, whereas fruits and vegetables have relatively less amounts. Sixty percent (60%) of the daily phosphorus intake of North Americans comes from milk and meat against only 10% from fruits and fruit juices (1). Legumes, cereals, and grains are also good sources of phosphorus and contribute almost 20% of the dietary intake. Phosphorus consumption from foodstuffs is increasing, despite an overall decline in the consumption of red meat and milk, because of the steadily increasing consumption of cheese (especially processed types), poultry, and fish (2).

Phosphate additives, the most rapidly growing source of phosphorus in the U.S. diet, may contribute to as much as 30% of overall phosphorus intake (3). This source of the mineral remains largely unnoticed by consumers because the phosphate content of a food product is not required on the label. Many salts containing phosphorus are

used by the food industry to preserve moisture or color, as emulsifiers and sequestrants, or to enhance and stabilize frozen foods. Such processing of foods, now commonplace in the United States, adds significant amounts of phosphate to daily intakes—an estimated minimum of 200 to 300 mg/day. Approximately 125 phosphate additives on the generally accepted as safe (GRAS) list are commonly used; those with up-to-date toxicology information are listed in Table 1. Common foods that contain phosphate additives are soft drinks, processed cheese, luncheon meat, products with leavening agents (like waffles), frozen foods (like pizza with added flavorings), and fast food items. As more and more phosphate-containing additives enter the food supply, largely unnoticed, the effects of lower calcium to phosphorus ratios in the diet need to be considered as potentially negative to the retention of skeletal mass (see actions of phosphate).

Many dietary supplements now contain phosphorus. One such supplement commonly used by athletes is creatine phosphate. This substance, found naturally in muscle fibers, can be used to generate adenosine triphosphate (ATP) and serves as a “quick energy” source. Creatine phosphate, promoted as a way to increase muscle strength during workouts, is commonly used by athletes and body builders. Health professionals do not promote the product, because research has failed to show any real beneficial effects. Many other “muscle-building” formulas are also high in phosphorus because they contain large amounts of animal protein.

Complete nutritional supplements, nutritionally balanced in macronutrients and micronutrients and consumed predominately by older individuals, contain

Table 1 Commonly Used Phosphate-containing Food Additives

Ammonium phosphate
Monoglyceride/diglyceride derivatives
Sodium aluminum pyrophosphate
Calcium phosphate
Phosphoric acid
Sodium acid pyrophosphate
Dipotassium phosphate
Potassium phosphate
Sodium phosphate
Ferric phosphate
Potassium pyrophosphate
Sodium tripolyphosphate
Magnesium phosphate
Potassium tripolyphosphate
Modified food starches, distarch phosphate

Table 2 Dietary Reference Intake (DRI) for Phosphorus (mg/day)

Life stage group	Males	Females
9–18 yr	1250	1250
19–>70 yr	700	700

Source: From Ref. 4.

calcium and phosphorus at a ratio of approximately 1:1. Infant formulas have a ratio of greater than 1:1. These products are not likely to contribute to excessive phosphorus intakes.

The recommended intakes of phosphorus for U.S. and Canadian citizens have recently been revised (4). The Dietary Reference Intakes of phosphorus for men and women older than 19 years is 700 mg/day (Table 2). Phosphorus is usually consumed with the protein fraction of food. Generally, every gram of protein consumed is accompanied by 15 mg of phosphorus. The rate of intestinal phosphorus absorption, 50% to 70% on average, is high in relation to the rates of other minerals.

OVER-THE-COUNTER PHOSPHATE SUPPLEMENTS

The Physicians' Desk Reference (5) includes a few entries on phosphate salts, but these are typically combined with other nutrients, particularly calcium. Common over-the-counter supplements generally contain little or no phosphate.

INTESTINAL ABSORPTION OF PHOSPHORUS AS PHOSPHATE IONS

The intestinal absorption of phosphorus as an inorganic phosphate (P_i) is highly efficient, particularly in infants where up to 80% to 90% of P_i may be absorbed. The absorption efficiency in adults is lower but may still be in the range of 50% to 60% or even higher. In contrast, the intestinal absorption of calcium is usually considered to be between 25% and 30% in adults.

The absorption of organic phosphorus in phospholipids and other molecules may occur, but phosphate groups are typically split out in the gut lumen or on cell surfaces by phosphatases and phospholipases, which are either secreted by the pancreas or exist on the surface of intestinal absorbing cells.

BLOOD CONCENTRATIONS OF PHOSPHATE IONS

Phosphorus circulates in the blood both as a component of organic molecules, primarily phospholipids, and as inorganic phosphate. Inorganic phosphate can exist in several different ionization states, including PO_4^{3-} , HPO_4^{2-} , and $H_2PO_4^-$. Because of the relative solubility of the different forms of P_i , approximately 44% of total P_i is in the form of free $H_2PO_4^-$, whereas 10% is present as free HPO_4^{2-} . The remaining 46% is bound to either serum proteins (12%) or complexed with cations (34%), primarily calcium.

PHOSPHATE HOMEOSTASIS

The serum P_i concentration is regulated by most of the same processes that regulate serum ionized calcium. However, the homeostasis of serum P_i is not as rigorous as that of calcium. The hormonal regulation of serum P_i primarily involves parathyroid hormone (PTH), FGF-23 (a phosphatonin, i.e., a hormone that acts to increase renal excretion), and 1,25-dihydroxyvitamin D, but many other hormones, including calcitonin, insulin, glucagons, growth hormone, estrogens, adrenaline, and adrenal corticosteroids, also may affect P_i homeostasis. A meal rich in phosphate or a phosphate supplement, results in increases of serum PTH and FGF-23 that reduce renal phosphate ion reabsorption. Although a direct feedback mechanism has been proposed for P_i concentration in the regulation of 1,25-dihydroxyvitamin D synthesis, most of the regulatory feedback for PTH is believed to involve the concentration of serum ionized calcium. The use of calcium ion concentration in regulation is understandable given the well-known tendency of serum phosphate and ionized calcium to move in opposite directions. Because phosphate and calcium ions readily form a complex with each other, an increase in phosphate will decrease the concentration of ionized calcium, while a decrease in the phosphate concentration will allow more calcium to circulate in its free or ionized form. Thus, regulation of P_i in serum is mediated through changes in ionized calcium resulting from renal hormonal action.

Phosphate homeostatic mechanisms primarily involve renal regulation. If the kidneys decline in function, as in chronic renal failure, phosphate cannot be efficiently excreted and the serum phosphate ion concentration increases, perhaps even to levels twice as high as the serum calcium concentration. Dysregulation of calcium may have several deleterious effects, including arterial and heart valve calcification.

URINARY PHOSPHATE EXCRETION

A major regulatory mechanism for control of the serum P_i concentration is renal excretion of P_i . Free P_i from serum passes freely through the glomeruli as part of the urinary filtrate. The reabsorption process, which is under the control of PTH, can return most of the filtered P_i to the serum. PTH reduces the efficiency of the reabsorption and increases the excretion of P_i , thus lowering the circulating serum P_i concentration even when the efflux of P_i from bone is increased. Because renal excretion of P_i is the major regulatory mechanism to control the concentration of this ion, a decrease in glomerular filtration rate during the development of renal failure results in a characteristic increase in serum P_i concentration. As the increased serum P_i complexes more ionized calcium in the serum, the resulting hypocalcemia stimulates secretion of PTH, contributing to an increased movement of calcium and phosphate from bone into serum. The increased load of P_i acts to worsen the hyperphosphatemia. Eventually the chronic elevation of PTH can cause a high-bone-turnover lesion known as osteitis fibrosa. The formation of such bone lesions and others

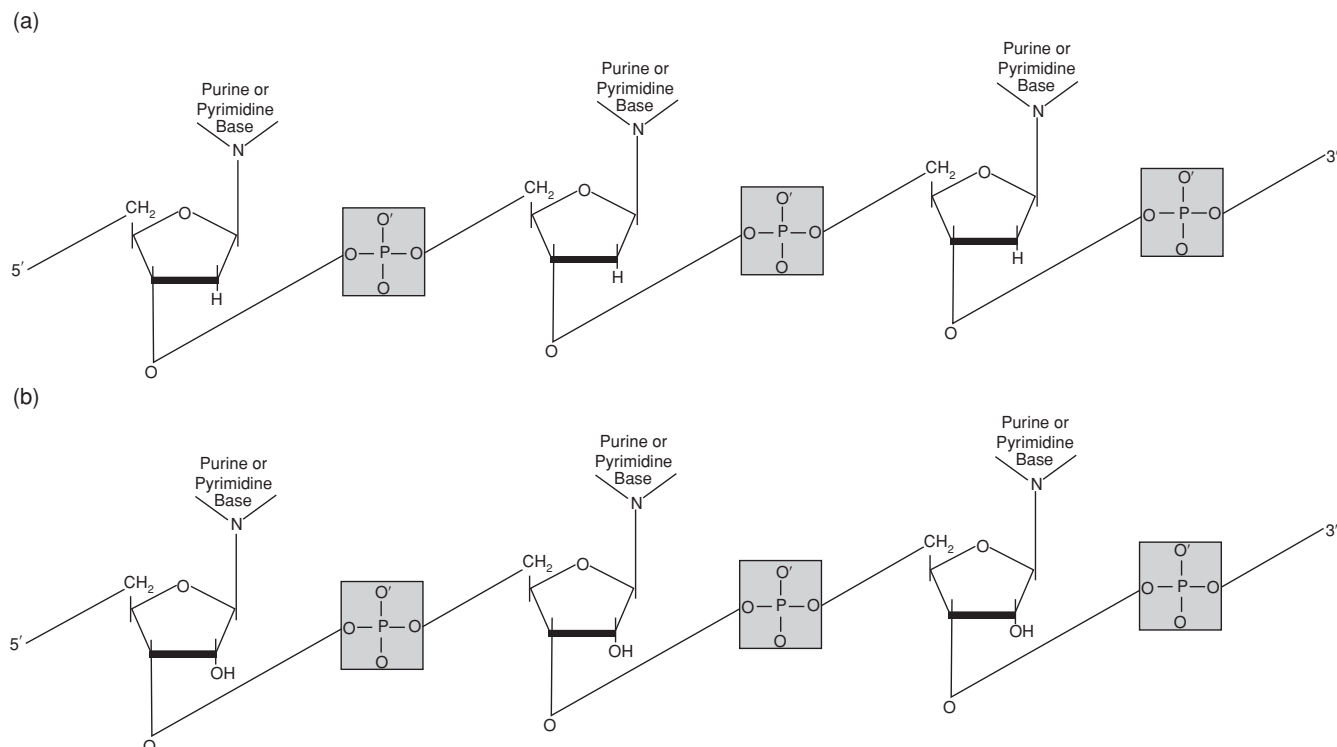


Figure 1 Nucleic acids. Phosphate groups (in the shaded boxes) serve to link the deoxyribose and ribose sugar molecules in (A) deoxyribonucleic acid (DNA) and (B) ribonucleic acid (RNA).

resulting from chronic renal failure is described as renal osteodystrophy.

An important aspect of treating chronic renal failure is the control of the elevated serum P_i . The usual approaches to this control are decreased dietary P_i intake and treatment with phosphate binders, such as calcium carbonate. Aluminum-based phosphate binders, such as aluminum hydroxide, were often used in the past, but the toxic effects of aluminum on bone and the nervous system have greatly decreased their use. Newer phosphate binders, such as sevelamer, are more effective and less toxic.

ACTIONS OF PHOSPHATES

The widespread biological use of phosphate groups makes these anions essential for both organic and inorganic components within cells and in extracellular structural tissues such as bones and teeth. About 600 g (19.4 mol) of phosphorus is present in the adult human body: 85% of which is in the skeleton, 14% in the soft tissues, and 1% in the extracellular fluids, intracellular structures, and cell membranes. The small amount of phosphate ions in extra- and intracellular fluids serves as the compartment to which dietary phosphorus is first added and from which the kidneys clear phosphate ions. Excretion of phosphate ions permits additional hydrogen ions to be secreted by renal tubules, which acidifies the urine. Phosphate ions that are

resorbed from bone also enter this fluid compartment. The concentration of phosphorus in adult plasma ranges from 2.5 to 4.5 mg/dL (0.81–1.45 mmol/L), but this concentration gradually declines with age (6). Phosphate anions participate in numerous cellular reactions and physiological processes and they are key components of essential molecules such as the phospholipids, ATP, and nucleic acids.

Phosphate ions interact with calcium ions in the body and thereby influence the secretion of PTH. Excessive absorption of dietary phosphate lowers the serum calcium ion concentration, which in turn signals the parathyroid glands to increase PTH secretion. If PTH secretion remains elevated continuously because of a low dietary calcium-to-phosphorus ratio, bone resorption may also be continuously upregulated, which may lead, over a period of months to years, to a significant reduction in bone mass and density. This potential scenario of a low calcium–high phosphate ratio has only been observed experimentally for short periods, up to as long as a month, with continuous elevation of PTH in young healthy adult women (7).

High phosphate intakes contribute to acid generation and to an acidic urine. Such an increase in dietary acid load may require buffering by bone (8), which may result in the loss of bone mass and density.

Phosphate ions are essential to life for both their cellular roles and their extracellular uses such as the mineralization of bones and teeth. Excessive amounts of

dietary phosphorus plus a low calcium intake may have adverse effects on skeletal retention of mineral and, therefore, strength.

PHOSPHATE IN BONE MINERALIZATION

Phosphate ions move in and out of the bone fluid compartment from the extracellular fluid, including blood, in large amounts over a 24-hour period. These bi-directional fluxes relate to bone formation and resorption. In the growth phases of life, especially in children, a net gain of phosphate occurs as bone mass increases; in late life when resorption predominates over formation, the phosphate flux out of bone is greater. Both phosphate ions and calcium ions are required for the mineralization of matrix, and the skeletal ratio between the two remains constant throughout life.

PHOSPHORYLATION REACTIONS INVOLVED IN CELL REGULATION

The phosphorylation of specific intracellular protein molecules plays a large role in the cellular regulation of many functions, including transcription, translation, and cell signalling. The amino acids commonly phosphorylated by phosphorylating enzymes (protein kinases) are serine and threonine because of their side-chain hydroxyl groups. Tyrosine kinases are especially important in the transfer of phosphate groups from ATP to the regulatory proteins. The same amino acids may have phosphate groups removed by phosphatase enzymes. Thus, the on and off states involving phosphates are critical for many cellular regulatory activities.

Besides proteins, a number of other molecules incorporate phosphate groups in their structures. These molecules include nucleotides and nucleic acids (DNA and RNA), ATP, phospholipids, creatine phosphate, and others. A few of these molecules are illustrated in Fig. 1–3.

INDICATIONS AND USAGE

Limited therapeutic uses of phosphates exist. Treatment with phosphate salts is not recommended except for a few clinical situations. Premature babies or failure-to-thrive infants who are deficient in phosphorus, as measured by serum inorganic phosphate, will need phosphate salts to survive. [A single plasma P_i measurement of less than 6.0 mg/dL would require a confirmatory measurement to establish deficiency (the acceptable lower limit for newborns and infants within six months of age is 7.0 mg/dL).] Management of any type of adult phosphate depletion, e.g., abuse of aluminum-containing antacids or vitamin D-resistant hypophosphatemic rickets or osteomalacia, would also require oral phosphate supplementation or possibly intravenous therapy (9). The same may be stated for a patient who is hypercalcemic; phosphate salt administration and plasma calcium concentrations need to be carefully monitored.

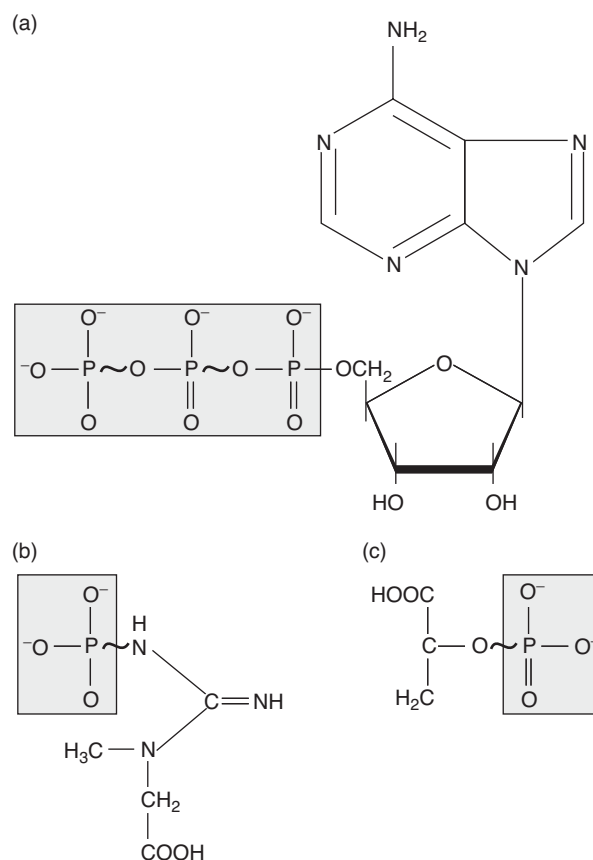


Figure 2 Energy-storing molecules. Phosphate groups (in the shaded boxes) provide the high-energy bonds in (A) adenosine triphosphate (ATP), (B) creatine phosphate, and (C) phosphoenol pyruvate, which are used to store energy in a bioavailable form.

Although phosphate deficiency remains rare in the United States, it may be present in approximately 5% of the elderly (10) who may be truly undernourished with respect to protein, energy, and most micronutrients. The need for additional phosphates is complicated by the needs for practically all macronutrients and micronutrients, so that these individuals should be provided increased amounts of nutrient-rich foods before considering phosphate supplementation, much as undernourished prisoners of war have been rehabilitated in the past. Elderly may also be losing phosphate ions because of a renal “leakage.” A postulated scenario of low phosphate dietary status leading to renal phosphate leakage and bone loss is illustrated in Fig. 4.

CONTRAINDICATIONS

Phosphate supplementation as phosphate salts is not typically recommended because of concern about the calcium to phosphorus ratio of the diet and the potential increase in PTH. With the exception of appropriate medical use of supplementary phosphate, this statement applies across the life cycle.

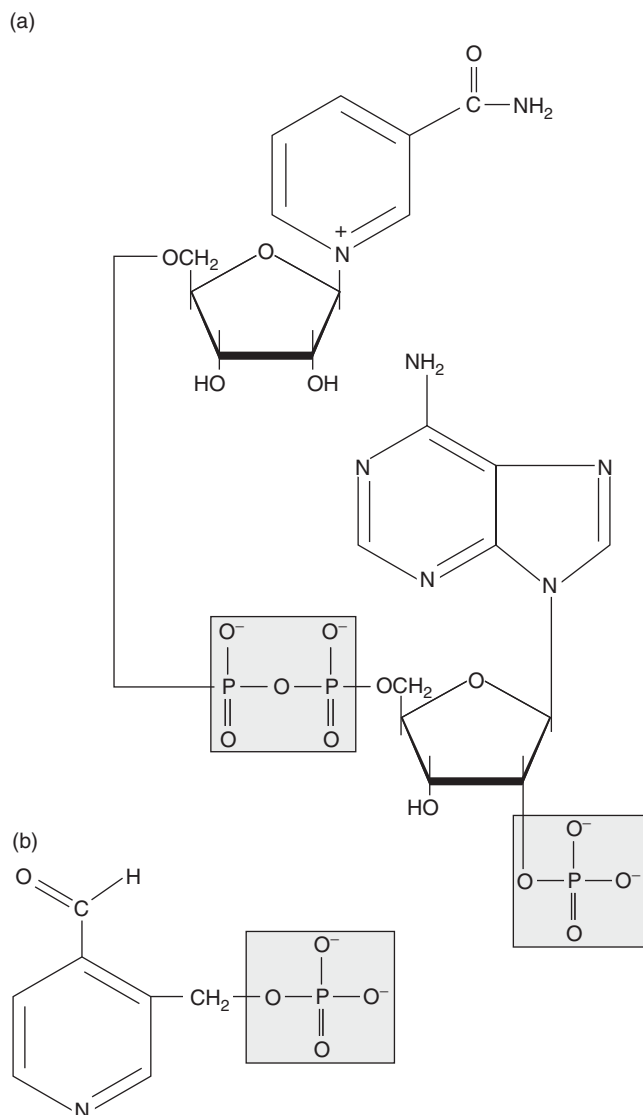


Figure 3 Enzyme cofactors. The enzyme cofactors (A) nicotinamide adenine dinucleotide phosphate (NADP) and (B) pyridoxal phosphate each contain one or more phosphate groups (in the shaded boxes).

PRECAUTIONS AND ADVERSE REACTIONS

Subjects supplementing with creatine phosphate, used as an ergogenic aid by athletes, may consume excessive amounts of phosphorus in a day over a considerable time period. Reports of adverse reactions from high phosphorus intakes have been very few, but the FDA has been concerned about potential deleterious actions of creatine phosphate. So, creatine phosphate is currently on the "watch" list for potential adverse effects.

OVER-DOSAGE

The upper limit for phosphorus is 4 g (4000 mg) per day for adults up to 70 years, but it is very difficult to achieve this

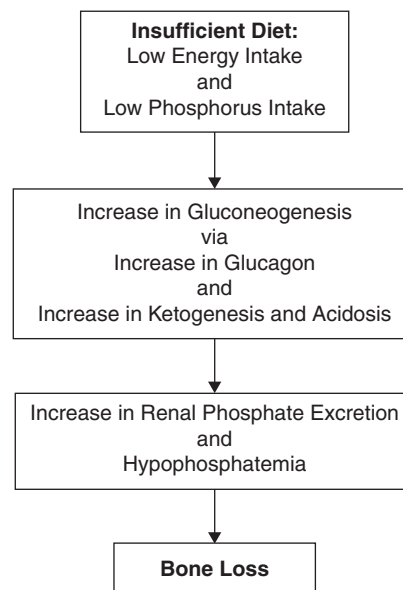


Figure 4 The postulated sequence of events leading to the renal leakage of phosphate ions.

level without taking supplements, such as creatine phosphate. The total daily intakes for adult males from foods, both naturally occurring phosphates and phosphate additives, approximate 1300 to 1800 mg in males and 900 to 1300 mg in females (Table 3). Therefore, adult intakes without supplements should not come anywhere near the upper limit of 4000 mg.

COMPENDIAL/REGULATORY STATUS

Phosphorus, as phosphate salts, is on the GRAS list of FDA additives. Because of the longstanding safe use of phosphate additives, they appropriately belong on this list, but concern about excessively low calcium-to-phosphorus intake ratios makes it desirable for the FDA and other federal agencies to review the status of phosphate additive use vis-a-vis low calcium intakes. If the calcium:phosphate ratio goes below 1:4 (0.25) on a chronic dietary pattern, a chronic increase in PTH will certainly follow and contribute to an increase in bone resorption and the loss of bone mass and density.

CONCLUSIONS

In general, phosphate supplements are not needed because the diet provides sufficient amounts of phosphate anions; to the contrary, healthy individuals may be put at risk by taking phosphate supplements because of the downward regression of the calcium:phosphate ratio. When the ratio of a typical dietary pattern is reduced to 1:4 (0.25), the excessive parathyroid secretion may lead to sufficient bone loss and may compromise skeletal integrity.

Table 3 Mean Calcium (Ca) and Phosphorus (P) Intakes, with Means of Calcium:Phosphorus Ratio

	1994 USDA CSFII Data*	2005–2006 USDA Data
Life stage	Calcium (mg) Phosphorus (mg) Ca:P Ratio	Calcium (mg) Phosphorus (mg) Ca:P Ratio
Males and Females		
0–6 months	457 322 1.42:1	---
7–12 months	703 612 1.15:1	---
1–3 years	766 926 0.83:1	947 1034 0.92:1
4–8 years	808 1059 0.76:1	961 1145 0.84:1
Males		
9–13 years	980 1359 0.72:1	1023 1321 0.77:1
14–18 years	1094 1582 0.69:1	1256 1681 0.75:1
19–30 years	954 1613 0.59:1	1141 1656 0.69:1
31–50 years	857 1484 0.58:1	1145 1727 0.66:1
51–70 years	708 1274 0.55:1	991 1492 0.66:1
>70 years	702 1176 0.60:1	878 1270 0.69:1
Females		
9–13 years	889 1178 0.75:1	942 1176 0.80:1
14–18 years	713 1097 0.65:1	843 1067 0.79:1
19–30 years	612 1005 0.61:1	851 1120 0.76:1
31–50 years	606 990 0.61:1	886 1197 0.74:1
51–70 years	571 966 0.59:1	795 1106 0.72:1
>70 years	517 859 0.60:1	759 985 0.77:1
Pregnancy	1154 1581 0.73:1	1237 1484 0.83:1

*Source: From Ref. 10.

If phosphate supplements are deemed by a physician to be essential to correct for phosphate deficiency, such supplements are truly indicated. Such supplementation is clearly rare and a physician's diagnosis of phosphate deficiency must be documented. Self-supplementation by consumers may place them at risk because of the potential for chronic elevation of PTH and

bone loss. So, phosphate supplementation, while rare, should only result from a clinical diagnosis of established deficiency.

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Polyphenols Overview

Navindra P. Seeram

According to chemical nomenclature, a “phenolic” compound contains at least one aromatic ring bearing a hydroxyl (-OH) group and thus, a “polyphenol” contains multiple aromatic rings and hydroxyl groups (1–3). Phenolics/polyphenols are secondary metabolites (or natural products) of widespread occurrence in the plant kingdom. Over 8000 different polyphenolic structures have been reported (1–3). Most are derived from intermediates of the shikimic acid pathway which gives rise to a large number of aromatic compounds related to phenylalanine and tyrosine (4). Several types are of interest for their potential biological effects ranging from the lower molecular weight phenolic acids to larger complex polymers such as the condensed and hydrolyzable tannins (5–7).

Polyphenols can be classified by their number and arrangement of carbon atoms and many are found naturally in conjugated forms (1–3). Although the most common conjugates are sugars/glycosides, it is not unusual to encounter polyphenols that are acylated with aliphatic and organic acids. In addition, polyphenol monomers can conjugate with themselves to form oligomers and polymers as well as undergo chemical and enzymatic changes when they are extracted and/or processed. Therefore, the possible combinations of polyphenolic structures are limitless which explains their vast structural diversity. Because of this wide diversity, and for simplification of discussion, polyphenols are grouped in this overview into two chemical subclasses, namely *flavonoid type* and *nonflavonoid type*. Figure 1 shows representative chemical structures of some of these polyphenols and they are individually discussed below.

Polyphenols discussed in separate chapters elsewhere in this encyclopedia include “Aloe Vera,” “Cascara Sagrada,” “Cranberry,” “Echinacea Species,” “Elderberry,” “Feverfew,” “French Maritime Pine,” “Ginger,” “Ginkgo,” “Grape Seed Extract,” “Green Tea Polyphenols,” “Hawthorn,” “Isoflavones,” “Milk Thistle,” “Pau d’Arco,” “Proanthocyanidins,” “Quercetin,” “Red Clover,” “St. John’s Wort,” and “Valerian.”

FLAVONOID-TYPE POLYPHENOLS

As a subclass, flavonoids constitute the vast majority of polyphenols (1,3–7). Their structure is based on fifteen carbons consisting of two aromatic (C_6) rings connected by a three-carbon (C_3) bridge (1). Thus, flavonoids are commonly referred to as having a $C_6-C_3-C_6$ (= 6-carbon ring or A ring; 3-carbon ring or C ring; 6-carbon ring or B ring) skeleton. The central C_3 ring in the majority of flavonoids is an oxygen heterocycle. Numbering of the fifteen carbon

skeleton starts from the oxygen atom, proceeds to the A ring, and is followed by the B ring. The basic flavonoid skeleton, as described, can have a wide variety of substituents at any number of different positions and many exist naturally in healthy plant tissues as glycosides. It is common to find hydroxyl groups at the 5, 7, and 4' positions. Whereas substituents such as hydroxyls and glycosides cause flavonoids to be more water-soluble than their corresponding aglycones, others, for example, methoxyls (OCH_3 ; OMe), impart lipophilic properties.

On the basis of several types of chemical modifications of the central C ring, including the presence and/or absence of carbonyl ($C=O$), hydroxyl, and unsaturation (carbon-carbon double bonds; $C=C$), flavonoids can be further categorized into: *flavonols* (2), *flavones* (3), *isoflavones* (4), *flavanones* (5), *flavanols* (also, *flavan-3-ols* or *catechins*; 6), and *anthocyanidins* (7). These are the major polyphenol constituents in human diet; ubiquitous in fruits, berries, vegetables, herbs, spices, and many other plant-derived products; and beverages including cocoa, chocolate, cider, coffee, fruit juices, red wine, and tea. A short description of each of these flavonoid subgroups follows.

The first, *flavonols*, is the most extensively distributed and abundant of dietary flavonoids. They occur with immense structural variations and are commonly substituted with hydroxyl and carbonyl groups at the 3- and 4-positions, respectively. Further, the 3-position is often conjugated with O-glycosides although, substitutions are common at other positions including the 5, 7, 3', 4', and 5'-carbons. As can be imagined, this gives rise to tremendous structural diversity depending on what substituents are present and the particular substitution pattern. Despite this inherent structural diversity, several common flavonol aglycones are known as quercetin (most ubiquitous), kaempferol, and myricetin. Although the number of flavonol aglycones may be limited, the number of possible conjugates is enormous. For example, over two hundred structural variations of sugar conjugates of quercetin alone have been reported.

The second subgroup, the *flavones*, may be regarded as flavonols that lack hydroxyl substitution at position 3. These polyphenols also have a large variation in substitution pattern including hydroxylations, methoxylations, and O- and C- alkylations and glycosylations. It is also common to find glycosylation at position 7. Well-known flavones include apigenin and luteolin, present in parsley and celery, and the polymethoxylated derivatives, tangeretin and nobletin, common to citrus.

As their name suggests, the third subgroup, *isoflavones*, may be regarded as isomers of flavones

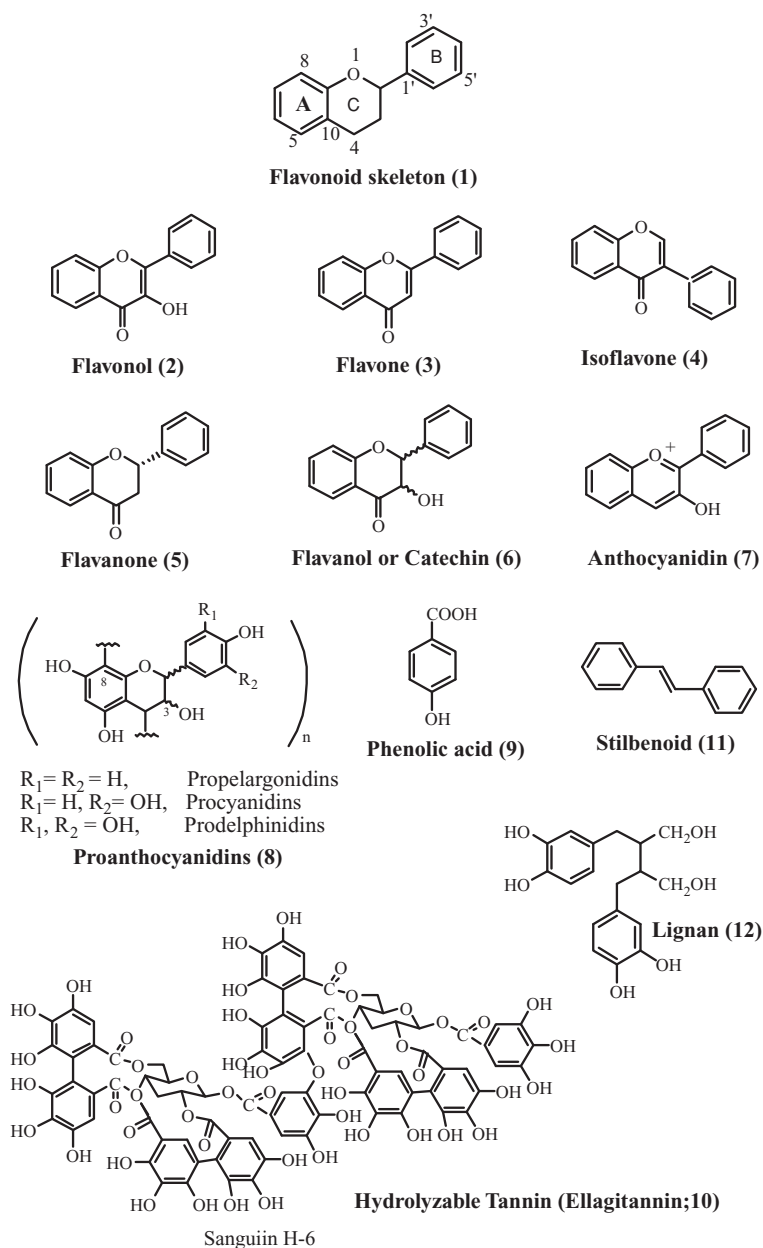


Figure 1 Examples of chemical structures of some polyphenols.

wherein the B ring is located at the 3-position instead of at the 2-position, as observed for the majority of the other flavonoids. The occurrence of isoflavones is restricted to leguminous (Fabaceae) plants and soya (*Glycine max*) is a recognized source, as is the Japanese Kudzu plant (*Pueraria lobata*). Depending on the absence or presence of a hydroxyl group at the 5-position, two common isoflavone aglycones encountered are daidzein and genistein, respectively. Glucosylation at the 7-position of these aglycones forms daidzein and genistein, respectively.

The fourth subgroup, *flavanones*, differs from the majority of other flavonoids in that while ring B is similarly attached to position 2 of the C ring (excluding isoflavones), it is in an α -orientation thus forming a chiral center. Among

common plant foods, flavanones and their glycosides are predominantly found in citrus fruits and include hesperidin and naringin.

The fifth subgroup, *flavanols* (also known as *flavan-3-ols* or *catechins*), are the only flavonoid subclass which are not found naturally in glycosylated forms. They lack unsaturation at C_2 – C_3 , and immense structural diversity results because of this nonplanarity, chirality, and stereoisomerism. In fact, flavanols are arguably the most structurally complex of the flavonoid subclasses. They may be found occurring naturally as simple monomers such as the isomers, (+)-catechin and (–)-epicatechin, hydroxylated to form gallocatechins or esterified with gallic acid to form larger polyphenols such as epigallocatechin

gallate (all found in green tea). Importantly, flavanol monomers may condense and link to form oligomeric and polymeric proanthocyanidins (PACs) (8), which are also known as condensed tannins. Here, it should be noted that although PACs are tannins, they are grouped within the *flavonoid type* of polyphenols. This separates PACs from the other class of tannins, i.e., hydrolyzable tannins, which are grouped within the *nonflavonoid type* of polyphenols (discussed later). As previously mentioned, the inherent chirality within flavanol monomer units of a PAC structure leads to enormous structural variability due to stereoisomerism/enantiomerism in these molecules. In addition, connections between different positions and/or types of linkages (for e.g., oxidative C–C couplings between C₄ and C₆/C₈, ether linkages between C₂–O₅/O₇, etc.) further diversify PACs. Thus, PACs with ether linkages are referred to as A-type PACs (common in cranberries and cinnamon), while those with C–C linkages are B-type PACs (common in grape skin and seeds, blueberries, green tea, and cacao/chocolate). PACs may occur as polymers in excess of 50 monomer units reaching molecular weights exceeding 1000 Daltons. PACs may also be grouped into constituent units that are produced, after acid hydrolysis, on the basis of the nomenclature system established for anthocyanidins (discussed later). For example, those PACs which release cyanidin on acid hydrolysis are called procyanidins. These are the most common PACs in plants and are based exclusively on (epi)catechin units. Similarly, PACs based on (epi)afzelechin or (epi)galocatechin units, are known as propelargonidins and prodelphinidins, respectively. Finally, flavanol monomers may be extensively transformed because of chemical and enzymic changes encountered during processing and extraction of plant-derived foods. These are also referred to as *derived polyphenols* and examples include those complex polyphenolics found in aged wines and black tea (contains thearubigins and theaflavins).

The last major subclass of flavonoids discussed here is the *anthocyanidins*, which can be regarded as aglycones of *anthocyanins* (i.e., glycosylated anthocyanidins). In fact, anthocyanins are the naturally occurring forms of anthocyanidins found in plant tissues. They are water-soluble pigments and are notorious for imparting the blue, red, and purple colors to berries, many other fruits, and vegetables. They are also the only flavonoid subclass that bears a positive charge (i.e., found as oxonium ions). Despite several hundred anthocyanin structures reported, most are based on the skeletons of six common anthocyanidins, namely cyanidin (most ubiquitous), delphinidin, pelargonidin, malvidin, petunidin, and peonidin. These anthocyanidins are distinguished by different numbers and substitutions of hydroxyl and/or methoxyl groups on the B ring. Although sugar conjugation at the 3-position is very common, glycosylation may also be observed at positions 5, 7, 3', and 5'. In addition, further diversification in anthocyanin structure may be achieved by conjugation with phenolic and organic acids.

Finally, apart from the aforementioned *flavonoid/bioflavonoid-type compounds*, other biologically important polyphenols, albeit minor constituents in plant foods, include coumarins, chalcones and dihydrochalcones, aurones, naphthoquinones, anthraquinones, and xanthenes.

These polyphenols may also be present in botanical dietary supplements depending on the plant source.

NONFLAVONOID-TYPE POLYPHENOLS

These compounds include *phenolic acids* (9), of which a notable one is gallic acid, the biosynthetic precursor of *hydrolyzable tannins* (10), as well as *stilbenoids* (11), and *lignans* (12). These are briefly described below.

Phenolic acids have a C₆–C₁ skeleton and may be regarded as derivatives of hydroxybenzoic acid. Common phenolic acids include gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid. They may also be esterified with organic acids, such as tartaric or quinic acids, to form chlorogenic acid derivatives.

Hydrolyzable tannins, similar to the other class of tannins, namely condensed tannins or PACs (discussed earlier), constitute a large class of phenolic polymers. They are found as glucose esters of gallic acids, called *gallotannins*, or of hexahydroxydiphenic acid, which on hydrolysis form ellagic acid (hence referred to as *ellagitannins*). The latter group, as aptly named, hydrolyzes during processing and extraction of plant materials to release ellagic acid, a bioactive polyphenol.

Stilbenes which are based on a C₆–C₂–C₆ skeleton include the popular bioactive polyphenol, resveratrol (3,4',5-trihydroxystilbene) present in grape and red wine. Because of the carbon–carbon double bond connecting the two C₆ rings, geometric isomerism that is *cis* and *trans* isomerism results. Further substitution by hydroxyl, methoxyl, and glycosides on the aromatic rings forms a number of stilbene derivatives. In addition, stilbene monomer units may oxidize to form dimers and polymers such as the viniferins.

Lignans (distinct from lignins, the constituent of plant cell walls) are polyphenols formed from phenylpropanoid units linked by the central carbon atoms of their side chains. They are commonly found in plants as dimers such as secoisolariciresinol and matairesinol in flax and sesame. However, more complex lignan oligomers may also be present in plants.

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Proanthocyanidins

Catherine Kwik-Urbe, Rebecca Robbins, and Gary Beecher

INTRODUCTION

Name and General Description

Proanthocyanidins, also named condensed tannins, are oligomers and polymers of monomeric flavonoids. More specifically, they are polyflavans: condensed molecules of those flavonoids with a saturated "C" ring (Fig. 1A). Fifteen subclasses of proanthocyanidins have been identified (1), however, only a few of these are prominent in foods and supplements that are generally consumed. The various subclasses are named on the basis of the conversion of the "interior" monomeric units (M) to the corresponding anthocyanidin during acid-catalyzed depolymerization; hence, this broad class of polymers is named *pro*anthocyanidins. Examples include conversion of (epi)catechin monomers to *cyanidin* (*procyanidins*) and (epi)gallocatechin monomers to *delephinidin* (*prodelephinidins*). In these tannins, the monomeric units are primarily linked through single 4→6 or 4→8 carbon-carbon bonds (B linkages), or through 4→8 carbon-carbon and 2→7 ether bonds (A linkages) (Fig. 1). Other linkages have also been identified, but have been isolated from nonfood plants or they constitute minor compounds of foods such as cocoa (1). Proanthocyanidins range in size from dimers (Degree of Polymerization, DP = 2) through very large polymers (DP > 10) and are found in many plant-based foods and dietary supplements.

CHEMISTRY

Proanthocyanidins are secondary metabolites of plants, that is, they are not required for the structural or metabolic integrity of the organism. Proanthocyanidins, however, do serve important biological functions for plants. Specifically, proanthocyanidins help in the protection of plants from invasion and predation by microbes, fungi, and animals. One of the earliest biochemical properties of proanthocyanidins to be realized was their ability to bind to and denature proteins. Their use in the conversion of animal hides into leather, a process called tanning (protein denaturation), led to the generic name of tannins for these compounds. The interaction between proanthocyanidins and proline-/hydroxyproline-rich proteins and other polymers is very strong (2). As collagen, a prominent protein in animal skin and hides, is rich in proline and hydroxyproline, the interaction of proanthocyanidins with these hydroxyl-containing amino acids serves as the basis for the tanning effect of these natural plant constituents.

The unique polyhydroxy phenolic nature of proanthocyanidins and the resulting electronic configuration allows relatively easy release of protons and hydrogen radicals, and as a result, they have been shown to have substantial antioxidant activity in vitro. Employing many antioxidant systems, investigators have shown that proanthocyanidins have high antioxidant and radical scavenging activity in vitro (3–7), usually greater than vitamins C and E, the "gold standards." In addition, these unique chemical structures bind divalent cations, such as iron and copper, reducing the availability of these prooxidant metals. In doing this, proanthocyanidins may work indirectly to reduce the oxidative stress and damage caused by such redox active metals. The antioxidant activity is indirect because both iron and copper stimulate oxidative type reactions (Fenton reaction), but by effectively reducing the concentration of these cations (through binding), the extent of oxidative activity can be greatly reduced by the proanthocyanidins. Conversely, the role that proanthocyanidin-cation binding has on the bioavailability of such minerals as copper, iron, or aluminum is uncertain (2). The biological relevance of these antioxidant effects of proanthocyanidins will be discussed later in this chapter.

ANALYSIS

Content analysis research for proanthocyanidins, although often not in the forefront, is directly connected with clinical and health investigations. To gather accurate data for food composition analysis and dietary intake levels, robust, reproducible quantitative methods are a necessity. The natural diversity of proanthocyanidins in foods and the inherent complexity of food matrices cause great difficulties in the accurate analysis of proanthocyanidins. In plant materials, these compounds are known to exist in free aglycone and conjugated forms with sugars and organic acids, as well as in a diverse array of oligomeric and polymeric forms (and typically also accompanied by flavanols, the monomeric building blocks of proanthocyanidins). Additionally, proanthocyanidins can occur in soluble, suspended, colloidal, or in covalent combinations within cell wall components. This structural diversity, solubility, and interaction with the matrix (plant or food matrix) impose a significant challenge in extraction, isolation, and analysis in foods and dietary supplements (8). Further difficulties arise because of the fact that proanthocyanidins are highly reactive and demonstrate general

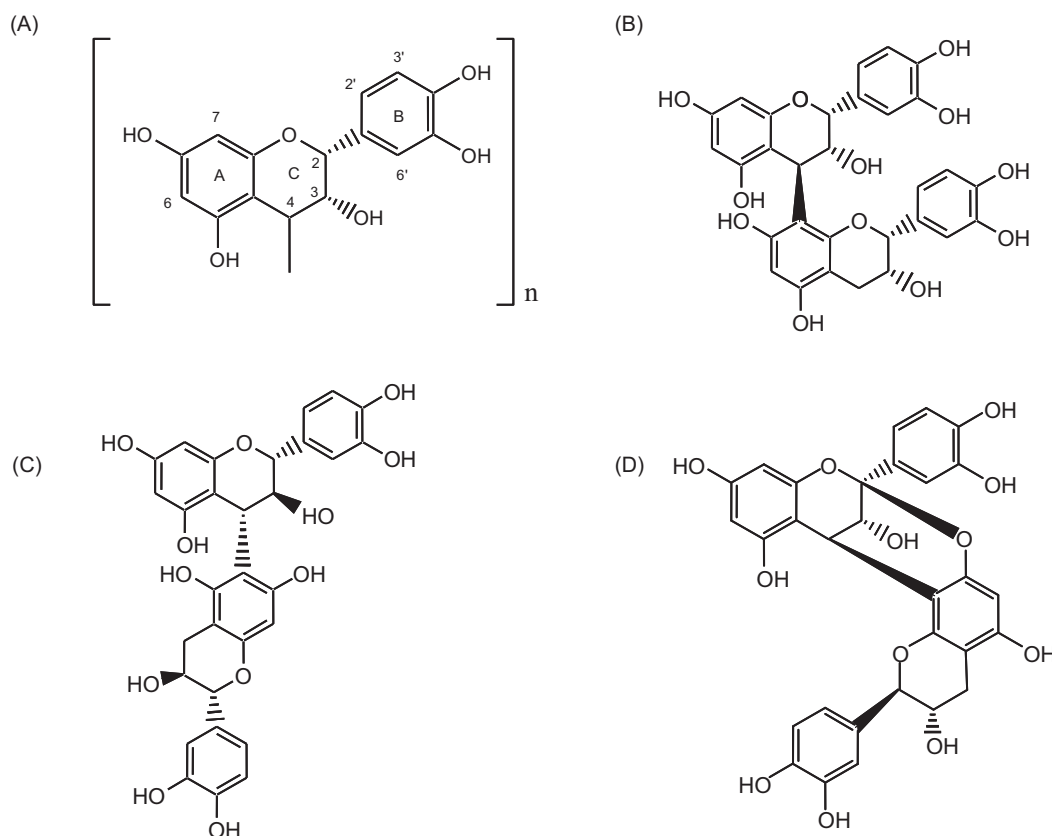


Figure 1 Representative linkages within proanthocyanidin molecules. (A) Monomeric representation with carbon 4 and 8 shown as potential linkages. Structure of (–)-epicatechin shown as example. Letters within rings identify individual phenolic or heterocyclic ring. n may equal 2 (dimer) to ~50. (B) Example of B type (4→8) linkage. Specific compound is procyanidin B2 (dimer), epicatechin-(4 β →8)-epicatechin. (C) Example of B type (4→6) linkage. Specific compound is procyanidin B5 (dimer), epicatechin-(4 β →6)-epicatechin. (D) Example of A-type (2 β →O7; 4 β →8) linkage. Specific compound is procyanidin A2 (dimer), epicatechin-(2 β →O7; 4 β →8)-epicatechin.

instability. These molecules are subject to enzymatic oxidation, electrophilic, nucleophilic, and single electron-mediated chemical reactions. All these challenges mean that extraction and sample preparation for analysis are investigations in their own right and contribute to the very limited availability of commercially available reference standards necessary for quantification (9,10).

Yet, even with these many challenges, there exist a number of techniques that have been developed for the quantification of proanthocyanidins, each providing different levels of content information (11 and references therein). Two broad categories of analysis include colorimetric and chromatographic methodologies. Common colorimetric assays for phenolics and proanthocyanidins are the Folin Ciocalteu (FC) method, Vanillin, 4-(dimethylamino)-cinnamaldehyde (DMAC) and hydrochloric acid–butanol assays. Generally speaking, colorimetric methods yield results that are empirical and not specific. For example, the FC reagent reacts broadly with the phenol functional group, rather than specifically with proanthocyanidins; therefore, the measurement is for all phenolics. The vanillin assay is more specific to flavanols, but still does not distinguish between monomeric, oligomeric, or polymeric compounds (12).

Although these colorimetric approaches are rapid and relatively easy, they provide gross, nonspecific estimates rather than the detailed information required for research focused on the composition of foodstuffs (including native foods, food ingredients, and supplements) and studies on health effects of specific chemical constituents. Colorimetric measurements do not physically separate compounds, that is, assays are performed on mixtures. The quantitative results are most often stated as catechin equivalents (sometimes gallic acid or epicatechin equivalents), making comparisons of content levels and data interpretations between studies confusing.

A wide variety of chromatographic analytical procedures have been employed for the bulk measurement of proanthocyanidins (13). Both reversed-phase and normal-phase chromatographic separations exist for proanthocyanidins in a variety of foods, food parts and dietary supplements. Individual dimers and trimers traditionally have been quantified with reversed-phase high performance liquid chromatography systems (HPLC) (14–16). Reversed-phase separations, however cannot separate and measure the larger polymeric proanthocyanidins.

Normal-phase chromatography can separate proanthocyanidins based on DP. Quantification of individual

proanthocyanidin oligomers ($DP \leq 12$) have been achieved using normal-phase HPLC techniques coupled with sophisticated detection instrumentation (17–19). Higher molecular weight proanthocyanidins ($DP > 12$) are not chromatographically resolved from one another and have been quantified together as a single chromatographic peak (19). More recently, an alternate HPLC method employing environmentally friendly normal-phase solvents and a diol stationary phase was developed and expanded to a multilaboratory assessment (20,21). Using a diol stationary phase with nonhalogenated mobile phases, flavanols and procyanidins in cocoa and chocolate can be quantified. In this method, polymeric materials greater than $DP 10$ were not measured. Often, to obtain higher levels of characterization of proanthocyanidins in complex materials, more than one type of technique or method is employed on the same sample (22). In a more comprehensive analysis of grape seed and pine bark extracts, Weber et al. (23) employed reversed-phase liquid chromatography with UV detection as a tool to fingerprint (profile) components, and also used Atmospheric Pressure Chemical Ionization liquid chromatography/mass spectrometry for further identification of monomers, dimers, and trimers. Gel permeation chromatography (GPC) has also been used to generate a molecular weight profile, along with gas chromatography/mass spectrometry for analysis of volatile components and, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for identification of the polymeric species (23). Such comprehensive analyses are often conducted to capture detailed information about the content of a broad range of molecules in complex food extracts.

PHYSIOLOGY

Foods or supplements containing high levels of proanthocyanidins are characteristically recognized as being astringent. This is due in part to the binding of these dietary constituents to proline-rich salivary proteins (2). As a result, formulation of palatable foods and supplements containing substantial levels of proanthocyanidins has been a challenge for food technologists and supplement formulation experts.

Although binding of proanthocyanidins to digestive enzymes has been a concern in animal nutrition where dietary concentrations of these components may be as high as a few percent, human foods contain much lower levels and as a result, interference with digestive enzymes is of little concern (2). Despite the low natural proanthocyanidin content of the human diet, in recent years there has been some research interest in exploring the potential weight loss benefits of exaggerated concentrations of proanthocyanidin-rich plant extracts. In vitro and in vivo studies, including a small probe trial in healthy adults, have demonstrated the ability of these proanthocyanidin-rich extracts to alter lipase activity and in turn, fat absorption and metabolism (24–26). Though these data suggest that plant-derived proanthocyanidin-containing extracts may be effective agents in reducing the energy density of the diet by reducing fat absorption, larger and more conclusive studies are warranted.

Early findings regarding the absorption of intact proanthocyanidins were mixed, with some animal studies reporting proanthocyanidin absorption (reviewed in (5) and others failing to demonstrate absorption of these food components in rats (27,28), chicken, or sheep (5). In recent years, the absorption of both intact A-type and intact B-type proanthocyanidins (see chapter “Polyphenols Overview”) has been reported. Shoji et al. (29), detected intact procyanidin dimers and trimers in rat plasma following the acute oral administration of apple-derived procyanidin oligomers. Interestingly, molecular size as well as stereochemical structure appeared to influence bioavailability. In the study, apple-derived dimers were more bioavailable than the larger trimeric oligomers, and the B2 dimer (epicatechin-($4\beta \rightarrow 8$)-epicatechin) appeared more bioavailable than the structurally related B1 dimer (epicatechin-($4\beta \rightarrow 8$)-catechin). Work by Appeldoorn et al. (30) further explored the influence of stereochemistry on bioavailability by comparing the absorption and metabolism of B-type and A-type procyanidin dimers. Using in situ perfusion of the rat small intestine, the absorption of individual procyanidin dimers [A1: epicatechin-($2\beta \rightarrow O7$; $4\beta \rightarrow 8$)-catechin; A2: epicatechin-($2\beta \rightarrow O7$; $4\beta \rightarrow 8$)-epicatechin; B2: epicatechin-($4-8$)-epicatechin] or a mixture of A-type, these and other larger oligomers was compared. Like the work reported by Shoji (29), stereochemical configuration did influence bioavailability. Appeldoorn et al. (30) showed that the A-type dimers were found to be more readily absorbed than dimer B2 and that A-type trimers were not absorbed. Interestingly, when administered as the pure chemical, dimer B2 was not detected in rat plasma; however, when administered in combination with A1 and an enriched A-type tetrameric fraction, B2 could be detected in rat plasma, suggesting that the co-consumption of different procyanidin oligomers may influence bioavailability. Information on the biotransformation, specifically glucuronidation, methylation, and sulfation, are sparse, yet some evidence of biotransformation has been reported (29).

In contrast to the large number of animal studies, studies on the bioavailability of proanthocyanidins in humans have been limited. Similar to what has been reported in animal models, intact procyanidin dimers have been detected in human plasma. Following the consumption of proanthocyanidin-containing grape seed extract (31) and cocoa powder (32), procyanidin dimer B1 (grape seed extract) and B2 (cocoa) were detected in plasma within two hours of consumption. In these studies, plasma procyanidin dimer concentrations were reported to average in the range of 10 to 40 nM, markedly less than μM concentrations that have been reported for the monomeric procyanidin subunits, that is, epicatechin and catechin. To date, no human studies have reported the absorption of intact procyanidins larger than dimers.

In addition to investigations into the absorption of proanthocyanidins, there has been research into the catabolism of these compounds by gut microflora. Metabolism of monomeric polyphenols by microflora of the lower gastrointestinal (GI) tract has been recognized for many years (33). In vitro experiments employing human colonic microflora demonstrated that purified (34) and semipurified proanthocyanidins (primarily

hexamers and heptamers, but free of monomers, dimers, and trimers); (35) were readily catabolized. The primary products of these experiments were monohydroxylated derivatives (*meta* and *para* isomers) of phenylacetic, phenylpropionic, and phenylvaleric acids, which are similar to those resulting from the metabolism of monomeric flavonoids (33). Studies in rats support the extensive catabolism of proanthocyanidins by gut microflora, with a variety of phenolic acid metabolites identified in urine, indicating that these catabolites are readily absorbed (34).

Though only a limited number of studies exist, there is research to support similar gut effects in humans. Studies utilizing cocoa (36), apple (37), and grape seed (38) proanthocyanidins demonstrate that following consumption, a variety of phenolic acids can be detected in urine. As suggested by *in vitro* and *in vivo* animal models, the microflora within the human GI tract are capable of extensive catabolism of the proanthocyanidins, generating a number of low-molecular-weight phenolic acids which are readily absorbed and are thus possible contributors to the putative health benefits ascribed to diets rich in proanthocyanidin-containing foods.

The lack of availability of purified individual oligomers and polymers limits the feasibility of experiments with well-characterized materials in the variety of plant extracts that are readily utilized in studies. Nonetheless, investigations are repeatedly undertaken in which natural plant extracts are administered (*in vitro* and *in vivo*) and thus great care must be taken in the interpretation of these published findings as the experimental repeatability and relevance to human health must be questioned when the analytical characterization of these materials is missing or incomplete.

ALTERATION OF BIOLOGICAL MARKERS ASSOCIATED WITH CHRONIC AND OTHER DISEASES

Proanthocyanidins as Antioxidants

Many life processes generate free radicals. The resulting reactive oxygen and nitrogen species (ROS, RNS), if left unchecked, have the potential to cause oxidative damage to DNA, lipids, and proteins, resulting in a cascade of degradative effects that may contribute to human disease pathophysiology (39). Free radical scavengers and/or antioxidants may protect cells against oxidative damage (40). Proanthocyanidins and their purported digestion products, hydroxylated phenolic acids, have high antioxidant activity *in vitro* (41,42). In the case of proanthocyanidins, major contributions to this activity are the presence of a catechol group (hydroxyl groups adjacent to one another) on the B-ring, and the stability of the reduction products, semiquinones and quinones (41).

Though *in vitro* studies have demonstrated the direct antioxidant activity of proanthocyanidins, the relevance of these findings to humans is questionable. As noted previously, the absorption of intact proanthocyanidins is limited, with nothing larger than dimers having been detected in human plasma following the consumption of proanthocyanidin-containing cocoa or a grape seed extract. Though there is limited understanding of the metabolism of these compounds in humans, animal work suggests that methylation and glucuronidation of

absorbed proanthocyanidins are possible. Assuming that similar processes may occur in humans, such chemical modifications would markedly reduce the hydrogen-donating capacity of a given proanthocyanidin. Furthermore, given that only low nM concentrations of dimers have been detected in plasma (31,32), the ability of plasma proanthocyanidins to effectively compete with conventional plasma antioxidants such as vitamin C, urate, and glutathione, which are present as micromolar /millimolar concentrations in plasma, is highly unlikely (43). Finally, adding to the limited support for direct antioxidant benefits associated with proanthocyanidins are the recent results of multiple human intervention studies with proanthocyanidin-containing foods which fail to demonstrate any improvements in markers of oxidative stress and damage (44–47).

Given that such low concentrations of proanthocyanidins, specifically procyanidin dimers, have been reported in the circulation, it seems unlikely that these compounds have any direct antioxidant effect *in vivo*. One exception to this may, however, be in the lumen of the gastrointestinal tract. From the dietary constituents themselves and as a consequence of digestive processes, the gastrointestinal tract is exposed to a variety of reactive oxygen and nitrogen species (48–50). As a consequence of their limited bioavailability and reported stability, at least through the initial phase of digestion (31,32), it is possible that micromolar concentrations of these compounds can be achieved within the GI tract following the consumption of proanthocyanidin-rich foods. Thus, the direct hydrogen-donating and metal-chelating capacities of proanthocyanidins may allow native proanthocyanidins to serve an important role in protecting the GI tract from oxidative stress, particularly through the digestive process. Human intervention studies with grape (48) and cocoa (51) products provide some evidence that postprandial oxidative stress may be reduced as a consequence of the consumption of these proanthocyanidin-containing food components.

Cancer

Many *in vitro* and *in vivo* systems have been employed to investigate the effects of proanthocyanidins on cancer processes, with a number of these studies suggesting that these compounds may offer preventative, and even potential therapeutic benefits in the management of cancer. Work with a variety of proanthocyanidin-enriched materials as well as highly purified proanthocyanidins demonstrate the ability of these compounds to inhibit cell growth and promote cell death (52–55) *in vitro*. One challenge in the interpretation of these (and other) studies is the stability of these compounds *in vitro* under the environmental conditions and the duration of incubation (hours to days) that are commonly employed. Proanthocyanidins have been shown to be unstable under commonly employed cell culture conditions, resulting not only in the loss of the intact proanthocyanidin (56,57), but also in the generation of hydrogen peroxide, semiquinone, and quinone species that may be the actual mediators of the observed response (58). The anticancer potential of proanthocyanidins, however, cannot be completely discounted, because there is evidence that when efforts are taken to minimize artifact

formation, select proanthocyanidins can be cytotoxic to a number of human cancer cell lines (55).

The influence of proanthocyanidins has been investigated on biological markers for cancer in several animal models. Proanthocyanidins fed as a condensed tannin extract of red alder bark or as grape seed extract significantly inhibited the multiplicity, size, and distribution of chemically induced colonic aberrant crypt foci in mice and rats (59,60). Experiments with proanthocyanidins isolated from cacao liquor and fed to Sprague-Dawley rats showed substantial inhibition of the initiation of 2-aminomethyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced pancreatic carcinogenesis (61). In vitro studies suggested that proanthocyanidins also directly inhibited the mutagenic activity of PhIP, perhaps through nonspecific binding. Feeding proanthocyanidins extracted from grape seeds to SKH-1 hairless mice also decreased both UVB-induced skin carcinogenesis and malignant transformation in terms of incidence, multiplicity, and size (62). A suggested mechanism for the inhibition of carcinogenesis is the antioxidant activity conferred by the dietary proanthocyanidins. Grape seed proanthocyanidins fed to mice or rats, however, were not effective in curtailing chemically induced mammary tumorigenesis (60,61). Several foods also contain proanthocyanidins; however, there is a paucity of observations on their effect on carcinogenic processes. Although black and green teas have been extensively investigated for their anticancer activity, green teas contain only limited proanthocyanidins (63) whereas black teas have substantial concentrations of derived tannins (theaflavins, thearubigins, and others), which are a heterogeneous mixture of oxidation products of monomeric flavonoids and structurally different from proanthocyanidins (64).

To date, no human intervention trials investigating the potential preventative or therapeutic benefits of proanthocyanidins have yielded conclusive evidence (65); however, multiple trials are currently registered which examine the potential application of various plant-derived polyphenols and flavonoids, including grape seed proanthocyanidins, in the prevention and management of specific cancers (the reader may search <http://clinicaltrials.gov/> for details on these registered trials). Interestingly, epidemiological studies do support the notion that the intake of proanthocyanidin-rich foods is inversely associated with the risk for development of non-Hodgkins lymphoma (66) and colorectal cancer (67). Though not causal, these studies suggest that the regular inclusion of proanthocyanidin-rich foods in the diet may offer protection from the development of certain types of cancer.

Atherosclerosis

Atherosclerosis is an inflammatory disease process (68,69), and today it is well accepted that a very early event in the atherosclerotic process is a disruption in the proper functioning of the cells that comprise the lining of blood vessels—the endothelium. The importance of blood vessels in the regulation of vascular homeostasis is well recognized today (reviewed in (70)). Furthermore, it is well recognized that a disruption in the function of the endothelium is an early indicator of the health of the vascular system, and there is growing evidence that assessing the

function of these endothelial cells may provide prognostic data and serve as an independent predictor of cardiovascular disease risk (reviewed in (70,71)). As a result of both in vitro and in vivo studies, there is evidence to suggest that exposure to proanthocyanidins may positively influence various steps in this complex disease process.

Inhibition of LDL Oxidation

Experiments investigating the interaction between synthetic liposomes and cocoa proanthocyanidins revealed that liposome oxidation originating in the aqueous phase was inhibited most effectively by flavan-3-ol monomers, as well as proanthocyanidin dimers and trimers (72). Conversely, protection was greatest with higher polymers (DP 3–6) when oxidation was initiated in the lipid phase (73,74). In vitro studies with isolated LDL (low density lipoprotein) particles have shown that individual isolated procyanidins (monomer through hexamer) or several natural products rich in proanthocyanidins (cranberry extract, grape seed extract) inhibited chemically induced oxidation of LDL (72,75–77). In a copper-catalyzed LDL system, equimolar concentrations of individual proanthocyanidins indicated antioxidant activity that was proportional to the DP of the procyanidins (75). Employing a similar system, isolated fractions from cranberries rich in proanthocyanidin oligomers (DP 3–9) and containing one to three A-type linkages were also effective in delaying LDL oxidation (76). When results from an 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH)-induced LDL conjugated diene formation system were expressed on a monomer equivalent basis, inhibitory activity of the various polymers was similar, suggesting that antioxidant capacity was a function of the number of available catechol groups (75). Studies with fractions containing mixed oligomers gave similar results in terms of antioxidant capacity, but higher polymers (DP 5–9) appeared to have greater affinity for LDL particles than oligomers with a lower DP (77). Similar studies in a cellular system (endothelial cell-mediated LDL oxidation) changed preference of antioxidant to monomeric catechin and dimers rather than higher polymers of proanthocyanidin (75). It is difficult to interpret results with polymeric proanthocyanidins and chemically induced LDL oxidation in terms of biological activity, because studies to date suggest oligomers larger than DP 2 are only minimally absorbed and circulated in the blood stream.

A controlled, double-blind, randomized, crossover human study demonstrated that the daily inclusion of proanthocyanidin-containing cocoa powder and chocolate in the context of an average American diet was shown to be effective in decreasing LDL oxidation susceptibility and slightly increasing serum total antioxidant capacity as well as high density lipoprotein cholesterol (HDL-C) levels (78). Similar results of cocoa ingestion on LDL oxidation susceptibility were observed in additional studies for which dietary control was less rigorous (79) and references therein). Pycnogenol® brand pine bark extract (150 mg/day) fed to healthy individuals for six weeks did not alter LDL oxidizability, but reduced LDL-cholesterol and increased HDL-C levels in the plasma of two-thirds of the subjects (80). However, the same extract (360 mg/day) given to patients with chronic venous insufficiency decreased total cholesterol and LDL-C values, but did not

alter HDL-C levels (81). Addition of grape seed extract to the diet of hypercholesterolemic subjects for eight weeks substantially reduced the level of antibodies to oxidized LDL (measure of oxidized LDL), compared to results of the placebo control group (82). Results from in vitro studies suggested that isolated cocoa-proanthocyanidins were inhibitors of mammalian 15-lipoxygenase-1, an enzyme that oxygenates LDL to an atherogenic form (83). Studies with red wine or red wine polyphenol-containing diets (rich in proanthocyanidins) gave mixed results in terms of plasma antioxidant capacity and resistance to ex vivo LDL oxidation (5,79).

Inhibition of Inflammatory Response

Studies with isolated or purified cyclooxygenase-1, cyclooxygenase-2, and 5-lipoxygenase demonstrated that their activities were inhibited by flavanols and oligomeric proanthocyanidins from cocoa at concentrations similar to drugs used for the same purpose, for example, indomethacin (83–85). Short-term (6 hr) in vivo experiments with human subjects fed proanthocyanidin-rich chocolate resulted in increased plasma levels of prostacyclin, decreased concentrations of leukotrienes, and a decreased leukotriene/prostacyclin ratio, all measures of the proinflammatory/anti-inflammatory eicosanoid balance (86). Similar results were observed with treated aortic endothelial cells in vitro. Longer-term studies (4 and 6 wk) with subjects consuming a daily combination of cocoa powder and dark chocolate plus an average American or a low-flavonoid diet failed to alter the urinary excretion of F_2 isoprostane, thromboxane B_2 , 6-keto-prostaglandin $F_{1\alpha}$, or their ratio (78,87). Consumption of proanthocyanidin-containing purple grape juice, but not several other juices or coffee devoid of proanthocyanidins, significantly increased 6-keto-prostaglandin $F_{1\alpha}$ at two hours postconsumption (88). Pycnogenol (200 mg/day) consumption reduced thromboxane B_2 levels in smokers but did not alter levels in nonsmokers (89). Results from these experiments suggest proanthocyanidins and proanthocyanidin-containing foods and supplements may alter eicosanoid metabolism in favor of an anti-inflammatory environment. However, environmental interactions as well as time course and magnitude of this response require further investigation.

Endothelial injury causes increased expression of cellular adhesion molecules (CAMs) (i.e., ICAM-1 [intracellular CAM], VCAM-1 [vascular CAM], E-selectin) that mediate recruitment of monocytes and their subsequent differentiation into phagocytic macrophages (77). Employing HaCaT cells (human keratinocyte), Pycnogenol pretreatment inhibited IFN gamma-induced adherence of these cells to Jurkat T cells and expression of ICAM-1 (90). Pycnogenol also inhibited NF κ B activation and VCAM-1 and ICAM-1 expression in tumor necrosis factor α (TNF- α) treated human umbilical vein endothelial cells (HUVECs) (91). A gene that codes for an oxidized LDL receptor directly linked to foam cells and atherosclerosis, CD36, was found to be downregulated by grape seed proanthocyanidin extract (GSPE) in TNF- α -induced HUVECs (82). In vitro studies with peripheral blood mononuclear cells (PBMC), isolated from human subjects that had low production of transforming growth factor (TGF)-beta-1, showed that TGF β -1 production was

greatly stimulated by dimeric and tetrameric proanthocyanidins isolated from cocoa, compared to higher polymers (DP > 5) (92). In contrast, TGF β -1 secretion from high producing PBMC at baseline was inhibited by all cocoa proanthocyanidin fractions tested (DP 2–10). A study with cultured vascular smooth muscle cells demonstrated that exposure to red wine polyphenolic compounds inhibited both the mRNA expression of vascular endothelial growth factor, as well as the release of vascular endothelial growth factor in response to platelet-derived growth factor AB, TGF β -1, or thrombin (93). Elucidation of the mechanism suggested that the redox-sensitive activation of the p38 mitogen-activated protein kinase had been inhibited. Though such in vitro studies appear promising, the limited-to-overt lack of bioavailability of most of these proanthocyanidins calls into question the physiological relevance of these findings in the context of foods and supplements.

Human intervention trials examining the impact of proanthocyanidin-rich foods on various circulating markers of inflammation and adhesion have been completed. Overall, the findings have been mixed. Some studies using wine (94,95) and cocoa (96,97) reported significant improvements in these endpoints, while other human intervention trials reported no significant effects (78,87,98). This variability in results may be attributable to a number of elements such as the health status of the study participants (i.e., healthy and disease-free versus those with known CV disease), small sample size, and duration of the studies. As such, further studies are needed in order to draw firm conclusions regarding the potential anti-inflammatory effects in vivo.

Decreased Platelet Aggregation

In vitro experiments with whole blood showed that cocoa procyanidin trimers and pentamers as well as dealcoholized red wine increased expression of platelet activation markers (fibrogen binding conformation of GPIIb-IIIa and P-selectin) in unstimulated platelets but suppressed platelet activation response to epinephrine (99). Both short-term (2–6 hr) studies and a long-term (28 day) study with human subjects demonstrated that consumption of proanthocyanidin-rich cocoa beverage lowered P-selectin expression and platelet aggregation (ADP-, collagen-, epinephrine-induced) in ex vivo experiments (47,75,99–101). The effects observed were qualitatively similar to aspirin, but less profound (75). There is also evidence that in addition to a direct action on platelets, the consumption of proanthocyanidin-containing foods can modulate the function of leukocytes (101). Other food sources of proanthocyanidins (and minor constituents) such as purple grape juice, combined extracts of grape seeds and grape skins, but not citrus juices, also were active in the reduction of platelet aggregation when administered to dogs, monkeys, or humans beings (77,102). Extract of *Ginkgo biloba* (120 mg/day for 3 mo) fed to healthy volunteers modulated collagen-, but not PAF-mediated platelet aggregation (103). However, on giving the same extract to subjects with type 2 diabetes decreased platelet aggregation stimulated by both systems.

An in vivo model based on cyclic flow reductions caused by platelet aggregation in the partially occluded circumflex coronary artery of anesthetized dogs has been

employed to test platelet activity and platelet–vessel wall interactions (102) and references therein). Several of the same dietary sources of proanthocyanidins (red wine, purple grape juice) that were active *in vitro*, were also active in preventing thrombus formation in this model. A similar model, based on experimental venous thrombosis in spontaneously normolipidemic rats fed a cholesterol-rich diet, demonstrated that dealcoholized red wine added to their diet reversed the prothrombotic effect of the hyperlipidemic factors (104).

Animal Models

Two animal models have been developed to study dietary and other effects on progression of atherosclerosis. Golden Syrian hamsters, when fed diets of high cholesterol and coconut oil for ten weeks, have a lipid profile similar to hypercholesterolemic humans beings. This treatment also results in the formation of foam cells on aorta walls, the extent of which has been used as a biomarker of the early stages of atherosclerosis (atherosclerotic index) (82). Addition of grape seed extract to hypercholesterolemic hamster diets (50 mg or 100 mg/kg body weight) resulted in a substantial and significant reduction of the atherosclerotic index. In addition, total plasma cholesterol and triglyceride levels also were significantly reduced in the GSPE-fed animals.

New Zealand White rabbits fed hypercholesterolemic diets respond with high plasma total cholesterol levels (400+ mg/dL) and the formation of Sudan-positive stained lesions (fatty streaks) on the walls of their aorta (biomarker of atherosclerosis potential) (105). Addition of a grape seed extract (Leucoselect Phytosome®) to hypercholesterolemic diets of a group of rabbits reduced aortic arch lesions to nearly control levels (3%), whereas atherosclerotic diets alone resulted in lesions that covered 18% of the vessel wall.

Currently, there are limited data available to support the translation of these effects from animal models into humans. Studies with human beings who consumed a combination of cocoa powder and dark chocolate for relatively long periods (4 and 6 wk) only slightly, but significantly, increased HDL levels in one experiment (78), but did not significantly alter plasma cholesterol, triglyceride, or other lipoprotein concentrations (78,87). In contrast cinnamon, which contains a series of unique trimeric and tetrameric procyanidins with A-type linkages (106), significantly decreased plasma levels of triglycerides as well as total and LDL-C, when administered (1–6 g/day) for only 20 days (107). Grape seed proanthocyanidins fed to rats along with high-cholesterol diets also reduced serum cholesterol levels compared to nonproanthocyanidin-fed controls (5). Studies with proanthocyanidin-rich cranberry juice powder fed to familial hypercholesterolemic pigs significantly lowered plasma total cholesterol and LDL, and slightly raised HDL (77). However, the same powder fed to normocholesterolemic pigs did not alter levels of circulating cholesterol fractions.

Nitric Oxide-Dependent Vasodilation

The enzyme nitric oxide synthase (NOS) uses L-arginine and oxygen as substrates to produce NO, which interacts with smooth muscle cells to cause vasorelaxation. A common inhibitor of NOS, N^G-nitro-L-arginine methyl ester,

when infused, nullified vasodilation observed with treatments that stimulate NO production (45,104,108) thereby validating the action of NOS and role of NO in vasodilation. Three distinct NOS isozymes have been identified: endothelial, the critical isoform relative to maintenance of vascular function; neuronal; and an inducible form found in a number of cell types, including macrophages and vascular smooth muscle cells (109).

In vitro studies demonstrated that red wine and Pycnogenol, but not white wine, improved vasodilation and simultaneously increased endothelial NO production (102). Further characterization of proanthocyanidin fractions isolated from red wine showed that vasodilation activity was greatest in the presence of low-molecular-weight oligomers (DP 2–3), whereas higher polymers were inactive. Examination of the mechanism of increased NO production with rat aorta ring strips and *G. biloba* extract suggested inhibition of Ca²⁺ influx through Ca²⁺ channels, thereby activating NO release (110). Contrary to the above findings, proanthocyanidins isolated from female inflorescences of hops (*Humulus lupulus*), a common ingredient of beer, were strong inhibitors of neuronal nitric oxide synthase activity, with procyanidin dimer B2 having the highest inhibitory activity (111). Procyanidin dimer B3, an isomer of B2, was noninhibitory in this system. An explanation for the differential action of these isomers on two isoforms of NOS is not apparent at this time.

Two noninvasive *in situ* systems have been developed to test the efficacy of various dietary components, drugs, and environmental conditions on vasodilation. A study in patients with coronary artery disease showed improved flow-mediated vasodilation of the brachial artery when purple grape juice was consumed compared to beverages that did not contain proanthocyanidins (77). Similar studies have been done utilizing proanthocyanidin-rich cocoa product, supporting both acute (45,108,112,113) as well as sustained improvements (46,114,115) in endothelial function. In several of these studies, the positive effects on endothelial function were linked to the production of nitric oxide, a potent vasoactive molecule key to regulation of vascular tone (44,45,114).

Vasoconstriction

Angiotensin II is a vasoconstrictor that is produced in the pulmonary capillaries by angiotensin converting enzyme (ACE) and can be involved in the development of hypertension and atherosclerosis (77). Several proanthocyanidins and preparations containing them inhibited angiotensin converting enzyme activity in both *in vitro* and *in vivo* experiments. These included Pycnogenol, proanthocyanidins isolated from red grapes, and extracts of *Erythroxylum laurifolium* (endemic species on Reunion Island in the Indian Ocean) and fruits of *Cupressus sempervirens* L. (Italian cypress).

Reperfusion

Induced ischemia–reperfusion studies in hearts isolated from laboratory animals simulate myocardial infarction and recovery in human beings. This model permits investigation of various dietary interventions and other environmental and circulatory alterations on recovery of hearts postischemia. Hearts from grape seed extract, red wine-, or red wine proanthocyanidin-fed rats were more

resistant to ischemia–reperfusion injury than hearts from control animals (82,116). Blood flow parameters were improved, whereas infarct size, formation of hydroxyl radicals, and malondialdehyde levels of heart perfusate were all modulated as a result of feeding animals proanthocyanidins or proanthocyanidin-containing ingredients to animals. These same dietary treatments also reduced the levels of proapoptotic factors JNK and c-Jun, as well as the proportion of apoptotic cardiomyocytes. Similar studies with a short-term recovery (12 min) showed opposite effects of *G. biloba* extract (EGB 761) pretreatment in terms of decreased inducible nitric oxide synthase mRNA expression and NO production (117).

Other Metabolic Alterations

Bacterial Antiadhesion

Anecdotal observations and recent critical evaluation of the scientific literature provides some evidence that consumption of cranberries or its products is effective in the prevention of urinary tract infections (reviewed in 118). Although the therapeutic effect was long thought to be increased urinary acidity due to hippuric acid excretion (119), it is now attributed to a family of unique proanthocyanidins and/or their catabolites, which have been characterized as containing a high proportion of A-type linkages (76,120,121). In the case of urinary tract infection, the primary effect is inhibition of cellular adherence of P-type (mannose-resistant) uropathogenic strains of *Escherichia coli* (119,122,123). In addition, evidence has been presented to the effectiveness of cranberries for similar responses with *Helicobacter pylori* to gastric epithelial cells (124) and a host of organisms commonly found in the oral cavity (125). Proanthocyanidin extracts from cocoa and cranberries have also been reported to have bacteriostatic properties (126,127).

Diabetes, Glucose, and Insulin Metabolism

Impaired glucose uptake and insulin resistance are subtle but common metabolic alterations that may be general etiologies for several age-related disorders and chronic diseases (128). Thus, identification of dietary components and natural products that have the potential to maintain these metabolisms throughout life has a highly favorable risk/benefit ratio. Several foods, biological materials, and synthetic preparations, such as tea, several spices, GSPE, and niacin-bound chromium, have been found to be effective (128–130). However, the chromium content of natural materials (long associated with insulin potentiating activity) was not associated with improved insulin action or glucose metabolism (131), which suggested that other biologically active components were responsible. Relative to proanthocyanidins, an extract of cinnamon, which contained a series of two trimers and a tetramer of flavan-3-ols, each with an A-type linkage (106), was effective in significantly reducing fasting blood glucose in a group of type 2 diabetic patients (107). Several studies with proanthocyanidin-containing cocoa products also support the potential for these types of cocoa products to positively influence insulin metabolism (115,132). Longer term intervention studies, and mechanistic investigations are still needed to verify these findings and determine what role proanthocyanidins may play in these processes.

Immune Function

Nonspecific or innate immune response of the immune system is one of the first lines of defense of the body to a host of environmental challenges. Many dietary components and drugs stimulate this system to an elevated level of preparedness. Besides those components of the immune system associated with atherosclerosis, the effect of proanthocyanidins also has been tested, in vitro, in PBMC. In a series of experiments investigating the effects of isolated individual proanthocyanidins from cocoa on resting PBMC, higher molecular weight fractions (DP 5–10) stimulated interleukins (IL)-1 β (proportional to DP), IL-4 production, and IL-1 β gene expression (135, 136), whereas intermediate-sized polymers (DP 4–8) were most active in the stimulation of TNF- α release (133,134). Employing a similar system, IL-2 and IL-5 secretion was unresponsive to isolated proanthocyanidin treatment (133–136).

The influence of Pycnogenol has been studied on some of the components of the immune system in cell culture. In RAW 264.7 macrophages, Pycnogenol treatment of LPS-stimulated cells reduced production of IL-1 β and its mRNA levels in a dose-dependent manner (137). In the same cell line, Pycnogenol blocked the activation of NF κ B and activator protein-1, two transcription factors involved in IL-1 β gene expression, and abolished LPS-induced I κ B degradation. Collectively these results suggest Pycnogenol treatment of this cell line can inhibit expression of proinflammatory cytokine IL-1 through the regulation of redox-sensitive transcription factors. When individual proanthocyanidins were investigated in the same cells induced by interferon gamma, monomers and dimers repressed NO production, TNF- α secretion, and NF κ B-dependent gene expression, whereas procyanidin C2 (trimer) and Pycnogenol enhanced these parameters (138). These latter two treatments also increased TNF- α secretion in unstimulated RAW 264.7 macrophages. Studies with stimulated Jurkat E6.1 cells indicated that Pycnogenol depressed IL-2 mRNA expression, but that the mechanism of transcriptional regulation was different from regulation of IL-1 β (137).

Using isolated proanthocyanidin fractions from *Ecdysanthera utilis* Hayata & Kawak. (du zhong teng, a Chinese medicinal plant) and a PHA-stimulated PBMC system, procyanidin A1 (dimer with A-type linkage) inhibited IL-2 and interferon-gamma production, which may have caused suppression of PBMC proliferation (139). Two newly identified trimers, each with an A-type linkage, failed to alter the response of cytokines or factors from PBMC. A polyphenol-rich fraction isolated from cocoa liquor inhibited mitogen-stimulated proliferation of T cells and polyclonal Ig production by B cells (140). In addition, this cocoa-liquor fraction also inhibited IL-2 mRNA expression and IL-2 secretion by T cells. Specific to cranberries, potential viral antiadhesion properties have also been suggested; a high molecular weight-containing fraction from cranberry inhibited hemagglutination of A (H1N1) and B (H3N2) virus strains as well as decreased viral infectivity (141), suggesting a potential therapeutic potential of these plant compounds.

Given the limited bioavailability of proanthocyanidins, these in vitro findings are likely primarily limited in their application to the mucosal immune system of the gut. To date, a limited number of in vitro studies have

been conducted with colonic or intestinal epithelial cell lines and animal models of intestinal inflammation; however, extracts of proanthocyanidin-containing plants including French maritime pine bark, cocoa, grape seeds, and pomegranate fruit peel were found to modulate various markers of intestinal inflammation (142) and references therein). Human studies are needed to provide clear evidence of the potential benefits of dietary proanthocyanidins in the gut immune response.

DIETARY SOURCES AND INTAKE

Dietary Sources

Foods

A wide variety of analytical procedures have been employed for the measurement of "total" proanthocyanidins (see analysis section). Employing normal-phase HPLC procedures, a large number of food samples, selected on the basis of market share and demographics within the United States (143), were analyzed for proanthocyanidin content (19). These data and others have been combined into a database of values for foods available online from the USDA Nutrient Data Laboratory at <http://www.nal.usda.gov/fnic/foodcomp>. Data for the proanthocyanidin content of selected foods containing substantial amounts are tabulated in Table 1. The data for red grapes reported in Table 1 are for seedless "eating" grapes, whereas cultivars of red-wine grapes and their wines have higher proanthocyanidin contents (144,145). This is reflected in the data for several red wines common in Spain, which contained dimers through polymers DP 13 and represented 77% to 84% of total flavanols (144). In general, a large number of vegetables, many spices, and some fruits (particularly citrus) had undetectable levels of proanthocyanidins (19,146). Fifty-six different kinds of common Spanish foods have been analyzed for flavanols, including dimers and trimers, but not higher oligomers (147). Results indicated procyanidin B2 was the most abundant dimer or trimer, and flavanols were very low or not detected in most vegetables.

Supplements

There are a number of commercially available proanthocyanidin-containing dietary supplements in the market. However, rigorous qualitative and quantitative data on the proanthocyanidin content of these di-

etary supplements is less precise than for foods because these dietary components have not been subjected to the same rigorous sampling and analysis programs. Though rigorous assessments of most of the commercially available proanthocyanidin-based dietary supplements are lacking, there are some exceptions. Some commercially available extracts of Maritime pine bark, grape extracts (Meganatural[®], Activin[®], Gravinol[®]), apples (Applephenon[®]), and lychee (Oligonol[®]) have been characterized, and putative health benefits and aspects of safety investigated.

Dietary Intake

Foods

Based on proanthocyanidin content for over 60 U.S. foods and daily food intake data [USDA Continuing Survey of Food Intakes by Individuals (CSFII) for 1994–1996], consumption by individuals in the United States was calculated for the first time in 2004 (146). The mean intake for all ages (> 2 years old) was estimated at 54 mg/day/person for all proanthocyanidins with DP of 2 or more. Detailed examination of intakes for age/sex groups indicated a bimodal high intake phenomenon for children (2–5 yr and 6–11 yr) and older males (40–59 yr and > 60 yr) each of whom consumed 59 mg/day or more. Proanthocyanidin consumption among adults ranged from 46 mg/day (20–39 yr, female) to 66 mg/day (> 60 yr, male). As outlined earlier, these data do not include proanthocyanidins that might be included in the consumption of red wines or other commonly consumed foods that have substantial polymer content but were not analyzed. Nonetheless, these results provided the scientific community with the first estimates of proanthocyanidin consumption.

With the release in 2004 of the USDA database for the proanthocyanidin content of selected foods, it is now more easily possible to gain better estimates of intakes within populations and to examine the relationship between proanthocyanidin intakes and health and disease endpoints. In 2007, the largest survey of flavonoid intake, including proanthocyanidins, and the relationship of intake to cardiovascular disease mortality was published (148). A survey of nearly 35,000 postmenopausal women in the Iowa's Women's Health study revealed much wider range of intakes from what had been reported in 2004. In this population, the average proanthocyanidin intake in the lowest quintile was estimated to be 62 mg/day

Table 1 Proanthocyanidin Content of Selected Foods (mg/100 g food)

Food/Spice	Dimers ^a	DP 3–10 ^a	DP >10 ^a	Total	Type ^b
Apples, red delicious, with peel	14	64	38	116	B, PC
Blueberries	7	40	129	176	B, PC
Chocolate, baking	207	680	551	1438	B, PC
Chocolate, milk	26	105	33	164	B, PC
Cinnamon, ground	256	5319	2509	8084	A, B, PC, PP
Cranberries	26	152	234	412	A, B, PC
Grape seed (dry)	417	1354	1100	2817	B, PC
Grapes, red	2	19	59	80	B, PC
Pecans	42	211	223	476	B, PC, PD
Plums, black	16	100	115	231	A, B, PC

^a Dimers, DP 2; DP 3–10, trimers through decamers summed; DP > 10 indicates values for polymers larger than decamers which eluted as a single chromatographic peak.

^b Linkage type (A, B) and proanthocyanidin subclasses (PC, procyanidin; PD, prodelphinidin; PP, propelargonidin) identified.

while the top quintile reported an average intake of 524 mg/day. Amazingly, daily intakes of over 3 g of proanthocyanidins were reported in this study. Though there was no statistically significant association between proanthocyanidin intake and cardiovascular disease mortality, the intake of several proanthocyanidin-containing foods including chocolate, red wine, pears, apples, and strawberries was found to be inversely associated with a reduction in cardiovascular disease mortality. Two additional studies examining the relationship between the intake of specific flavonoids, including proanthocyanidins, and the risk of esophageal cancer (149) and non-Hodgkin lymphoma (66), reported intakes in the range of 3 mg to ~350 mg/day. Though intakes were variable, these data provided invaluable information regarding proanthocyanidin intakes among various population groups.

Supplements

Because of the dearth of analytical data for proanthocyanidin content in supplements, botanicals and herbals, comprehensive intakes from these dietary sources are not available.

ADVERSE BIOLOGICAL EFFECTS

Traditionally, condensed tannins (proanthocyanidins) have been considered antinutrients in animal nutrition due to their astringency (reduced feed intake) and ability to bind several macronutrients, thus reducing their digestion and absorption (2,150). Although Pycnogenol has been shown to bind selected purified intracellular enzymes (151), the precise role of these polymers in the alteration of the metabolic equilibrium in the gastrointestinal tract of human beings is unknown. Toxicological studies on long-term (90 days) oral administration of grape seed extract to rats established a no-observed-adverse effect of 1.4g/kg of body weight per day for males and 1.5g/kg of body weight per day for females (152). Similarly, the LD₅₀ of a single oral dose of grape seed extract IH636 was greater than 5g/kg of body weight for both male and female rats (153). Feeding IH636 at the rate of 100 mg/kg/day to male B6C3F1 mice for a year or 500 mg/kg/day to female mice for six months had no detectable adverse effects on the pathologies of vital organs or on serum chemistries (153). In terms of dermal irritation, IH636 was rated as moderately irritating and the no-observed-effect level for systemic toxicity was set at 2 g/kg for male and female albino rats (153). Observations in both rats and human subjects consuming FastOne, a herbal supplement containing extracts of kola nut, grape, green tea, and *G. biloba*, suggested an increased risk of colorectal cancers as substantiated by induced activity of CYP1A2 (154).

In a review of potential drug–dietary supplement interactions, about one-half of patients taking prescription medication and at least one dietary supplement had potential for an “interaction of significance” (155). Of these patients, only 6% had the potential of a severe interaction. Investigation into the impact of various commercial supplements on P450-CYP3A4 activity revealed that specific plant extracts could alter enzyme activity (156,157), suggesting that the coconsumption of some

herbal supplements with certain medications could alter drug metabolism and thus drug effectiveness. Given the concerns that herbal supplements may antagonize or enhance the effects of medications (158), the reporting of herbal/botanical usage to family physicians and other health care professionals is encouraged.

RESEARCH NEEDED

Although there are many areas of research on proanthocyanidins that can be identified for emphasis, three are critically important for substantial advancement of the association of these dietary components with human health:

1. Identify biologically active compounds that are absorbed and their tissue distribution. Studies with human subjects demonstrate that in addition to the absorption of monomeric flavanols, humans can absorb dimeric proanthocyanidins. To date, there is no evidence that higher oligomeric proanthocyanidin species are capable of being absorbed intact. Even though dimeric proanthocyanidins can be absorbed, current evidence demonstrates that circulating levels are in the low nanomolar concentration range and thus questions remain as to the biological relevance of these findings. In contrast to the intact molecules, there is considerable evidence of the catabolism of proanthocyanidins in the lower GI tract to many different phenolic acids (35,36) and evidence that these phenolic acids are readily absorbed and metabolized. A phenolic acid, 3, 4-dihydroxyphenylacetic acid, whose concentration was raised in plasma after consumption of diets rich in fruits and vegetables, significantly modulated platelet activity at concentrations observed in plasma (69), supporting the concept that catabolites of proanthocyanidins are putative bioactives that may contribute to the physiological improvement noted following the consumption of proanthocyanidin-containing foods. Characterization of metabolites and catabolites and their concentrations in various tissues will be of great advantage in terms of designing in vitro studies for the elucidation of mechanisms of action of these dietary constituents.
2. Assess intake of proanthocyanidins from dietary supplements. The recent development of robust analytical techniques for the measurement of proanthocyanidins resulted in the analysis of a large number of foods, the development of a database of values for foods, and estimates of intakes of these components from foods (17–19,146,159). Similar efforts must be applied to those botanicals and herbals known to contain proanthocyanidins. In addition, accurate estimates of supplement consumption (especially botanicals and herbals) must be included in National Nutrition Surveys so that the contribution of these dietary sources can be calculated (160,161).
3. In order to truly establish the health benefits of proanthocyanidins, larger, longer, and more robust human intervention trials are required. Ideally, these studies should be randomized, double-blind, controlled investigations that examine the impact of multiple levels of proanthocyanidins. Foundational to these studies is the

use of analytically well-characterized food products, as well as the use of study endpoints that are biologically relevant within the context of human health. It is only with such a methodical approach will progress be made towards establishing clear recommendations to the public regarding the consumption of specific proanthocyanidin-containing foods.

CONCLUSIONS

There is emerging science to support a range of potential health benefits associated with proanthocyanidin-containing foodstuffs. In vitro and in vivo studies using animal models provide evidence in support of a range of biological effects. Efforts are still needed in understanding how these effects translate to humans, and to understand the overall impact of these effects to human health. Importantly, critical research is still needed to clearly identify the relevant proanthocyanidins with biological activity so that mechanisms of action at the tissue, cellular, and subcellular level can be elucidated. Fundamental to this biological research is the detailed analysis of proanthocyanidins, because the accurate and reliable measurement and characterization of these components in the materials used in research is necessary for understanding and substantiating the purported health effects.

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Pygeum

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INTRODUCTION

Pygeum africanum (also called *Prunus africana*) is a tree belonging to the Rosaceae family. It grows in tropical and humid equatorial mountain zones, at altitudes between 1000 and 2400 m. The tree is commonly found in countries such as Cameroon, Kenya, Madagascar, Congolese Democratic Republic, Equatorial Guinea, Uganda, Tanzania, Angola, South Africa, Ethiopia, Burundi, Rwanda, Malawi, and Nigeria.

The origin of the use of *P. africanum* bark is documented back to at least the early 19th century. The ground bark was used in a water, tea, or milk mixture as a drug, the use being triggered by its flavoring effects (hydrocyanic acid). The bark was used by the Zulus, who had observed beneficial effects on urinary symptoms. Other tribes from Africa and Madagascar used it for the relief of symptoms such as gastric pain, urinary disorders, and also for its aphrodisiac properties. Such uses are, however, poorly documented, and are based on extracts whose contents and properties might differ both between modes of extraction and from the pharmaceutical standardized extract.

The standardized *P. africanum* extract is used to alleviate lower urinary tract symptoms (LUTS) including those accompanying benign prostatic hyperplasia (BPH).

Recent pharmacological and clinical studies have demonstrated that *P. africanum* extract quantitatively and qualitatively improves bladder as well as prostate-related parameters and symptoms causing urinary disorders.

P. africanum is featured in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) II list of endangered species, imposing strict regulations on its harvest and trade. The exploitation of the bark is done within the framework of durable development. Only adult trees, with a diameter of at least 30 cm, undergo partial bark harvest (opposite quarters) that does not compromise the tree's survival. Reforestation and forest enrichment could contribute to conservation and sustainability.

PHARMACEUTICAL DESCRIPTION

Because the origin of the *P. africanum* bark and the extraction and standardization processes play a role in the final quality and content of the extract, results obtained with a given preparation might not necessarily apply to others. Most investigations performed and published were done using a standardized extract, which allows easier comparison of results.

This extract is obtained by a process of maceration and solubilization of the *P. africanum* bark in organic solvent. The solvent is eliminated and the extract is purified. The extract has a soft-to-hard consistency, a dark brown color, and a very strong aromatic odor. It is freely soluble in chloroform but is insoluble in water.

The *P. africanum* bark extract contains numerous constituents, including saturated and unsaturated fatty acids (C12–C22), phytosterols (β -sitosterol, β -sitosteryl glucoside, and β -sitosterone), pentacyclic triterpenoids (ursolic acid, 2 α -hydroxyursolic acid, and oleanolic acid), alcohols (n-tetracosanol and n-docosanol), and carbohydrates (triacontane and nonacosane). The pharmaceutical properties are documented in the European Pharmacopoeia (Monograph no. 07/2002:1986). The American Pharmacopoeia mentions *P. africanum* bark, extract, and capsules in its Pharmacopeial Forum 29 (4) July–August 2003.

CLINICAL STUDIES

Benign Prostatic Hyperplasia

BPH is a very common finding in aging men. Its prevalence above the age of 50 varies from 50% to 75% in most cases. Transurethral and open surgical adenomectomy are the most widely used treatments for BPH patients who have severe symptoms. However, because of the clinically significant incidence of complications associated with surgery, such as blood loss, urinary tract infections, urethral stenosis, incontinence, impotence, and the need for reintervention after prostatic surgery (1), the management of BPH and LUTS has been rapidly changing. In addition, a large proportion of patients with LUTS do not have prostate enlargement and do not need surgical intervention. Medical approaches used in the treatment of BPH include α -adrenoceptor-blockers, 5- α -reductase inhibitors, and plant extracts.

α -Blockers partially alleviate symptoms of BPH (2) by reducing the α -adrenergic tone of the smooth muscle. However, they may have significant cardiovascular side effects that may limit their therapeutic application.

The 5- α -reductase inhibitors were developed from an elegant series of medicinal chemistry studies. Through a reduction in prostate size, they are supposed to act on symptoms by a direct effect on the mechanical component of obstruction. However, there is certain delay in the onset of the improvement (6–12 mo) and an important incidence of side effects, in particular, on sexual function (3). The limitations of 5- α -reductase inhibitors and α -blockers in treating bladder outlet obstruction might be related to the

functional and structural remodeling of the bladder that also develops as the disease progresses. BPH is largely considered to be pathologically a disease of the prostate, while being symptomatically a disease of the bladder. The analogy of an enlarged prostate impinging upon bladder function still holds true in general, but numerous individuals see no improvement in urinary function in spite of objective reduction in prostate size. Still others see clear improvement in LUTS independent of any discernable change in prostate size (4). Modifications in the prostate do not necessarily evolve in parallel with bladder remodeling. The efficacy of the various treatment options depends, in part, on the judicious use of the appropriate treatment corresponding to the stage of advancement of the disease. Treatment directed at improving bladder function may be more efficacious in older individuals, while that designed to solely affect the prostate may be more beneficial in relatively younger patients. BPH results from progressive enlargement of the transition zone of the prostate and involves both glandular and stromal prostate tissue. Stromal elements contain smooth muscle, and contraction is mediated by α -1-adrenergic receptors. Hyperplasia of the transition zone is responsible for the organ enlargement. Prostate enlargement also mechanically and physically affects bladder dysfunction by a progressive denervation via damage to intramural nerves and synapses, as seen in animal models and in obstructive dysfunction in men (5–7). The involvement of specific bladder components indicates that effective treatment should not solely target the prostate, but must also be directed at the bladder. Still, the origin of the modification of bladder function in BPH remains the prostate, as witnessed by the paucity of symptoms in castrated individuals (8).

More than 2000 patients were enrolled in clinical trials with *P. africanum* extract (9). These studies were conducted either in a double-blind placebo-controlled manner (10–12) or as open-labeled studies (13). There are 18 published randomized controlled trials comparing preparations of *P. africanum* with placebo or medical therapy for more than 30 days in men with symptomatic BPH. Thirteen trials included comparison of *P. africanum* extract with placebo (14,15). Most often, the treatment regimen was 100 mg/day (50 mg t.i.d.) for one to two months. The persistence of the effect and the long-term safety profile over more than five years has been investigated in an observational study (16). The effects observed in those studies have demonstrated that:

- More than 67% of patients reported “excellent,” “very good,” or “good” results.
- Mean maximum urinary flow rate was improved in all studies in which it was measured.
- Nocturia was improved in 50% to 100% of the patients in whom it was measured.
- Daytime frequency was improved in 50% to 95% of patients in whom it was measured.
- Hesitancy, urgency, weak stream, and dysuria were improved in the majority of studies.
- Quality of life scores were improved. Standardized assessment scores such as International Prostate Symptoms Score (IPSS) confirmed these results.
- Both clinical and urodynamic improvements were maintained during long-term treatment in a high proportion of patients.
- Both clinical and urodynamic improvements were maintained after 12 months of treatment.

The most frequently used dosage of *P. africanum* extract is 50 mg b.i.d. One prospective clinical trial (12,17) demonstrated the equivalence of effects of the extract 100 mg/day given either as 50 mg t.i.d., or as a single daily dose of 100 mg in men with moderate-to-severe urinary symptoms associated with BPH. Both daily dosage modalities of *P. africanum* extract (100 mg/day and 50 mg t.i.d.) decreased the total IPSS according to an equivalent pattern and to a similar extent (41% and 37.5%, respectively). Mean maximum flow rate increased after two months of treatment by 16% in the 50-mg b.i.d. group and by 18.5% in the 100-mg q.d. group. Similar effects were observed on all components of the condition and on quality of life scores.

MECHANISM OF ACTION

Effects on In Vitro and In Vivo Growth Factor-Mediated Prostate Growth

Normal prostate growth is controlled by an orchestration of growth factor-mediated autocrine and paracrine communication acting on epithelial and stromal prostatic tissue. Mutually distinct biochemical and suborganelle perturbations within the prostate can predispose toward BPH or prostatic carcinoma (18). The BPH affects predominantly stromal and glandular cell growth, where basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) are major regulators along with insulin-like growth factor. Keratinocyte growth factor is expressed in epithelial tissue and has a paracrine effect on stromal cells, whereas transforming growth factor- β 1 has been shown to inhibit prostatic fibroblast proliferation. Finally, testosterone via dihydrotestosterone (DHT) has a causal role in controlling prostate growth and affects the expression of the aforementioned growth factors and their receptors indirectly. In advanced BPH, the role of androgens may be no more than permissive, whereas in prostatic carcinoma, androgen receptor signaling is not strictly a function of DHT levels. Furthermore, in men 50 years old or more, the role of testosterone relative to estrogen appears to diminish (19). Following an initial observation of an effect of *P. africanum* extract on bFGF- and EGF-mediated proliferation of 3T3 fibroblasts in vitro (20), a detailed series of experiments were performed comparing the potential of the extract to affect prostatic stromal cell proliferation mediated by various prostate-derived growth factors in rats (21). At concentrations devoid of cytotoxicity, *P. africanum* extract was shown in vitro to affect stromal fibroblast cell proliferation induced by EGF, bFGF, and insulin growth factor-I as well as with protein kinase C activators, but not by keratinocyte growth factor. Furthermore, the inhibition of cell proliferation was observed with IC50 values in the range of 10 mg/mL. The similarity of the individual IC50 values of *P. africanum* extract on the various growth factors suggests that a locus common to the three growth

factors was being affected (22). Furthermore, the in vitro studies demonstrated that stromal fibroblast proliferation was inhibited via a site that converged near the level of protein kinase C.

The results were not limited to rodent prostatic cells, as *P. africanum* extract was also shown to inhibit proliferation of fibroblasts from human hyperplastic prostate and bladder as well (23).

The precise molecular component(s) of *P. africanum* extract responsible for the in vitro effects is not known. Therefore, these results cannot infer in vivo activity per se, as it is not possible to establish a correlation between the in vitro IC₅₀ value and a C_{max} value for the same metabolite following oral administration. Therefore, in an attempt to confront this problem, subsequent in vivo studies (24) focusing on the rat ventral prostate, which is approximately equivalent to the transition zone of the human prostate, showed that *P. africanum* extract affected adenyl cyclase-mediated cross-talk in cell signaling pathways also at the level of protein kinase C. Finally, ventral prostate hyperplasia induced by DHT treatment in vivo in rats was reversed by oral administration of the extract, while the latter had no effect on dorsal prostate enlargement (25).

The molecular mechanism(s) underlying the in vitro and the in vivo effects of *P. africanum* extract are similar. The extract exhibited an effect on in vitro fibroblast proliferation of both rodent as well as human cells, which correlated with a growth factor profile signature corresponding to stromal cell proliferation, and this was paralleled by a preferential in vivo effect of *P. africanum* extract on ventral (stromal) prostate hyperplasia in rodents.

Effects on Bladder Function

In man as well as in selected animal models, bladder obstruction induced by enlargement of the prostate or partial outlet obstruction of the urethra leads to a progressive increase in urethral resistance. The latter is initially compensated by an increase in bladder wall thickness, increased pressure generation, and alterations in flow parameters that limit the immediate deterioration in bladder function. This phase of compensated bladder function is slowly, though inexorably, followed by a decompensated bladder with a marked loss of contractility. The initial phase displays impaired detrusor smooth muscle function characterized by neuronal degeneration leading to reduced postsynaptic innervation, mitochondrial dysfunction, and loss of intracellular Ca²⁺ homeostasis that collectively compromise myosin contractility.

The preceding section described in vitro data corroborated by in vivo results that demonstrated an effect of *P. africanum* extract on molecular aspects of prostate growth mediated by prostate-derived growth factors. Growth factor-mediated fibroblast proliferation is common to both the hyperplastic prostate as well as the hypertrophic bladder secondary to (26,27) similarly involving bFGF, EGF, and transforming growth factor- β . This common link was the rationale for investigating the effect of *P. africanum* extract in various models of impaired bladder function (28). Indeed, initial results showed that the extract inhibited bFGF-stimulated fibroblast proliferation (23) and modestly reduced bladder weight in vivo in a preliminary

study that was confirmed in larger subsequent studies. Pretreatment of rabbits with *P. africanum* extract prior to partial outlet obstruction reduced contractile dysfunction in both a time- and dose-dependent fashion, determined by measuring the contractile responses to field stimulation, bethanechol and KCl. At a dose of 100 mg/kg, it improved the response to field stimulation by 50% and to KCl by 70%, and completely normalized the response to bethanechol when subsequently determined three weeks following partial outlet obstruction. Of the three parameters measured, partial outlet obstruction affects field stimulation to a greater extent, reflecting marked deterioration of synaptic function. Therefore, the initial experiment was complemented by a series of investigations that first determined the time course of the effects of *P. africanum* extract and correlated this to specific perturbations in synaptic and postsynaptic membranes coupled to alterations in key mitochondrial enzymes and calcium homeostasis. The results of the time-course study indicated that *P. africanum* extract was able to normalize the response to field stimulation after two weeks of treatment. These results were superior to those obtained after three weeks and may indicate a reduced efficacy of *P. africanum* extract after prolonged obstruction or an irreversible deterioration of synaptic function over time in this model.

In an attempt to mimic the clinical situation, wherein bladder function is already compromised when treatment is initiated, it was then shown that the efficacy of *P. africanum* extract was maintained when administered only after the application of partial outlet obstruction. Specifically, the effects of *P. africanum* extract in restoring the contractile response to field stimulation, carbachol, and KCl were qualitatively identical when administered before or after partial outlet obstruction (28,29).

Contractile dysfunction ultimately results in reduced force generation and alterations in myosin isoforms. In parallel with the improved contractile dysfunction, *P. africanum* extract was able to partially normalize the expression of myosin isoforms in line with improved contractility. These studies were based on the observation that alternative post-transcriptional splicing of myosin mRNA generates two isoforms of myosin, SM-A and SM-B, with lower and greater actin-activated adenosine triphosphate hydrolysis, respectively, and hence force generation. Following obstruction, detrusor smooth muscle SM-A myosin isoform expression increases threefold corresponding to reduced force generation, whereas treatment with *P. africanum* extract normalizes the SM-B/SM-A ratio in parallel with the improvement in field stimulation (30).

Contractile dysfunctions of the obstructed bladder are directly related to ischemia (reduced blood flow) and detrusor hypoxia (31). Thus, short-term ischemia is a relevant model that recapitulates pathological aspects of contractile dysfunction inherent in partial outlet obstruction originating surgically or via prostatic enlargement. In this model, unilateral ischemia provokes direct and irrevocable ischemic insult to one side of the bladder, while partially compromising the nonischemic side. In agreement with what was observed following partial outlet obstruction, *P. africanum* extract pretreatment protected the nonischemic side of the bladder from the

development of contractile dysfunction. This protective effect was further correlated with an enhanced expression of Hsp70 and *c-myc*. The clinical pharmacological relevance of these animal data was established by Valentini et al. (32), who demonstrated in a blinded clinical study that *P. africanum* extract improved detrusor contractile function after two months of treatment.

The contractile dysfunctions induced by partial outlet obstruction in animal models, and by BPH-induced obstructive dysfunction in men, are secondary to denervation, mitochondrial dysfunction, and calcium storage dysfunction, which in turn is mediated partially by ischemia-generated free radicals and calcium-activated hydrolytic enzymes. One hypothesis is that *P. africanum* extract acts in part by protecting neuronal and subcellular membranes from ischemia-induced damage, and by this means protects the contractile function of the bladder (7,33).

Testosterone, in addition to its well-known action on stimulating prostate growth, also affects the bladder, and in the rat, administration of DHT significantly affects urodynamic parameters including frequency and volume. In DHT-stimulated rats, *P. africanum* extract, in addition to the aforementioned selective effect on ventral prostate growth, also significantly improves bladder frequency and volume (34). Collectively, these results clearly demonstrate that *P. africanum* extract directly affects bladder function.

Among the limitations of the animal models employed is the lack of a concerted pathophysiology strictly representative of human BPH. The anatomic separation of prostate and bladder function in these models is, nonetheless, an advantage when attempting to demonstrate independent effects of *P. africanum* extract on the two organs. Furthermore, animal studies have the obvious advantage of being devoid of a placebo effect that complicates the design and interpretation of clinical trials in this indication.

Treatment of LUTS is complicated by the multifactorial and multiorganelle origin, the slow evolution of the disease process, as well as the high placebo response in this patient population, which collectively limit the perceived efficacy of monotherapy in short-term clinical trials.

P. africanum extract has demonstrated a reproducible efficacy in a variety of pharmacological studies addressing key aspects of lower urinary tract pathophysiology, thus altering the often encountered perception of plant extracts as poorly defined mixtures acting in an ambiguous manner to that of a reproducible molecular effector.

The precise molecular component(s) responsible for the effects of *P. africanum* extract have not been identified.

A limitation of the animal data described is the use of short-term treatment periods and the rapid evolution of the pathology in animals to investigate what in man is a chronic disease. The treatment duration of most clinical trials has also been limited, and the patient population not always ideally chosen to demonstrate beneficial effects on the bladder. The optimal patient population for showing the efficacy of an α -blocker, a 5- α -reductase inhibitor, and *P. africanum* extract could vary, reflecting different stages of the disease process and related symptoms.

The sum of the in vitro and in vivo pharmacological studies suggests a pharmacological mechanism of action of *P. africanum* extract affecting independently:

- Prostate hyperplasia via a downstream target common to bFGF, EGF, as well as androgen-mediated cell proliferation at or near the level of protein kinase C. The signature of growth factor-mediated inhibition by *P. africanum* extract on in vitro and in vivo prostatic cell proliferation suggests that in vitro results are predictive and correlated to in vivo activity.
- Bladder function with improvement in contractile dysfunction mediated via myosin isoform expression, lessened synaptic denervation and improved mitochondrial function.

ADVERSE EFFECTS

Routine preclinical safety trials performed in various animal species by oral and parenteral routes, with single and repeated administrations, studied *P. africanum* at doses greater than 50 times the therapeutic doses. In such studies, no target organ could be identified as to potential toxic effects. *P. africanum* extract is devoid of any mutagenic potential.

Most published open-label and placebo-controlled studies mentioned a good tolerance of the extract. Of particular note is the absence of any hormone-related adverse effects, confirming that the extract does not exert any hormonal effect. No interactions with concomitant medications such as antihypertensive agents, lipid-lowering agents, or anti-arrhythmics were reported. No significant changes were observed in biochemical safety parameters in those published studies where this is documented (10,12,13).

In a recently published review, 13 of 18 randomized controlled trial studies provided information on specific adverse events. Side effects due to *P. africanum* were generally mild in nature and similar in frequency to placebo. The most frequently reported were gastrointestinal and occurred among seven men in five trials (15). In the most recently completed study (12), the type and overall frequency of adverse effects had similar distributions between dosage modalities during the comparative phase and were comparable for both phases of the trial.

Light-to-moderate gastrointestinal effects, such as nausea, constipation, or dyspepsia, which are known to be treatment-specific, were reported most frequently (5.4% and 9.8% of patients who participated in the comparative and the extension phase, respectively). The majority of the serious adverse effects were related to the urogenital system (1.3% and 3.5% of patients who participated in the comparative and the extension phase, respectively) and appeared more related to the natural evolution of the disease than to the medication itself. It has been observed that very few severe emergent adverse effects (SEAE) appeared during the study, and the risk of presenting an SEAE during the long-term follow up was very low and constant in time. The rate of the SEAE-free patients at one year was 90% with 95% of CI 85% and 95%. Similar observations were made for the treatment-related emergent adverse events (TREAE), with the rate of the TREAE-free patients at one year equal to 92% [95% CI (88%, 96%)]. Few side effects were responsible for patients' withdrawal from the study (15 patients during the comparative phase and 8 patients during the long-term phase). No

significant changes were noted in blood or urine analyses in either group or during the two phases of the study. There was no statistically or clinically significant variation of the prostate-specific antigen level at 12 months compared to the baseline value. There is no report of any unwanted effect on sexual function with *P. africanum*.

The cardiovascular effects were studied after a trial of 12 months of treatment with *P. africanum* (12). The treatment was not associated with any unwanted cardiovascular effects in this study.

PRODUCTS AND DOSAGE

P. africanum extracts are available worldwide under various formulations. One of them, *P. africanum* extract V1326, is the most common preparation *Pygeum africanum* Extract 573, and is marketed under the TadenanTM trade name as a prescription drug. Other preparations are available in various countries, containing *P. africanum* extract either as a single component (such as PronitolTM, BidrolarTM, FoudarilTM, KunzleTM, Neo UrogeninTM, NormobrostTM, NormoprostTM, ProlitrolTM, ProvolTM, etc.) or in combination with other components such as vitamins or minerals (such as ProFlowTM, PotenziaTM, Super Prostate FormulaTM) to name a few.

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Quercetin

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INTRODUCTION

Quercetin (3',4',5,7-tetrahydroxyflavonol, 3,3',4',5,7-pentahydroxyflavone, 2-(3,4-dihydroxy-phenyl)-3,5,7-trihydroxy-chromen-4-one, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one) is a flavonol belonging to a class of naturally occurring flavonoids (1–4). It is composed of 3,5,7-trihydroxy-4H-1-benzopyran-4-one (A and C) and a 3,4-dihydroxyphenyl ring (B) (Fig. 1).

Synonyms for quercetin include: C.I. Natural Yellow 10; C.I. 75670; cyanidelonon 1522; flavin meletin; quercetine; quercetol; quertin; quertine; sophoretin; xanthaurine; 3,3',4',5,7-pentahydroxyflavone; 3,5,7,3',4'-pentahydroxyflavone; 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one.

BIOSYNTHESIS AND NATURAL SOURCES

Quercetin belongs to the class of flavonoids providing flavor, color, and other functions to plants including fruits and vegetables. More than 5000 different flavonoids have been isolated, and on the basis of chemical structures, these are generally classified into several subgroups: flavanones (e.g., naringenin, hesperidin), flavones (e.g., apigenin, luteolin), flavonols (e.g., quercetin, myricetin), flavans (e.g., epicatechin, gallocatechin), anthocyanins (e.g., cyanidin, pelargonidin), and isoflavones (e.g., genistein, daidzein). In fact, quercetin belongs to the subgroup of flavonol, because of its hydroxylation at 3-position of the C ring. (1,2). Quercetin is synthesized in plants via multiple enzymatic processes from phenylalanine and

malonyl-CoA. Briefly, 4-coumaroyl-CoA and acetyl-CoA from phenylalanine and malonyl-CoA, respectively, yield chalcone, a precursor for flavonoids including quercetin (1,5). Quercetin present in plants is mainly found conjugated to sugars as glycones (e.g., hyperin, isoquercitrin, avicularin, quercitrin, and rutin) (1,3). Besides glycosylation, it is also found modified by prenylation, acetylation, and methylation (1,3,6). Quercetin is commonly found in numerous dietary sources (onions, apples, black tea, green tea, red wine, beans, grapes, berries, vegetables, and fruits), and its dietary intake is associated with various potential health benefits (7–10).

ABSORPTION, METABOLISM, AND BIOAVAILABILITY

There is great interest in the potential health benefits of flavonoids because of their potent antioxidant, free-radical scavenging, and other biological activities observed in vitro (9,10). Quercetin has potent antioxidant and other activities, which might contribute beneficial health effects on chronic diseases such as inflammation, cardiovascular diseases, and some types of cancers (9–11). Most beneficial health effects of quercetin would necessitate its absorption into the human body, which is interconnected with its metabolism and bioavailability. Data about these processes are to some extent available, but they are yet to be elucidated extensively.

Absorption

Quercetin occurs as glycones or aglycones, but in plants mainly as glycones such as rutin (quercetin rutinoside). Quercetin aglycone and glycones are likely to be quite different in their absorption and pharmacokinetics. Studies indicate that the overall kinetic behavior of quercetin changes following the ingestion of quercetin aglycones or glycones. This includes properties such as C_{\max} (the highest level at a given dose) and T_{\max} (time to reach C_{\max}) (12).

Currently, there are two proposed hypotheses on the absorption mechanisms of quercetin glycosides across the small intestine. One is an active uptake of quercetin glycoside by the sodium-dependent glucose transporter (e.g., SGLT1) with subsequent deglycosylation within the enterocyte by cytosolic beta-glucosidase (CBG). The other is the absorption by passive diffusion of quercetin after luminal hydrolysis of its glycones by lactase phlorizin hydrolase (LPH). Both methods seem to be utilized for the uptake of individual quercetin glycones with substrate selectivity. For instance, quercetin-4'-glucoside

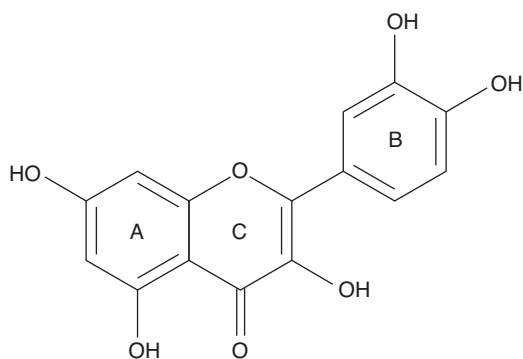


Figure 1 The chemical structure of quercetin.

involves both an interaction with SGLT1, which is not able to transport aglycone quercetin, and a luminal hydrolysis by LPH, whereas quercetin-3-glucoside appears to be absorbed only following hydrolysis by LPH (13,112). The deglycosylation step seems critical for the absorption of quercetin glycosides in humans and is mediated by glucosidases such as LPH and CBG. The significant variation in their activity between individuals was even suggested as a potential cause for differences in flavonoid (quercetin) bioavailability (14). Even though the hydrolysis of the glycoside moiety from quercetin glycones is strongly believed to be a prerequisite process for quercetin absorption, there are also reports indicating that a fraction of quercetin may be absorbed as its glycones via passive diffusion and/or unidentified transporters (15,16). The discrepancy of absorption patterns between quercetin and its glycones can be accounted for by hydrolysis process of its glycoside and its diverse absorptive processes.

Metabolism

Quercetin was reported to be metabolized mainly in the liver and the intestine. Absorbed quercetin is mainly metabolized in the liver, whereas the unabsorbed form can be metabolized in the gut by intestinal microorganisms. *Eubacterium ramulus* was reported as a microorganism involved in degrading quercetin in the intestine. This strictly anaerobic bacterium can cleave the ring structure of quercetin into 3,4-dihydroxyphenylacetic acid (17,18). Intestinal metabolism of quercetin by the microorganism may provide an alternative pathway for quercetin absorption in the gut, even though this absorption is likely to be insignificant and irrelevant to quercetin bioavailability. Primary metabolism of quercetin absorbed occurs in the liver, even though some minor metabolic processes take place in various human cells. Investigation of metabolites of quercetin in the plasma and urine revealed that the flavonol is metabolized by glucuronidation, hydroxylation, methylation, and sulfonylation. Glucuronidation occurs usually during passage across the epithelium as well as in the liver (19). The enzymes responsible for this process are UDP-glucuronosyltransferases (UGT) such as UGT1A9 (in human liver) and UGT1A1 and UGT1A8 (in intestinal epithelium) (15,20). In intestinal epithelial cell, quercetin was reported to metabolize mainly into quercetin-3- and quercetin-7-glucuronides (21). Quercetin glucuronides were also reported to metabolize further via two pathways: first, methylation of the catechol functional group of both quercetin glucuronides by methyltransferases; and second, hydrolysis of the glucuronide by endogenous beta-glucuronidase, followed by sulfation to quercetin-3'-sulfate (22,23). Quercetin is also methylated, sulfonylated, and hydroxylated in the liver (22,24). It is also found in plasma as unconjugated quercetin aglycone, even if quercetin glucuronides are considered as main circulating metabolites in humans. Occurrence of the quercetin aglycone within tissues is likely to result from the deconjugation of flavonoid glucuronides by the enzyme beta-glucuronidase. Indeed, some human tissues from the small intestine and the liver, and neutrophils, exhibit beta-glucuronidase activity against quercetin glucuronides (25). Multiple processes

of quercetin metabolism may yield quercetin metabolites with different biochemical properties and produce some metabolites with more or less purported beneficial effects (26).

Bioavailability

Bioavailability is the physiological availability of a compound in a given amount. In other words, it is the proportion of the administered amount that is absorbed into the bloodstream. Therefore, bioavailability depends mainly on the initial administered amount, absorption, metabolism, tissue distribution, and excretion. Pharmacokinetic studies are performed using several different doses and routes to determine the bioavailability of an administered compound, which can be used as a good guideline for safe human intake and a valuable criterion for verifying purported effects in *in vitro* studies. However, most pharmacokinetic studies of quercetin seem fragmentary and require further research to provide complete information regarding quercetin bioavailability.

Originally, quercetin aglycone was thought to be able to pass through the gut wall better than its glycones. However, pharmacokinetic studies suggest that quercetin glycones are absorbed better than its aglycone (10,27,28). The studies also revealed that quercetin glycosides could show significant differences in absorption rate and bioavailability, depending on the glycosylation sites (27,28). Some pharmacokinetic studies of quercetin were performed using quercetin (aglycone) and its glycones to determine bioavailability. In the study using quercetin aglycone, it was reported that after a single 4 g dose of quercetin administered orally in humans, the flavonol was detected in the plasma. The highest peak plasma concentration (C_{\max}) of quercetin was less than 100 ng/mL, and that all quercetins detected in the plasma were in the form of glucuronidated and sulfated quercetins rather than quercetin aglycone (1,29). In the same study, following single intravenous (100 mg) administration of quercetin, the highest peak plasma concentration (C_{\max}) of quercetin was around 3000 ng/mL, and the time to reach C_{\max} (T_{\max}) was 10 minutes (1,29). Human absorption of quercetin can be enhanced by quercetin conjugation with glucose (30). For instance, following ingestion of quercetin glycosides (in fried onions) equivalent to 64 mg of quercetin aglycone, C_{\max} was reported to be 196 ng/mL after 2.9 hours, which is higher than that following 100 mg of quercetin aglycone (31). The sugar moieties and positions of quercetin O-glycosides seem to influence their bioavailability as well (32–35). The difference in bioavailability between quercetin glycosides can also be attributed to their different solubility influencing their accessibility for absorption and to enzymes involved in the absorption (36,37). Currently, the average human intake of quercetin (glycones and aglycone) is less than 60 mg daily. On the basis of this amount, the highest concentration achieved in the plasma (C_{\max}) is less than 200 ng/mL (0.6 μ M), which includes quercetin and all its metabolites. *In vivo* effects of quercetin reflect its bioavailability, which can be changed on the basis of a given dose of the flavonol. Unfortunately, data on bioavailability in multiple doses are currently not available, requiring future investigation.

CELLULAR AND MOLECULAR ACTIONS

Effects of quercetin in humans are manifested in a dynamic environment. For instance, ingested quercetin (glycone and aglycone) undergoes absorption, metabolism, tissue distribution, and excretion. However, cellular and molecular actions of quercetin reported in *in vitro* studies are observed in a rather static environment, excluding many key physiological conditions. Also, the quercetin concentrations used in the many experiments discussed below are relatively too high to be achieved by dietary ingestion. Therefore, some biological activities reported *in vitro* should be regarded as having potential for future application of pharmacologic quercetin or quercetin analogs in preventing and/or treating human diseases, rather than as indicating direct use of quercetin and/or metabolites in human diseases. Flavonoids (quercetin) were once named vitamin P or vitamin C2 due to their abilities to decrease capillary permeability or spare vitamin C activities (2). Since then, quercetin has been viewed as a compound with both beneficial and harmful effects (38–40). However, it is currently recognized as a compound that is more helpful than deleterious, because epidemiological, cellular, and molecular studies of quercetin have suggested this (41,42). The antioxidant properties of quercetin are believed to explain its positive effects in a major way. The 5,7,3',4'-hydroxyl groups on quercetin are capable of donating electrons to quench various radical oxygen species (ROS) and other radical species (43,44), which have the potential to influence pathogenesis or treatment of chronic human diseases such as inflammation, cardiovascular diseases, and cancer.

Antioxidant Properties

Oxygen radicals (superoxide, hydrogen peroxide, hydroxyl radicals, and other related radicals) have been reported to be involved in initiating and/or promoting inflammation, cardiovascular diseases, cancer, aging, and other chronic diseases (44). The radicals are quenched by endogenous antioxidant systems, including antioxidant compounds, which balance cellular redox status involved in cellular processes for cell homeostasis, such as proliferation, signaling transduction, and apoptosis (45,46). Therefore, it is proposed that improper redox balance can contribute to the progression of chronic human diseases such as inflammation, heart diseases, and cancer, and adequate intake of antioxidant chemicals from fruits and vegetables may afford significant protection against them. Generally, three criteria are considered to assess the antioxidant activity of flavonoids *in vitro*: first, B ring with two hydroxyl groups (adjacent); second, C ring with 2,3-double bond, 4-oxo, and 3-hydroxyl group; and third, A ring with 5,7-dihydroxyl groups (2). Quercetin meets all three criteria, indicating stronger antioxidant activity than flavonoids that do not meet the criteria. Accordingly, the flavonol was reported to prevent radicals from damaging carbohydrates, proteins, nucleotides, and lipids (47). Quercetin is metabolized and found in the plasma as quercetin glucuronide conjugates, and other metabolites. What about their antioxidant properties? The glucuronide conjugates found in the plasma were also reported to have potent antioxidant activity, indicating that the activity may be

retained depending on conjugation positions (48). The antioxidant activity of quercetin is believed to contribute to its beneficial effects in a significant way.

Effects on Inflammation

Inflammation is the first and necessary response of the immune system to infection and others. Inflammation is supposed to fight against on-going infection as well as initiate healing processes. However, when inflammation is not properly under control, it may attribute to developing chronic diseases (e.g., arthritis, allergy, asthma, atherosclerosis, cancer, and aging) (49–51). Inflammation processes are complex and highly orchestrated with numerous biological factors including eicosanoids, cytokines, and other immune factors. Eicosanoids are chemical mediators synthesized by cells in response to local tissue damage as well as hormonal and immunological stimuli (52,53). Eicosanoids (e.g., prostaglandins and thromboxanes) are involved in inflammatory processes via binding to their cognate receptors on a wide variety of tissues throughout the body (53). Cyclooxygenase (COX) is the key enzyme metabolizing arachidonic acid to generate prostaglandin H₂, which is converted by downstream enzymes to other prostaglandins and thromboxanes (54). Therefore, COX enzyme has been a molecular target for developing anti-inflammatory drugs. COX enzymes consist of two COX isoforms: COX-I and -II (55,56). COX-1 is constitutively expressed in nearly all tissues, but its activity is most strongly associated with prostaglandin production in gastric mucosa and thromboxane production in platelets. Meanwhile, COX-2 expression is upregulated in response to inflammatory stimuli. Increased prostaglandin levels are often considered as a part of inflammatory responses (56). Several *in vitro* studies indicated that quercetin is able to inhibit COX-I and COX-II enzymes, thereby potentially providing anti-inflammatory effects via modulating eicosanoid effects on immunological and other processes (49–51). Also, inflammation mediates various immunological effects in infected tissues. For instance, tumor necrosis factor- α (TNF- α) generated by activated macrophages induces several pathophysiological conditions during acute and chronic inflammation. Quercetin was reported to inhibit TNF- α overproduction and attenuate pathophysiological conditions during acute and chronic inflammation (57–60). The inhibition is not surprising at all, because quercetin is believed to inhibit a transcription factor NF- κ B involved in the production of various immunological molecules such as TNF, IL-1 β , and iNOS. However, this seems not a specific action of only quercetin (61,62). Asthma is another well-known inflammatory disease, characterized by constriction of lung airways. During asthma attack, the activation of mast cells and basophils by allergen releases chemical mediators and synthesizes cytokines leading to inflammatory conditions. Among these cytokines, interleukins IL-4, IL-13, and IL-5 are major ones involved in allergic inflammation. Quercetin was reported to inhibit the expression and syntheses of these cytokines in human basophils (63). In one study, a metabolite of quercetin, 3-O-methylquercetin (3-MQ), was even reported to provide beneficial effects on asthma by inhibiting cAMP- and cGMP-phosphodiesterase (PDE), counteracting harmful

effects of the cytokines, etc. (64). Although some reports related to beneficial effects of quercetin on inflammation are available, the reported effects of quercetin are still preliminary, necessitating future studies prior to any firm conclusions.

Effects on Cardiovascular Diseases

Cardiovascular disease, also known as coronary artery disease, represents the class of diseases related to the heart and/or blood vessels such as arteries and veins where inflammation is deeply involved. One representative example of cardiovascular diseases is atherosclerosis of the coronary arteries. Atherosclerosis is a condition in which the arteries become clogged and narrowed, and the restriction of blood flow to the heart occurs. There are accumulating data indicating that quercetin is associated with beneficial effects on cardiovascular diseases with inflammatory complications. Some epidemiologic studies show that eating a diet rich in flavonoids is associated with decreased incidence of cardiovascular diseases. Quercetin has biological properties consistent with its purported effects on the cardiovascular system. For instance, quercetin has been shown to protect low-density lipoprotein (LDL) from oxidation and prevent platelet aggregation (65). It was also reported to inhibit the proliferation and migration of smooth muscle cells. These findings provide new insights and a rationale for the potential use of quercetin for preventing cardiovascular diseases. Currently, there are numerous reports supporting beneficial effects of quercetin on cardiovascular diseases. For instance, quercetin was reported to significantly lower the plasma lipid, lipoprotein, and hepatic cholesterol levels, inhibit the production of oxLDL produced by oxidative stress, and protect an enzyme that can hydrolyze specific lipid peroxides in oxidized lipoproteins and in atherosclerotic lesions (66–68). Quercetin was also reported to even induce endothelium-dependent vasorelaxation in rat aorta via increasing nitric oxide production (69). These data suggest that the protective effects of quercetin on heart diseases may result from its arterial, venous, and coronary vasodilator effects (70). The cardiovascular protective effects of quercetin may also play significant roles in attenuating hypertension. Hypertension (high blood pressure) is a condition in which the force of blood flow against artery walls is too strong. Hypertension and its related events are able to damage arteries, heart, and kidneys, leading to the progression of several types of cardiovascular diseases including atherosclerosis and stroke (71,72). Angiotensin-converting enzyme (ACE) is a well-known peptidase in the renin-angiotensin system (RAS) regulating blood pressure via controlling extracellular volume and vascular constriction through converting angiotensin I to angiotensin (Ang) II. Quercetin and its glycosides were reported to inhibit the ACE activity and also suppress Ang II-induced c-Jun N-terminal kinase activation inducing vascular smooth muscle cell (VSMC) hypertrophy (73,74). These findings suggest that the positive health effects of the flavonol on heart diseases are executed via inhibiting signal transduction pathways leading to the diseases including atherosclerosis and hypertension (75,76). Quercetin was also reported to inhibit platelet aggregation that can be beneficial for lessening conditions of cardiovascular disease (77,78). On

the basis of all these findings, quercetin seems to have potential in the prevention and/or treatment of cardiovascular diseases. However, some effects may not be feasible or negligible in physiological conditions, because concentrations of quercetin in most studies are often too high to be achieved by dietary ingestion of quercetin. In summary, there are beneficial effects of quercetin in relation to heart disease, but many areas of uncertainty exist.

Effects on Diabetes

Diabetes is a disease condition in which the body does not produce or use insulin properly, a hormone needed to convert glucose, carbohydrates, etc., into energy needed for daily life (79–83). There are two major types of diabetes, type 1 and type 2. Both types can cause blood sugar levels higher than normal. However, their mechanisms causing diabetes are different. Type 1 diabetes (insulin-dependent diabetes or juvenile diabetes) is attributed from the inability of the pancreas to produce the hormone insulin. It happens probably because the person's own immune system jeopardizes the insulin producing cells in the pancreas. However, type 2 diabetes (non-insulin-dependent diabetes or adult onset diabetes) is different from type 1 diabetes. In type 2 diabetes, there is an inability of tissues to respond to insulin normally, even though insulin is produced (81,82). The cause of type 2 diabetes is yet to be elucidated in detail, even though there are data suggesting that both genetics and environmental factors such as obesity and lack of exercise may play roles. Patients with type 2 diabetes represent the largest portion by far of total diabetic patients compared to other types. In type 2 diabetes, the age of onset and disease progression are variable, and complications are related to control of blood glucose. There are a great number of reports indicating that flavonoids including quercetin may attenuate health conditions and side effects derived from diabetes (84–86). In fact, potential effects of flavonoids on diabetes were highly speculated originally on the basis of an old phlorizin study (87). In the study, the reabsorption of glucose in kidney was greatly attenuated upon the intravenous administration of a relative high dose of phlorizin, even suggesting that phlorizin may be a toxic compound. Phlorizin is a glycosylated form of chalcone phloretin belonging to the subclass of flavonoids. Therefore, flavonoids have been believed to contain similar effects on glucose absorption in intestinal and other organs. In fact, quercetin-*O*-glycosides were proposed to interact with glucose transporters (88,89). Quercetin glycosides [quercetin-3-glucoside (isoquercitrin) and quercetin-4'-glucoside (spiraeosid)] were reported to inhibit mucosal uptake of the nonmetabolizable glucose analog methyl- α -D-glucopyranoside (MDG) (89). In another study, quercetin and several flavonoids were stated to prevent glucose uptake by blocking sodium-independent glucose transporters (Glut-1 and -3) (90). Glucose and dehydroascorbic acid (oxidized vitamin C) uptakes (see chap. 92, "Vitamin C") are interconnected in some ways, such that some sodium-independent glucose transporters are involved in both glucose and dehydroascorbic acid uptake. In HL-60, U937, and Jurkat cells, quercetin was reported to inhibit the intracellular accumulation of ascorbic acid by blocking uptake of both

dehydroascorbic acid and ascorbic acid (vitamin C). These data may indicate new understanding of the biological effect of flavonoids on glucose and vitamin C uptake in human cells, and future application of quercetin on diseases involving abnormal glucose utilization (91,92). However, potential effects of quercetin on intestinal glucose absorption have not been investigated completely, thereby necessitating more future studies.

Effects on Cancer

Human cancers are caused by numerous oncogenic and other factors, and oxidative stress is believed a key culprit implicated in the initiation and propagation of cancer. Many epidemiological studies suggest that dietary intake of quercetin may have beneficial effects on various types of human cancers, mostly via inhibiting oxidative stress (93). Lately, numerous studies have been performed for elucidating potential anticancer effects of quercetin in each type of human cancer to bolster findings from epidemiological studies. Deoxynucleotide acid (DNA) damage by oxygen radicals can be detrimental for normal cells and can change them into cancer cells. Routinely, 8-hydroxy-2'-deoxyguanosine and related compounds are used as biomarkers to assess levels of DNA damage. Quercetin was shown to increase resistance of lymphocyte DNA to strand breakage, thereby decreasing the level of urinary 8-hydroxy-2'-deoxyguanosine (41). In several reports, quercetin has been described to have potent anticancer effects against various cancers by inducing programmed cell death (apoptosis). In human myelogenous leukemia cells, quercetin was reported to arrest growth of the cell by an increase in the uptake of vincristine, a chemotherapeutic agent (94). In pancreatic tumor cells, the flavonol was reported to induce cell death via inhibiting epidermal growth factor receptor (EGFR) tyrosine kinase activity and decreasing protein phosphorylation (95). In prostate cancer cells, quercetin was stated to inhibit cell growth via suppressing protein phosphorylation (96). Quercetin was also shown to induce growth inhibition and apoptosis in MCF-7 human breast cancer cells. All these data suggest that quercetin may induce growth inhibition and apoptosis in human cancer cells by inhibiting receptor and cell cycle-related kinases and others (97). However, quercetin may have other actions against cancer cells. For instance, gastrointestinal cancer is a cancer associated with dietary and lifestyle factors. In an animal study, quercetin increased both small and large intestinal UGT enzyme activities, thereby helping detoxify compounds with carcinogenic potentials, and preventing gastrointestinal cancer. There are also several reports indicating that quercetin can have beneficial effects on colon and skin cancers (98–100). Taken together, quercetin may have capability to protect cells against mutagenic agents as well as to suppress cancerous growth via modulating numerous biological molecules via anti- and/or pro-oxidant properties. Although quercetin seems to have potential as an anticancer agent, future studies are needed. Human data come mainly from epidemiologic association studies rather than from intervention trials. Most experiments in vitro used quercetin concentrations that were too high to be achieved by dietary ingestion. In addition, beneficial

effects of quercetin and cancer are not consistently found in animal and human studies.

Effects on Bone Formation

Bone formation is a balance between two major processes: bone formation by osteoblasts and bone resorption by osteoclasts. Many hormonal and biologic factors are involved, such as growth hormone, thyroid hormone, parathyroid hormone, calcitonin, estrogens, androgens, cytokines, vitamin D, and transcription factors (e.g., nuclear factor kappa B) (101–103). Several studies suggest that the receptor activator of nuclear factor kappa B (NF- κ B) ligand (RANKL) expressed on the cell surface of osteoblasts is an important factor in response to bone-resorptive processes, because osteoclast precursors possess RANK (a receptor for RANKL), and the cells can differentiate into mature osteoclasts via a mechanism of RANK–RANKL recognition (103,104). During bone resorption, prostaglandin E2 (PGE2) is also produced by osteoblasts, acting as a potent stimulator of bone resorption (104). Inflammatory cytokines such as IL-1 and IL-6 are known to induce PGE2 production by osteoblasts, and the produced PGE2 stimulates adenylate cyclase to accumulate cellular cAMP in osteoblasts, which induces the expression of RANKL for osteoclast differentiation (101). Quercetin and its conjugate were reported to inhibit the receptor activator of RANKL-induced osteoclast differentiation and the RANKL-stimulated expression of osteoclast-related genes. As mentioned above, quercetin and its glycosides are able to inhibit COX-I and COX-II, as well as NF- κ B, which may be involved in modulation bone-resorption processes. Although a number of reports related to potential effects of quercetin on bone homeostasis are available, the reported effects are premature, necessitating future studies before any firm decision.

Other Effects on Human Health

Besides the beneficial effects mentioned above, there are also some interesting biological effects of quercetin on cognitive functions, aging, UV ray protection, and others (112–115). Nonetheless, their effects requires more data, particularly epidemiologic studies, before supporting the claimed actions.

INDICATIONS AND USAGE

Quercetin is widely distributed in plant-derived dietary sources such as onions, apples, black tea, green tea, red wine, beans, and grapes (7). Humans have consumed quercetin (mostly quercetin glycosides) from dietary food sources, and the estimated average daily intake of quercetin by an individual in the United States is estimated to be less than 60 mg. Currently, no dietary recommendations regarding estimated average requirement (EAR), recommended dietary allowance (RDA), adequate intake (AI), and upper limit (UL) have been set for quercetin. Also, epidemiological studies exploring its role in human health are inconclusive (105). Future research is required because of the many biological activities

attributed to quercetin, some of which could be beneficial or detrimental depending on the circumstances.

Potential Uses

Quercetin intake is associated with benefit in some chronic human diseases including cardiovascular diseases, inflammatory diseases, and some cancers. Some epidemiologic studies reveal an inverse association between quercetin intake and cardiovascular diseases, and the flavonol was shown to contain biological properties consistent with its purported effects on the cardiovascular system (106). However, the beneficial effects of quercetin on heart diseases are still inconclusive, and further studies are needed to prove these unambiguously (107). Quercetin was also reported to have anti-inflammatory potential through numerous in vitro studies. Epidemiological studies regarding effects of quercetin on inflammation are solid but still incomplete. Therefore, further research is necessary to demonstrate that inflammation can be benefited by quercetin intake. Quercetin was also stated to have anti-cancer potential through numerous in vitro studies. Epidemiological studies regarding effects of quercetin on cancer are less comprehensive than those on cardiovascular diseases and inflammation. In some experiments, the positive correlation between quercetin intake and risk of cancer was found, but none was found in others. Therefore, further research is necessary to demonstrate that the risk of cancer can be lowered by quercetin intake. Quercetin was also claimed as a potential compound for other human diseases such as diabetes mellitus, bone formation, and cognitive functions. However, these claims have little scientific evidence confirming its efficacy against these diseases. To note, the solubility of quercetin is comparatively lower than other well-known flavonoids (e.g., catechins, anthocyanins), which may pose a significant disadvantage in using the compound as a potential dietary supplement. In summary, the potential uses discussed herein do not suggest that quercetin supplements have therapeutic use for the prevention and treatment of the diseases. Its health effects require further study.

ADVERSE EFFECTS

Although current studies emphasize beneficial effects of quercetin on cardiovascular diseases, inflammation, and cancer, its high doses are believed to have mutagenic and genotoxic activities as demonstrated in in vitro systems (108–110). Even though in vitro experiments indicate that there might be adverse effects, the concentrations in the experiments may have been too high. From animal experiments, there was also uncertainty regarding side effects of quercetin. One study was conducted using F344/N rats that were fed daily quercetin doses of 40, 400, and 1900 mg/kg, for longer than one year. No toxicity occurred at one year. However, after a longer duration, some male rats had renal tubule cell adenomas, but female rats did not. It was not clear whether quercetin induced these adenomas. Because of the uncertainties, it is suggested that quercetin should be consumed from dietary food sources, and caution should be exercised when taking high doses

of quercetin supplement, because its adverse effects are yet to be determined in humans (111).

Compendial/Regulatory Status

Not applicable.

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Red Clover

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INTRODUCTION

Red clover (RC) is a herbaceous perennial plant that inhabits temperate and subtropical areas throughout the world. Native Americans traditionally valued RC for treatment of external skin problems and lung, nervous, and reproductive system ailments. Herbalists have employed it as a blood cleanser, expectorant, alterative, and sedative. With the recognition of its high content of mildly estrogenic isoflavones, the plant has gained popularity as a treatment for menopausal symptoms. Clinical evidence is presently lacking to support the efficacy of semipurified RC isoflavone extracts for alleviation of vasomotor and prostate cancer/benign prostatic hyperplasia (BPH) symptoms. Limited evidence suggests possible efficacy in prevention of osteoporosis and improvement of arterial compliance, a risk factor for atherosclerosis. Current RC isoflavone products do not contain a protein fraction, which precludes analogous comparison with isoflavone studies utilizing dietary soy [*Glycine max* (L.) Merr.] or soy protein isolates (SPIs) for various clinical outcomes. RC isoflavone extract preparations may also differ from soy isoflavone extracts in content of minor chemicals, many of which have not yet been identified, quantified, or tested for biological activity. The need for long-term studies of RC isoflavone supplements is great as placebo effects, especially for menopausal symptoms, and may persist several weeks. Also, thyroid disease and cancer patients may face a potential, yet undefined, risk from long-term exposure to isoflavones and other compounds in RC.

BOTANICAL NAME

Trifolium pratense L. (Fabaceae or Leguminosae).

COMMON NAMES

Bee bread, cleaver grass, clover-grass, clover rose, cow clover, creeping clover, honeysuckle, klevor lugovoi, ladies' posy, meadow clover, meadow trefoil, purple clover, red clover, rozheva konyushina, sweet clover, three-leaved grass, treboil, trefoil, trevor, wild clover, and wild red clover (Fig. 1).

BOTANICAL DESCRIPTION

Trifolium pratense L. is a low-growing perennial herb that originated in the Mediterranean area and is now

widespread around the globe. It has been cultivated since the 4th century AD and is used as livestock fodder and sometimes as green fertilizer to replenish the soil. Consumption as a food is not widespread, except occasionally as young sprouts or cooked greens.

The general habit of RC is described as having "several stems arising from the same root, ascending, somewhat hairy, and varying much in its height. The leaves are ternate; the leaflets oval or obovate, entire, nearly smooth, often notched at the end, and lighter colored in the center. Stipules ovate and mucronate" (1). Flowers occur "in short, dense, ovate, sessile spikes or heads. Corollas unequal, monopetalous; lower tooth of the calyx longer than the four others, which are equal, and all shorter than the rose-red corolla" (1). Flower heads are "globose or ovoid, from 1.5 to 3 cm in length, consisting of numerous purplish red or pinkish brown papilionaceous flowers, about



Figure 1 Drawing of RC. Source: From the USDA-NRCS PLANTS Database; Britton, N.L. Brown, A. Illustrated Flora of the Northern States and Canada; 1913; Vol. 2, 355.

10 mm in length; calyx pubescent, and with subulate teeth shorter than the corolla; odor fragrant; taste somewhat sweetish and bitter" (2).

Trifolium pratense should not be confused with the similarly named yellow or white "sweet clovers," *Melilotus officinalis* (L.) Pall. and *Melilotus alba* Medikus. The flowers of *M. officinalis* "are in small spike-like racemes with a papilionaceous corolla and about 3 mm in length and when fresh, yellow, but on drying, a yellowish brown. The odor is fragrant, resembling coumarin, and the taste slightly bitter and pungent" (2). *Melilotus alba* has white flowers and is similar in appearance to *M. officinalis*.

FOLKLORIC, HISTORIC, AND ETHNOMEDICAL USES

External

Red clover blossoms were incorporated into ointments or decocted to make compresses for "ulcers," believed by more recent authors to be cancerous lesions or growths. These preparations are also used to treat burns, bites, wounds, gout, and fungal infections. The expressed juice has been used for eye diseases.

Internal

Cherokee Indians made a tea of the flowers or above-ground parts to treat fevers, "Bright's disease" (nephritis), and leukorrhea (3). The Iroquois referred to a decoction of RC flowers as a "blood medicine." The Ute of Nevada used a decoction as an abortifacient (4). Tea or tincture was utilized for the spasmodic coughs of whooping cough, measles, bronchitis, laryngitis, and tuberculosis in the 19th and 20th centuries (1). Red clover cigarettes were a treatment for asthma according to the *National Formulary*. Decoctions and infusions are still used as expectorants, alteratives, sedatives, and remedies for rheumatism, ulcers, and skin conditions. Less frequently, its utility for normalization of menses, lactagogue action, or as a fertility tonic has been reported.

In 1900, a product named "Extract of *Trifolium* Compound," produced by the Wm. S. Merrell Chemical Company (Cincinnati, OH), contained potassium iodide plus extracts of the following plants: *T. pratense* L., *Stillingia sylvatica* L., *Lappa minor* Hill, *Phytolacca decandra* L., *Cascara amara*, *Berberis aquifolium* Pursh, *Podophyllum peltatum* L., and *Xanthoxylum carolinianum* (1). This preparation was recommended for treatment of syphilis, scrofula, chronic rheumatism, and glandular and various skin afflictions.

The formula for the Hoxsey internal cancer remedy has likely changed over time and been customized for individual patients. However, it has probably contained, at one time or another, the following plants (plus potassium iodide): *Phytolacca americana* L., *Arctium lappa* L., *B. vulgaris* L., *Rhamnus frangula* L., *S. sylvatica* L., *Zanthoxylum americanum* Mill., *C. sagrada* and/or *C. amara*, *Glycyrrhiza glabra* L., *Medicago sativa* L., and *T. pratense* L.

Flor-Essence™, currently manufactured by Flora, Inc. (Lynden, Washington, D.C.) and Flora Manufacturing & Distributing, Ltd. (Burnaby, British Columbia, Canada), is sometimes used by cancer patients and contains the following plant extracts: *A. lappa* L., *T. pratense* L., *Cnicus benedictus* L., *Ulmus rubra* Muhl., *Rumex acetosella* L.,

Rheum palmatum L., *Laminaria digitata* Lmx., and *Nasturtium officinale* R. Br.

CHEMICAL CONSTITUENTS

Red clover contains several general classes of compounds but is particularly rich in isoflavones, flavones, and flavonols. Both soy and RC contain the isoflavones genistein and daidzein, and soy may contain small amounts of formononetin and biochanin A. However, RC contains substantially more formononetin and biochanin A, relative to genistein and daidzein, when compared to soy.

Under storage conditions of greater than 13% moisture, RC may become contaminated with the fungus *Rhizoctonia legumicola*, which produces the indolizidine alkaloids slaframine and swainsonine. The latter alkaloid causes lysosomal storage disease and may precipitate "locoism" (i.e., weakness, lack of coordination, trembling, and partial paralysis) in livestock. Slaframine is activated by liver metabolism to form a ketoimine that stimulates muscarinic receptors, causing excessive salivation, lacrimation, frequent urination, diarrhea, bradycardia, and bradypnea in livestock.

Compounds Used for Standardization

The isoflavones genistein, daidzein, formononetin, and biochanin A are currently used to standardize chemical content of commercial RC products (Fig. 2). Standardization is based on the rationale that these four compounds exhibit significant in vitro estrogenic activity. However, RC contains various other isoflavones and compounds from distinct structural classes, some with unknown biological activity.

PHARMACOKINETICS AND METABOLISM

The RC isoflavones exist in the plant primarily as inactive glucosides (genistin, daidzin, ononin, and sissotrin) or malonated glucoside forms. Microflora in the human digestive tract hydrolyzes these conjugates to their bioactive, aglycone counterparts, which are readily absorbed from the intestine. Formononetin and biochanin

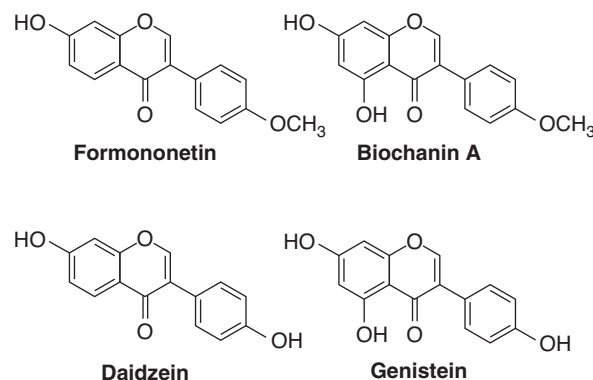


Figure 2 Chemical structures of the four isoflavones used in standardization of RC products.

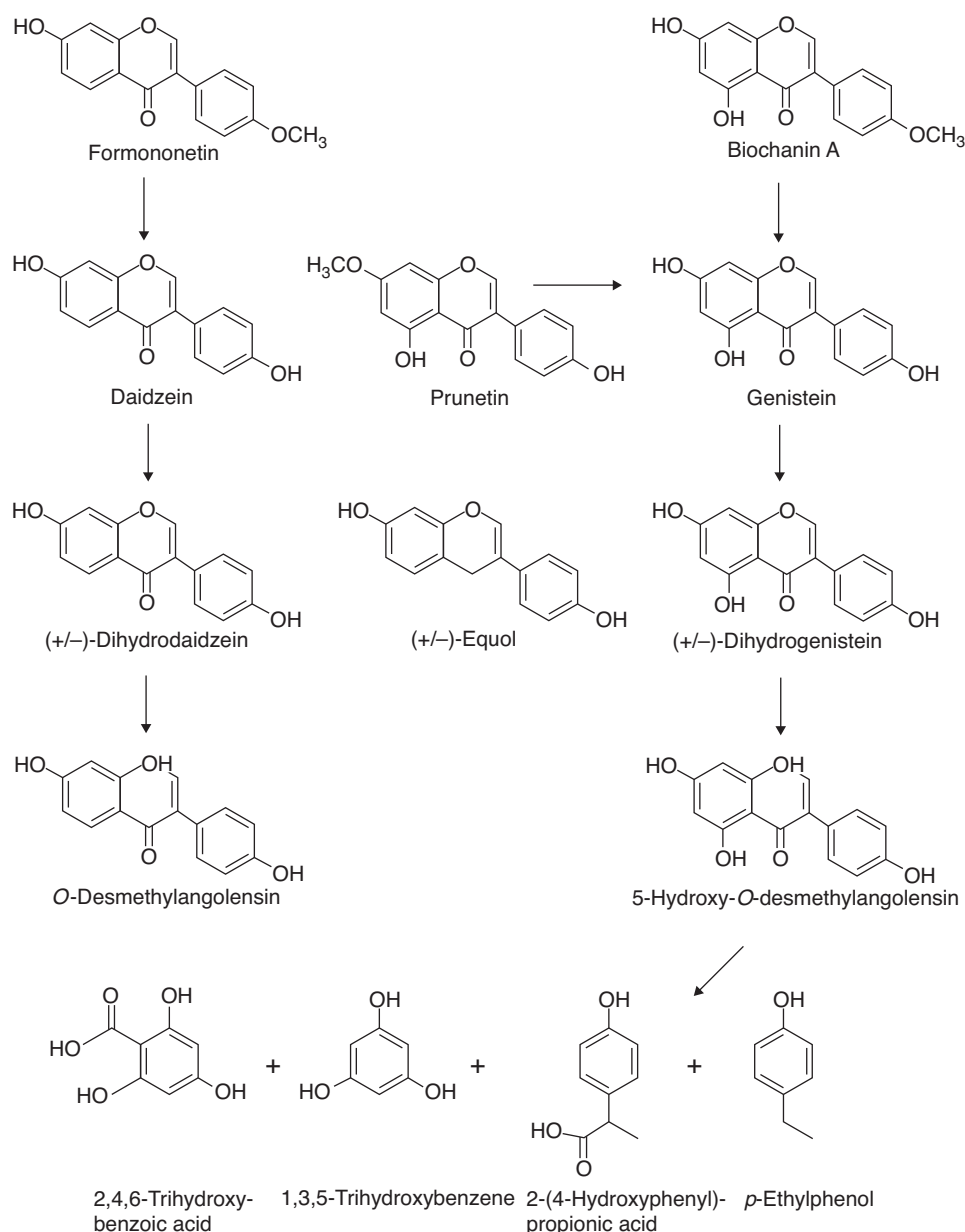


Figure 3 Structures and metabolism of the main isoflavones in RC.

A are demethylated to daidzein and genistein, respectively in the gut and the liver (Fig. 3). Prunetin, a minor component of RC, may also be converted to genistein. Only 30% to 40% of individuals can metabolize (+/-)-dihydrodaidzein, a daidzein metabolite, to the potent estrogen equol. Hepatic phase II enzymes catalyze formation of isoflavone glucuronides and sulfates. Human hepatic microsomal enzymes, like gut bacteria, demethylate the 4'-O-methylated isoflavones in vitro.

Isoflavones circulate in the plasma mainly as conjugates and then are excreted in urine or bile, or undergo enterohepatic circulation. Data on the tissue and fluid distribution of isoflavones in humans are limited, but isoflavones and/or isoflavone conjugates are present in prostatic fluid and secreted into breast milk. Interindi-

vidual variability is substantial, but it is estimated that isoflavone (98–99% conjugated) plasma concentrations will reach 1 μM in a 70-kg individual who has consumed a single 50 mg dose (5). The pharmacokinetic parameters associated with long-term administration of RC isoflavones suggest that once-daily dosing is adequate.

PHARMACOLOGICAL ACTIVITY

Hormonal Effects

Estrogen Receptor

Research into RC mechanism of action has largely focused on interactions with estrogen receptors (ER), ER- α and ER- β . Crude, hydroalcoholic extracts of RC inhibit binding of

(3) H-17 β -estradiol to endogenous and purified recombinant ER in a number of test systems. Components of RC extracts bind preferentially to ER- β over ER- α , earning RC its categorization as a natural selective estrogen receptor modulator (SERM). Pure isoflavones also bind preferentially to ER- β but exhibit competitive binding at both receptor subtypes with the same rank order of potency: genistein > daidzein > biochanin A \sim prunetin > formononetin.

Pike et al. (6) resolved the crystal structure of genistein bound to the ER- β ligand-binding domain. The ER- β :17 β -estradiol structure remains unsolved, but the ER- α :17 β -estradiol complex is often used as a basis of comparison to deduce the mode of binding of genistein with ER- β . Comparison of the ER- β :genistein structure with the previously published ER- α :genistein structure enabled identification of two conservative amino acid changes, which may contribute to isoflavones' ER- β selectivity. The new structure also explains the observation that genistein is only a partial agonist. Like 17 β -estradiol, genistein is buried within the hydrophobic core of the binding cavity, but Helix 12 adopts an antagonist orientation similar to that seen for raloxifene. Figure 4 illustrates the ligand-binding modes of genistein and 17 β -estradiol coupled to the ligand-binding domains of ER- β and ER- α , respectively.

RC exhibits a complex array of (anti)estrogenic activities in various in vitro test systems (7). Crude extracts of RC upregulate the estrogen-inducible genes for progesterone receptor (PR) and the trefoil peptide (TFF1/*pS2*)

and induce alkaline phosphatase (AP) activity in various cell lines. RC downregulates ER levels in T-47D (ER+/PR+) breast cancer cells, an effect that cannot be reversed in the presence of RU486. Preparations of RC and purified isoflavones usually stimulate the proliferation of ER+ breast cancer cells in steroid-depleted media yet inhibit steroid-stimulated growth at midrange to high micromolar concentrations. Modulation of bone cell homeostasis by isoflavones is believed to occur via ER-dependent mechanisms.

In vivo animal studies generally support the picture of RC as a weak estrogenic agent with tissue-dependent effects. A survey of various clover and alfalfa fodders associated content of biochanin A and formononetin with uterotrophic activity in the immature rat model. Exposure of ovariectomized ewes and heifers to formononetin and RC silage, respectively, caused enlargements in teat, vulval, and uterine size and production of milky fluid by the mammae. More recently, a standardized RC extract proved weakly estrogenic by affecting uterotrophic and vaginotrophic outcomes in the ovariectomized rat model, but it did not stimulate breast cell proliferation. In a rat model of endometrial cancer, genistein upregulated estrogen-responsive genes but did not promote tumor growth. Consistent with ER- β selectivity, isoflavone preparations improve bone density and protect against cardiovascular disease in most ovariectomized rat and prepubertal rhesus monkey models, although bone results may vary with concentration, timing, and length of exposure.

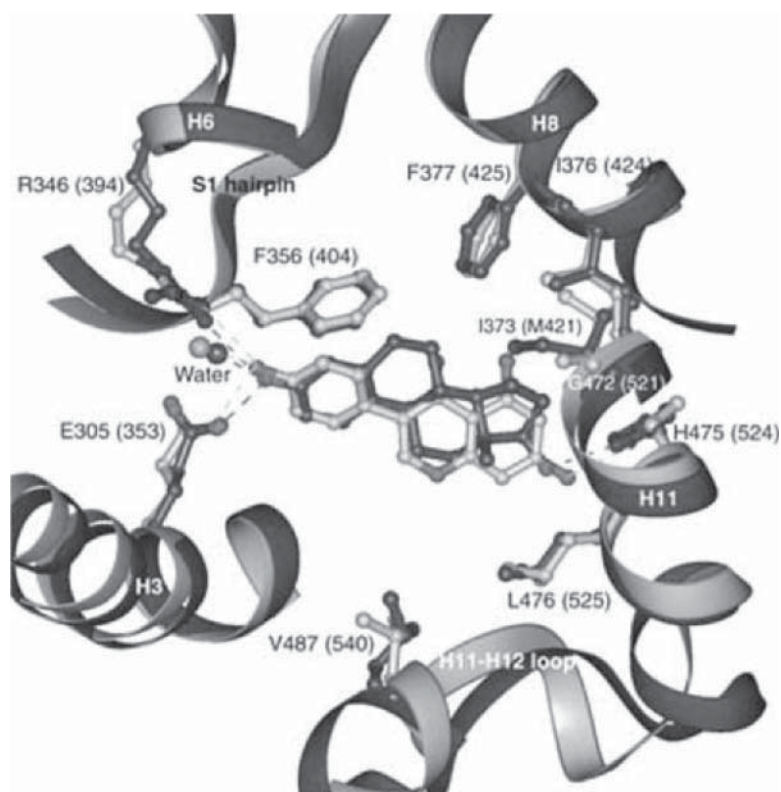


Figure 4 Comparison of hER- β -LBD:genistein and hER- α -LBD:17 β -estradiol ligand-binding modes. Crystal structures of genistein in the human (h) ER- β ligand-binding domain (LBD) (hER- β -LBD:genistein, light grey) and 17 β -estradiol in the ER- α -LBD (hER- α -LBD:17 β -estradiol, dark grey) are superimposed. Hydrogen bonds are drawn as dotted lines. Primary protein residue labels correspond to the hER- β -LBD sequence. The corresponding hER- α -LBD sequence number and residue, if different, is provided in parentheses. [This figure was kindly prepared by Barbara Calamini and Andrew Mesecar (University of Illinois at Chicago, Illinois).]

Isoflavone concentrations in humans routinely exceed endogenous estradiol concentrations. However, RC is not overtly estrogenizing, suggesting that alternate molecular targets must mediate the clinical actions of the isoflavones.

Nonestrogen Receptor

In addition to weak estrogenic activity, RC extracts exert weak antiprogesterational and antiandrogenic activities. A 50% hydroethanolic extract did not stimulate, but instead almost completely blocked, progestin-induced AP activity in T-47 cells (8). More recently, antiandrogenic action of a 5% RC extract appeared responsible for reduction in benign prostate enlargement in aromatase knockout mice. Affinity constants for the four major isoflavones at the progesterone and androgen receptors fall in the millimolar range and adhere to the same rank order of potency for both steroid receptors: biochanin A > genistein = daidzein > formononetin. A recent study of receptor binding and transactivation activities of RC isoflavones and their metabolites found that androgen and progesterone were low, but several isoflavone metabolites provided interesting ER α and ER β binding and transactivating properties: equol, and *O*-desmethylanagolensin (both from daidzein), and the reduced metabolite of formononetin, angolensin (9).

Receptor Independent

A number of receptor-independent mechanisms of action influence hormone status. Isoflavones tend to lower steroid hormone levels in part through inhibition of several enzymes involved in steroid biosynthesis (10): aromatase (biochanin A > genistein), 5- α -reductase type 2 (genistein, daidzein, and biochanin A), and 17- β -hydroxysteroid dehydrogenase (coumestrol, biochanin A, and genistein). Daidzein sulfates inhibit the sulfatase and sulfotransferase enzymes that (i) regulate sulfation of endogenous and environmental estrogens; (ii) help determine the availability of hormones to tumors; and (iii) have been hypothesized to minimize bioactivation of xenobiotic procarcinogens. All four isoflavones—biochanin A being the most potent—expedite the elimination of inactive hormone conjugates through stimulation of UDP-glucuronosyltransferase. Genistein stimulates the production of sex hormone-binding globulin (SHBG) in human hepatocarcinoma cells. Equol producers exhibit enhanced urinary ratios of 2-hydroxyestrone to 16 α -hydroxyestrone, a metabolic measure that is negatively correlated with breast cancer risk.

Cancer-Related Effects

Antioxidant

Crude RC extracts display antioxidant activity in several in vitro experimental systems. Known antioxidant compounds in RC include genistein, daidzein (precursor to equol), biochanin A, genistin, daidzin, formononetin, clovamide, and texasin. In addition to exerting their influence through direct chemical interactions, isoflavones lower oxidative stress via indirect mechanisms such as induction of antioxidant scavenging enzymes. In certain biological

environments, genistein may act as a pro-oxidant with potential mutagenic and/or genotoxic consequences.

Antiproliferative

At pharmacological doses, isoflavones inhibit the proliferation of both hormone-dependent and hormone-independent cancer cells. This phenomenon has been attributed, in part or in whole, to a wide range of ER-independent effects (11): the inhibition of enzymes such as tyrosine kinase (genistein, biochanin A) and DNA topoisomerases I and II (genistein, biochanin A, equol, and orobol); regulation of growth factors and their receptors (genistein); effects on cell cycle regulatory proteins (genistein and biochanin A); regulation of stress response genes (genistein); apoptosis (genistein); and inhibition of nitric oxide synthesis (biochanin A). Biochanin A is largely responsible for the inhibition by RC of benzo(a)pyrene metabolism in hamster embryo cell culture. The isoflavones generally exert a chemoprotective effect if administered to rodents before puberty, although genistein will stimulate or suppress tumor growth depending on the cancer model employed.

Other

Tumor progression and metastasis may be checked by the following actions of genistein: inhibition of angiogenesis, cell adhesion effects; tyrosine phosphorylation of membrane proteins that mediate cellular invasion; and collagenases/metalloproteases (12,13).

Inflammation and Immune Function

Several lines of evidence suggest that the isoflavonoids in RC modulate inflammatory (12) and immune responses (14). In the ovariectomized mouse model, genistein in the diet or by injection induces thymic atrophy and suppresses cell-mediated immunity. Injected genistein also decreases humoral immunity. In the hairless mouse model, genistein, equol, isoequol, and dihydroequol protect against UV-induced inflammation and immunosuppression. An aqueous extract of RC strongly inhibited (82% at 0.25 mg/mL) platelet activating factor-induced exocytosis in human neutrophils. The isoflavones modulate anti-inflammatory responses in animal models of chronic ileitis, inflammation-induced corneal neovascularization, and ischemic reperfusion injury.

Thyroid Function

Speculation that the isoflavones, and RC by extension, trigger thyroid disease has been based on both in vitro and in vivo findings (15). In vitro, genistein and daidzein competitively inhibit thyroid peroxidase (TPO) to form iodinated isoflavones and irreversibly inhibit the enzyme in the absence of iodide, albeit at IC₅₀s above typical circulating levels of free isoflavones. Rat studies have confirmed the relevance of these in vitro findings. Dietary exposure of Sprague-Dawley rats to genistein that achieved serum concentrations comparable to those seen in humans caused intrathyroidal accumulation of genistein and inactivation of TPO. Paradoxically, the rodents did not present as hypothyroid. Thyroxine, tri-iodothyronine (T3), and thyroid stimulating hormone levels remained

unchanged, and thyroid sections revealed no pathologies. In the ovariectomized ewe model, however, exposure to RC silage resulted in elevated free and total T3 levels, increased thyroid follicle size, and thyroid gland immunoreactivity to ER- α .

Glucose and Lipid Metabolism

Isoflavone supplements and RC extracts have been postulated to have beneficial effects on obesity and diabetes mellitus, and in vitro and in vivo experiments provide some support for this contention. Genistein increases glucose-induced insulin release from either pancreatic β -cells or insulinoma cell lines. Also contributing to the antidiabetic effect, genistein and, to a lesser extent, daidzein inhibit insulin-stimulated glucose uptake from the intestine and other cells, and isoflavones protect against glucose-induced oxidation of low-density lipoproteins (LDL). Anabolic effects of RC isoflavonoids have been observed in mice, rats, and cattle.

At least three rabbit studies have demonstrated beneficial effects of isoflavones or RC (16) on lipid metabolism and/or progression of atherosclerosis, although reduced LDL peroxidation rather than changes in serum lipids was hypothesized to account for improvements in one study. Genistein exerts lipolytic/antilipogenic effects in ovariectomized mice.

Other Biological Activities of RC Compounds

Genistein weakly antagonizes the A1, A2a, and A3 adenosine receptors at low micromolar concentrations. Genistein and daidzein inhibit GABA_A receptor-mediated chloride currents. The two isoflavones, and certain structural analogues, also block calcium channels in human platelets, inhibiting thrombin-induced $[Ca^{2+}]_i$ elevation. The isoflavone actions at ligand-gated ion channels occur independent of tyrosine kinase inhibition at low- to midrange micromolar concentrations. The RC isoflavones serve as effective antimicrobials (genistein, daidzein, biochanin A, and formononetin) and fungicides (genistein, biochanin A, and formononetin). See Table 1 for a noncomprehensive list of selected RC compounds with interesting biological activity.

USAGE

Human Clinical Studies

Caveats

Inferences about RC based on soy isoflavone studies may not be valid. RC and soy not only differ in their balance of individual isoflavones, but soy foods and SPIs also contain a unique protein fraction that is not present in RC semipurified isoflavone extracts. To date, no reports have been published regarding the clinical activity of RC protein. Research of dietary soy isoflavone effects on hormone status is complicated by the relative amounts of fiber and nonstarch carbohydrates in the diet, as these influence the gut flora populations responsible for metabolism of isoflavones in the colon. In addition, it is currently assumed that the isoflavones are the only "active" constituents present in RC extracts. This may or may not eventually be shown to be true, as 20 or more minor compounds may be present in the semipu-

Table 1 A Noncomprehensive Listing of Compounds Found in RC

Compound (compound class)	Selected biological activities of potential interest
Calycosin (isoflavonoid)	Antiandrogenic, cell differentiation induction, hemoglobin induction
Caryophyllene (sesquiterpene)	Antispasmodic, ambulatory behavior stimulation, choleric
Citrulline (amino acid)	DNA damage prevention, prolactin inhibition
Coumestrol (coumarin)	Prolactin stimulation, estrogenic and antiestrogenic effects in vivo, bone resorption inhibition, apoptosis induction
Daphnoretin (coumarin)	Protein kinase C stimulation, platelet aggregation inhibition
Fraxidin (coumarin)	Negative chronotropism
Glycitein (isoflavone)	Antiestrogenic in vivo, nitric oxide synthesis inhibition, osteocalcin stimulation, prostaglandin E ₂ inhibition
Glycitin (isoflavone glucoside)	Bone resorption inhibition
Hyperoside (flavonol)	Anti-ischemic, Ca ²⁺ uptake inhibition, antihemorrhagic, positive chronotropism, coronary vasodilator, negative inotropism
Medicarpin (pterocarpan)	Apoptosis induction, cell differentiation, hemoglobin induction
Orobol (flavonol)	15-lipo-oxygenase inhibition, topoisomerase II induction
Pectolinarigenin (flavone)	Antiatherosclerotic, antihyperlipemic, Ca ²⁺ -ATPase inhibition
Pratensein (isoflavonoid)	Antihypercholesterolemic
Prunetin (isoflavone)	Antihypercholesterolemic, aromatase inhibition, estrogenic in vivo
Scoparol (flavonol)	Antispasmodic, cAMP inhibition, lipo-oxygenase inhibition, platelet aggregation inhibition, tumor necrosis factor α release inhibition
Scopoletin (coumarin)	Bronchodilator, CNS depressant, platelet aggregation inhibition, uterine relaxant, negative chronotropism/inotropism
Texasin (isoflavonoid)	Lipid peroxidation inhibition
Trigonelline (alkaloid)	Analgesic, antimutagenic, cell cycle disruption, neuron sprouting
Xanthotoxol (coumarin)	Antiarrhythmic, antispasmodic, cell differentiation, negative chronotropism, neural transmission inhibition

Selected biological activities of potential interest are presented (17).

Source: From Ref. 69.

rified extracts used in clinical trials. Studies reviewed here are limited to clinical trials of RC isoflavone supplements and/or pure isoflavones, and the above-mentioned caveats should be borne in mind when interpreting the results presented. Refer to Table 2 for details regarding specific RC isoflavone products.

Menopause

Hot flashes and menopausal symptoms Initial studies administering semipurified RC preparations to women to relieve menopausal hot flashes have generally been of short duration (≤ 12 weeks) and effects, when present, take eight weeks to manifest. Trials broadly demonstrate a significant placebo effect during the first four weeks that may persist throughout the investigation (18,19). More recent studies have incorporated a two- or four-week run-in period to assess this placebo effect (18,20,21). Evidence

Table 2 Table of RC Products^a Evaluated in Clinical Trials

Product name	Delivery form and dosage	Effective ingredients	Isoflavone ratio (genistein + biochanin A:daidzein + formononetin)	Indications
Promensil ^b	Tablet; 40 mg total isoflavones (genistein + daidzein + formononetin + biochanin A)	Dried aqueous alcoholic extract of <i>T. pratense</i>	1.9	Relief of menopausal symptoms such as hot flashes and night sweats; maintenance of bone and cholesterol health; general well being
Trinovin ^b	Tablet; 40 mg total isoflavones (genistein + daidzein + formononetin + biochanin A)	Dried aqueous alcoholic extract of <i>T. pratense</i>	1.9	Maintain normal prostate and urinary function. May assist in the relief of medically diagnosed benign prostatic hypertrophy
Rimostil ^b	Tablet; 57 mg total isoflavones (genistein + daidzein + formononetin + biochanin A)	Dried aqueous alcoholic extract of <i>T. pratense</i>	0.15	Maintain bone and cholesterol health in postmenopausal women
P-07 ^c (not commercially available)	Tablet; 40 mg total isoflavones (genistein + daidzein + formononetin + biochanin A)	Dried aqueous alcoholic extract of <i>T. pratense</i> ; principal isoflavone is biochanin A	3.8	N/A
P-07(b) ^c (not commercially available)	Tablet; 40 mg total isoflavones (genistein + daidzein + formononetin + biochanin A)	Dried aqueous alcoholic extract of <i>T. pratense</i> ; principal isoflavone is biochanin A	3.5	N/A
P-083 ^c (not commercially available)	Tablet; 40 mg total isoflavones (genistein + daidzein + formononetin + biochanin A)	Dried aqueous alcoholic extract of <i>T. pratense</i> ; principal isoflavone is formononetin	0.2	N/A
Menoflavin ^b (MF11RCE)	Capsule; 40 mg total isoflavones (genistein + daidzein + formononetin + biochanin A)	Dried aqueous alcoholic extract of <i>T. pratense</i> ; principal isoflavones are biochanin A and formononetin	1.12	Relief of menopausal symptoms including hot flashes and vaginal dryness
N/A Developed by UIC/NIH Botanical Center ^{c,d}	Capsule; 116.6 mg total isoflavones (genistein + daidzein + formononetin + biochanin A)	Dried aqueous ethanol extract of <i>T. pratense</i> ; principal isoflavones are biochanin A and formononetin	1.03	Formulation developed for clinical trial—relief to menopausal symptoms

^aRC manufacturer: Novogen Ltd.^bContent has been independently verified. (See Refs. 70–72.)^cContent is per producer's claim and has not been independently verified.^d From Ref. 73.

for RC efficacy in reduction of hot flashes is not compelling, and long-term trial results have varied from trials of shorter duration.

One trial with a duration of 12 weeks (after a 4-week run-in period), using 80 mg of isoflavone (Promensil[®] 82 mg isoflavones/day, high genistein + biochanin A, Novogen Ltd., Australia) recorded a statistically significant decrease in hot flashes of 44% at week 12 among the 15 participants in the treatment group (20). Another 12-week trial, with a 2-week run-in period with a total of 246 participants in three groups testing two commercially available RC products (Promensil and Rimostil[®], 57 mg isoflavones/day, high daidzein + formononetin; Novogen Ltd., Australia) versus placebo, demonstrated no clinically important effect on hot flashes or other symptoms of menopause by either RC formulation (21). Both treatment groups showed higher response in women with body mass index (BMI) greater than 25.1.

Promensil (1 mg genistein, 0.5 mg daidzein, 16 mg formononetin, 26 mg biochanin A) was evaluated in a trial, with a duration of one year, taken daily by 117 women aged between 49 and 65 years. Results demon-

strated no statistically significant changes in mean number of hot flashes or menopausal symptoms with the treatment group compared with placebo (22). Only one study has directly compared RC to a pharmaceutical proven to alleviate hot flashes. In that study RC did not show a statistically significant difference in the number of vasomotor symptoms, hot flashes, or intensity of hot flashes compared to placebo in this trial of one year that compared red clover to the active control—conjugated equine estrogens with medroxyprogesterone (CEE/MPA) (23). A subset of participants from this study participated in a separate study of objective measures of hot flashes using an ambulatory hot flash monitor. The subjects in the CEE/MPA group saw hot flashes decrease significantly; however red clover participants exhibited a 33% decline in hot flashes compared to placebo, which experienced a 0% decline in hot flashes (24).

Vaginal/sexual Health

In a 12-week randomized, double-blind placebo-controlled trial of 36 postmenopausal women evaluating 40 mg or 160 mg of RC (Promensil, 40 mg per

tablet, genistein 4.0 mg, daidzein 3.5 mg, biochanin 24.5 mg, and formononetin 8.0 mg) relative to placebo, for menopausal symptoms, there were no changes in vaginal wall smear or vaginal pH relative to baseline for any group (19). There have been two reports from a six-month randomized, double-blind, placebo-controlled, crossover trial of 53 postmenopausal women, which evaluated RC [Menoflavon[®] (MF11RCE), 40 mg total isoflavones, Melbrosin International/Meldex International, U.K.] on vaginal health and sexuality. Results showed improvements in karyopyknotic, cornification, and basal cell maturation indices compared to placebo, which correlated with a decrease in dyspareunia, vaginal dryness, and decreased libido (25,26).

Memory and Mood

A six-month randomized, placebo-controlled trial in postmenopausal women found no significant short-term effects of daily RC tablets (2-tablet dose; 25 mg formononetin, 2.5 mg biochanin A, < 1 mg daidzein + genistein per tablet, Rimostil) on memory and compared to placebo showed a trend toward deterioration of digit recall. However, participants did demonstrate a trend toward improvement in the block design test (27). In contrast, postmenopausal women who participated in a one-year study of botanicals, which included a RC arm (2-capsule dose; 57.5 mg biochanin A, 56.6 mg formononetin, 1.6 mg genistein and 0.9 mg daidzein per capsule) for cognitive function compared to CEE/MPA and placebo, did not experience improvement in visuospatial abilities or decreased digit recall (24). The RC group in this study did not show a change in verbal memory, which differed from placebo. All treatment groups showed a significant decline from baseline with the CEE/MPA group showing the greatest decline.

Another six-month randomized, placebo-controlled, crossover trial of 113 postmenopausal women were enrolled to examine the effect of RC (2-capsule dose; standardized to 40 mg isoflavones per capsule in a proprietary blend of biochanin A, formononetin, genistein, and daidzein, MF11RCE) on anxiety and depressive symptoms. Instruments used were the HADS (Hospital Anxiety Depressive Scale) and SDS (Zung's Self-rating Depressive Scale). Subjects were measured at baseline, 90, and 187 days and were shown to exhibit significant decreases of 76.9% for the HADS score and 80.6% for the SDS score. In contrast, total HADS and SDS scores also decreased significantly compared to baseline after placebo, but at an average of 21.7% (28).

Breast Cancer

No studies have directly evaluated the effects of RC isoflavone supplements in breast cancer patients. In a study of high-risk women, 177 subjects (49–65 years) with Wolfe P2/DY mammographic breast patterns received Promensil daily for one year and exhibited no statistically significant changes in estradiol, follicle stimulating hormone (FSH), or luteinizing hormone (LH) levels (22). Differences between densities of breast patterns were not significant between treatment and placebo groups. In a three-year study to assess the safety and tolerability of RC (40 mg tablet Promensil) taken once daily by premenopausal, perimenopausal, and postmenopausal

women with a first-degree relative with breast cancer, it was found that RC had no estrogenic effect on breast density. In this study, 18% of the 320 women who enrolled eventually withdrew; eight because they developed cancer. There was no significant difference between the treatment group and placebo for those developing cancer. On the basis of a risk calculation for each first-degree relative, 4.6 breast cancers could be expected during the study. Although there were 8 observed (5 from the placebo group), the increase (8 compared to 4.6) was not significant ($P = 0.8$) (29).

Excretion of daidzein, genistein, and equol (from dietary sources, including soy) is reduced in women with breast cancer compared to case controls. Equol production is associated with lower concentrations of testosterone, androstenedione, dihydroxyepiandrosterone (DHEA), DHEA sulfate, and higher levels of SHBG, regardless of isoflavone consumption. Female equol producers tend to have lower midluteal phase plasma estrone, estrone sulfate, and progesterone and higher FSH levels versus nonproducers.

Cyclical Mastalgia

A study for relief of cyclical mastalgia first admitted subjects to a two menstrual cycle placebo run-in period. Those with less than 30% average decrease in pain compared to baseline levels were randomized and administered 40 or 80 mg RC isoflavones (Promensil) over three menstrual cycles (30). A three-day increase in menstrual cycle length was noticed in the 80-mg group compared with the placebo group. Breast pain was significantly reduced in the 40-mg group compared with the placebo group.

Endometrial Effects

A three-month study of 50 mg RC isoflavones/day (product P-07, Novogen Ltd.) in perimenopausal women found no change in the Ki-67 proliferative index of endometrial biopsies taken during the late follicular phase nor was there change in plasma estradiol, FSH, progesterone, or endometrial thickness (31). One study (32) discovered a significant inverse association between endometrial cancer risk and dietary consumption of daidzein and total isoflavones, especially at 1.2 to 1.7 mg isoflavones/day. Doses up to 85.5 mg/day of Rimostil in postmenopausal women for six months did not cause increased endometrial thickness or breakthrough bleeding. A 90-day, randomized, double-blind, placebo-controlled crossover study of 109 postmenopausal women was undertaken to examine the effects of 80 mg RC/day (2 daily capsules, MF11RCE) on the endometrium (33). Transvaginal ultrasound examinations revealed that endometrial thickness was significantly decreased with RC compared to placebo. In addition, there were no significant effects seen on hormone measurements of E₂, FSH, and SHBG; however, testosterone levels were significantly increased with RC and there was a nonsignificant decrease of FSH seen with placebo. Also, a significant reduction in LH was seen for placebo, but not RC. Interestingly, in an assay of transactivational potency of MF11RCE, it was shown to be equivalent 79 µg E₂ per gram for ERβ, and 17 µg for ERα (34).

In a three-year study of women with a family history of breast cancer who took one 40-mg tablet of Promensil daily, transvaginal ultrasound examinations of

postmenopausal women were conducted annually and revealed no significant differences between the placebo and RC groups (29).

Prostate Cancer

It is currently unclear what role, if any, serum and tissue levels of isoflavones play in prostate cancer and BPH. One study found that prostate cancer patients had higher serum levels of isoflavones compared to cancer-free controls. However, there were more equol producers in the control group versus the cancer group. Another experiment collected plasma and prostatic tissue specimens from BPH patients and bladder cancer patients with normal prostates. Prostatic genistein was lower in the BPH group, whereas equol and daidzein concentrations were similar across both groups. Plasma isoflavone concentrations were similar for both cohorts.

Three clinical studies have examined the effect of RC extracts on male prostate health. The first study, unpublished but described in another report, administered 40 or 80 mg RC isoflavones/day for three months to BPH patients (exact product and methodology not provided). The International Prostate Symptom Score decreased 23.3%, urinary flow rate increased 9.8%, and quality of life improved 17% for both treatment groups. A study (35) of Trinovin™ (Novogen Ltd.) administered four 40 mg tablets daily to men with prostate cancer for 7 to 54 days before radical prostatectomy. Apoptosis of prostate cancer cells was more common in tissues from the treatment group and was especially evident in regions of low-to-moderate grade cancer. No differences were seen pre- and posttreatment for serum prostate-specific antigen (PSA), Gleason score (grade of cancer severity), or serum testosterone. A study in healthy men using Trinovin as above showed no effects on plasma testosterone, androstenedione, dehydroepiandrosterone sulfate, androsterone, epiandrosterone sulfate, cortisol, or SHBG, but dihydrotestosterone levels increased, which is possibly a detrimental change.

Colorectal Cancer

Two studies examining the effects of RC on colorectal cancer risk have been conducted; epidemiological studies have indicated that increased insulin-like growth factor IGF-I concentrations have been linked to colorectal cancer risk. In vitro and in vivo animal studies have shown that soy isoflavones may decrease IGF-I concentration. A six-month placebo-controlled, double-blind, crossover trial of 37 men with a personal history of colorectal adenomas or at least one first-degree family member with a history of colorectal cancer who were randomized to two daily tablets of RC (Promensil; 25 mg biochanin, 8 mg formononetin, 4 mg genistein, and 5 mg daidzein) exhibited no effect on serum IGF-I nor were free IGF-I, IGF-II, IGFBP-1, IGFBP-2, or IGFBP-3 concentrations significantly altered (36). A similar six-month study of 34 postmenopausal women was conducted by the same investigators and produced generally similar results: RC did not significantly affect serum total IGF-I or IGF-II, and the mean or median relative differences in IGFBP-1, IGFBP-2, and IGFBP-3 between isoflavone and placebo did not deviate from zero (37).

Osteoporosis Prevention and Treatment

Results from clinical studies of RC in prevention and treatment of osteoporosis are promising but complicated by varying length of bone remodeling cycles in individuals and the biphasic effects of isoflavones. Background hormonal milieu in the body also plays a role, as does basal metabolic index, which is inversely related to rate of bone loss in postmenopausal women.

Seven studies have examined the effects of RC on bone. Refer to Table 3 for a summary of these studies. Three trials in postmenopausal women observed favorable effects in terms of preservation of bone mineral density (BMD). One year of treatment with Promensil (43.5 mg total isoflavones) significantly decreased the loss of lumbar spine BMD in pre- and perimenopausal women (38). There was no effect in postmenopausal women nor was there an effect on hip BMD for any group. A six-month study documented increased BMDs of the proximal forearm (2.9%, 4.1%, 3.0% increases, respectively) but not the distal forearm, for 25, 50, or 75 mg RC isoflavones/day (Rimostil), after a one-month placebo run-in period (39). Another six-month study found increases in BMD of the proximal radius and ulna in postmenopausal women taking Rimostil at 57 or 85 mg RC isoflavones/day (40). The fourth study reported no measured changes in N-telopeptide and osteocalcin bone markers in perimenopausal women taking 50 mg RC isoflavones/day (product P-07, Novogen Ltd.) for three months (31). The fifth study also reported no effect of either Promensil or Rimostil on serum osteocalcin and urinary N-telopeptide levels after daily use for 12 weeks by menopausal women (41).

The sixth study also reported no statistically significant difference in N-telopeptide; bone alkaline phosphatase (BAP) rose in the placebo group at 6 and 12 months but not in the RC (Promensil, 40 mg daily) group. Osteocalcin was marginally higher in the RC group, but did not change significantly from baseline. Serum beta CTx was higher in the RC group compared to baseline. There were no significant differences in percent change in BMD versus placebo (29). The seventh trial compared the effects of four commercially available isoflavone products (two soy products, red clover, and kudzu) with estradiol + medroxyprogesterone on reduction in bone resorption (42). The soy product with the highest content of genistein produced the highest reduction and had about 5× the genistein as the RC (Rimostil, Novogen Ltd.) product. However, all of the isoflavones were variously efficacious at preventing bone loss.

Early postmenopausal women taking 54 mg genistein/day showed increased bone AP, bone Gla protein levels, and increased BMD in the femur and lumbar spine.

Cardiovascular Disease Risk

Vascular Effects

(a) *Arterial compliance*: Arterial stiffness is related to the presence of atherosclerotic plaques, and this parameter has been evaluated in two studies of RC. The first (43) administered 40 mg Promensil (4 mg genistein, 3.5 mg daidzein, 8.0 mg formononetin, and 24.5 mg biochanin A; reported content differs from manufacturer specifications) daily for five weeks. The dose was then doubled to 80 mg/day for five more weeks. Treatment groups (both doses) showed increases in arterial compliance, the

Table 3 Summary of studies evaluating effects of RC extracts on bone in women

Reference	Product and dosage	Study length (mo)	No. of subjects	Significance	Study design
Novogen Ltd. Patent: (39) WO 00/64438; PCT/AU00/00384	Not stated (15:1 to 2:1 ratio of formononetin to the sum of daidzein + genistein + biochanin A); 25, 50, or 75 mg total isoflavones given	6	50	50 mg group had 4.1% increase in proximal forearm BMD; 25 and 75 mg groups had 2.9%, and 3.0% increase. No significant effect seen on distal forearm BMD	Postmenopausal women; 1-mo placebo run-in followed by isoflavone tablet(s) daily for 6 mo
Clifton-Bligh et al. (40)	Rimostil, 28.5, 57, 85.5 mg isoflavones (daidzein + genistein + formononetin + biochanin A)	6	46	57, 85.5 mg groups showed significant (4.1%, 3.0%, respectively) increase in proximal radius and ulna; no significant response in 28.5 mg group	Perimenopausal women; 1-mo run-in period followed by 1-mo placebo period, then double-blind treatment for 6 mo
Hale et al. (31)	50 mg of Novogen Ltd.'s P-07 RC isoflavone formulation containing high amount of biochanin A	3	30	No changes in <i>N</i> -telopeptide or osteocalcin bone markers	Pre- and perimenopausal women; double-blind, randomized, placebo- controlled
Atkinson et al. (38)	43.5 mg total isoflavones (26 mg biochanin A, 16 mg formononetin, 1 mg genistein, 0.5 mg daidzein)	12	205; 177 completed trial	Reduced loss of lumbar spine bone mineral content and BMD in treatment group; significant increase in bone-specific AP and <i>N</i> -propeptide of collagen type I; no significant effect on hip BMD/ mineral content or boneresorption markers	Pre-, peri- and postmenopausal women; double-blind, randomized, placebo-controlled
Schult et al. (41)	Promensil, 41 mg isoflavones (24.5 mg biochanin A, 8 mg formononetin, 4 mg genistein, 5 mg daidzein) and Rimostil, 28.6 mg isoflavones (2 mg biochanin A, 25 mg formononetin, trace amount genistein + daidzein)	3	252; 245 completed trial	No changes in urinary <i>N</i> -telopeptide or serum osteocalcin bone markers	Peri- and postmenopausal women; double-blind, randomized, placebo- controlled
Powles et al. (29)	Promensil 40 mg tablet containing defined amounts of isoflavones genistein, daidzein, formononetin and biochanin from red clover, tablet taken daily.	36 – 12 for bone markers	401 – 77 Postmen- opausal	No significant difference in percentage change in BMD between RC and placebo; NTx/Cr ratio showed no significant difference from baseline at 6 or 12 mos; BAP ↑ in placebo at 6 & 12 months, but not in RC group. RC with lower level of serum beta CTx compared to placebo at 6 mos	Randomized, double-blind, placebo-controlled trial
Weaver et al. (42)	Four products were evaluated for their antiresorptive effects on bone: Soy Cotyledon, soy germ, kudzu, red clover (Rimostil Biochain a 6.40 mg, formononetin 31.10 mg, genistein 0.562 mg, and daidzein 1.75 mg and) estradiol + MPA	50 days/ intervention with 50 day washout period	11	Outcome measure was 41Ca (a marker of bone resorption in urine). Serum alkaline phosphatase was lower during the RC intervention than at baseline. Serum genistein levels were highest for soy cotyledon, most effective isoflavone of the four products for suppressing bone resorption.	Randomized-order, crossover, blinded trial

magnitude of which was comparable to results seen in studies of hormone replacement therapy. A six-week randomized, double-blind crossover, placebo-controlled study (44) administered two tablets of each of two different products to men and women daily: one significantly enriched in biochanin A [P-07(b)] or another in formononetin (P-083). Isoflavone treatment resulted in significant improvements in systemic arterial compliance (SAC) and pulsed wave velocity (PWV) compared to placebo. However, the formononetin-enriched product had a stronger adjusted trend toward favorable effect on SAC compared to the biochanin A-enriched product.

(b) *Vascular endothelial function*: After six weeks in the previously mentioned study (44), plasma levels of vascular cellular adhesion molecule-1 (VCAM-1) were reduced in the group receiving 80 mg/day of the formononetin-enriched RC extract (P-083). Administration of up to 85.5 mg/day isoflavones (Rimostil) to postmenopausal women for six months did not result in altered levels of serum factor V, VII, VIII, antithrombin III, or fibrinogen (45). An unpublished study observed no adverse changes in intravascular coagulation (factor VIIc), platelet activation (P-selectin), or endothelial activation (von Willebrand factor) compared to placebo after five weeks of treatment with 40 mg/day of RC isoflavones (product unspecified), although no details were provided about the patients.

A small, four-month double-blind randomized, placebo-controlled, crossover study in 16 type 2 diabetic postmenopausal women showed efficacy for reducing mean ambulatory daytime systolic and diastolic blood pressure taken every 30 minutes. Study participants took two RC tablets per day, each containing 25 mg formononetin, 2.5 mg biochanin and less than 1 mg of genistein and daidzein (Rimostil, Novogen Ltd.) for four weeks. Some subjects controlled their diabetes with diet alone, and others were taking oral diabetic agents. Other physiological measures (BMI, clinic blood pressures, heart rate, glycated hemoglobin, fasting blood glucose, total-C, HDL-C, and LDL-C) did not differ significantly between RC and placebo therapies. Forearm vascular response was tested and shown to be significantly greater with L-NMMA (46). Another trial in peri- and postmenopausal women taking 43.5 mg RC isoflavones daily did not demonstrate an effect on systolic or diastolic blood pressure (47).

Despite the mechanisms by which RC extracts act on the vasculature having not been definitively characterized, studies on pure isoflavone compounds yield interesting clues. Orally administered genistein caused increased vasodilation in postmenopausal women, presumably via increasing basal nitric oxide (NO) levels and reducing levels of the vasoconstrictor endothelin-1 (ET-1). Two trials support this hypothesis. The first administered 54 mg genistein/day for 6 months, and plasma levels of breakdown products of NO nearly doubled compared to either the placebo group or baseline levels. Endothelin-1 levels dropped by approximately 50%. Forearm blood flow and brachial artery diameter were significantly increased during reactive hyperemia after genistein treatment. The second administered the same genistein regimen or 17 β -estradiol/norethisterone acetate (1 mg/0.5 mg) for 1 year. Genistein again improved brachial artery flow-mediated dilation, and improvements in NO breakdown products and ET-1 in the genistein group were of similar magnitude

as the results for the hormone group. It remains to be seen whether these vascular effects will also be observed for RC extracts. A recent study concludes that RC acts as an anti-atherogenic and anti-inflammatory agent by reducing the expression of leukocyte adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (48).

Lipid Effects

Several studies have investigated the effects of RC preparations on serum lipoprotein levels. In premenopausal women, consumption of 86 mg RC isoflavones per day (product P-07, Novogen Ltd., 51.4 mg biochanin A, 18.6 mg formononetin, 8.6 mg genistein, 7.4 mg daidzein; reported content differs from the manufacturer's claim) for two menstrual cycles had no effect on total cholesterol or triacylglycerol levels. A second study using the same product (P-07, Novogen Ltd.) and dosing regimen over three menstrual cycles found no effects on total cholesterol, LDL, high-density lipoprotein (HDL), triacylglycerol, lipoprotein(a), glucose, or insulin levels. A one-month placebo-controlled crossover study in pre- and postmenopausal women taking two Promensil tablets (43 mg isoflavones/tablet, 25 mg biochanin A, 8 mg formononetin, 4 mg genistein, 5 mg daidzein) had a significant effect on HDL in postmenopausal women, but no effect on insulin-like growth factor (IGF) in either group (49).

Postmenopausal women receiving 40 mg and then 80 mg Promensil for five wk/dose had no change in HDL, LDL, triglyceride, or total cholesterol levels. A randomized double-blind ascending dose study administered one or two tablets (26 mg biochanin A, 16 mg formononetin, 0.5 mg daidzein, and 1 mg genistein/tablet; Promensil; content differs slightly from the manufacturer's specifications) for 4 wk/dose and also found no effect on plasma lipids (50). A three-month study in peri- and postmenopausal women taking 2 tablets Promensil (24.5 mg biochanin A, 8 mg formononetin, 4 mg genistein, 5 mg daidzein/tablet) or Rimostil (2 mg biochanin A, 25 mg formononetin, trace genistein + daidzein/tablet) had no effect on plasma lipids, but did decrease triglycerides in women with high baseline levels (41). Another study administered 28.5, 57, or 85.5 mg of RC isoflavones/day as Rimostil for 6 months. HDL for all treatment groups increased at least 15%. Apolipoprotein B declined in all groups by at least 9%. One-year treatment with 43.5 mg RC isoflavones daily decreased triglycerides and plasminogen activator inhibitor type I (PAI-1) in perimenopausal but not in postmenopausal women (47). Differences between RC extract formulations may account for some of the observed clinical variation.

A randomized, placebo-controlled, parallel crossover, double-blind trial in men and postmenopausal women compared effects of a biochanin A-enriched RC product [P-07(b), Novogen Ltd.] versus a formononetin-enriched one (P-083, Novogen Ltd.) (51). The former, but not the latter, lowered LDL by 9.5% in men compared to baseline levels. Neither product affected plasma lipids in the postmenopausal group.

A four-month randomized, placebo-controlled, parallel study of 25 premenopausal women taking 2 tablets RC (25.7 mg biochanin A, 4.3 mg genistein, 9.3 mg

formononetin, and 3.7 mg daidzein per tablet, Novogen, Australia) was conducted over four menstrual cycles and showed that RC had no significant impact on mean serum lipid and lipoproteins in normocholesterolemic or mildly hypercholesterolemic subjects. Lipid concentrations during the follicular and luteal phases were not affected by RC supplementation. In addition, RC had no effect on Lp(a) levels (52).

Fifty-three postmenopausal women completed a six-month, randomized, double-blind, crossover study of 80 mg RC (Menoflavon 40 mg total isoflavones per capsule) to evaluate effects on serum lipids, finding that mean baseline TC, LDL-C, and TG levels were lowered by 4.4%, 11.5%, and 9.5%, respectively; however, TGs alone decreased significantly. Interestingly, serum LpA levels decreased significantly after both RC and placebo (25). A second analysis of the same trial reports results for the 35 subjects who had increased BMI values ($>25 \text{ kg/m}^2$) (53). For this group, RC significantly reduced baseline TC, LDL-C, and LpA 4.6%, 15.6%, and 63.8%, respectively. Women with higher BMIs had higher TG levels at baseline and showed a nonsignificant decrease with supplementation.

When 54 mg/day of pure genistein was administered to postmenopausal women for six months, no effects on serum lipids were observed.

Homocysteine and Folate Effects

Homocysteine levels in 23 premenopausal women taking RC were evaluated in a double-blind, randomized, parallel pilot study during four menstrual cycles. All subjects took placebo during the first menstrual cycle; following randomization the intervention group then received 86 mg RC (Novogen®; formononetin 9.3 mg, biochanin A 25.7 mg, genistein 4.3 mg, and daidzein 3.7 mg) daily. Mean monthly values of folate and homocysteine averaged from weekly blood samples resulted in no significant changes from baseline in the RC or placebo groups (54).

Dosage and Extract Preparation

Current standardized preparations of RC are based on total aglycone content of the main four isoflavones. Typical products incorporate dried aqueous alcoholic extracts of RC to deliver ≥ 40 mg isoflavones per dose recommended on the label. These extracts may be hydrolyzed during processing for greater isoflavone aglycone content. Standardized products are available in tablet and capsule form, and clinical doses generally range from 40 to 160 mg isoflavones per day, given in a single dose. Isoflavone doses less than 80 mg/day are considered to be higher than isoflavone exposure received by eating a diet containing soyfoods and isoflavone-containing legumes.

SAFETY, TOXICITY, AND ADVERSE EFFECTS

Adulteration Issues

Heavy Metal Contamination and Pesticide Residues

Although RC does not have a particular tendency to preferentially absorb heavy metals under normal conditions, when it is grown on contaminated soil, it can accumulate high levels of Cd, Cu, Pb, As, and Zn to varying amounts, depending on the soil pH and metal solubility. It is recommended that source material and/or any resultant extracts

be assayed for the presence and level of Pb, As, Cd, and Hg, and the country of origin be required to provide quality control documentation. Imported products containing the ingredients realgar (arsenic) and cinnabar (mercury) should be avoided (55,56). The first supplement to the *U.S. Pharmacopoeia/National Formulary 2003* recommends a limit of not more than 10 ppm of heavy metals be present in RC products.

The following pesticides have been designated by the United Nations as hazardous and are banned by some countries, including the United States: aldrin/dieldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), heptachlor, lindane, malathion, and parathion. These chemicals were used on RC fields in the United States during the 1950s and 1960s. Residues persist in the soil for extended periods of time and are still present in everyday foods at low but detectable levels. It is unknown whether proprietary extraction processes may concentrate these residues in the botanical extracts that are used to make dietary supplements. Some of these pesticides remain in use in other countries and are a potential contaminant of imported plant material and extracts. Limits (mg/kg) of 34 specific organophosphorus, organochlorine, and pyrethroid pesticides for RC are given in Table 4 under method section (561) of the *U.S. Pharmacopoeia/National Formulary 2004* (57).

Botanical Misidentification

Trifolium pratense shares the common name "sweet clover" with the plants *M. alba* Medikus and *M. officinalis* (L.) Pall. This shared common name is unfortunate but physical misidentification is avoidable; the flowers of RC are pinkish-purple, whereas those of *M. alba* and *M. officinalis* are white and yellow and can be easily distinguished from one another. See botanical description section for more botanical characteristics of *M. officinalis*.

Presence of Coumarins

There are more than 3400 naturally occurring coumarins present throughout at least 160 plant families. Many do not have anticoagulant effects in vivo; many more have unknown effects. Red clover has been reported to contain some coumarins. Dicoumarol, a 4-hydroxycoumarin derivative that is known to inhibit blood coagulation, was isolated in 1941 from *M. alba* Medikus and/or *M. officinalis* (L.) Pall. (58). It is a fungal metabolite formed by *Penicillium* species growing in diseased *M. alba* and *M. officinalis*. Although *Melilotus* and *Trifolium* species are closely related, there are no reports of dicoumarol occurring in *Trifolium* species. Coumestrol, daphnoretin, fraxidin, xanthotoxol, medicagol, and scopoletin are present in trace amounts in some RC extracts (≤ 100 ppm) (59).

A randomized, double-blind, placebo-control trial of botanicals, which included an RC arm, for the management of vasomotor symptoms examined prothrombin time of all participants at randomization and at the one-month safety visit and found no increase in prothrombin time in women in the RC group (23). But RC has never been evaluated for long-term anticoagulant effects, or herb-drug interactions with blood-thinning drugs such as warfarin. Usual clinical doses of RC extracts are such that exposure to any particular coumarin present would

likely be below the threshold where any (hypothetical) clinical anticoagulant effects should manifest.

Inhibition of Cytochrome P450 (CYP450) Enzymes

In vitro experiments with human microsomes have shown that RC extracts exhibit selective inhibition of CYP2C9, marginal inhibition of CYP1A2 and CYP3A4, and nominal inhibition of CYP2A6 and CYP2D6. Genistein and daidzein, as well as genistin and daidzin, inhibit CYP1A1 as measured by reduction of enzyme activity in a mouse hepatoma cell culture system. In other experiments, genistein and equol did not cause significant induction of xenobiotic-metabolizing enzymes in mouse (ethoxyresorufin *O*-deethylase, *p*-nitrophenol oxidase, glutathione *S*-transferase, CYP1A2, CYP2E1, or CYP3A1) or human hepatic cells (CYP1A1, glutathione *S*-transferase λ a, or xenobiotic response elements). A recent report of the structure–activity correlation on the inhibitory effects of flavonoids on cytochromes P450 3A activity concludes that daidzein and genistein inhibited CYP3A activity in a concentration-dependent manner (60). In a study of MCF-7 breast cancer cells, biochanin A inhibited the enzyme activity and suppressed the transcriptional control of CYP19 (61). There are no reports of clinically significant RC–drug interactions.

Thyroid Function

Red clover products are used by menopausal women, a group that is prone to hypothyroidism and autoimmune thyroiditis and could be particularly susceptible to the antithyroid actions of the isoflavones. Individuals in this patient population on chronic RC regimens should be monitored for thyroid function. In terms of potential benefit, the San Francisco Bay Area Thyroid Cancer Study recently concluded that isoflavone intake is associated with reduced thyroid cancer risk in both pre- and postmenopausal women (62).

Safety for Cancer Patients

While RC and the isoflavones exhibit anticancer activities in vitro and affect SERM-like effects in vivo, insufficient evidence exists to support their use by patients with active cancer, at elevated risk for ER+ cancer, or recovering from cancer. RC has not been rigorously tested in cancer populations, and the theoretical possibility remains that isoflavone supplementation could promote or cause progression of hormone-dependent tumors. The isoflavones also have the potential to compete with antiestrogenic chemotherapeutic agents, and their antioxidant properties may interfere with radiation and chemotherapies. Significant in vivo CYP450 interactions appear unlikely but could prove problematic in the context of chemotherapy.

Safety for Pregnant Women and Children

The safety of RC or isoflavone supplements for pregnant or (the children of) lactating women has not been established, although RC is considered a class 2b herb by the American Herbal Products Association and as such is contraindicated during pregnancy (63). Red clover supplementation is also discouraged for those younger than 18 years, as the long-term consequences, if any, of high

isoflavone consumption early in life have not yet been elucidated.

Adverse Effects Reported in Clinical Trials

Because the exact chemical content of commercial RC isoflavone products is proprietary, and total isoflavone content (or a ratio of summed isoflavone content) is often reported rather than individual chemical content, it is difficult to estimate clinical doses of individual isoflavones. This vague content labeling hinders correlation of clinical effects with specific RC compounds. More trials involving chronic exposure of large patient populations to RC isoflavone extracts are needed to assess long-term risk.

Novogen Ltd. provides a list of side effects, occurring at doses as low as 40 mg isoflavones/day, in their online clinical monograph for Promensil. These effects include breast tenderness, swollen neck glands, increased thyroid function, migraine/headache, dizziness, vertigo, tremor, hypertension, acne, rash, pruritus, psoriasis, bloating, constipation, diarrhea, nausea, mouth ulcer, sore throat, myalgia, osteoarthritis, bronchitis, low platelets, reflux (80 mg), epistaxis (80 mg), menstrual bleeding (80 mg), urinary tract infection (120 mg), and vaginal thrush (80 mg).

Additional adverse events reported in two trials using oral administration of 54 mg/day genistein included the following: (symptomatic) hypotension, vertigo, paresthesiae, temporary return of abbreviated menses, vaginal bleeding, hot flushes, and endometrial thickness greater than 5 mm.

Compounds and mechanisms responsible for triggering adverse events are currently unknown. Use of the lowest RC dose possible for treatment, with upward titration as necessary, is recommended to decrease the probability of side effects occurring.

COMPENDIAL/REGULATORY STATUS

Red clover is included on the U.S. FDA generally recognized as safe (GRAS) list, and “RC isoflavones” is an approved herbal components name (HCN) designated by the Therapeutic Goods Administration of Australia (64). The flower heads are listed in the *British Herbal Compendium* (65), the *British Herbal Pharmacopoeia* (66), and in *Martindale: The Extra Pharmacopoeia* (67). It appears in the UK’s General Sale List, Schedule 1 of Statutory Instrument 1994 No. 2410 (68).

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Reishi

Solomon P. Wasser

INTRODUCTION

Reishi or Ling Zhi (*Ganoderma lucidum*), a popular medicinal mushroom, has been used in China, Japan, and Korea for the promotion of longevity and health since ancient times. Over the years, fables and legends revered *G. lucidum* as a “heavenly herb,” which connotes auspiciousness, blissfulness, and happiness. This mushroom has become more popular all over the world in recent years. The application of modern analytical techniques has revealed the mushroom to contain numerous bioactive compounds including polysaccharides, triterpenes, and immunomodulatory proteins. This review collates the publications detailing with the activities and compounds of *G. lucidum* while considering the most valid claims of effectiveness; it also presents a scientific understanding of Reishi’s beneficial functions on human immune, endocrine, nervous, and cardiovascular systems. Reishi has been used in conjunction with treatment for cancer patients, chronic bronchitis, hyperlipidemia, hypertension, diabetes, hepatitis, dermal and urological dysfunctions, and viral and bacterial diseases. Reishi also has nutraceutical applications; it is a well-known dietary supplement that can help improve general health and wellness. The latest available estimates put the annual value of Reishi products worldwide at more than 3 billion USD. Further expansion of the market for Reishi products will require the introduction of more protocols for mushroom production and downstream processing to improve quality control and assure the public of medicinal mushroom benefits. It is widely grown on a commercial scale and is commonly purchased for its medicinal and spiritual properties.

BACKGROUND

Name and General Description

In Latin, *lucidum* means shiny or brilliant and aptly describes this mushroom’s fruiting body, which has a modeled, sculptured, varnished appearance. The Chinese and Koreans know it as Ling Zhi (mushroom of herb and immortality), whereas the Japanese call this mushroom Reishi or mannentake (10,000-year mushroom). The virtues of *G. lucidum* extracts, handed down from generation to generation, include it as a “cancer cure” and a symbol of happy augury, good fortune, good health, longevity, and even immortality. Beginning with the Yuan Dynasty (AD 1280–1368), *G. lucidum* has been endlessly represented in art—in paintings, carvings of jade and deer’s

antlers, furniture and carpet designs, balustrades, jewelry, women’s hair combs, perfume bottles—in short, wherever an artistic urge found an outlet. The earliest mention of Ling Zhi was in the era of the first emperor of China, Shing-huang of the Ch’in Dynasty (221–207 BC). Subsequently, depictions of this fungus proliferated through Chinese literature and art. The mushroom is known by many in North America and Europe as one of the “artist’s conk” fungi (the true artist conk is *Ganoderma applanatum*). The mushroom is too tough to be edible.

A detailed description of the Reishi mushroom and its taxonomy can be found in Refs (1,2). (Fig. 1).

Habitat

This annual mushroom grows on a wide variety of dead or dying trees, for example, deciduous trees especially oak, maple, elm, willow, sweet gum, magnolia, and locust (*Quercus*, *Acer*, *Alnus*, *Betula*, *Castanea*, *Corylus*, *Fagus*, *Fraxinus*, *Populus*, *Pyrus*, *Magnolia*, *Tilia*). *G. lucidum* is less frequently found on coniferous trees (e.g., *Larix*, *Picea*, *Pinus*) in Europe, Asia, and North and South America (in temperate rather than subtropical regions). In the

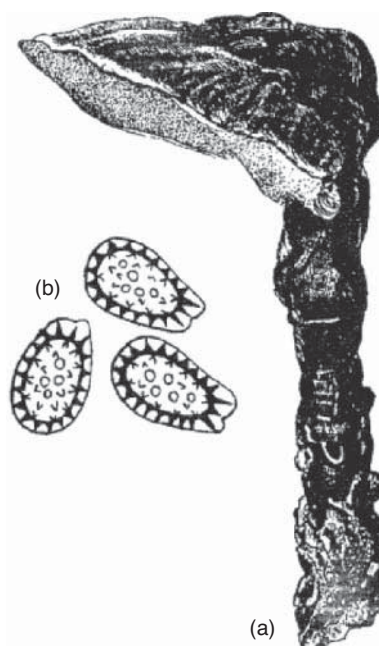


Figure 1 *Ganoderma lucidum*: (A) fruit body, (B) spores.

Orient, it grows primarily on plum trees. It is also found on stumps, generally near the soil surface, and occasionally on soils arising from buried roots.

Related Species and Artificial Cultivation

Ling Zhi encompasses several *Ganoderma* species, which are widely used for medicinal purposes, for example, *G. lucidum*, *G. luteum* Steyaert, *G. atrum* Zhao, Xu and Zhang, *G. tsugae* Murrill, *G. applanatum* (Pers.: Wallr.) Pat., *G. australe* (Fr.) Pat., *G. capense* (Lloyd) Teng, *G. tropicum* (Jungh.) Bres., *G. tenue* Zhao, Xu and Zhang, and *G. sinense* Zhao, Xu and Zhang. According to two famous Chinese plant medical books, *Shen Nong Ben Cao Jing* (25–220 A.D., Eastern Han Dynasty) and *Ben Cao Gang Mi*, by Li Shi-Zhen (1590 A.D., Ming Dynasty), six Ling Zhi species/varieties were known in China at that time. Worldwide, more than 250 *Ganoderma* species have been described (1,3). However, in therapeutic practices and literature citations, *Ganoderma* usually refers to the species of *G. lucidum*.

Besides being treasured for its medicinal value in China for more than 1000 years, the lack of availability of *G. lucidum* was also largely responsible for it being so highly cherished and expensive. During ancient times in China, any person who picked the mushroom from the natural environment and presented it to a high-ranking official was usually well rewarded. Even in the early 1950s, it was presented to Chinese leaders in Mainland China and Taiwan, following the occasional discovery in the wild. In the past, *G. lucidum* grew in small quantities only in the wild; therefore, it was very expensive.

Artificial cultivation of this valuable mushroom was successfully achieved in the early 1970s, and since 1980, production of *G. lucidum* has developed rapidly, particularly in China and the United States. The process of producing *G. lucidum* fruiting bodies is the same as for other cultivated edible mushrooms and can be divided into two major stages. The first involves the preparation of the fruiting culture, stock culture, mother spawn, and planting spawn, while the second entails the preparation of growth substrates for mushroom cultivation. Currently, the methods most widely adopted for commercial production are the wood log, short wood segment, tree stump, sawdust bag, and bottle procedures (for cultivation details, see Refs (4,5)).

History and Traditional Uses

G. lucidum has been used in folk medicine of China and Japan, especially in the treatment of hepatopathy, chronic hepatitis, nephritis, hypertension, arthritis, neurasthenia, insomnia, bronchitis, asthma, and gastric ulcers (2,6–9). In China, *G. lucidum* has been cherished for over 4000 years as a longevity-promoting tonic (6). According to Hikino (10), “the most important elixirs in the Orient” are ginseng (*Panax ginseng* C.A. Meyer) and the fruit bodies of *G. lucidum*.

Fascination with *Ganoderma* began under the name of *ling chih*, later transliterated to *reishi* in Japanese. The fungus first appeared in Chinese literature during the Han Dynasty (206 BC–AD 220). Emperor Wu associated growth of the fungus in an inner chamber of the Imperial Palace with a plant of immortality—known simply as the chih

Table 1 The Six Types of Ling Zhi Mushroom

Color	Taste	Japanese name	Use
Blue	Sour	Aoshiba	Improves eyesight and liver function; calms nerves
Red ^a	Bitter	Akashiba	Aids internal organs; improves memory; enhances vitality
Yellow	Sweet	Kishiba	Strengthens spleen function; calms the “spirit” (shen)
White	Hot (or pungent)	Shiroshiba	Improves lung function; gives courage and strong will
Black	Salty	Kuroshiba	Protects kidneys
Purple	Sweet	Murasakishiba	Enhances function of ears, joints, muscles; helps improve complexion

^aThe red-colored variety of *G. lucidum* is generally regarded as the most potent and medicinal. (20).

plant or chih fungus (11). The Han Dynasty chronicler, Pan Ku, wrote a poem using the term ling chih (11). However, the association between the original chih fungus and *G. lucidum* had been clearly derived from legends of an earlier mysterious chih fungus or chih plant of immortality recorded in India. Indeed, versions of Indian legends concerning this mushroom are found later, in almost identical form in the Chinese literature, in reference to what would be ling chih (Reishi), while the identity of the true chih plant or fungus of immortality remains in dispute (11). In addition to its medicinal properties, Reishi has been used in the Orient as a talisman to protect a person or home against evil (6).

Medicinal uses of *G. lucidum* in ancient Far East countries included the treatment of neurasthenia, debility from prolonged illness, insomnia, anorexia, dizziness, chronic hepatitis, hypercholesterolemia, mushroom poisoning (antidote), coronary heart disease, hypertension, prevention of altitude sickness, treatment of “deficiency fatigue,” carcinoma, and bronchial cough in the elderly (1,2,6,8,9,12). Chinese research during the past decade has focused on much the same uses, whether in the fields of antiaging/life prolongation, brain ischemia/reperfusion injury, chronic viral hepatitis, male sexual dysfunction, hypercholesterolemia, immunological function in the elderly, chemotherapy-induced toxicity, narcotic-induced immunosuppression, anticarcinogenic and antitumor activity, and immunostimulation (6,7,13–19). Different types of *G. lucidum*, according to Traditional Chinese Medicine, have different tastes and thus affect different organs. Based on their color, six different types of *G. lucidum* have been classified (20), each with different uses (Table 1).

CHEMISTRY

General Nutritional Components of *G. Lucidum*

G. lucidum contains mainly protein, fat, carbohydrate, and fiber. The artificially cultivated variety has similar contents of nutritional components compared with wild types, and the extraction significantly increases the amounts of crude protein and carbohydrates and deleted crude fiber. Mizuno (21) reported the composition of *G. lucidum* extract (% of dry weight), which consisted of folin-positive material (68.9%), glucose (11.1%), protein (7.3%), and metals (10.2%) (K, Mg, and Ca are the major components

with Ge having the fifth highest metal concentration at 489 $\mu\text{g/g}$). These results generally agree with those reported by other authors (2,4,5). However, there are qualitative and quantitative differences in the chemical composition of *G. lucidum* products depending on the strain, origin, extracting process, and cultivation conditions (1,2,5,9,21).

Major Bioactive Constituents

Over 300 reports have been published concerning the chemical constituents of *G. lucidum* and related species. The fruiting body, mycelia, and spores of *G. lucidum* contain approximately 400 different bioactive compounds, which mainly include triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins/peptides, and trace elements (9,16,19,21,22).

Triterpenes

At least 140 different triterpenes have been identified in *G. lucidum* (1,2,6,9,21,22). The majority are bitter tasting and largely occur as ganoderic acid (22). A new triterpenoid, named ganosporeric acid A, was recently isolated from the ether-soluble fraction of the spores (23). Min et al. (24) reported the isolation of six new lanostane-type triterpenes, and also from the spores (ganoderic acids γ , δ , ϵ , ζ , η , and θ). Preliminary studies indicate that the spores contain considerably higher contents of ganoderic acids than other parts of the fungus and that triterpene composition of the fruit body varies according to the area in which it is grown (23). The spores also contain triterpene lactones (22), and documented triterpenoids have been divided into 10 groups based on the structural similarities and known biological and medicinal properties (Fig. 2).

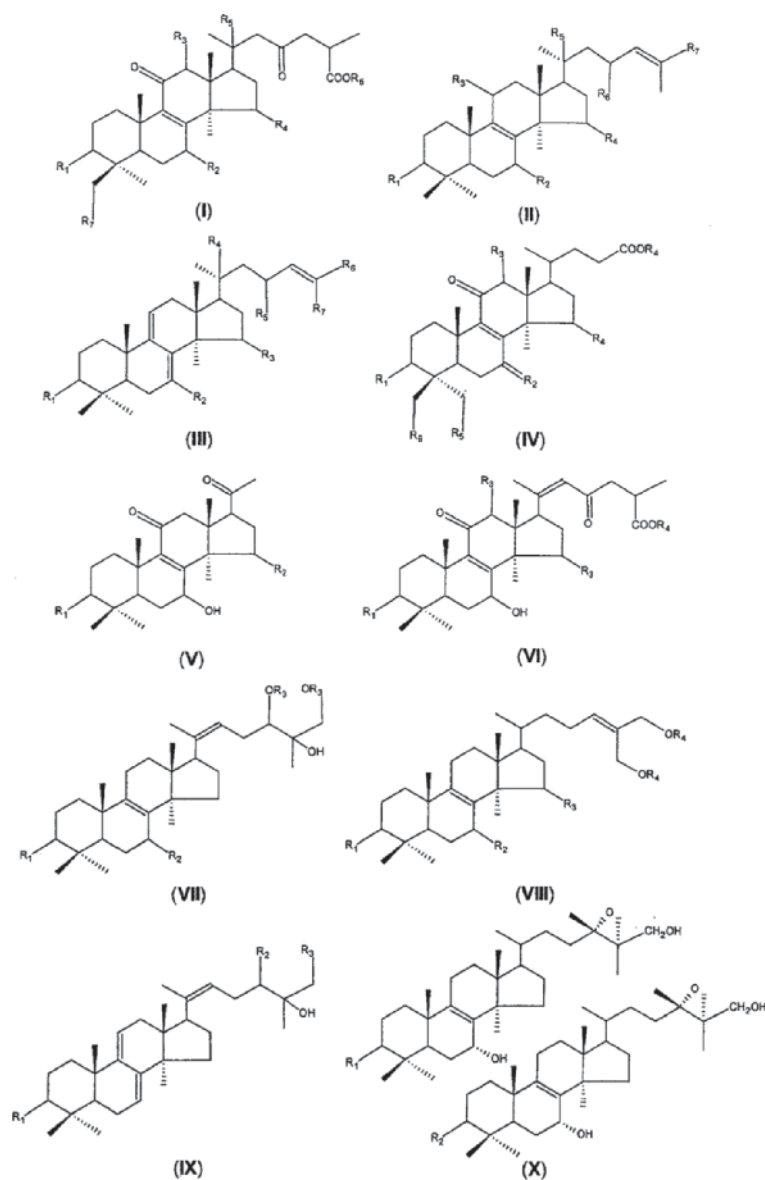


Figure 2 The lanostane-type triterpenoids of *Ganoderma lucidum*. These triterpenoids are divided into ten groups on the basis of structural similarity.

Polysaccharides

More than 100 types of polysaccharides have been isolated from the fruiting body, spores, and mycelia, or separated from the broth of a submerged liquid culture of *G. lucidum*. Most have a molecular weight ranging from 4×10^5 to 1×10^6 in the primary structure. They comprise one of the major sources of *G. lucidum*'s pharmacologically active compounds.

G. lucidum polysaccharides such as β -D-glucans, heteropolysaccharides, and glycoprotein have been isolated and characterized and are considered the major contributors of bioactivity of the mushroom. β -D-glucans consist of a linear backbone of β -(1 \rightarrow 3)-linked D-glucopyranosyl groups with varying degrees of branching from the C6 position. In addition to water-soluble β -D-glucans, β -D-glucans also exist with heteropolysaccharide chains of xylose, mannose, galactose, uronic acid, and β -D-glucans-protein complexes that are present at 10% to 50% in dry *G. lucidum* (16,25–27). Some protein-bound polysaccharides and fucose-containing glycoprotein with bioactivity have been isolated (18,28,29).

Proteins

Some proteins with bioactivity have also been isolated from *G. lucidum*. The LZ-8 is one such protein isolated from *G. lucidum*, which was shown, by sequencing studies, to be similar to the variable region of the immunoglobulin heavy chain in its sequence and in its predicted secondary structure. Major biological activities of LZ-8 resemble those of lectins, with mitogenic capacity toward mouse spleen cells and human peripheral lymphocytes and agglutination of sheep red blood cells in vitro. Neither was inhibited by the mono- or dimeric sugars examined, indicating that LZ-8 is not a lectin per se. It did not agglutinate human red blood cells but could function as a potent suppressor of bovine serum albumin-induced anaphylaxis in CFW mice in vitro. It appears to be related to an ancestral protein of the immunoglobulin superfamily (30).

Nucleotides and Nucleosides

Nucleosides include adenosine and 5-deoxy-5'-methylsulfinylnosine (21).

Other Constituents

G. lucidum also contains sterols, amino acids, soluble proteins, oleic acid, cyclo-octasulfur, an ergosterol peroxide (5,8-epidioxy-ergosta-6,22E-dien-3-ol), and the cerebrosides (4E',8E)-N-D-2'-hydroxystearoyl-1-O- β -D-glucopyranosyl-9-methyl-4-8-sphingadienine, and (4E,8E)-N-D-2'-hydroxypamitoyl-1-O- β -D-glucopyranosyl-9-methyl-4-8-sphingadienine (1,9,17,18,21).

Regarding the inorganic ions, the mushroom contains Mg, Ca, Zn, Mn, Fe, Cu, and Ge. The spores themselves contain choline, betaine, tetracosanoic acid, stearic acid, palmitic acid, ergosta-7, 22-dien-3-ol, nonadecanoic acid, behenic acid, tetracosane, hentriacontane, ergosterol, and β -sitosterol. One of the lipids isolated from *G. lucidum* is pyrophosphatidic acid (13,17,21).

PRECLINICAL STUDIES

G. lucidum has been reported to have a number of pharmacological effects including immunomodulating,

antiatherosclerotic, anti-inflammatory, analgesic, chemopreventive, antitumor, radioprotective, sleep-promoting, antibacterial, antiviral (including anti-HIV), hypolipidemic, antifibrotic, hepatoprotective, diabetic, antioxidative and radical-scavenging, anti-aging, hypoglycemic, and anti-ulcer properties (1,2,6,8,9,16,19,26,31).

Antitumor Effects

Polysaccharides (β -D-glucans, heteropolysaccharides, and glycoproteins) isolated from *G. lucidum* demonstrated antitumor activity against Sarcoma 180 in mice (1,1,2,2,13,16,21,26,28,29,31). Triterpenoids, such as ganoderic acids T-Z isolated from *G. lucidum*, showed cytotoxic activity in vitro on hepatoma cells (32). A lanostanoid, 3 β -hydroxyl-26-oxo-5 α -lanosta-8,24-dien-11-one, and a steroid, ergosta-7,22-diene-3 β ,3,9-triol, isolated from fruiting bodies of *G. lucidum*, demonstrated potent inhibitory effects on KB cells and human PLC/PRF/5 cells in vitro (33).

The polysaccharide-mediated potentiation of immune function is thought to be the major mechanism of antitumor action by *G. lucidum*. Among the multiple polysaccharides, active β -D-glucans are responsible for the antitumor effect (1,2,9,13,21,29,31). This polysaccharide appears to act by binding to leukocyte surfaces or serum-specific proteins leading to activation of macrophages, T-helper, natural killer (NK), and other effector cells (34–36). All of these increase the production of cytokines such as tumor necrosis factor (TNF- α) interleukins (IL) and interferon (IFN), nitric oxide (NO), and antibodies by the activated effector cells. Tumor regression in various animal models can be ascribed to vascular damage to tumor blood flow and necrosis caused by T cells and local TNF- α production.

In addition to host defense potentiation, other mechanisms are also involved in the antitumor effect. A compound from *G. lucidum* suppressed the growth of K562 leukemic cells in a dose- and time-dependent manner and induced their differentiation into more mature erythrocytic cells (37). The conditioned medium from PS-stimulated human blood mononuclear cells (PSG-MNC-CM) significantly inhibited the growth of U937 cells and induced their differentiation into mature monocytes/macrophages, which had functions of phagocytosis and of producing cytoplasmic superoxide (38). Inhibition of DNA polymerase and posttranslational modification of oncoproteins may contribute to the antitumor activity of *G. lucidum* (39). The organic germanium may also contribute to its antitumor activity (40). Active constituents from *G. lucidum* may operate through several mechanisms including enhancement of detoxification of carcinogens, increased expression and activity of Phase II enzymes, inhibition of organ exposure of carcinogens due to reduced absorption or increased excretion, decreased expression and activity of Phase I (e.g., CYPs) enzymes, decreased formation of toxic metabolites and adduct formation with macromolecules, enhanced host immune responses (e.g., activation of macrophages, T lymphocytes, and natural killers producing various cytokines such as TNF- α , IFNs, and ILs, which improve immunosurveillance and kill preneoplastic and cancer cells), antioxidative and radical-scavenging effects, antipromotion effect, antiproliferation,

apoptosis induction of tumor cells, induction of differentiation, direct cytotoxicity, induction of cell-cycle arrest, antiproliferation and modulation of signaling transduction molecules, antiproliferation and tumor growth inhibition, antimetastasis, and antiangiogenesis (16).

In summary, animal studies have demonstrated the antitumor activity of *G. lucidum* administered by different routes at different stages of tumor growth (1,2,16,21). Polysaccharides and triterpenoids are the major contributors to the anticancer effect of *G. lucidum*, but other constituents, such as proteins, also play a role (21). Possible molecular pathways that may provide an explanation for the cancer preventive and anticancer effect of *G. lucidum* are shown by Gao et al. (16).

Chemopreventive and Radiopreventive Effects

The chemo- and radiopreventive effects of *G. lucidum* may result from its effects on the immune system. *Ganoderma* polysaccharides restored the TNF- α production inhibited by cyclophosphamide to normal levels in mice. Both the *G. lucidum* extract and krestin (protein-bound β -glucan isolated from *Trametes versicolor*) were beneficially effective in the recovery of cellular immunocompetence, measured by [3 H] thymidine incorporation with splenic cells stimulated through mitogens, such as phytohemagglutinin and concanavalin A. The extract appears more effective than krestin in repairing the damage of subset T cells in the spleens of γ -irradiated mice, as the relative thymus weight and CD4 and CD8 splenocytes were higher in *G. lucidum* extract-treated mice compared with krestin-treated mice (16).

In morphine-dependent mice, a polysaccharide peptide from *G. lucidum* could restore several immunologic parameters depressed by morphine treatment to normal levels or even beyond (41). Both *c-myc* and *c-myc* mRNA expression in splenocytes of repetitive morphine-treated mice was significantly decreased, and the polysaccharide peptide could induce the expression of these genes indicating that the one from *G. lucidum* could be of a potential application in controlling abuse of opiate-induced immunodeficiency.

Enzyme-Inhibiting Activity

Triterpenoids of *G. lucidum* have been reported to exert various enzyme inhibitory activities. Inhibitors of farnesyl protein transferase have been demonstrated to inhibit Ras-dependent cell transformation and thus represent a potential therapeutic strategy for the treatment of human cancers. Ganoderic acids A and C were identified to be inhibitors of farnesyl protein transferase (42). Ergosterol peroxide, 5,8-epidioxy-5 α , 8 β -ergosta-6,22E-dien-3 β -ol, from *G. lucidum*, was reported to selectively enhance the inhibitory effect of linoleic acid on DNA polymerase- β , but not on DNA polymerase- α . Ergosterol peroxide itself was ineffective but completely blocked rat DNA polymerase- β in the presence of linoleic acid (39). Inhibitors of phospholipase A₂ can be developed as potential anti-inflammatory agents for the treatment of rheumatic arthritis, asthma, and psoriasis. Ganoderic acid T was found to inhibit secreted phospholipase A₂ from pig pancreas, human synovial fluid, and bee venom, but no such effect was

observed with ganoderic acids AA, O, R, S, T-OH, and T-OH-H₂ (16).

Immunomodulating Effects

The major immunomodulating effects of active substances derived from *G. lucidum* include mitogenicity and activation of immune effector cells such as T lymphocytes, macrophages, and NK cells leading to the production of cytokines including ILs, TNF- α , and IFNs. Other effects, such as inhibition of mast cells, activation of B lymphocytes, and the complement system have also been reported (15).

Mitogenic Activity

Extracts from *G. lucidum* (e.g., polysaccharide fractions, methanolic extracts, and LZ-8) have mitogenic effects on mouse splenocytes and human peripheral blood mononuclear cells (PBMCs) in the presence of various immunostimulating or immunosuppressive agents (e.g., phytohemagglutinin and 12-O-tetradecanoylphorbol 13-acetate) (43,44). Treatment of the PBMCs with cyclosporin A (CsA) led to blockage of the cell proliferation. The methanolic fraction from *G. lucidum* recovered the CsA-induced inhibition of the cell proliferation, which might be due to the inhibition of the protein kinase C signal pathway and acceleration of the CsA signal pathway.

Effects on Immune Effector Cells

Splenocytes

In vitro and in vivo studies in mice indicated that *G. lucidum* water extract stimulates the production of IL-2 by splenocytes in the presence of hydrocortisone (1,2,9,12).

T Cells

Extracts from *G. lucidum* are potent activators of T cells, inducing the production of a number of cytokines, in particular IL-2. In human PBMC (primarily T cells) in vitro, the crude *G. lucidum* water extract induced the expression of cytokines including IL-10 and TNF- α , IL-1 β , IL-6, and IL-2 (44). Crude polysaccharide fractions isolated from fresh fruiting bodies potentiated the release of IFN- γ from human T cells (38). A polysaccharide fraction (GL-B) promoted the production of IL-2 in a dose-dependent manner and markedly enhanced the cytotoxicity of cytotoxic T lymphocytes, which was increased by 100% at a concentration of 200 μ g/mL. GL-B also restored the mixed lymphocyte response to alloantigen, automatic proliferation, and IL-2 production of splenocytes in aged mice declined as compared with that in young adult mice in vitro.

LZ-8 is also a potent T-cell activator mediating its effects via cytokine regulation of integrin expression. Stimulation of human peripheral blood lymphocytes with LZ-8 resulted in the production of IL-2 and a corresponding upregulation of IL-2 receptor expression (45). In addition to T-cell proliferation, microscopic examination of LZ-8-stimulated peripheral blood lymphocytes revealed that LZ-8 induced cellular aggregate formation. This formation correlated with a dramatic rise in ICAM-1 expression and an increased production of IFN- γ , TNF- α , and IL-1 β , molecules associated with regulation of ICAM-1 expression. Both the aggregate formation and the proliferative

effects of LZ-8 were blocked by the addition of a monoclonal antibody to either CD18 or CD11a, the counter-receptor complex components for ICAM-1. Furthermore, addition of neutralizing antibodies to both IL-2 receptor and TNF- α blocked aggregate formation, cellular proliferation, and ICAM-1 expression.

Natural Killer (NK) Cells

A water-extracted polysaccharide fraction from *G. lucidum* enhanced the cytotoxicity of splenic NK cells in tumor-bearing mice (1,16,38).

Macrophages

Macrophages are responsible for killing pathogens in the body. Activation of macrophages by substances from *G. lucidum* results in the release of cytokines, NO, and other mediators (38,46). All of these responses are associated with the antitumor, antimicrobial, and anti-inflammatory effects of *G. lucidum*.

Polysaccharides from *G. lucidum*, in particular β -D-glucans, are potent stimulators of murine and human macrophages in vitro and in vivo (38,46). CR3 receptors on macrophages are bound by β -D-glucans and internalized, priming a series of molecular events. Crude water-extracted polysaccharides isolated from fresh fruiting bodies of *G. lucidum* potentiated the production of cytokines including IL-1 β , IL-6, IFN- γ , and TNF- α by human macrophages, which were antiproliferative, differentiated and apoptosis inductive to the HL-60 and the U937 leukemic cells (38). IFN- γ and TNF- α released from macrophages act synergistically to inhibit the growth of leukemic cells as shown by the antibody-neutralization studies. GLB7, a *G. lucidum* polysaccharide, decreased the production of oxygen-free radicals and antagonized the respiratory burst induced by PMA in murine peritoneal macrophages. These observations suggest that GLB7-decreased production of oxygen-free radicals in murine peritoneal macrophages plays an important role in the anti-aging effect of *G. lucidum* polysaccharides (46).

Ganoderan (GAN), a β -D-glucan isolated from *G. lucidum*, enhanced the production of NO in the RAW 264.7 macrophages (46). The ability of GANs to produce NO was based on differences in the chemical composition of GANs obtained from the mycelium on various carbon sources and mycelial fractionation. The highest NO production was observed in the polysaccharide, which was extracted from the mycelial wall. Partial removal of the protein in the extracellular GAN by TCA treatment did appreciably reduce its capacity to secrete NO. The cell proliferation of GAN-treated RAW 264.7 cell lines was inhibited compared to its control. Of the culture supernatant of macrophage activated by this glycan, the percentage of cytotoxicity against mouse leukemia L1210 cells was slightly dependent on the amount of NO in the culture supernatants of the activated macrophages. These results indicate that the β -glucan-related polysaccharides of the higher fungus activate macrophages and release NO, which is an important chemical messenger for the induction of many biological responses.

A protein-polysaccharide fraction (GLB) from the growing tips of *G. lucidum* is a strong stimulator to the macrophages (47). When analyzed using a flow cytometer, GLB increased the phagocytic activity of the

BALB/c mouse peritoneal macrophages as well as chicken macrophage BM2CL cells against FITC-labeled *Candida albicans* by 55.2% and 21.2%, respectively. It also enhanced the spreading and expression of MHC class II molecules of BM2CL cells as well as the mouse peritoneal macrophages.

Mast Cells

Some substances from *G. lucidum* can act on mast cells. A water extract of the fruit body had inhibitory activity on histamine release from rat peritoneal mast cells, induced by compound 48/80 or antigen (egg white albumin)-antibody reaction and on passive cutaneous anaphylaxis reaction in guinea pigs and rats. Two ganoderic acids (C and D) isolated from the fruit body by methanol inhibited the histamine release from rat mast cells, induced by compound 48/80 and concanavalin A. A chloroform extract from *G. lucidum* broth also significantly inhibited histamine release from rat peritoneal mast cells induced by A-23187 and compound 48/80. The mechanism for the inhibitory activity on histamine release from mast cells was further studied. Palmitic acid, stearic acid, oleic acid, and linoleic acid were isolated from the active fractions. Of these, oleic acid induced membrane stabilization in model membrane systems. Cyclo-octasulfur extracted from the culture medium of *G. lucidum* may decrease calcium uptake from the extracellular medium by a disulfide exchange reaction in the cell membrane leading to inhibition of histamine release from mast cells (1,2,9,14,16).

Complement System

An alkali extract isolated from cultured mycelium of *G. lucidum* activated classical and alternative pathways of a complement system. Activated complement C3 was observed by crossed immunoelectrophoresis in mice. This fraction also activated the reticuloendothelial system of mice in the carbon clearance test and increased hemolytic plaque-forming cells of the spleen. The alkali extract consisted of 10% carbohydrate and 49% proteins.

Histamine Release Inhibition

The fruiting bodies have been traditionally used as anti-inflammatory agents for the treatment of asthma or allergy. In the course of a screening test for the inhibition of histamine release from rat mast cells, it was found for the first time that ganoderic acids C and D inhibited histamine release from rat mast cells (that were induced by compound 48/80 and concanavalin A). Other than the triterpenoid compounds, cyclo-octasulfur from this fungus also effectively inhibited histamine release from rat peritoneal mast cells and interacted with membrane proteins to inhibit Ca uptake causing a blockade of histamine release (12,13,19).

Hepatoprotective Activity

G. lucidum has been widely used for the treatment of chronic hepatopathy of various etiologies. Data from in vitro and animal studies indicate that *G. lucidum* extracts (mainly polysaccharides or triterpenoids) exhibit protective activities against liver injury induced by toxic chemicals (e.g., CCl₄) and Bacillus Calmette-Guerin plus lipopolysaccharide. Reishi also showed antihepatitis B virus (HBV) activity in a duckling study. The mechanisms

of the hepatoprotective effects of *G. lucidum* have been largely undefined. However, accumulating evidence suggests several possible mechanisms. These include antioxidant and radical-scavenging activity, modulation of hepatic Phase I and II enzymes, inhibition of β -glucuronidase, antifibrotic and antiviral activity, modulation of NO production, maintenance of hepatocellular calcium homeostasis, and immunomodulating effects (17). The mushroom could represent a promising approach for the management of various chronic hepatopathies. Further studies are needed to explore the kinetics and mechanisms of action of its constituents with hepatoprotective activities.

Antidiabetic Effect

Animal studies have demonstrated that the polysaccharide fractions of *G. lucidum* have potential hypoglycemic and hypolipidemic activities.

A water extract of Reishi reduced the increase in blood glucose and blood insulin levels in rats (50 mg p.o.) following oral glucose test. Following adrenaline (IV) or oral glucose in rats, the mushroom inhibited increases in blood glucose without raising blood insulin levels. Glycans (ganoderans B and D) have shown significant hypoglycemic activity in mice.

Cardiovascular and Circulatory Functions

Cholesterol and Lipid Metabolism

The powdered mycelium of Reishi, at 5% of the diet of spontaneously hypertensive rats for four weeks, caused plasma total cholesterol to decrease significantly (by 18.6%) compared to controls. Total liver triglyceride and total liver cholesterol levels were also significantly lower in the Reishi-fed group (by approximately 46% and 56%, respectively) (48,49).

Hypertension

A water extract of the mycelium administered to rats and rabbits produced significant hypotensive effects; an activity the researchers suggested is secondary to the primary effect that suppresses sympathetic outflow of the central nervous system (50). The powdered mycelium of *G. lucidum*, at 5% of the diet of spontaneously hypertensive rats for four weeks, caused systolic blood pressure to be significantly lower without causing a significant difference in the heart rate (48).

Antibacterial and Antiviral Value

Antibacterial Effect of *G. lucidum* on Gram-Positive and Gram-Negative Bacteria

Recently, more studies demonstrated that *G. lucidum* contained antibacterial constituents that are able to inhibit gram-positive and/or gram-negative bacteria (1,2,2,5,5,17,51). The aqueous extract from the carpophores of *G. lucidum* inhibited 15 types of gram-positive and gram-negative bacteria. Further studies indicate that the antimicrobial combinations of *G. lucidum* extract with four antibiotics (ampicillin, cefazolin, oxytetracycline, and chloramphenicol) resulted in additive effects in most instances: synergism in two instances when combined with cefazolin against *Bacillus subtilis* and *Klebsiella oxytoca* (52), and antagonism in two instances.

Helicobacter pylori

Helicobacter pylori is associated with human gastroduodenal diseases such as gastritis, peptic ulcer, and gastric carcinoma. The extracts of many mushrooms inhibited the growth of this bacterium (17,53). The extract of *G. lucidum* and some other species of higher Basidiomycetes arrested the growth of this pathogen. When their extracts were fractionated, the ether fractions of *G. lucidum* and *Agaricus bisporus* (J. Lge) Imbach were the most effective. Among seven components separated from the ether fraction of *G. lucidum* extract by silica gel column chromatography, P3 was the most potent with a minimum inhibitory concentration of 200 μ g/mL.

It appears that some constituents such as ganomycin, triterpenoids, and aqueous extracts from *Ganoderma* species have a broad spectrum of in vitro antibacterial activity against gram-positive and gram-negative bacteria and *H. pylori*. Thus, it is possible that the antibacterial activity of *Ganoderma* species may be beneficial for those patients with chronic infection (e.g., chronic bronchitis) and those with *H. pylori*-positive peptic ulcer diseases, though clinical studies are required to confirm this.

Antihuman Immunodeficiency Virus (HIV) Activity

HIV was isolated as an etiological agent of acquired immunodeficiency disease syndrome in 1983 (54). Acquired immunodeficiency syndrome caused by HIV infection has recently become an important social and medical problem. Anti-HIV therapy by nucleoside analogues, such as 3'-azido-thymidine, is the major effective approach for the treatment of acquired immunodeficiency syndrome (55). These agents are potent inhibitors of HIV reverse transcriptase (RT) and protease (56). However, the emergence of drug-resistant variants of HIV and toxicities severely limits the long-term effectiveness of these drugs. Recent studies have indicated that many natural products are active as anti-HIV agents. These compounds belong to a wide range of different structural classes, for example, coumarins, flavonoids, tannins, alkaloids, lignans, terpenes, naphtho- and anthraquinones, and polysaccharides (57).

In vitro studies indicate that various triterpenoids from *G. lucidum* had potent inhibitory activity against HIV. Lucidenic acid O and lucidenic lactone, isolated from the fruiting body of *G. lucidum*, not only inhibited the activities of calf DNA polymerase- and rat DNA polymerase- β , but also those of HIV-1 RT (17). Ganoderiol F and ganodermanontriol isolated from the fruiting bodies of *G. lucidum* are active against HIV-1 growth (2,9,17). Ganoderic acid B and ganoderiol B showed potent inhibitory effect on HIV protease. Other triterpenoids including ganoderic acid C1, 3 β -5 α -dihydroxy-6 β -methoxyergosta-7,22-diene, ganoderic acid-, ganoderic acid H, and ganoderiol A had moderate activity against HIV-1 protease (2,9,17,58). In addition, ganoderic acid- β , lucidumol B, ganodermanontriol, ganodermanontriol, and ganolucidic acid A showed significant anti-HIV-1 protease activity (23). Ganoderic acid A, B, and C1 had minor inhibitory activity against HIV protease with IC₅₀ values of 140–430 μ M. It appears that there is a structure-activity relationship for triterpenoid showing anti-HIV protease activity. The C3, C24, or C25 atoms are vital for the anti-HIV activity (23).

The aqueous low-molecular-weight fraction extracted from *G. lucidum* also exhibited anti-HIV activity using the XTT [2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2 H-tetrazolium hydroxide] antiviral assay, which can quantitatively measure cytopathic effects of HIV-1 on CEM cells, a human T lymphoblastoid cell line (59,60). The IC₅₀ and EC₅₀ values were 125 and 11 µg/mL, respectively, resulting in a therapeutic index of 11.4. This aqueous low-molecular-weight extract was further fractionated to eight subfractions by methanol: GLA (methanolic extract), GLB (hexane soluble), GLC (acetic ether soluble), GLD (water soluble), GLE (neutral), GLF (acidic), GLG (alkaline), and GLH (amphoteric). All subfractions except GLD, GLF, and GLH exhibited anti-HIV activity with IC₅₀ and EC₅₀ values of 22–44 µg/mL and 14–44 µg/mL, respectively. GLC and GLG inhibited HIV RT. Showing consistency, incubation of GLC at 50 µg/mL or GLG (100 µg/mL) with Jurkat T cells gave a 75% and 66% inhibition of HIV growth, respectively. However, the high-molecular-weight fraction did not inhibit any HIV-induced cytopathic effect. Both low-molecular-weight and high-molecular-weight fractions from *G. lucidum* had negligible toxicities to CEM cells. The results indicate that the aqueous low-molecular-weight fraction from the fruiting bodies of *G. lucidum*, and the neutral and alkaline subfractions from the methanolic extract might contain small molecular weight polysaccharides (61).

Epstein-Barr Virus

Virus-induced carcinogenesis is considered a complicated process with multiple steps involving a number of cellular signaling pathways. A few polyoxygenated lanostanoid triterpenes isolated from *G. applanatus* inhibited the 12-O-tetradecanoylphorbol-13-acetate induced Epstein-Barr virus early antigen in Raji cells. Similar effects have been observed with *Zingiberaceae rhizomes*, a commonly used traditional medicine in Malaysia. These results indicate that herbal medicines, such as *Ganoderma* species, may behave as antitumor promoters (17,62).

Other Viruses

The antiviral effects of two water-soluble substances (GLhw and GLlw) and eight methanol-soluble substances (GLMe-1–8) isolated from the carpophores of *G. lucidum*, were investigated on influenza A virus strains and vesicular stomatitis virus Indiana and New Jersey in vitro. These activities were evaluated by the cytopathic effect inhibition assay and plaque reduction assay using Vero and HEp-2 cells. Five substances, GLhw, GLMe-1, -2, -4, and -7 significantly inhibited the cytopathic effects of vesicular stomatitis virus. GLMe-4 did not exhibit cytotoxicity up to 1000 µg/mL, while it displayed potent antiviral activity on the vesicular stomatitis virus New Jersey strain with a therapeutic index of more than (5,44,60,62–64).

CLINICAL STUDIES

Reishi has now become recognized as an alternative adjuvant in the treatment of leukemia, carcinoma, hepatitis, and diabetes (2,8,9,14–19,26,31). Clinical studies, to date, lack the controls needed to make a scientific assessment

of its efficacy in a given application, a situation expected to change with increasing interest from Western scientific communities. It was only since the last decade that clinical trials on the use of *G. lucidum* preparation used to treat cancer and other diseases have been reported in international peer-reviewed journals (62,64).

Cancer

In clinical studies, *G. lucidum* products have been widely used as a single agent or in combination with other herbal medicines or chemotherapeutic drugs for many years, mainly in Asian countries. However, randomized, placebo-controlled and multicancer clinical studies using *G. lucidum* alone have rarely been reported.

G. lucidum as a Single Agent

In a randomized, placebo-controlled clinical study, 143 patients with advanced previously treated cancer were given an oral *G. lucidum* polysaccharide extract (Ganopoly) of 1800 mg three times daily for 12 weeks (16). Twenty-seven patients were not assessable for response and toxicity, because they were lost in the follow-up or refused further therapy before the 12 weeks of treatment. Of the 100 fully assessable patients, 32.2% had progressive disease before or at the 6-week evaluation point (range: 5 day–6 wk).

Sixteen subjects developed progressive disease between 6 and 12 weeks of therapy. No objective (partial or complete) responses were observed, but 26.6% had stable disease for 12 weeks or more (range: 12–50 wk). There was no significant change in the Functional Assessment of Cancer Therapy-General (FACT-G) scores in 85 assessable patients. However, palliative effects on cancer-related symptoms, such as sweating and insomnia, have been observed in many subjects. In the group with stable disease, FACT-G scores improved in 23 patients, were unchanged in five, and declined in one. Within this group, the median change from the baseline score to the 6- and 12-week score was +7.6 and +10.3, both statistically significant ($P < 0.05$). For the 38 patients with stable disease (SD), the median change from the baseline score was 28.1 ± 10.2 weeks. The prostate-specific antigen (PSA) levels in the five prostate cancer patients were reduced significantly ($P < 0.05$) during SD. Ganopoly was well tolerated with five moderate adverse events recorded. The results indicate that Ganopoly may have an adjunct role in the treatment of patients with advanced cancer although objective responses were not observed in this study.

G. lucidum-Containing Herbal Mixture: PC-SPES

Several recently published reports have found that *G. lucidum* or *G. lucidum*-containing herbal mixtures (PC-SPES) had biological activities (e.g., cancer biomarker alteration) and beneficial effects (e.g., palliative effects in cancer patients), although striking objective responses were not observed (16,65). PC-SPES has been used as an alternative in the treatment of prostate cancer (65). Several clinical trials have been completed with patients having advanced prostate cancer (66,67). Small et al. (67) included 70 subjects with androgen-dependent ($n = 33$) and androgen-independent ($n = 37$) disease, which was refractory to surgery, radiotherapy, and hormone therapy. Treatment of PC-SPES at a dose of three capsules (320-mg cap) orally resulted in $\geq 80\%$ decrease in PSA levels in all 32 patients

with androgen-dependent cancer, while it was undetectable in 26 patients (81%). The median duration of PSA response was 57 weeks. In the 35 patients with androgen-independent cancer, 19 (54%) had a PSA decrease of $\geq 50\%$ with median duration of PSA response of 18 weeks. The study by Pfeifer et al. (66), which included only 16 patients with androgen-independent disease for just a 20-week followup, showed an improvement in quality of life for the patients. PC-SPES was generally well tolerated by prostate cancer patients, but they exhibited a dose-dependent toxicity similar to that of diethylstilboestrol (67). Side effects include reduced libido, hot flashes, diarrhea, dyspepsia, leg cramps, nipple tenderness, and gynecomastia (66,67). More life-threatening adverse events are pulmonary emboli in 4% to 5% of patients and deep vein thrombosis in 2% of patients. Overall, the clinical responses to PC-SPES compare favorably with second-line hormonal therapy with agents, such as estrogens and ketoconazole (58,62). However, it must be noted that the adulteration of PC-SPES products has become a serious problem. Further details may be obtained at the website of the NIH National Center for Complementary and Alternative Medicine at <http://nccam.nih.gov/health/alerts/spes/>.

Complement System

A clinical study in elderly patients with insomnia and palpitations recently showed that taking *G. lucidum* essence for four to six weeks increased their serum C3 levels (1,2,21,62).

Dosage Forms

G. lucidum is usually prescribed in various forms. It may be injected as a solution of powdered spore. It may be ingested as a soup, syrup, tea, tablets, capsules, tincture, or bolus (powdered medicine in honey). The dose in tincture form (20%) is 10 mL three times daily, that of tablet is one g tablet three times daily, and syrup is 4 to 6 mL/day. As an antidote for ingestion of poisonous mushrooms, dried *G. lucidum* (120–200 g) is decocted in water and given as a drink three to five times daily (1,2,5,9).

Safety Profile

Contraindications. None known.

Drug Interactions

Because Reishi potentiates the immune system, caution is advised for those receiving immunosuppressive therapies.

Side Effects

In oral treatments, some patients, when initially taking a powder extract of Reishi, have experienced temporary symptoms of sleepiness, thirst, rashes, bloating, frequent urination, abnormal sweating, and loose stools (49,62). Large oral doses of vitamin C taken at the same time as Reishi powder extract reportedly counteracted loose stools (2,8,9,49,62).

The inhibition of platelet aggregation by *G. lucidum* (1,2,9) may present an additive effect in those taking blood-thinning medications such as daily aspirin or warfarin.

Synergistic antimicrobial activity was shown with an aqueous extract of *G. lucidum* in combination with cefazolin against *Klebsiella oxytoca* ATCC 8724 and *Bacillus subtilis* ATCC 6603, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25933, and *Salmonella typhi* ATCC 6509 (56).

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Riboflavin

Richard S. Rivlin

INTRODUCTION

New metabolic roles for riboflavin are continually emerging since its discovery in the early part of the 20th century (1–3). We are now defining the mechanisms of action of riboflavin at the molecular, physiological, and clinical levels. In particular, we are beginning to appreciate how riboflavin may play a role in pathogenesis of chronic diseases, such as cancer, cardiovascular disease, metabolic bone disorders, inflammation, and infections.

BACKGROUND

The structure of riboflavin was identified (4,5), its synthesis achieved (6,7), and its coenzyme derivatives described in 1937 (8) and 1938 (9,10). More recently, the role of this vitamin in homocysteine metabolism has become more widely appreciated; acting in concert with folic acid, vitamin B6, and vitamin B12, to lower serum levels of homocysteine (11).

Riboflavin deficiency, when it occurs, has traditionally been attributed to a daily diet that contains inadequate amounts of this vitamin. In our view, insufficient attention has been paid to the many factors, both physiological and pathological, that influence the utilization of this vitamin in health and disease. Thus, a physiological state of riboflavin deficiency can result from the effects of certain drugs, hormones, and other factors in addition to a poor diet. Newer aspects of this subject are reviewed in this chapter.

BIOCHEMISTRY AND FUNCTIONS

Chemically, riboflavin is 7,8-dimethyl-10-(1'-D-ribityl)-isoalloxazine. The isoalloxazine ring is a planar structure that is also shared by the two major coenzyme derivatives formed from riboflavin, namely flavin mononucleotide or riboflavin-5'-phosphate (FMN) and flavin adenine dinucleotide (FAD). These structures are shown in Figure 1.

A small proportion of the flavin coenzymes are linked covalently with tissue proteins (12), including some vital enzymes, such as sarcosine dehydrogenase, succinic dehydrogenase, and monoamine oxidase. Unlike humans, who cannot synthesize ascorbic acid from its precursors, some species of mammals have large amounts of the microsomal ascorbic acid synthesizing enzyme, L-gulonolactone oxidase, which contains covalently bound flavins.

The sequence of events in the synthesis of the flavin coenzymes from riboflavin is that the first biosynthetic enzyme, flavokinase, catalyzes the initial phosphorylation of riboflavin by ATP to FMN (Fig. 2). A fraction of FMN is directly utilized in this form as a coenzyme. The largest fraction, however, combines with a second molecule of ATP to form FAD, the predominant tissue flavin, in a reaction catalyzed by FAD synthetase, also known as FAD pyrophosphorylase. The covalent attachment of flavins to specific tissue proteins occurs after FAD has been synthesized (13,14). A sequence of phosphatases reconverts FAD to FMN and FMN, in turn, to riboflavin (15). Most flavoproteins utilize FAD rather than FMN as coenzyme for a wide variety of metabolic reactions. Microsomal nicotinamide adenine dinucleotide phosphate-cytochrome P450 reductase is highly unusual in containing both FMN and FAD as coenzymes in equimolar ratios.

Riboflavin is yellow and has a high degree of natural fluorescence when excited by UV light, a property that can be utilized conveniently in its assay. There are a number of variations in structure of the naturally occurring flavins. Riboflavin and its coenzymes are sensitive to alkali and acid, particularly in the presence of UV light. Under alkaline conditions, riboflavin is photodegraded to yield lumiflavin (7,8,10-trimethylisoalloxazine), which is inactive biologically. Riboflavin is degraded by light to form lumichrome (7,8-dimethylalloxazine) under acidic conditions, a product that is also relatively inactive (10).

Thus, an important physical property of riboflavin and its derivatives is their sensitivity to UV light, resulting in rapid inactivation. Therefore, prolonged phototherapy of neonatal jaundice and of certain skin disorders may promote the development of systemic riboflavin deficiency. The structure–function relationships of the various biologically active flavins have been comprehensively reviewed (14). One physical property of riboflavin needs emphasis, namely, its very limited water solubility, which greatly restricts its use as a parenteral supplement and as an oral supplement as well.

The flavin coenzymes, FMN and FAD, as well as the fraction of flavins bound covalently to tissue proteins, function in a wide array of processes in intermediary metabolism, most notably in oxidation–reduction reactions. FAD is an inherent component of the respiratory chain and therefore is closely involved in the generation of energy. Flavin coenzymes participate in drug and steroid metabolism together with the cytochrome P450 enzymes. Flavins have critical roles in fat metabolism. One-electron transfers and two-electron transfers from substrate to FMN and FAD constitute the major redox functions of these flavin coenzymes (10). Other reactions

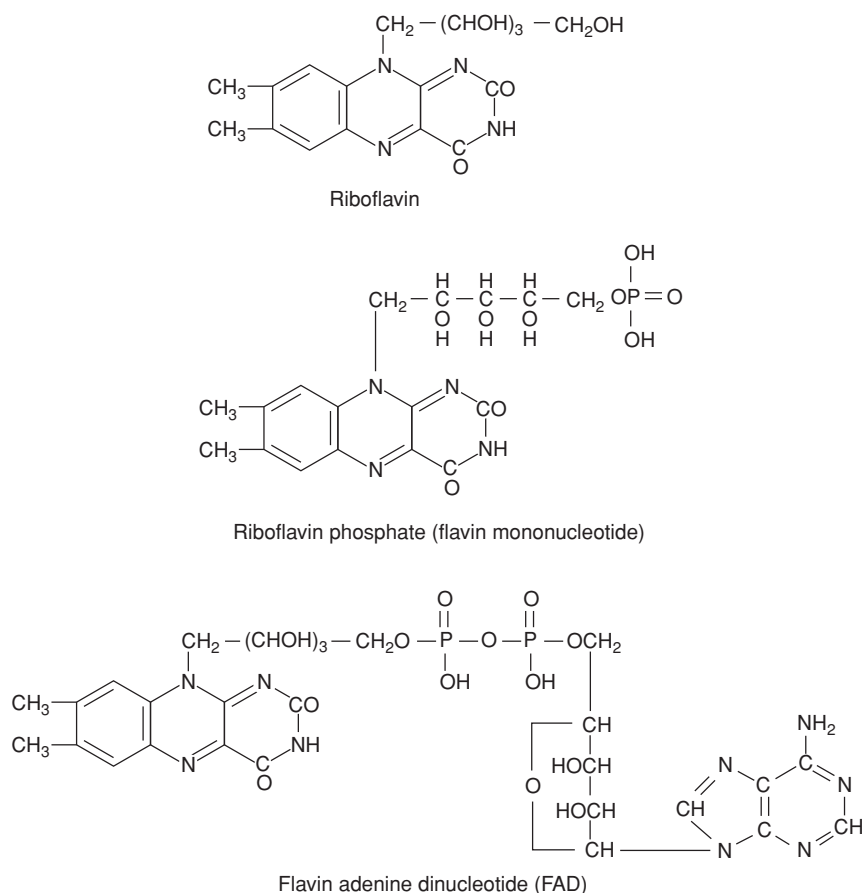


Figure 1 Structures of riboflavin, riboflavin-5'-phosphate (flavin mononucleotide, FMN), and flavin adenine dinucleotide (FAD).

catalyzed by flavoproteins include dehydrogenation, oxidative decarboxylation, dioxygenation, and reduction of oxygen to hydrogen peroxide.

RIBOFLAVIN DEFICIENCY

With the onset of riboflavin deficiency, there are a number of metabolic adaptations that occur to conserve the limited

reserves. One of the adaptations is a fall in the small hepatic pool of free riboflavin to nearly undetectable levels, with a relative sparing of the pools of FMN and FAD (16). These coenzymes are needed to fulfill critical metabolic functions, whereas the vitamin itself has little biological activity.

Another adaptation to deficiency in its early stages is an increased de novo synthesis of reduced glutathione (GSH) from its amino acid precursors (16). This effect

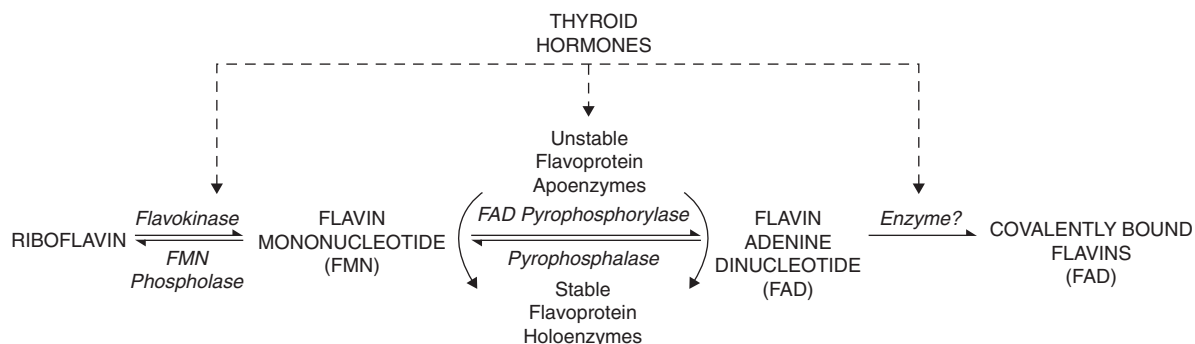


Figure 2 Sequence of events in the formation of FMN, FAD, and covalently bound flavins from riboflavin and its regulation by thyroid hormones. Thyroid hormones stimulate the activity of flavokinase and FAD pyrophosphorylase as well as the formation of covalently bound flavins. The combination of unstable apoenzymes with their flavin cofactors converts them into stable flavoprotein holoenzymes.

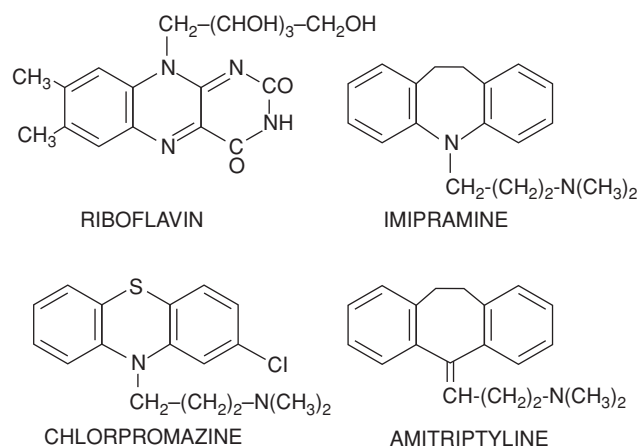


Figure 3 Structural similarities among riboflavin, chlorpromazine, imipramine, and amitriptyline.

may occur in response to the diminished reconversion of oxidized glutathione (GSSG) to its reduced form. In riboflavin deficiency, the activity of glutathione reductase, a key FAD-requiring enzyme, is greatly lessened. Reduced glutathione levels may be maintained if the increased capacity to synthesize GSH *de novo* is adequate to meet the mounting needs.

There is increasing evidence for the emerging concept that dietary inadequacy is not the only cause of deficiency and that certain endocrine abnormalities, such as adrenal and thyroid hormone insufficiency, certain drugs, and diseases may interfere significantly with vitamin utilization (17,18). Psychotropic drugs, such as chlorpromazine, antidepressants (including imipramine and amitriptyline (19)), cancer chemotherapeutic drugs (e.g., adriamycin), and some antimalarial agents (e.g., quinacrine (20)) impair riboflavin utilization by inhibiting the conversion of this vitamin into its active coenzyme derivatives. Figure 3 shows the structural similarities among riboflavin and the psychoactive drugs imipramine, chlorpromazine, and amitriptyline.

Riboflavin deficiency commonly occurs in patients who abuse alcohol chronically. Alcohol causes shortage of the vitamin by inhibiting both its digestion from dietary sources, which are largely in the form of FAD, and its intestinal absorption (Fig. 4) (21). These findings suggest that improvement of the riboflavin nutrition of alcoholics can be accomplished more rapidly and effectively by administering vitamins in pure form, as in supplements, rather than entirely from food sources. Furthermore, in riboflavin-deficient animals, decreased GSH concentrations as well as decreased activities of GSH peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase occur. These findings strongly indicate that the combination of riboflavin deficiency and alcohol administration not only lowers hepatic GSH concentrations, but also inhibits enzymes controlling GSH metabolism and therefore may intensify the hepatic injury induced by excessive alcohol consumption. The consequences of a poor diet in a patient abusing alcohol may be exacerbated by the use of certain drugs for prolonged periods.

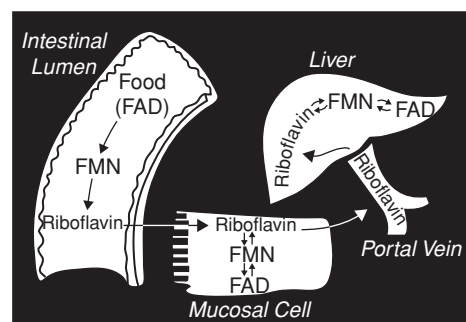


Figure 4 Digestion of food sources of flavins to riboflavin, which is transported across the intestinal mucosa and rephosphorylated in the mucosal cell. Riboflavin bound to serum proteins is transferred to the liver, where it is rephosphorylated to FMN and FAD.

In experimental animals, hepatic architecture is markedly disrupted in riboflavin deficiency. Mitochondria in riboflavin-deficient mice increase greatly in size, and cristae increase in both number and size (22). These structural abnormalities may disturb energy metabolism by interfering with the electron transport chain and metabolism of fatty acids. Villi decrease in number in the rat small intestine; villus length increases, as does the rate of transit of developing enterocytes along the villus (23). These findings of structural abnormalities together with accelerated rate of intestine cell turnover (24) may help to explain why dietary riboflavin deficiency leads to both decreased iron absorption and increased iron loss from the intestine.

Riboflavin deficiency has many other effects on intermediary metabolism, particularly on lipid, protein, and vitamin metabolism. Of particular relevance to vitamin metabolism is the fact that the conversion of vitamin B6 to its coenzyme derivative, pyridoxal-5'-phosphate, may be impaired (25). Riboflavin deficiency has been studied in many animal species and has several consequences, foremost of which is failure of growth. Additional effects include loss of hair, skin disturbances, degenerative changes in the nervous system, and impaired reproduction. Congenital malformations occur in the offspring of female rats that are riboflavin deficient. The conjunctiva becomes inflamed, the cornea is vascularized and eventually opaque, and cataract may result (26).

Changes in the skin consist of scaliness and incrustation of red-brown material consistent with changes in lipid metabolism. Alopecia may develop, lips become red and swollen, and filiform papillae on the tongue deteriorate. During late deficiency, anemia develops. Fatty degeneration of the liver occurs. Important metabolic changes occur, so that deficient rats require 15% to 20% more energy than control animals to maintain the same body weight. In all species studied to date, riboflavin deficiency causes profound structural and functional changes in an ordered sequence. Early changes are very readily reversible. Later anatomical changes, such as formation of cataract, are largely irreversible despite treatment with riboflavin (26).

Clinically, riboflavin deficit is not detectable at the bedside by any unique or characteristic physical features. The classical symptoms of glossitis, angular stomatitis,

and dermatitis are not specific to riboflavin deficiency and may be observed in other vitamin deficiencies as well. When dietary deficiency of riboflavin occurs, it is almost invariably associated with multiple nutrient deficits (27).

The syndrome of dietary riboflavin deficiency in humans has many similarities to that in animals. Until recently, there was one notable exception. The spectrum of congenital malformations observed in rodents with maternal riboflavin deficiency had not been clearly identified in humans (28). It now appears from several independent reports that riboflavin deficiency in humans is indeed associated with congenital abnormalities. The National Birth Defects Prevention Study, which evaluated 324 newborn infants who had transverse limb deficiency, concluded that inadequate maternal dietary riboflavin intake is a risk factor (29). Furthermore, Smedts et al. (30) reported that reduced maternal dietary intake of riboflavin and nicotinamide increases the risk of congenital heart disease.

Antioxidant Potential

As a precursor to FMN and FAD, riboflavin is a significant contributor to antioxidant activity. Riboflavin itself has little inherent antioxidant action, but the glutathione redox cycle (31) has a major protective role against lipid peroxides. Glutathione peroxidase degrades reactive lipid peroxides. This enzyme requires GSH as a substrate, which is regenerated in vivo by reduction from its oxidized form (GSSG) by glutathione reductase, a well-recognized FAD-containing enzyme, as noted above.

It is for this reason that riboflavin deficiency is expected to lead to reduced antioxidant defense capabilities, as has been demonstrated in several studies (32–34). Increased lipid peroxidation has been reported in experimental riboflavin deficiency, with a return towards normal after supplementation with this vitamin (32,35). Both basal and stimulated lipid peroxidation are increased in deficiency of the vitamin (36).

Furthermore, the reducing equivalents provided by nicotinamide adenine dinucleotide phosphate, the other substrate required by glutathione reductase, are primarily

generated by an enzyme of the pentose monophosphate shunt, glucose-6-phosphate dehydrogenase. Taniguchi and Hara (34), as well as our laboratory (35), have found that the activity of this enzyme is significantly diminished during riboflavin deficiency. This observation provides an additional mechanism to explain the diminished glutathione reductase activity in vivo during riboflavin deficiency and the eventual decrease in antioxidant capacity (36).

By sensitizing cells to light, riboflavin may intensify the tissue damage caused by solar radiation. One possible mechanism for this effect may be that UV-A radiation together with administration of riboflavin was reported to increase gene mutations sevenfold compared to UV-A radiation without added riboflavin (37).

Homocysteine Metabolism

There is much contemporary interest in the increasingly persuasive evidence that homocysteine has a role in the pathogenesis of vascular disease, including cardiovascular, cerebrovascular, and peripheral vascular disorders (11,38). A simplified sequence of homocysteine metabolism is shown in Figure 5, illustrating the sites of action of vitamins B₆ and B₁₂, folic acid, and riboflavin. Blood levels of folic acid sensitively determine serum homocysteine concentrations (39). *N*-5-Methyltetrahydrofolate is a cosubstrate with homocysteine in its inactivation by conversion to methionine. Methylcobalamin is also a coenzyme in this enzymatic reaction. Vitamin B₆ is widely recognized for its importance in the inactivation of homocysteine by serving as coenzyme of two degradative enzymes, cystathionine-β-synthase and cystathioninase.

However, in our view, there is insufficient appreciation of the fact that riboflavin also plays a vital role in homocysteine metabolism. The flavin coenzyme FAD is required by methylenetetrahydrofolate reductase, the enzyme responsible for converting *N*-5,10-methylenetetrahydrofolate to *N*-5-methyltetrahydrofolate. Thus, the efficient utilization of dietary folic acid requires adequate riboflavin nutrition. As expected, therefore, riboflavin deficiency reduces the activity of

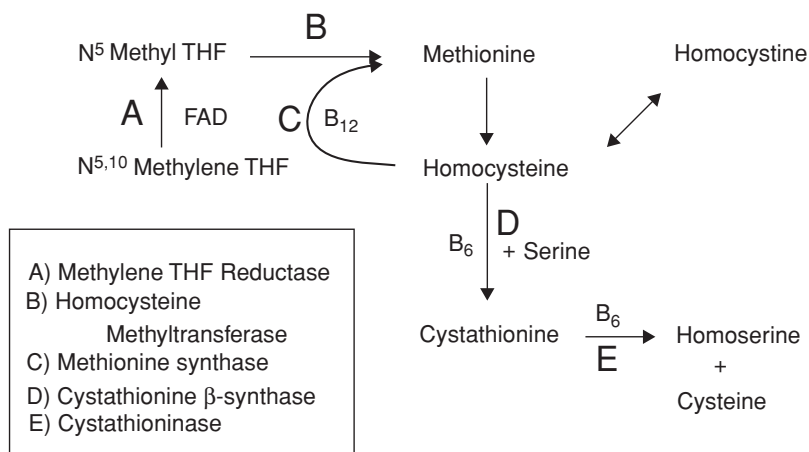


Figure 5 Simplified representation of homocysteine metabolism to illustrate the sites of action of vitamins B₆, B₁₂, folic acid, and riboflavin.

methylenetetrahydrofolate reductase and inhibits folic acid metabolism in rats (40). As a consequence of this effect, plasma homocysteine levels increase (41). In a large cohort of subjects in the Framingham Offspring Study, the more deficient the individual, as measured by the erythrocyte glutathione reductase activity coefficient (EGRAC), the higher the serum homocysteine concentration, particularly in those with compromised folate status (42).

It is relevant to note in this context that there is a genetic variant of the methylenetetrahydrofolate reductase gene (677→T) that is common in the Caucasian population (43). Individuals homozygous (TT) for this gene have approximately half the normal activity of the enzyme and are predisposed to develop elevated serum concentrations of homocysteine (41). It is of interest that a group of investigations found an inverse correlation between plasma homocysteine and plasma riboflavin in individuals both with and without the genetic variation (44). Further research is required to determine whether the serum levels of homocysteine and the prevalence of vascular disease can be correlated directly with indices of marginal as well as overt riboflavin deficiency.

Elevated serum levels of homocysteine in both hypothyroid rats (13) and hypothyroid humans are likely due to diminished conversion of dietary riboflavin to its coenzyme derivatives, flavin mononucleotide and flavin adenine dinucleotide. Treatment of hypothyroid adults with thyroid hormones without increasing dietary riboflavin intake completely corrects these defects (18).

Fat Metabolism

The vital role of riboflavin in fat metabolism has been highlighted by demonstrations that in certain rare inborn errors, administration of the vitamin may be therapeutic. In acyl-CoA dehydrogenase deficiency, infants present with recurrent hypoglycemia, lipid storage myopathy, and increased urinary excretion of organic acids. Clinical improvement has occurred rapidly after riboflavin supplementation (45,46). Three varieties of the disorder occur, all of which involve flavoproteins of various types. Several patients with a mitochondrial disorder associated with NADH dehydrogenase deficiency showed improvement with riboflavin treatment (47).

In patients with HIV infection, riboflavin administered together with thiamin has been noted to prevent elevation of levels of lactic acid and lactic acidosis (48). In other studies of patients with HIV, riboflavin has potential in managing lactic acidosis induced by treatment of the underlying disease with nucleoside analogs (49,50). In addition, riboflavin has been beneficial to cases of ethylmalonic acid encephalopathy (51). Therefore, this vitamin as a supplement may have a role in the management of certain rare inborn errors of fat metabolism.

Anemia

Anemia is a characteristic feature in many vitamin deficiencies and it is usually multifactorial in pathogenesis. Nevertheless, there appears to be a relatively specific anemia occurring in riboflavin-deficient individuals that responds to supplementation with the vitamin (52). The anemia is associated with erythroid hypoplasia, and as a consequence, there is diminished reticulocytosis.

A major effect of riboflavin deficiency in the pathogenesis of anemia appears to be that on iron metabolism (53). It influences tissue mobilization of iron from ferritin, particularly in the gastrointestinal mucosa (54). In addition, it now appears that the deficiency leads to a decrease in the intestinal absorption of the element (55) as well as an increase in its loss from the gastrointestinal tract (56).

These mechanisms have been elucidated in rodents. Their relevance to the pathogenesis of anemia in humans remains to be firmly established. It is evident, nevertheless, that riboflavin supplementation does improve hematological function in humans, and likely has an effect on improving the metabolism of iron (53).

Carcinogenesis

More recent studies (57) confirm earlier reports (58) that riboflavin deficiency may favor cancer formation by increasing the activation of certain carcinogens, particularly nitrosamines. The flavin vitamin may possibly provide protection against the damage to DNA caused by a number of carcinogens through its action as a coenzyme with a variety of cytochrome P450 enzymes. Furthermore, deficiency enhances the covalent binding of carcinogens to DNA (59).

It is important to establish more firmly the potential role of riboflavin as a dietary factor capable of preventing carcinogenesis while at the same time determining the full implications of the photosensitizing actions of flavins on mutagenesis and carcinogenesis. There are reports from China (60,61) and Russia (62) raising the possibility that deficient riboflavin nutritional status, together with shortages of other vitamins, may possibly enhance development of precancerous lesions of the esophagus.

Because of their photodynamic actions, flavins may have potential efficacy as adjuncts in cancer treatment. Blue light has been reported to inhibit the proliferation of B16 melanoma cells grown in culture, as well as those transplanted to rodent models (63). It has both cytostatic and cytotoxic properties.

Riboflavin is the only vitamin that has been observed to increase the degree of cell necrosis induced by blue light. The efficacy of riboflavin is concentration dependent and antagonized by catalase. Ohara et al. have postulated that riboflavin, by reacting with blue light to form active oxygen intermediates, may cause a greater degree of cell necrosis than blue light alone (63).

Large-scale studies (64,65) have largely been disappointing in demonstrating benefits of multivitamin or multimineral supplements on cancer prevention. Nevertheless, there have been some intriguing reports that merit further study. DeSouza Quiros et al. in a preliminary report (66) observed an antiproliferative and antimetastatic effect of irradiated riboflavin in solid tumors.

Other investigators have reported modifications in the riboflavin carrier protein in human prostate cancer (67) and proposed a role for vitamin-binding proteins in prostate cancer (68). This group of scientists has applied for a patent for treatment of prostate cancer with antagonists of riboflavin-binding protein (69).

These considerations suggest that riboflavin deficiency may possibly play a role in carcinogenesis. Clearly, more research needs to be done before this vitamin can

be recommended for cancer prevention for populations at risk.

ASSESSMENT AND DIETARY RECOMMENDATIONS

A variety of methods are available for the analysis of riboflavin and its coenzyme derivatives. Fluorometric procedures take advantage of the inherent fluorescent properties of flavins (70). Some degree of purification of the urine or tissues may be required before analysis is undertaken. There is often significant interference by other natural substances that leads to quenching of fluorescence and methodological artifacts. Riboflavin can be measured by competitive protein binding which is applicable to studies in human urine (71). Riboflavin binds specifically to the avian egg white riboflavin-binding protein (72) and thereby provides the basis for quantitative analysis. Currently, procedures using high-performance liquid chromatography have been widely applied as they have a high degree of precision and can be utilized for the analysis of riboflavin in pure form as well as in biological fluids and tissues (73). High-performance liquid chromatography is the method most widely employed for the determination of flavins in the blood and in other tissues.

In clinical studies that involve individual patients as well as population groups, the status of riboflavin nutrition is generally evaluated by determining the urinary excretion of riboflavin and EGRAC (74). Urinary riboflavin determinations may be done in the basal state, in random samples, in 24-hour collections, or after a riboflavin load test. Normal excretion in the urine is approximately 120 $\mu\text{g/g}$ creatinine per 24 hour or higher (74). It is useful to express the value in terms of creatinine to verify the completeness of the collection and to relate excretion to this biological parameter. Expressed in terms of the total amount, riboflavin excretion in the normal adult is about 1.5 to 2.5 mg/day, which is very close to the recommended dietary allowance (RDA) of the National Academy of Sciences (75). In deficient adult individuals, outflow with urine is reduced to about 40 $\mu\text{g/g}$ creatinine per 24 hours.

Individuals deficient in riboflavin have reduced urinary excretion, reflecting diminished dietary intake and depleted body stores. Excretion is reduced with age and is stimulated by elevated body temperature as well as treatment with certain drugs, and by various stressful conditions associated with negative nitrogen balance (74). Data from urine analysis must therefore be interpreted with these factors in mind. Another potential drawback to utilizing urinary riboflavin excretion as an assessment of nutritional status of this vitamin is that the amount excreted reflects recent intake very sensitively. Thus, if an individual has been depleted for a long time but consumes food items high in riboflavin, the level of the vitamin in urine as determined a few hours later may not be in the deficient range, but is likely to be normal or even elevated.

It is important, therefore, to utilize assessment techniques that more accurately reflect long-term riboflavin status. The method most widely employed and that largely meets these needs is assay of EGRAC. The principle of the method is that the degree of saturation of the apoenzyme with its coenzyme, FAD, reflects the body stores of the latter. In deficient individuals, relative un-

saturation of the apoenzyme leads to decreased basal activity of the enzyme. Therefore, the addition of FAD to the enzyme contained in a fresh erythrocyte hemolysate from deficient individuals will increase activity in vitro to a greater extent than that observed in preparations from well-nourished individuals, in whom the apoenzyme is relatively more saturated with the coenzyme. The EGRAC is the ratio of enzyme activity with, to that without, addition of FAD in vitro. In general, most studies indicate that an activity coefficient of 1.2 or less indicates adequate riboflavin status, 1.2 to 1.4 borderline-to-low status, and greater than 1.4 riboflavin deficiency (74).

It must be kept in mind that a number of physiological variables influence the results of this determination as well. In the inherited disorder of glucose-6-phosphate dehydrogenase deficiency associated with hemolytic anemia, the apoenzyme has a higher affinity for FAD than that of the normal erythrocyte, which will affect the measured EGRAC. Thyroid function affects glutathione reductase activity, the coefficient being elevated in hypothyroidism (75). This disorder has many biochemical features in common with those of riboflavin deficiency, as discussed earlier (75).

The RDA for riboflavin issued by the Food and Nutrition Board (76) calls for adult males aged 31 to 50 years to consume 1.3 mg/day and those aged 51 to 70 years, 1.1 mg/day. Adult females aged 31 to 50 years should consume 1.1 mg/day and the same by women aged 51 to 70 years. It is recommended that in women aged 19 to 50 years, intake be increased to 1.4 mg/day during pregnancy and 1.6 mg/day during lactation.

There has been some concern as to whether these figures are applicable to other population groups around the world. The Chinese tend to excrete very little riboflavin, and their RDA may be lower than that of Americans (77). Adults in Guatemala appear to have a similar RDA in individuals older than 60 compared to those 51 years or younger (78). This finding may not necessarily be relevant to populations of other countries. The RDAs of various national groups require further study. Environmental factors, protein-calorie intake, physical activity, and other factors may have an impact on riboflavin status. More research is needed on the requirements of the extremely old, who form an increasingly large proportion of the population. They are also the group that consumes the largest number of prescribed and over-the-counter medications.

A point of interest is whether riboflavin requirements are elevated in individuals who exercise compared to those who are sedentary. In women aged 50 to 67 years who exercised vigorously for 20 to 25 min/day, 6 days a week, both a decrease in riboflavin excretion and a rise in the EGRAC were noted, findings consistent with a marginal riboflavin-deficient state (79). Supplementation with riboflavin did not, however, improve exercise performance. These investigators observed compromised riboflavin status in young women exercising vigorously as well (80). Similarly reduced urinary riboflavin excretion and elevated EGRAC were observed in young Indian males who exercised actively (81).

To determine whether the status of riboflavin nutrition influences metabolic responses to exercise, blood lactate levels were determined in a group of physically active college students from Finland before and after the exercise

period. A number of the students were initially in a state of marginal riboflavin deficiency. Following supplementation with vitamins, including riboflavin, that produced improvement in the elevated EGRAC, the blood lactate levels were unaffected and were related only to the degree of exercise (82).

Thus, to date, while it is known that exercise may lead to biochemical abnormalities in riboflavin metabolism, it has not been shown that these abnormalities lead to impaired performance; nor has it been shown that riboflavin supplementation improves exercise performance.

SAFETY AND ADVERSE EFFECTS

There is general agreement that dietary riboflavin intake at many times the RDA is without demonstrable toxicity (10,83,84). Because riboflavin absorption is limited to a maximum of about 25 mg at any one time (10), the consumption of megadoses of this vitamin would not be expected to increase the total amount absorbed. Furthermore, classical animal investigations showed an apparent upper limit to the tissue storage capacity of flavins that cannot be exceeded under ordinary circumstances (85). This storage capacity is probably limited by the availability of proteins providing binding sites for flavins. These protective mechanisms prevent tissue accumulation of excessive amounts of the vitamin. Because riboflavin has very low solubility, even intravenous administration of the vitamin would not introduce large amounts into the body. FMN is more water soluble than riboflavin but is not ordinarily available for clinical use.

Nevertheless, the photosensitizing properties of riboflavin raise the possibility of some potential risks. Phototherapy in vitro leads to degradation of DNA and increase in lipid peroxidation, which may have implications for carcinogenesis and other disorders. Irradiation of rat erythrocytes in the presence of FMN increases potassium loss (86). Topical administration of riboflavin to the skin may increase melanin synthesis by stimulation of free-radical formation. Riboflavin forms an adduct with tryptophan and accelerates its photo-oxidation (87). Further research is needed to explore the full implication of the photosensitizing capabilities of riboflavin and its phosphorylated derivatives.

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Saw Palmetto

Edward M. Croom and Michael Chan

INTRODUCTION

Saw palmetto fruit extracts are frequently consumed for relief of the lower urinary tract symptoms (LUTS) associated with benign prostatic hyperplasia (BPH). Clinical trials of saw palmetto for symptom relief of LUTS have yielded mixed results that have been attributed to product differences and clinical trial design concerns. Multiple clinical trials of extracts of saw palmetto have been shown to be superior to placebo and to have fewer side effects than α -blocker and 5 α -reductase inhibitor drugs for relief of symptoms in men with mild-to-moderate BPH. Because there are chemical differences in saw palmetto products and variations in the scientific substantiation including efficacy of products to improve prostate health, consumers and health care professionals must learn the scientific basis for their safety and effectiveness to make an informed product choice. When evaluating the totality of the scientific evidence including observational trials and small trials with limited information on the quality of the trial design, saw palmetto appears to have few safety concerns for any serious side effects and to be more beneficial than watchful waiting or placebo for relief of LUTS associated with BPH.

BACKGROUND

Serenoa repens (W. Bartram) Small (Arecaceae; also known as Palmaceae, Palmae) is commonly known as Saw palmetto. Botanical synonyms include *Corypha repens* W. Bartram; *Brahea serrulata* (Michaux) H. Wendland; *Chamaerops serrulata* Michaux; *Corypha obliqua* W. Bartram; *Sabal serrulata* (Michaux) Nuttall ex Schultes & Schultes f.; *Serenoa serrulata* (Michaux) G. Nicholson.

The stems of the palm are usually prostrate, branched, and sometimes upright to a length of 3 m or more. The stiff, fan-shaped leaves range in color from yellow green to green and grayish green to silver green (Fig. 1). The saw-toothed (serrate) petioles are from 0.5 to 1 m long and have fine to coarse teeth that account for the common name of this shrub-like, branching palm. The flower stalks are approximately the same length as the petioles. The small (4–5 mm), fragrant, spring flowers are creamy white, with three petals and six stamens. The pulpy, one-seeded fruits ripen from green to orange to black or bluish black (Fig. 2). Mature fruits are approximately 2 cm long and 1 cm in diameter, with some fruits being similar in shape and size to commercial black olives, having a large hard

seed inside the pulp. Flowering and fruit production are highly variable each year; most fruits mature in August and September. Saw palmetto is an important wild plant providing food and cover for many animals. The fruits are consumed by black bears, deer, raccoons, foxes, opossums, fish, and many species of birds (1–3).

Ecological Distribution

Saw palmetto is endemic to the southeastern United States. The native range is from the coastal plain of south-east South Carolina to Georgia, throughout the state of Florida, including the Florida Keys, and to the coastal plains of Alabama, Mississippi, and southeast Louisiana. Saw palmetto is a major understory plant, sometimes forming dense thickets in pinelands, dunes, sand pine scrub, mesic hammocks, and woodlands. The plant is one of the most abundant in Florida and is reported to be very well adapted to surviving fires (1–3).

Historical Uses

Saw palmetto leaves, stems, roots, and fruits have had a variety of historical uses (3). In the late 1800s, saw palmetto fruits were “lauded as the ‘old man’s friend,’ giving relief from the many annoyances commonly attributed to enlarged prostate.” Felter and Lloyd, writing in the *King’s American Dispensatory* in 1898, commented “We would rather regard it a remedy for *prostatic irritation* and



Figure 1 Saw palmetto leaves.



Figure 2 Saw palmetto fruits. Source: Courtesy of Renato Igüera.

relaxation of tissue than for a hypertrophied prostate.” As authorities of the eclectic school of medicine, they begin their discussion of saw palmetto with “Saw palmetto appears, from clinical reports, to be a nutritive tonic.” Indeed, at that time, saw palmetto was used as a tonic to treat a variety of maladies of the male and female reproductive organs and irritations of the mucous membranes, including coughs due to different medical disorders (4).

Saw palmetto fruits have been used in both traditional and homeopathic medicine for the treatment of urological symptoms associated with prostate hypertrophy. In the United States, at the beginning of the 20th century, alcohol extracts of the fruit made as tinctures and alcohol based fluidextracts were popular remedies for prostatitis and prostate hypertrophy. Although the plant was popular and was listed in the *United States Pharmacopeia* from 1906 to 1916 and in the *National Formulary* from 1926 to 1950, it is the more recent successful development of European pharmaceutical products that has stimulated more detailed studies on its pharmacology and potential mechanisms of action (5,6).

Saw Palmetto and Other Botanical Treatments

Plant-derived agents may account for approximately one third of total sales of all therapeutic agents used for treating BPH in Europe (7). Saw palmetto is sold in the United States as a dietary supplement through many sales channels, including the mass market, mail order, direct sales, and multilevel marketing, as well as specialty retail and health food stores. In 2008, saw palmetto was the fourth leading botanical in total consumer sales through all types of market channels in the United States, with sales of over \$17.4 million (8). In the United States and Europe, other popular phytotherapies and natural remedies include β -sitosterol (from the tuber of the South African plant *Hypoxis rooperi*), a grass pollen mixture (92% rye, 5% timothy, and 3% corn), stinging nettle root, pumpkin seed oil, and *Pygeum* bark (see chapter “*Pygeum africanum* Extract”) (9). Saw palmetto is the most popular botanical used for mild-to-moderate LUTS associated with BPH and has shown an advantage over standard α -blockers and 5 α -reductase

inhibitors of having fewer significant side effects such as impotence, ejaculatory disorders, and dizziness.

CHEMISTRY AND PREPARATION OF PRODUCTS

The dried drupe-like fruit is the plant part that has most frequently been used in traditional and allopathic medicine. The fruit consists of approximately 36% outer rind, 16% flesh, 10% seed shell, and 38% seed. The fleshy part contains large quantities of lipids, starches, polysaccharides, sugars and mannitol, and small quantities of ceramides and sphingolipids. The lipid content has been reported to consist of approximately 75% free fatty acids and 25% neutral fats. During the ripening and drying of the fruit, a lipase splits triglycerides into fatty acids. From the oil obtained by pressing fruits preserved in alcohol, C6–C18 fatty acids have been identified (5). Oleic, lauric, and myristic acids are the predominant ones, while palmitic, caproic, caprylic, and capric acids have been reported in smaller quantities in both 90% ethanol and CO₂ extracts (5,10). The characteristic smell of the oil has been attributed to the secondary formation in the fruit of ethyl esters of several of the fatty acids present (5). A systematic evaluation of the total fatty acids of fruits at different stages of ripeness, the seed, fruit pulp, fruit powder, and extracts, as well as mixtures, has shown that each product has a characteristic fatty acid profile that can be used for identification and standardization of products (11).

Lipophilic extracts of saw palmetto are widely used in BPH therapy. The most documented extracts, from a pharmacological and clinical point of view, are those obtained by two different extraction processes. The first process involves extraction from dried and finely ground fruits with hexane in an inert gas atmosphere and in the presence of an antioxidant such as ascorbic palmitate, and the second process involves extraction with CO₂ in supercritical conditions (5).

In the United States, common saw palmetto products include ethanol and CO₂ extracts. In Germany, fruit extracts obtained with 90% ethanol are very popular, and a hexane extract is a registered pharmaceutical. An analysis of two commercial saw palmetto extracts produced using 90% ethanol versus under an optimized CO₂ condition found that although the relative composition of free fatty acids and total fatty acids was very similar, the absolute percentage of free fatty acids was 69% in the ethanol extract versus 90% in the CO₂ extract (10). A second major difference found was that the ethanol extract contained a large amount of ethyl esters that were not present in the CO₂ extract (5).

A CO₂ extract was found to contain free fatty acids as well as their methyl- and ethyl esters, β -sitosterol, β -sitosterol-3-O- β -D-glucoside, campesterol, stigmasterol, lupeol, cycloartenol, 24-methylene-cycloartanol, long-chain saturated and unsaturated alcohols, including farnesol, phytol, and alcohols with C22, C23, C24, C26, and C28 chain lengths, and polyprenolic alcohols. Carotenoids, which give a marked orange color to the oily extracts, have also been isolated (5). Flavonoids, including rutin, isoquercitrin, kaempferol-3-O-glucoside, and apigenin-7-O-rhamnoglucoside, and anthranilic acid have been reported in alcohol extracts of the fruits.

Polysaccharide fractions from aqueous extracts have shown anti-inflammatory and immunomodulatory activities. Some of the polysaccharides found in saw palmetto are galactose, arabinose, xylose, mannose, rhamnose, glucose, and glucuronic acid (5).

There exists significant chemical variation in different saw palmetto products (12). This chemical variance may be a major cause of the discrepancies observed in results from various clinical studies. For instance, a study by Scaglione et al. demonstrated that the potency of 5 α -reductase inhibition, a proposed mechanism of action of the herbal, varied between brands and even batches of saw palmetto material sold in Italy (13).

PRECLINICAL STUDIES

Pharmacology and Mechanisms of Action

Although BPH is one of the most common diseases in aging men, its etiology is still not completely understood. The factors that cause the imbalance in prostate growth, which produce an enlarged prostate, are mostly unknown. Studies suggest that this imbalance may be related to inflammation and apoptotic mechanisms. Effective treatment of BPH is focused on the relief of the LUTS associated with the condition. The drugs currently used to relieve these symptoms are α -blockers and 5 α -reductase inhibitors. Research concerning potential mechanisms of action for saw palmetto has included investigations into antiandrogenic actions, anti-inflammatory activity, and factors related to prostate cell growth as well as the mechanisms that are generally attributed to α -blockers and 5 α -reductase inhibitors.

Symptom Relief

The use of α -blockers is based on the hypothesis that LUTS are partly caused by α_1 -adrenergic-mediated contraction of prostatic smooth muscle and bladder neck (14). The α -blockers decrease smooth muscle tone and contraction, which reduces bladder outlet obstruction by improving urinary flow through the prostatic urethra (14). A 90% ethanol extract has been shown to reduce norepinephrine-induced contractions of rat deferential duct, and potassium chloride-induced contractions of guinea pig ileum and bladder smooth muscle tissue. Both lipid and saponifiable fractions of saw palmetto reduced the norepinephrine-induced contractions of rat aorta, in vitro as well as potassium chloride-induced contractions of rat uterus. Vanadate-induced contractions of the rat uterus have also been reduced by a lipophilic (90% ethanol) saw palmetto extract (15).

Extracts of saw palmetto have potent noncompetitive inhibition of human prostatic α_1 -adrenoceptors in vitro (16,17). Suzuki et al. reported saw palmetto inhibiting the α_1 -adrenoceptors the prostate and spleen and also the muscarinic receptors in the bladder and submaxillary gland of rats (17). Inhibition of the muscarinic receptors in the bladder causes the detrusor muscle to relax and can reduce the frequency and intensity of contractions of the bladder. Results from a separate experiment by Suzuki et al. showed that organ selectivity was associated with the inhibitory action. Suzuki et al. reported that the muscarinic receptors in the prostate and bladder were prefer-

entially targeted for inhibition. The authors suspect that this specificity is due to saw palmetto extract preferring to accumulate in the prostate to a greater extent compared to other tissues (17,18).

The other class of drug used to treat BPH symptoms is the 5 α -reductase inhibitors. The major action of these drugs is to inhibit the enzymatic conversion of testosterone to dihydrotestosterone (DHT) by 5 α -reductase (14). Although both testosterone and DHT can activate the androgen receptors, DHT is the more potent androgen with a much greater binding affinity (19,20). The activation of the androgen receptors promotes protein synthesis and cellular growth and the overexpression of the two isoenzymes 5 α -reductase has been associated with BPH tissue (19,20).

The peripheral antiandrogenic activity of the saw palmetto extracts has been studied in vivo in mice and prepubertal rats. The animals underwent castration to remove the source of endogenous testosterone and were then given a subcutaneous (SC) injection of the hormone. Saw palmetto extract given orally for 12 days (300 mg/mouse) antagonized the stimulant effect of exogenous testosterone, reducing the weights of the ventral prostate, seminal vesicles, and the preputial glands in comparison to the control mice treated only with testosterone. Rats given saw palmetto extract (200 mg/animal) orally for six days showed similar results. Other organs were not affected (19). The antiandrogenic activity in castrated rats treated orally with a saw palmetto CO₂ extract has been shown to depend on both the temperature and pressure conditions and the dose of the extract (21). Arruzazabala et al. examined the effects oral dosages of saw palmetto, coconut oil, and sunflower oil had on the prostate and body weights of rats receiving a SC injection of testosterone. The authors reported significant inhibition of testosterone-induced prostate hyperplasia in the rats receiving saw palmetto extract and coconut oil at doses of 400 mg/kg but not in rats ingesting sunflower oil (22).

Unlike the synthetic steroidal 5 α -reductase inhibitors such as finasteride, which show much greater inhibitory activity and compete with testosterone for the active androgen binding site at nanomolar concentrations, saw palmetto does not appear to bind directly to these receptors or affect their binding affinities. In vitro and in vivo studies have shown that saw palmetto does not affect serum prostate specific antigen (PSA) levels (23–27). Habid et al. demonstrated that in the presence of both DHT and Permixon, LNCaP cells demonstrated no change in accumulation of PSA protein compared to DHT alone (24). In the presence of DHT and finasteride, PSA secretion was significantly inhibited and intracellular PSA levels were reduced (24). Bayne et al. developed a BPH model that allowed for the observation of PSA secretion and the expression of functional androgen receptors. This model showed that treatment with Permixon at a dose of 10 μ g/ml had no effect on PSA levels or on any other androgen-dependent processes that rely on binding of androgens to their receptors (25).

The antiandrogenic action of saw palmetto is due primarily through the inhibition of the two isoforms of 5 α -reductase, which inhibits the production of DHT. Bayne et al.'s model showed that Permixon was a potent

inhibitor of both isoforms of 5 α -reductase (25). In studies with cultured genital skin fibroblasts, the lipophilic extract of saw palmetto inhibits the enzymatic conversion of testosterone to DHT by 5 α -reductase and of DHT to androstenediol by 3 α -hydroxysteroid dehydrogenase. Both alcohol and CO₂ extracts have also been shown to inhibit 5 α -reductase. Biopsy cores from patients receiving a saw palmetto combination product showed significant reduction in DHT levels, whereas levels in men receiving placebo exhibited no significant change (28). Wadsworth et al. reported that although feeding TRAMP mice with a dose of 50 mg/kg, a dosage considered equivalent to the recommended human dose, did not significantly change prostate DHT levels, a dose of 300 mg/kg did show reduction in prostate DHT levels (29).

The free fatty acids are thought to be responsible for these inhibitory effects. In a study of the 5 α -reductase inhibiting activity of a CO₂ extract and fractions made from it, inhibition was limited to the fatty acid fraction. The activity for the entire extract and the main fatty acid components was comparable (30). A study by Raynaud et al. demonstrated that lauric acid inhibited both 5 α -reductase isoforms, with IC₅₀ of 16.7 μ g/mL for 5 α -reductase 1 and 18.6 μ g/mL for 5 α -reductase 2, whereas the unsaturated acids oleic and linoleic selectively inhibited 5 α -reductase 1 with IC₅₀ of 4 μ g/mL and 13 μ g/mL respectively. Myristic acid was also found to strongly inhibit 5 α -reductase 2 activity with an IC₅₀ of 4.3 μ g/mL (31).

Active 5 α -reductase enzymes are membrane-bound (30). It is suggested that saw palmetto lipids could inhibit the enzymes by changing their microenvironment through disruption of the nuclear membrane (25,30). Bayne et al. reported that Permixon appeared to disrupt the intracellular membranes, including the nuclear and mitochondrial membranes of prostatic epithelial and fibroblast cells (25). Permixon treated cells showed accumulation of lipid droplets in the cytoplasm and wide-ranging damage to the intracellular membranes. At higher magnifications polarization of the nucleus and condensation of chromatin was also observed (25).

Prostate Health and Underlying Disease Mechanisms

It is suggested that inflammation and factors influencing prostate cell growth and apoptosis could play a role in the development of BPH and prostate cancer. Experimental evidence has shown that saw palmetto extract affects inflammation, cell growth, and apoptotic mechanisms.

Evidence of the extract influencing cell growth and apoptosis in prostate cells has been detected through both direct observation of increased apoptotic rates versus control and through the observation of cellular apoptotic indicators. Studies on various cell lines have demonstrated that saw palmetto treated cells show increased levels of the protein p27^{kip1}, a cell cycle inhibitor that prevents cell cycle progression (27). Increased levels of cleaved Poly ADP ribose polymerase (PARP) fragments have also been observed in saw palmetto treated cells (27,32). Cleavage of PARP is indicative of Caspase-3 activity, part of the caspase cascade an essential sequence in the initiation of the execution-phase of cell apoptosis. Saw palmetto treatment appears to also decrease Akt phosphorylation thus inhibiting the P13 K/Akt1 signaling pathway, an im-

portant inhibitor of apoptotic processes and inductor of protein synthesis (32–34). The herb also inhibits Signal Transducer and Activator of Transcription 3 (STAT-3) signaling by preventing the phosphorylation of STAT-3 by Interleukin 6, another pathway that has antiapoptotic and proliferative effects (27).

Indications of apoptosis are also observed in *in vivo* settings. Wadsworth et al. reported that treatment with saw palmetto extract resulted in a significant shift in tumor pathology to less advanced grades in TRAMP mice versus control. A significant increase in detectable apoptotic cells in the TRAMP mice receiving saw palmetto was also observed (29). In a comparison between 10 patients taking 320 mg of Permixon and 15 control patients that were not prescribed Permixon, Vela-Navarrete et al. reported significantly lower levels of intact PARP in the Permixon group. The Permixon treated group had prostatic tissue with higher Bax-to-Bcl-2 ratios than those in the control group. Both Bax and Bcl-2 are proteins that belong to the Bcl-2 gene family, which encodes a number of proteins that can be either proapoptotic or antiapoptotic. Bax is thought to be a proapoptotic protein whereas Bcl-2 is considered antiapoptotic and the ratio of the two proteins is used as an indication of the susceptibility of cells to undergo apoptosis (20).

Petrangeli et al. observed increased apoptosis rates and decreased levels of phosphorylated Akt in human prostate cancer PC3 cell line treated with Permixon. These effects were initially caused by rapid changes in membrane composition, notably a decrease in cholesterol content and the subsequent disruption of lipid rafts that act as organizing centers for cell signalling pathways. A second phase of membrane changes was seen 24 hours following saw palmetto treatment. In this phase the majority of the initial changes in membrane components were reversed and returned to baseline, except for a large increase in the saturated fatty acid to unsaturated fatty acid ratio. This change in ratio was predominantly due to a net decrease of omega-6 fatty acids in the membrane, most noticeably arachidonic acid. The release of these unsaturated fatty acids are thought to be responsible for the continued increased apoptosis and decreased cell proliferation rates observed in the late stage (34).

It has been shown that free arachidonic acid has proapoptotic effects. The acid has been shown to cause opening of the mitochondrial permeability transition pores causing the release of cytochrome c and the activation of the caspase cascade (34,35). Baron et al. attributed the opening of the permeability transition pores in prostate cancer PC3 and LNCaP cell lines treated with Permixon to the fatty acids in the extract, noting that other studies with fatty acids have demonstrated these types of effects on mitochondria (32,34,36).

Because of the high activity of cyclooxygenase (COX) and 5-lipoxygenase (LOX) in the prostate, the level of free arachidonic acid in the prostate is generally low (36). These conditions may make these cells particularly sensitive to high levels of arachidonic acid release from the membranes and could explain the apparent selectivity for prostate cell lines of saw palmetto action that have been observed. Baron et al. noted that other studies have shown that different fatty acids can induce mitochondria depolarization in other cell lines. It is possible that the

variations in fatty acid content of different test materials could account for the reports of saw palmetto extracts inducing apoptosis in other cell lines.

Typically the high level activity of COX and LOX in prostate cells would result in arachidonic acid having an antiapoptotic effect (35). These enzymes readily convert the acid into eicosanoids such as prostaglandins and leukotrienes that mediate inflammatory reactions (35). Patients with malignant prostatic disease have significant reduction in arachidonic acid and docosapentaenoic acid in their prostatic tissue (37). There is evidence that indicates saw palmetto extracts can inhibit the activity of both LOX and COX. The acid lipophilic fraction of a saw palmetto CO₂ extract was found *in vitro* to be an inhibitor of both the COX (IC₅₀-value: 28.1 µg/ml) and LOX (IC₅₀ value: 18.0 µg/ml) pathways (38). In one study, malignant prostatic tissue converted radiolabeled arachidonic acid to prostaglandin PGE₂ at an almost 10-fold higher rate than BPH tissue. PGE₂ production from [³H]arachidonic acid by malignant prostatic tissue was investigated in the presence of various fatty acids, and oleic acid was found to be the most effective inhibitor (37). Oleic acid is among the most abundant of the free fatty acids in saw palmetto lipophilic extracts as it comprises approximately 30% to 35% of the free and total fatty acids in extracts obtained using 90% ethanol or supercritical CO₂ (10).

Saw palmetto extract, when administered orally in rodents, has shown antiedematous activity in a diversity of animal models, including centrifugation-induced tail edema in mice, histamine- and dextran-induced increase in microvascular permeability and edema in rats, IgE-dependent passive cutaneous anaphylaxis in rats, and UV erythema in guinea pigs. Because the antiedematous activity was also observed in an adrenalectomized rat model, glucocorticoids cannot be the source of activity (12).

The absence of estrogenic properties has been demonstrated by studying the effect of saw palmetto extract on the growth of the prepubertal female mouse uterus and on the changes in the estrus cycle of adult female mice. The absence of progestational activity has been investigated in ovariectomized female mice that were sensitized with estrone and treated daily with 100 to 400 mg saw palmetto extract orally or 100 µg of SC progesterone. On day 10 of treatment, histamine was administered in one of the uterine horns. In contrast to progesterone, the extract of saw palmetto did not cause an increase in the weight of the uterus.

Saw palmetto has both individual compounds and specific extracts with pharmacological activity that could be related to mechanisms that would provide symptomatic relief for LUTS associated with BPH. The lack of systemic hormonal and α-blocker activity should be important for the safe use of saw palmetto and points to the need for more detailed research on activity in prostate cells, tissues, and animals, as well as in humans, to determine whether multiple mechanisms of action are working in synergy or whether the main mechanism of action has yet to be discovered.

CLINICAL STUDIES

Primary Use

The current primary use of saw palmetto is for prostate health and relief of LUTS that are associated with BPH, a

condition that is common in men over 50 years. The only saw palmetto fruit extracts that have been subjected to multiple clinical trials in BPH are those produced under highly standardized conditions, by extraction with hexane or under hypercritical CO₂ conditions. They have a chemical content of 85% to 95% fatty acids, and have been stability tested to assure that throughout their shelf life the chemical contents are sufficient and that the capsules will disintegrate in conditions that mimic the acidity of the stomach. Clinical trials generally evaluated the ingestion of two 160 mg soft gel capsules or one 320 mg capsule per day. Evidence from clinical trials of standardized lipophilic saw palmetto extracts has been mixed but in general it has been seen that they are a viable therapy for the relief of BPH symptoms that has been shown to be as effective and better tolerated than the use of α-blocker and 5α-reductase inhibitors. Although saw palmetto may give relief as early as four to six weeks, three months or more of therapy may be required for some patients before the effects are felt.

Emerging Uses

Saw palmetto has been suggested for other conditions associated with the prostate including prostatitis and prostate cancer. A trial by Kaplan et al. found no long term benefit after 12 months of saw palmetto at a dosage of 325 mg/day for category III chronic nonbacterial prostatitis/chronic pelvic pain syndrome (39). Results, however, from two clinical trials of patients with chronic bacterial prostatitis suggest that the use of a combination of saw palmetto with an α-blocker or other natural products and antibiotics results in a lower incidence of disease recurrence, as well as quicker and more significant relief of symptoms when compared to antibiotic therapy alone. The results of these trials cannot be definitively attributed to saw palmetto as the combination therapy of one trial included the α-blocker alfuzosin and the other trial included curcumin, quercetin, and stinging nettle (40,41).

In vitro evidence demonstrating that saw palmetto can stimulate apoptosis and inhibit cell growth in multiple myeloma, breast, and prostate cancer cell lines has shown the possibility that the herb could be used to prevent or reduce the growth of specific types of prostate cancer cell lines (42). Results from Wadsworth et al.'s study indicated that a saw palmetto CO₂ extract at doses calculated to be equivalent to and six times the recommended human dose showed no signs of toxicity, increased apoptosis, or reduced tumor grade and frank tumor incidence (29). Thus, saw palmetto supplementation could possibly be effective in slightly delaying the onset and progression of prostate cancer through its antiandrogenic action and ability to increase epithelial cell apoptosis (29). A prospective cohort study suggested saw palmetto supplement use for several years had no association with the risk of developing prostate cancer (43). Residual confounding factors and misclassifications of variables due to the reliance on self-reported data were identified as factors that would limit the interpretation of the data in this study (43).

Benign Prostatic Hyperplasia

BPH is a noncancerous enlargement of the prostate that is associated with bothersome and irritative LUTS, including increased urgency and frequency, nocturia (arising from sleep at night to urinate), dribbling, hesitancy,

straining, and incomplete bladder emptying (44). In the United States, a reported 40% of men in their 50s and nearly 90% of men in their 80s have BPH (44). Also in the United States, in 2000, nearly 8 million visits to physicians resulted in a primary or secondary diagnosis of BPH (44).

Current Medical Treatment

There exist several guidelines on the diagnosis and treatment of BPH that were published by various institutions and associations (14). BPH is not always used to denote changes in the prostate but is mostly employed to describe a cluster of bothersome LUTS that increase in frequency and severity as men age. Associated mortality and even serious complications such as complete urinary retention are uncommon. Because some of the symptoms associated with BPH may also be due other medical conditions that cannot be rationally treated by saw palmetto, men should have a thorough diagnostic evaluation by a physician before and during any use of the herb to be certain that BPH and not a different disease is the source of the symptoms. All guidelines use a symptom score, the most commonly recommended is the International Prostate Symptom Score (IPSS), which has the patient answer questions concerning the severity and frequency of the main symptoms. The score on the IPSS or the identical American Urological Society (AUA) Symptom Score questionnaire is used to classify the severity of BPH as mild (0–7), moderate (8–19), or severe (20–36). Since some individuals may chose no therapy (“watchful waiting”) whereas others with the same symptom score may be sufficiently bothered to seek relief by pharmaceuticals or surgical intervention, there exists wide latitude in the treatment of choice for a condition that mainly concerns quality of life. The main drug therapies recommended for treatment are α -adrenergic blockers and 5 α -reductase inhibitors. None of the guidelines recommended the use of saw palmetto or other phytotherapies citing concerns such as variations of product consistency, lack of identification of the active compound, absence of a well-documented mechanism of

action, and the lack of long-term studies on the use of these therapies (14).

Phytotherapies, however, are prescribed and used to treat BPH, especially in Europe. In an observational, cross-sectional study Fourcade et al. reviewed the management of patients treated for BPH in primary care in four European countries and reported that phytotherapies did account for a significant number of prescriptions (45). An observational study by Hutchinson et al. found similar results (Table 1) (46). It should be noted that the data provided by both studies are based on prescriptions, and that the actual usage of the herb would be higher as patients can obtain phytotherapies over the counter without a prescription. Prescribing patterns are affected by a number of variables, not the least of which is drug cost. The complete lack of prescriptions for saw palmetto in the UK and the low numbers in Portugal are likely the result of the limited reimbursement policies for herbal therapies in those countries. In contrast, France, which has a more extensive reimbursement policy towards herbals, reported much higher phytotherapy prescription numbers.

The impact reimbursement has on prescription is especially prominent when Hutchinson’s results are compared to numbers reported in Fourcade et al.’s study a few years later. Fourcade et al. reported prescription numbers for the period between January 2005 and June 2006, a year after the German government implemented policies that eliminated reimbursement for nonprescription medications. In Germany, saw palmetto is classified as a nonprescription medication and is not eligible for reimbursement under the new policy and the number of prescriptions for the herb fell accordingly. In contrast, in what could be an indication of the growing acceptance and increasing popularity of herbal remedies, the percentage of prescriptions for herbal remedies for France, Spain, and Portugal all increased significantly. *S. repens* was the most popular phytotherapy. In France phytotherapy products were the most popular class of drug and *S. repens* was the most popular drug.

Table 1 Prescribing Patterns by Country for Newly Presenting LUTS/BPH Patients Prescribed a Drug from January 2000 to July 2002 and from January 2005 to June 2006

January 2000–July 2002	All Countries	France	Germany	Portugal	Spain	Italy	United Kingdom
Number of Patients	1516	241	106	656	248	199	66
Drug Class							
Phytotherapy	15.6%	25.3%	36.8%	15.9%	10.5%	3.5%	0
Alpha-blockers	79.2%	71.8%	60.4%	77.0%	85.9%	91.0%	98.5%
5-alpha reductase	5.1%	2.9%	2.8%	7.2%	3.6%	5.5%	1.5%
Named Drug							
<i>Serenoa repens</i>	5.7%	16.5%	16.2%	1.1%	5.8%	4.0%	
Tamsulosin	38.4%	46.0%	46.5%	24.3%	42.1%	69.5%	39.1%
January 2005–June 2006							
Number of Patients	446	141	139	49	117		
Drug Class							
Phytotherapy	25.3%	49.6%	5.0%	10.2%	26.5%		
Alpha-blockers	67.3%	46.1%	87.1%	63.3%	70.9%		
5-alpha reductase	4.0%	2.8%	4.3%	12.2%	1.7%		
Combination	3.4%	1.4%	3.6%	14.3%	0.9%		
Named Drug							
<i>Serenoa repens</i>	14.6%	27.0%	0	6.1%	20.5%		
Tamsulosin	35.9%	23.4%	49.6%	34.7%	35.0%		

Source: From Refs. 45 and 46.

Efficacy in Control of Symptoms Related to BPH

Using patient follow-up examinations Hutchinson et al. evaluated the efficacy of the different drug treatment regimes (46). The author's reported that 75.3% of the 83 patients prescribed saw palmetto had IPSS improvements and an overall mean reduction in IPSS of 3.2 (46). These results are significantly higher than the results of those patients that underwent no drug therapy where only 59.2% of the 559 patients reported IPSS improvements and a mean IPSS reduction of 1.4 (46). Patients taking the herb also reported higher improvements in Quality of Life scores than the "watchful waiting" group with a mean improvement of 1.0 point versus 0.4 points (46). Although the patients were not randomized to treatments and the numbers in some categories do not allow for robust statistical analysis, these findings still correspond to the general conclusion that *S. repens* provides improvement of urologic symptoms and flow measures in patients suffering from BPH that were made in various clinical trial reviews (44).

A recent large-scale study by Bent et al., however, has resulted in a re-assessment of the conclusions made in earlier reviews. Bent et al. investigated the effectiveness of saw palmetto, in the treatment of men suffering from moderate-to-severe BPH (23). A total of 225 men participated in the trial and were given 160 mg twice per day of either a CO₂ saw palmetto extract or a placebo over a 12-month period. The study indicated that neither the saw palmetto nor the placebo showed any reduction in AUASI scores or significant improvements in any of the other clinical parameters assessed (42). In light of these results, the most recent Cochrane review concluded that *S. repens* was not more effective than placebo for treatment of urinary symptoms consistent with BPH (44). In their discussion the reviewer's acknowledged the significant weight they placed on Bent et al.'s study in their analysis (44).

The lack of any improvements observed for either placebo or saw palmetto obtained from Bent et al. contradict many other trials that did report data suggesting saw palmetto did relieve urinary symptoms. As data from Hutchinson et al.'s observational studies indicate, improvements were observed even when patients underwent a "watchful waiting" therapy (23). The Cochrane review did state that the evidence for the efficacy of *S. repens* in relief of symptoms was mixed (44). It was noted that three of the four trials that provided full data showed evidence of mean changes of -4.4, -4.4, and -6.1 in IPSS scores (44). The other trial was the one performed by Bent et al., which showed no improvements in either the saw palmetto or placebo arms. The reviewers placed greater weight on the Bent et al. trial noting that two of the trials were of smaller size and duration and the third, by Debruyne et al., lacked a placebo arm (44).

Debruyne et al.'s study, which was of the same duration (12 mo) and of substantially larger size (542 patients completed the study) than Bent et al.'s trial, compared a saw palmetto hexane extract (Permixon), at a dose of 320 mg/day to Tamsulosin, the most widely prescribed therapeutic agent for BPH, at a dose of 0.4 mg/day (47). In both groups IPSS scores showed decreases by three months' time (47). At 12 months both treatments resulted in a similar decrease of 4.4 in IPSS scores and mean changes in peak urinary flow rate of 1.89 mL/s for Tamsulosin and 1.79 mL/s for saw palmetto (47). The only

meaningful difference in side effect profiles of the agents was the 4.2% ejaculation disorders reported for Tamsulosin against 0.6% for saw palmetto (47). The results from Debruyne's trial indicate that the effectiveness of saw palmetto is, at the least, equivalent to the effectiveness of Tamsulosin (47). A subsequent subset analysis by Debruyne et al. found that for those patients that had the most severe IPSS scores, saw palmetto was shown to be more effective than Tamsulosin in reducing the symptom score (48).

A study by Carballido et al. examined the direct healthcare cost of BPH diagnosis and treatment in Spain and concluded that pharmacological therapy is responsible for 42% to 74% of the disease's cost. Carballido et al. calculated that the annual cost of therapy, including the cost of treating any adverse events, with the Permixon was €135 less than the annual cost of therapy with Tamsulosin. Permixon was also found to be the more cost-effective option even when a generic Tamsulosin is used, costing approximately €32 less per year (49).

Product-to-product variability in the chemical profiles of saw palmetto product is common in the marketplace (12). The biological and clinical activities of different extracts are dependent on the presence and amount of all the known and unknown compounds in the extract. Differences in test materials used can result in very different clinical results. Thus, direct comparison of trials utilizing different plant extracts is only valid for the entire class of a botanical extract as the different trials are effectively comparing different therapeutic agents. When trial results are homogenous then the results for the entire group of extracts are clear, but when the results are mixed then the specific extracts must also be examined individually for safety and efficacy.

Although the well-designed LUTS/BPH trial by Bent et al. reported no improvement by saw palmetto, the overall clinical evidence from European observational studies and clinical trials shows that specific saw palmetto products can be a viable therapy for the relief of BPH symptoms that are more cost-effective and better tolerated than α -blocker and 5 α -reductase inhibitors in some patients. The important and unknown questions are whether saw palmetto can maintain prostate health for many years and prevent the worsening of LUTS symptoms or impact the initiation or slow the growth of prostate cancer in some patients.

Safety

Although not available for independent scientific review, hexane/CO₂ extracts of saw palmetto that have been registered as prescription drugs in Europe have had to undergo the same preclinical safety studies as drugs in the United States. The specific extracts and final pharmaceutical products registered would have been tested in vitro and in vivo for oral toxicity, teratogenicity, mutagenicity, peri- and postnatal toxicity, estrogenic activity, and effect on fertility. In the United States, saw palmetto products as dietary supplements are sold in combination with other plant products that have not undergone the safety, quality, and clinical efficacy testing required of drugs in Europe. Because saw palmetto products sold in the United States could have a different safety profile than those sold in Europe, their safety has been reviewed as a monograph by a special committee of the Food and Nutrition Board and

the Board on Life Sciences [part of the Institute of Medicine (IOM) and National Research Council of the National Academies of Science]. Considering the weight of the current scientific evidence, the report concludes that the consumption of saw palmetto fruit (powders and extracts) does not pose a safety risk for men at the currently recommended doses (18). The report notes that the toxicity of combination products (including the 8-herb combination product PC-SPES, which was removed from commerce because of adulteration with drugs, including the anticoagulant warfarin) was evaluated only in relation to the saw palmetto component (18). The IOM report notes that no drug interactions have been documented with saw palmetto, but that more systematic drug interaction studies are needed (18). A review of the modulating effects of various herbal medicines concluded that while evidence indicated saw palmetto inhibited cytochrome activity in vitro, in vivo studies failed to show any effect on cytochrome activity leading to the conclusion that saw palmetto was unlikely to have any herb-drug interactions (50).

The latest Cochrane review reported that the side effects associated with treatment with *S. repens* were generally mild and comparable in frequency to placebo. Their meta-analysis showed that there existed no significant difference in the relative risk of any adverse event between *S. repens* or placebo. In comparison to the 5 α -reductase inhibitor drug Proscar, saw palmetto had fewer adverse events, including impotence, the most frequent side effect for Proscar. Similar results were reported when comparing the herb to the α -blocker Tamsulosin, which had a statistically significantly higher frequency of headaches and ejaculation disorders (44).

In a detailed safety assessment of saw palmetto extract using the data obtained from Bent et al.'s study, Avins et al. concluded that there was no evidence that saw palmetto extract at a dose of 320 mg/day over a year was associated with any clinically important adverse effects (51). There were few serious adverse events reported and they were more common in the placebo arm. There was no significant difference in nonserious adverse events between the extract and placebo (51). Debruyne et al. had similar results, reporting that the overall incidence of adverse events were similar between both treatment groups with a slightly higher frequency of ejaculation disorders reported in the Tamsulosin group (4.2%) versus the saw palmetto group (0.6%) (47,48).

Although the results from Avins et al.'s assessment suggest that no important toxicities are associated with saw palmetto, the authors did stress that the study could not assess the association of rare serious adverse events with saw palmetto. The authors noted case reports of patients suffering from hepatitis, pancreatitis, and excessive intraoperative bleeding and prolonged bleeding time that had been associated with the herb (51). Few other adverse event reports have been submitted to the FDA. Although concerned about the prolonged bleeding time report, no clear causal relationship was found by the IOM Committee review (18).

Results from a small animal study by Singh et al. did not find any evidence of hepatotoxicity due to saw palmetto (52). Singh et al. reported that their enzyme assay data actually suggested that the saw palmetto extract at doses two to five times the recommended dose appeared to have a hepatoprotective effect (52). A study

by Tuncel et al. investigated the effects taking saw palmetto for five weeks prior to transurethral resection of the prostate would have on the intraoperative blood loss and microvessel density. The authors reported that there were no significant statistical differences in any of the variables that were measured in saw palmetto and control groups (53). A study by Beckert et al. investigated platelet function in 10 men taking saw palmetto extract (54). After two weeks of therapy, the authors could find no effect of the herb on in vivo platelet function (54). These results combined with investigations from the authors' literature review led to their conclusion that there was no evidence to support the concern of perioperative bleeding in users of saw palmetto (54).

The safe use of saw palmetto by pregnant and lactating women is more questionable, because drugs like finasteride that block the conversion of testosterone to DHT could impair the development of male genitalia in the fetus or feeding infant (18). The World Health Organization (WHO) monograph on saw palmetto states that "Owing to its effects on androgen and estrogen metabolism, the use of Fructus Serenoae Repentis during pregnancy or lactation and in children under the age of 12 years is contraindicated" (15). Because the safety and efficacy of all botanicals is based on the specific extract and final product formulation as the "active" ingredient, it is worth noting that, although rarely used today by women and children, the hexane and CO₂ extracts and final products registered as prescription drugs in Europe would have been tested like all drugs for potential detrimental effects on the fetus in animal models of fertility and teratogenicity. All chemical compounds that have been reported from saw palmetto are generally nontoxic in the quantities consumed from commercial products (18).

Overall, standardized lipophilic saw palmetto extracts at doses of 320 mg/day have proven risk-free in long-term clinical use in many European countries and in controlled clinical trials from 6 to 48 weeks. In the United States, saw palmetto, consumed at current levels in commercially available products, has also been very safe. For men, it should only be used to treat mild-to-moderate BPH after a complete medical examination to rule out more serious disorders. Saw palmetto products sold as dietary supplements have not undergone standard animal tests for teratogenicity and effects on fertility and therefore are contraindicated for use by pregnant or lactating women.

Pharmacokinetics

Unlike pharmacodynamic studies that measure physiological changes, pharmacokinetic studies in humans with saw palmetto are difficult to conduct because the most active compounds that would logically be measured are fatty acids and sterols that are common in the normal Western diet. The pharmacokinetics has been investigated in an open, randomized, crossover study of 12 healthy males who ingested one 320-mg capsule or two 160-mg capsules per day. The extract was absorbed rapidly, with a peak time (t_{max}) of 1.50 to 1.58 hour and peak plasma levels (C_{max}) of 2.54 to 2.67 μ g/mL. The area under curve value ranged from 7.99 to 8.42 μ g hr/mL. Because the plasma concentration-time profiles of both preparations were very similar, the preparations were considered

bioequivalent, but the validity of the methodology has been questioned (5,6).

In another study, the bioavailability and pharmacokinetic profile of a rectal formulation containing 640 mg of *S. repens* extract were determined in 12 healthy male volunteers. The rectal was similar to the oral one but showed a slower absorption, with a t_{\max} of 2.96 hour (5). In a study of the administration of a radioactive lipophilic sterol extract of saw palmetto in rats, tissue concentrations of lauric acid, oleic acid, and β -sitosterol were highest in abdominal fat tissue, the prostate, and the skin with lower concentrations in the liver and urinary bladder (18).

REGULATORY STATUS

Saw palmetto products are regulated in the United States as dietary supplements since the passage of the Dietary Supplement Health and Education Act (DSHEA) in 1994. Under the regulations of the DSHEA, saw palmetto is sold without FDA premarket approval since dietary supplements are sold as a category of food. Although clinical testing is not required for inclusion in the *United States Pharmacopeia* or *National Formulary*, identity, chemical content, and quality standards for saw palmetto fruits, powders, extracts, and capsules have recently been reintroduced into the dietary supplement section of the USP32-NF27 (55). Saw palmetto products sold as dietary supplements are not allowed to make a drug claim (such as for the treatment of urological symptoms associated with BPH) but are allowed to make structure function claims (such as "to support prostate health" or "support healthy prostate function"). Saw palmetto is included in the *Homeopathic Pharmacopoeia of the United States* and as such may be sold as a homeopathic over-the-counter drug at strength of 1X, which is similar to a full strength alcoholic (65%) extract (56).

Saw palmetto is sold in Canada as a natural health product that must be manufactured to pharmaceutical standards and meet other monograph standards, including the amount of the product to be taken and the form of the product that can be used, and is sold with labels that can state the more accurate claim, "Used in Herbal Medicine to help relieve the urologic symptoms associated with benign mild-to-moderate prostatic hyperplasia" (57). In France, it is a prescription drug reimbursable by the national health insurance. In Italy, a lipophilic hexane extract is sold as a prescription drug. The dried fruit, simple galenical preparations, and lipophilic extracts are approved as nonprescription drugs in Germany. In Belgium, saw palmetto is a prescription adjuvant for BPH, while in Sweden, it is sold as a natural remedy for self-medication after premarketing authorization. In Switzerland, it is sold without a prescription, but sales are limited to pharmacies, and in the United Kingdom, saw palmetto is an herbal medicine on the *General Sales List* that requires full product licensing (6).

CONCLUSIONS

Saw palmetto is a lipid-rich fruit and the fatty acids extracted from the fruit have been used to sooth irritations. In the late 1800s, Eclectic School of Medicine believed the extract was a nutritive tonic based on the high-energy

content of its lipids and the mild anti-inflammatory activity obtained from its combination of fatty acids. This still seems a rationale description of what is now widely sold as a dietary supplement.

Saw palmetto fruits and extracts, on the basis of the known chemistry, pharmacology, clinical trial data, and the low number of adverse event reports, appear to be very safe for men to consume in the amounts used in clinical trials and available in dietary supplement products on the market. Mild nausea or mild gastrointestinal upset appear to be the most frequent side effects from ingesting amounts currently consumed as dietary supplements or as drugs. Saw palmetto products that have not been tested for systemic estrogen and androgen effects pose a theoretical risk to the proper development of the fetus and infants; their use is therefore contraindicated in the WHO monograph for pregnant or lactating women and children under 12 years.

Based on the fact that different products vary in chemical content and amount of substantiation for efficacy, specific saw palmetto products could have different therapeutic effects. The use of saw palmetto for treating LUTS has shown mixed clinical results and it is most rationally used as a therapeutic agent on the basis of the excellent safety profile as initial therapy. If LUTS symptoms do not improve with saw palmetto, then α -blockers and 5- α reductase inhibitors or surgery are the main therapeutic options to be considered.

The most interesting scientific questions that still need to be answered include: what is the long-term impact of saw palmetto to improve human health; what is the long-term impact of saw palmetto on the progression of LUTS symptoms, BPH, as well as prevention or slowing the progression of prostate cancer; and because saw palmetto is generally used as a supplement of 320 mg of a specific mixture of fatty acids and plant sterols, what is its impact on the person's nutritional status, and even on the fatty acid content of a person's diet, as well as on the physiological activity of supplementing the diet with saw palmetto for LUTS, cancer progression, or other improvements of health? Although saw palmetto became popular to treat LUTS associated with BPH, the impact of lipophilic components on prostate health is an important scientific question because the nutritional requirements of a healthy prostate are not well understood.

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Selenium

Roger A. Sunde

INTRODUCTION

Selenium (Se) is an essential micronutrient that is incorporated into the primary structure of proteins in the form of selenocysteine. Selenoproteins facilitate redox reactions that underlie a number of biochemical processes. Among them are protection against oxidative damage, metabolism of thyroid hormones, support of DNA synthesis, and regulation of transcription factors. Selenium deficiency occurs in China and some other countries but not in the United States. The recommended dietary allowance (RDA) for selenium is 55 $\mu\text{g}/\text{day}$ for adults, and the recommended dietary upper limit is 400 $\mu\text{g}/\text{day}$.

BACKGROUND

Selenium was discovered by Berzelius in 1817 as a by-product of sulfuric acid production. Its importance in biology was established in the 1930s, when it was identified as the toxic principle in plants that poisoned grazing animals in certain parts of the Great Plains of the United States. In response to this problem, the U.S. Department of Agriculture mapped the selenium content of forage from all regions of the United States and produced a selenium map to help farmers avoid grazing their animals in areas that might produce toxicity (1). In addition, the metabolism of the nutrient was studied over the next two decades with the aim of determining its mechanism of toxicity. Studies carried out during that period established that its metabolism was intertwined with that of sulfur (2).

The essentiality of selenium in animals was recognized in 1957, when provision of the element to rats fed a yeast-based diet was shown to prevent the development of dietary liver necrosis (3). Vitamin E could also prevent the condition, and this fact has linked these two nutrients since that time. A number of naturally occurring animal diseases are caused by combined selenium and vitamin E deficiency. They include mulberry heart disease, nutritional muscular dystrophy, and nutritional liver necrosis of swine, exudative diathesis of chickens, gizzard myopathy of turkeys, white muscle disease of sheep, and male infertility of cattle. Reference to the selenium map produced by the U.S. Department of Agriculture shows that these conditions occur in areas where plant selenium concentrations are low (1).

Geological studies led to the recognition that selenium is distributed irregularly in soil. Soils subjected to

leaching from high rainfall are generally poor in selenium. Alkaline soils yield their selenium whereas acid soils withhold it from plants. Thus, alkaline soils derived from sedimentary rock that are in arid regions tend to transmit high levels of selenium to plants. Plants grown on acidic soils tend to have low selenium content. Areas of low selenium in the United States include the Pacific Northwest and the Midwest, and grazing animals in those regions require selenium supplementation. The South Island of New Zealand and much of Scandinavia also have low selenium levels in soil. In contrast, soils in the Dakotas arising from volcanic activity are high in selenium, and wheat grown in these regions is high in selenium (4).

Human selenium deficiency was firmly demonstrated in 1979, when Chinese scientists provided evidence that a cardiomyopathy occurred only in selenium-deficient children living in regions of China where the nutrient content in plants was very low. Blood and hair selenium content of inhabitants of regions where Keshan disease was found were the lowest levels reported in human beings (5). Thus, selenium is an essential nutrient for human beings.

In recent years, supplementation of selenium in pharmacological doses has been touted for a variety of health purposes. These include chemoprevention of cancer, delay of aging, and prevention of heart disease. These potential actions do not appear to relate to its nutritional effects. This brief review will focus on nutritional (physiological) functions of selenium. Other uses of the element will be addressed only briefly.

CHEMICAL FORMS

Selenium occupies a spot in the periodic table of elements just below sulfur and shares many chemical properties with that element. Its chemistry is covalent in biological systems.

Most selenium in biological material is present in amino acids (Fig. 1). Plants are not known to require selenium and incorporate the element nonspecifically into selenomethionine in place of sulfur. A major source of selenium in the diet is selenomethionine. Once ingested by an animal, it enters the methionine pool and is not distinguished from methionine. Thus, much of the selenium in animal tissues is selenomethionine that is nonspecifically incorporated in proteins at methionine positions. When selenomethionine is catabolized, its selenium becomes available to the selenium metabolic pool. Thus,

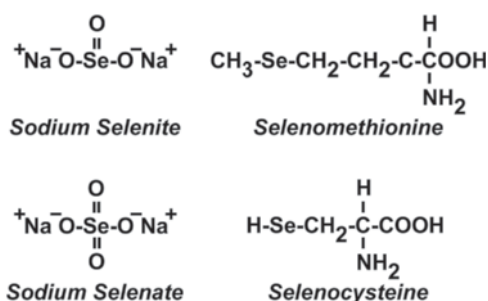


Figure 1 Common selenium supplementation forms and amino acid forms that account for most of the element in the body.

selenomethionine apparently has no biological function related to selenium other than serving as a dietary source of the element.

Selenocysteine (Sec or U) is present in stoichiometric amounts in selenoproteins and is necessary for their function. It is synthesized from the selenium present in the metabolic pool. Presence of selenocysteine at active sites of enzymes such as glutathione peroxidases generally increases their activities 1000-fold above that when cysteine is present.

The urinary metabolites of selenium that have been identified are trimethylselenonium ion and a methylated selenosugar (6). They are synthesized in the liver and kidney to regulate whole-body selenium.

Inorganic forms of selenium such as selenite and selenate (Fig. 1) are biologically available and are often used to supplement selenium intakes. They are well tolerated and inexpensive.

BIOCHEMISTRY AND FUNCTION

Selenoprotein Synthesis

Animal selenoproteins contain stoichiometric amounts of selenocysteine in their primary structures. The selenocysteine that is used for this process is synthesized in the cell by modification of a serine residue that has already been ligated to tRNA^{[ser]sec}. The resulting selenocysteinyl-tRNA^{[ser]sec} recognizes a UGA in the open reading frame of the selenoprotein mRNA with the aid of at least three transacting proteins. The process of selenoprotein synthesis is complex and costly to the organism. It requires at least six gene products in addition to the ones normally used for protein synthesis. Reviews describing selenoprotein synthesis are available (7,8).

Selenoproteins and Their Functions

Twenty-five genes that code for selenoproteins (Table 1) have been identified in the human genome (9). Because some genes produce more than one protein product, the number of selenoproteins in humans is probably between 25 and 50. In addition, one of the selenoproteins in humans is present in some other animals with cysteine rather than selenocysteine encoded in the gene, and a small number of selenoproteins found in other organisms are present as the cysteine-encoding genes in humans. Plants and fungi

Table 1 Human Selenoproteins

Group/name	Abbreviation
<i>Selenoproteins involved in thiol redox reactions</i>	
<i>Glutathione peroxidases</i>	
Cellular glutathione peroxidase	GPX1
Gastrointestinal glutathione peroxidase	GPX2
Extracellular glutathione peroxidase	GPX3
Phospholipid hydroperoxide glutathione peroxidase	GPX4
Odorant metabolizing glutathione peroxidase	GPX6
<i>Thioredoxin reductases</i>	
Cytosolic thioredoxin reductase	TXNRD1
Thioredoxin/glutathione reductase	TXNRD2
Mitochondrial thioredoxin reductase	TXNRD3
<i>Other U-C motif redox selenoproteins</i>	
Methionine-R-sulfoxide reductase	SELR
Selenoprotein 15 (ER resident ^a)	SEP15
Selenoprotein H (may regulate glutathione metabolism)	SELH
Selenoprotein M (ER resident)	SELM
Selenoprotein O (largest mammalian selenoprotein)	SELO
Selenoprotein T (ER resident)	SELT
Selenoprotein V (related to Sepw1; expressed in testes)	SELV
<i>Membrane selenoproteins</i>	
Selenoprotein I (may be phosphotransferase)	SELI
Selenoprotein K (ER resident)	SELK
Selenoprotein S (ER resident)	SELS
<i>Selenoproteins involved in thyroid hormone metabolism</i>	
Type I iodothyronine deiodinase	DIO1
Type II iodothyronine deiodinase (ER resident)	DIO2
Type III iodothyronine deiodinase	DIO3
<i>Muscle selenoproteins</i>	
Selenoprotein W (binds glutathione)	SEPW1
Selenoprotein N (ER resident)	SEPN1
<i>Selenocysteine synthesis selenoproteins</i>	
Selenophosphate synthetase-2	SEPHS2
<i>Transport Selenoproteins</i>	
Selenoprotein P	SEPP1

^aSelenoproteins that appear to be localized in the endoplasmic reticulum (ER).

Source: From Ref. 9.

lack selenoproteins, and marine organisms generally have more selenoproteins than land animals (10).

Selenoproteins Involved in Thiol Redox Reactions

There are two major systems that regulate thiol redox status in cells: the glutathione and the thioredoxin systems. Both of them depend on NADPH for their reducing equivalents and contain selenoproteins as components.

The glutathione peroxidases utilize reduced glutathione to catabolize hydroperoxides of various kinds. The active sites of these enzymes typically contain selenocysteine, although some of them function with cysteine in the active site. Of the seven glutathione peroxidase genes in the human genome, five code for selenoproteins. At least one of these genes produces three different protein products by the use of alternative translation start sites.

Three glutathione peroxidase genes have been knocked out in mice without obvious effects on health and reproduction (11,12). Only when these knockout mice are stressed do they show increased injury compared with wild-type animals (13). A knockout of another glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, causes embryonic lethality (14). One isoform of this latter enzyme has structural functions in spermatozoa (15). The above-mentioned considerations indicate that

these enzymes have a variety of biological functions and each will have to be evaluated separately.

Thioredoxin reductases maintain thioredoxin and some other substances, including ascorbate, in a reduced state. These enzymes contain selenium in mammals, but lower life forms sometimes have cysteine homologs. Thioredoxin is responsible for providing reducing equivalents to ribonucleotide reductase and to a number of other enzymes and transcription factors. Therefore, this family is important for gene expression, signaling, and oxidant defenses (16,17). Knockout of thioredoxin in mice causes embryonic lethality (18).

In addition to the glutathione peroxidases and the thioredoxin reductases, most selenoproteins contain selenocysteine-cysteine (U-C) redox motifs and thus appear to be involved in oxidation-reduction reactions. Methionine-R-sulfoxide reductase in humans contains selenium and can reduce oxidized methionine resulting from oxidative stress. Other family members have cysteine in place of selenocysteine and are specific for other forms of oxidized methionine. Selenoproteins W, V, T, H, M, and O also contain selenocysteine-cysteine motifs, although their functions are unknown (19).

At least three of the selenoproteins are membrane proteins and seven of the selenoproteins appear to be associated with the endoplasmic reticulum inside cells (20). Targeted loss of all selenoproteins in mouse brain results in impaired neurological function, clearly illustrating the important roles of selenoproteins in the body's normal processes (21,22). A full discussion of these enzyme families are beyond the scope of this chapter and can be found elsewhere (23,24).

Selenoproteins Involved in Thyroid Hormone Metabolism

Thyroxine, or T₄, is the hormone produced by the thyroid gland. It must be converted to triiodothyronine, or T₃, to exert biological activity. T₃ is inactivated by conversion to T₂. All these reactions are carried out by three selenoproteins known as deiodinases (25).

In animals, selenium deficiency leads to decreased activity of the deiodinases, but this is compensated for by a rise in T₄ levels. When T₄ production is compromised by iodine deficiency, the addition of selenium deficiency is not well tolerated (26). There have been reports of cretinism occurring in infants living in areas where combined selenium and iodine deficiency is found (27). Supplementation of selenium without iodine in these populations appears to further aggravate this disease (28).

Muscle Selenoproteins

The early discoveries of the association of muscle disease in animals with selenium deficiency are being substantiated as new selenoproteins are discovered. Selenoprotein W is abundant in human and primate muscle, and levels fall in selenium deficiency (29). Selenoprotein N is highly expressed in muscle, and mutations in selenoprotein N are associated with one form of congenital muscular dystrophy (30).

Selenocysteine Synthesis Selenoproteins

A selenoprotein is even required in the pathway for synthesis of selenocysteine from serine. Selenocysteine-containing selenophosphate synthetase-2 is a major en-

zyme in this pathway but a synthetase with threonine replacing the selenocysteine also has a role (31).

Selenium Transport Selenoproteins

Selenoprotein P is essential for normal selenium distribution (32). This extracellular selenoprotein contains 10 selenocysteine residues in its full-length form. It is produced in most tissues, but selenoprotein P in plasma appears to originate largely in liver. Recently, receptors for selenoprotein P have been identified in testes, brain, and kidney, thus providing targeted transport of selenium to these tissues (33,34).

Mice with selenoprotein P knocked out have been produced (35,36). Males have abnormal spermatozoa and have sharply reduced fertility. Both males and females develop neurological impairment when fed a diet containing normal amounts of selenium. They can be rescued by provision of a high-selenium diet (22,36). Knocking out the testes and brain selenoprotein P receptor results in the same defects as loss of selenoprotein P, further demonstrating the importance of targeted delivery of selenium to these organs (21,33,34).

Antioxidant Properties of Selenium

The element Se is not an antioxidant and itself may be a pro-oxidant. Already discussed are the redox activities of selenoenzymes and the relationship with vitamin E, which is a free radical scavenger. When rats that are vitamin E deficient are also made selenium deficient, they undergo massive lipid peroxidation and liver necrosis (37). This indicates that selenium-dependent proteins partially compensate for the lack of vitamin E, but that loss of both nutrients leads to severe oxidative damage in the liver.

A number of oxidant defense enzymes that do not contain selenium become induced in selenium deficiency. These include glutathione transferases, glutamate-cysteine ligase, NAD(P)H quinone reductase, heme oxygenase-1, and other phase-II enzymes (38). These changes may occur due to the presence of response elements in genes that sense changes in cellular oxidants in selenium deficiency (39).

Cancer Chemoprevention

A great deal of attention has been directed to the use of selenium to prevent cancer. The first data suggesting a correlation between low selenium intake and increased cancer incidence were presented in the 1970s. Since then, other observational studies have supported this correlation. However, they were unable to isolate selenium as the only factor responsible for the lower cancer incidence.

Intervention studies have attempted to evaluate the effect of selenium administration on cancer incidence. One such study was carried out in China (40). An overall reduction in cancer mortality of 13% was achieved in a rural population by giving combined supplements of selenium, vitamin E, and carotene. Unfortunately, no subjects were given selenium alone; so the protection cannot be ascribed to it.

Another study using high-selenium yeast that provided 200 µg of selenium per day was carried out in subjects who had a nonmelanoma skin cancer (41). The primary endpoint was development of another skin cancer.

Secondary endpoints of other cancers were introduced after the study had been initiated.

The initial analysis (10 yr of supplementation) of the primary endpoint showed no effect of the high-selenium yeast on skin cancer development, but it showed a decrease of 37% in overall cancer development. The diagnosis of prostate cancer was decreased 63% in the high-selenium yeast group. Concern was raised recently by a report on the entire blinded period (12 yr) of high-selenium yeast supplementation (42). It showed that the supplemented group had a higher incidence of squamous cell carcinoma of the skin than did the placebo group. Analysis of the full study also found a significantly higher diabetes incidence (hazard ratio 1.55) in Se-supplemented subjects versus placebo subjects, and a significant hazard ratio of 2.7 for Se supplementation in subjects in the highest tertile of baseline plasma Se level (43).

The initial report of this study stimulated a great deal of interest in selenium as a chemoprevention agent, which contributed to the initiation of the Se and Vitamin E Cancer Prevention Trial (SELECT). The SELECT trial enrolled 35,534 participants who consumed pills containing 200 µg Se as L-SeMet and/or 400 mg dl- α -tocopherol acetate or placebo pills. In 2008, however, the study was stopped because an independent monitoring committee found that Se and vitamin E, taken alone or together for an average of five years, did not prevent prostate cancer, and also there were suggestions of adverse effects due to single supplements (44). The discrepancy between these two trials is not understood, but may involve initial Se status of the two populations or the form of Se supplementation (selenized yeast vs. selenomethionine).

Because of the uncertainty that exists about the effect of pharmacologic amounts of selenium on cancer development, selenium supplementation cannot be recommended at present to prevent cancer. Furthermore, analysis of 67 intervention studies looking at low-bias risk trials also found no significant effect—positive or negative—of Se supplementation on all-cause mortality (45), further indicating that selenium supplementation is not a panacea.

METABOLISM

Selenium has chemical properties that account for its function in selenoproteins. Because these can also lead to catalysis of unwanted reactions, homeostatic control of selenium in the organism is necessary.

The major dietary forms of selenium are selenomethionine derived from plants and selenocysteine from animal selenoproteins. Both amino acids appear to be virtually completely absorbed by intestinal amino acid transporters. The inorganic forms that are often used for supplementation, selenite and selenate, are also well absorbed. Thus, absorption of selenium is very high and not subject to regulation by selenium status.

The selenium pool in the liver appears to be the site of homeostatic regulation. Absorbed selenium is removed from the portal blood by the liver, and selenomethionine is catabolized there by transsulfuration, releasing its selenium to the selenium metabolic pool.

Liver selenium is used to synthesize liver selenoproteins and selenoprotein P for export. Selenium in excess of that needed for these processes appears to be converted

into excretory metabolites (trimethylselenonium ion and a methylated selenosugar) that appear in the urine. When toxic amounts of selenium are present, dimethyl selenide appears in the breath. Thus, excretion is responsible for regulating the selenium content of the body.

The only transport form of selenium that has been identified is selenoprotein P (35,36). However, other form(s) must exist, because knockout of selenoprotein P does not affect selenium levels of many tissues.

Selenium storage is accomplished through two mechanisms. One is unregulated and consists of selenomethionine that is present in the methionine pool. As selenomethionine is catabolized, its selenium is fed into the selenium metabolic pool. The other storage mechanism relates to the most abundant glutathione peroxidase enzyme. This protein contains a greater fraction of whole-body selenium than any other selenoprotein. When selenium is in short supply, the mRNA of this glutathione peroxidase is degraded more rapidly, reducing synthesis of this selenoenzyme. This allows selenium to be directed to other selenoproteins that are presumably more important for survival (46).

In summary, selenium homeostasis is maintained by excretion of the element when it is present in amounts greater than what can be utilized for selenoprotein synthesis. When insufficient selenium is present for synthesis of all selenoproteins, hepatic cytosolic glutathione peroxidase is downregulated so that selenium can be directed to other selenoproteins.

DIETARY INTAKE AND DEFICIENCY

Sources and Regional Variation of Selenium

The amount of selenium in plants depends on the availability in the soil on which the plants are grown. This fact leads to a single food plant such as wheat having a selenium content that can vary by a factor of 10 or more, depending on where it is grown. This variation renders food tables for selenium in plants of reduced value.

Animals, on the other hand, require selenium and have homeostatic mechanisms to maintain predictable concentrations in their tissues. Thus, foods of animal origin are more reliable sources of selenium. In areas where the soil is poor in selenium, animal foods contain more selenium than plant foods.

Some marine fish such as tuna can have high levels of selenium. This selenium can diminish toxicity of high mercury levels that sometimes are found in predatory fish (47).

The lowest and the highest intakes of selenium in the world have been reported in China. They vary from less than 10 µg/day to over 1 mg/day (48). The cause of this wide variation is the reliance of the population on plant foods and the extreme variation in available soil selenium in different regions.

Intakes in other countries generally vary from around 30 µg/day in New Zealand and parts of Scandinavia to around 100 µg/day in North America. Intakes in Europe are in the range of 30 to 60 µg/day.

Keshan Disease and other Human Diseases

The only human disease that has been clearly linked to selenium deficiency is Keshan disease. It is a childhood

cardiomyopathy that occurs in low-selenium regions of China, where the intake of the element is approximately 10 µg/day. A double-blind placebo-controlled study that was carried out in the 1970s showed that selenium supplementation could prevent the development of Keshan disease (5). Because not all selenium-deficient children developed Keshan disease, a second stress was considered. Subsequent studies in mice have suggested that the second stress might be a viral infection (49).

The incidence of Keshan disease has declined in the last few decades and it is now rare. This is likely due to the improvement of the Chinese economy, with increased meat intake and exchange of foodstuffs between regions.

In addition to Keshan disease, muscle pain and wasting and inability to walk due to selenium deficiency occurred in TPN patients before these solutions were routinely supplemented with selenium (50), and some specific forms of male infertility are linked to selenium deficiency (51). Selenium deficiency may also contribute to a bone disorder, called Kaskin-Beck disease, that is found in certain low-selenium regions of China and Tibet (52).

Assessment of Selenium Status

The endpoint usually used to set recommended intake of selenium is optimization of selenoprotein concentrations. This is based on the assumption that full expression of selenoproteins will promote optimal physiological function and health. Inadequate dietary supply of selenium limits selenoprotein synthesis and results in depressed selenoprotein concentrations. When selenoproteins are optimized, provision of additional selenium will not cause their concentrations to increase. Instead, the additional selenium is excreted. Thus, optimization of the plasma selenoproteins (as representatives of all selenoproteins) has been used to determine the selenium requirement.

There are two selenoproteins in plasma, selenoprotein P and extracellular glutathione peroxidase. When optimized, these proteins contain 6.4 and 1.7 µg of selenium per 100 mL of plasma, respectively (53). Thus, the total selenium in the plasma selenoproteins is approximately 8 µg per 100 mL of plasma.

A third pool of selenium is present in plasma proteins. It is selenomethionine distributed nonspecifically in the methionine pool. In the United States, the amount of selenium in this form ranges from 1 to 12 µg per 100 mL of plasma. This leads to the total plasma selenium concentration in the United States to vary from about 9 to 20 µg per 100 mL. The mean serum selenium level of 18,597 persons reported by the Third National Health and Nutrition Examination Survey (NHANES III, 1988–1994) was 12.5 µg per 100 mL, with 5th and 95th percentiles of 10 and 15 µg per 100 mL, respectively (54).

On the basis of these results, plasma or serum selenium concentrations of 8 µg per 100 mL or higher in healthy people should indicate optimization of the plasma selenoproteins. Concentrations greater than this merely indicate that the subjects are consuming selenomethionine. People with diseases may have alterations in their selenoprotein concentrations caused by their conditions (55), so this value may not apply to them.

For reference purposes, plasma concentrations of selenium in low-selenium regions of China are generally 2 µg per 100 mL or less. In New Zealand, they are 5–8 µg

per 100 mL, and in Europe, 5–10 µg per 100 mL. Concentrations greater than 50 µg per 100 mL of plasma occur in high-selenium regions of China.

Selenium status regulates the mRNA levels of glutathione peroxidase and several other selenoproteins. In rats, these mRNA levels have been used as molecular biomarkers to determine selenium status and requirements (46,56), and to investigate selenium requirements in pregnancy and lactation (57). Future research may identify panels of molecular biomarkers that can discriminate between deficient, marginal, adequate, and supernutritional individuals and populations, and differentiate between individuals who will benefit versus who will be adversely affected by selenium supplementation.

Dietary Reference Intakes

Estimates of the human selenium requirement have been based on two studies. One was performed in China in the early 1980s (58). Men with a dietary intake of 11 µg of selenium per day were supplemented with selenium as selenomethionine for several months. The group that received a supplement of 30 µg of selenium per day optimized its plasma glutathione peroxidase activity. Thus, a total intake (diet plus supplement) of 41 µg/day optimized plasma selenoproteins.

The other study was carried out in New Zealand in a group with a dietary selenium intake of 28 µg/day (59). Its results were more difficult to interpret because of the high basal selenium intake. However, results indicated a similar requirement for optimization to that found in China.

In 2000, on the basis of these two studies, the Institute of Medicine set the RDA for selenium at 55 µg/day for adults of both sexes (60). Table 2 shows the corresponding values for other subjects as well. These recommendations are for healthy people and are meant to satisfy the biochemical selenium requirements of the body. Further research will be necessary to determine whether there are special populations that need a greater selenium intake. Also, this RDA does not take into consideration the possible pharmacologic use of selenium.

SUPPLEMENTATION

Several forms of selenium are available for use as supplements. The two inorganic forms, selenite and selenate, are often used in animal experimental diets because they cannot be converted to selenomethionine, and therefore

Table 2 Recommended Dietary Allowances (RDAs) for Selenium

Group	Amount (µg/day)
Children	
1–3 yr old	20
4–8 yr old	30
9–13 yr old	40
Adolescents (14–18 yr old)	55
Adults (≥ 19 yr old)	55
Pregnant women	60
Lactating women	70

Source: From Ref 60.

tissue selenium concentrations reflect only selenoproteins. These forms have similar bioavailability (50–90%), but selenite is subject to reaction with intestinal contents. Also, selenite is an oxidant and can be quite damaging when given in large quantities. Selenite is added to salt in some selenium-deficient regions of China and to fertilizer in Finland. Both these methods of supplementation have been shown to be effective in improving the selenium status of human populations.

Selenomethionine makes up a large fraction of the normal dietary selenium. It is virtually completely bioavailable. It is less likely to cause acute toxicity than are the inorganic forms, although its toxicity is approximately the same as that of those forms under steady state conditions. Administration of selenium as selenomethionine can complicate interpretation of tissue selenium levels, and it is more expensive than the inorganic forms.

High-selenium yeast preparations are available for selenium supplementation. These are proprietary products in which yeast is grown in a high-selenium medium. Much of the selenium in the yeast is in the form of selenomethionine, but many minor forms are also present in variable amounts, and there may be considerable variation due to differences in production. Producers and marketers of these yeast preparations make various claims, but there is no good evidence for their special efficacy. These are often expensive.

All these forms of selenium are effective as supplements in delivering selenium to human beings and animals. The inorganic forms have the advantage of being inexpensive. Selenomethionine allows the person to incorporate surplus selenium into protein thus raising tissue levels of selenium. Because high-selenium yeast contains selenomethionine, it shares this property. There is, however, no evidence of beneficial effects of higher selenium levels in tissues resulting from selenomethionine supplementation.

Scientific experiments generally use inorganic selenium or selenomethionine, depending on the design of the experiment.

Research is being performed on other forms of selenium such as Se-methylselenocysteine, and on high selenium foods such as onions or broccoli, to evaluate their health-promoting activity. Such forms are not generally available at present and cannot be recommended except for research purposes.

Toxicity

Selenium can be toxic. Manifestations range from severe acute multiorgan failure after ingestion of milligram-to-gram quantities of selenious acid (selenite) to loss of hair and nails caused by chronic ingestion of more than a milligram of selenium per day for long periods.

Misformulation of selenium-containing over-the-counter products, perhaps because of addition of milligram rather than microgram quantities, is a real issue for the American public. Recent (61) and past (62) events resulted in adverse effects including significant hair loss, muscle cramps, diarrhea, joint pain, deformed fingernails, and fatigue.

Studies carried out in a high-selenium region of China indicated that brittle nails and hair loss did not

occur at intakes below 1 mg/day. On the basis of this result, the Institute of Medicine set a safe upper limit of 400 µg/day for chronic selenium intake for adults (60). In practical terms, this is about 300 µg above the dietary intake of selenium in the United States. Thus, it allows room for supplements to be taken by those who believe they might be efficacious.

CONCLUSIONS

Selenium is an essential element that has a variety of biochemical functions. It is lacking in the food supply of many countries, but there is no such evidence in North America. There is a need for research into the effects of marginal selenium intakes such as those in New Zealand, Scandinavia, and Europe. Also, studies are needed to identify better biomarkers of selenium status and to determine whether genetic and/or disease conditions raise the selenium requirement. Finally, further studies are needed to determine if ingestion of pharmacologic amounts of selenium, and in what form, can modulate cancer incidence.

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INTRODUCTION

Shiitake mushroom, the common Japanese name for *Lentinus edodes* (Fig. 1), derives from the mushroom associated with the shii tree (*Castanopsis cuspidate* Schottky) and *take*, the Japanese word for mushroom. Because Japan was the world leader in the production of this species of mushroom, the mushroom is now widely known by this name. These mushrooms are renowned in Far East countries (e.g., Japan, China, and Korea) as a food product and medicine for thousands of years. In the year AD 199, Kyusuyu, a native tribe of Japan, offered the Japanese Emperor Chuai a shiitake mushroom. Even older documents record its use in ancient China, where it was referred to as “ko-ko” or “hoang-mo.” Now shiitake is the second most commonly cultivated edible mushroom worldwide. Shiitake is an important ingredient in Far East cuisine and is increasingly finding its way to the dining tables of North Americans, Europeans, and people of other countries. In this chapter the artificial cultivation and the physicochemical and pharmaceutical properties of shiitake mushroom are described in detail. The application of modern analytical techniques has revealed numerous bioactive compounds including polysaccharides, -D-glucans, heterogalactans, glycoproteins, immunomodulatory proteins, organic acids, dietary fiber, and low-molecular-weight compounds from this mushroom. The health benefits of the shiitake mushroom are rapidly increasing, demonstrating immunomodulating, antioxidant, antitumor, antidiabetic, cholesterol-regulating, antiviral, antibacterial, antifungal, and antiparasitic activities and beneficial cosmetic applications. All scientific studies have demonstrated that shiitake is one of the most bioactively safe mushrooms. Cancer is the second leading cause of death in technologically developed countries worldwide, and the proven protective effects and use of shiitake in combination with chemo- and radiation therapy in Japan may well increase its production and popularity. More randomized, double-blind, controlled studies are needed to clarify the benefits, dosages, and therapeutic regimes for the use of shiitake in the treatment of cancer and other diseases (1).

Mycological Data for Shiitake Mushroom

A detailed description of the shiitake mushroom (Fig. 1) can be found in literature (1–3). Formally known as *Lentinus edodes* (Berk.) Singer (family Pleurotaceae), its basynonym is *Agaricus edodes* Berk. Common synonyms are *Collybia shiitake*, *Armillaria edodes*, *Agaricus russaticeps*, *Lentinus tonkinensis*, *Lepiota shiitake*, *Mastaleucomyces edodes*, *Pleurotus russaticeps*, *Cortinellus shiitake*, *Tricholoma shi-*

itake, *Cortinellus edodes*, *Lentinula edodes*, whereas common names are English, Black forest mushroom, black oak mushroom, golden oak mushroom, snake butter, pasania mushroom, oakwood mushroom, Japanese forest mushroom; Japanese names include Shiitake; and in Chinese, Shiang-gu, Shing ku, Hua Gu, Xiang, Hoang-mo.

Habitat and Distribution

Gregarious on fallen wood of a wide variety of deciduous trees, especially shii, oak, chestnut, beech, maple, sweet gum, poplar (aspen, cottonwood), alder, hornbeam, ironwood, chinquapin, mulberry (*Castanopsis cuspidate*, *Quercus*, *Castanea*, *Fagus*, *Acer*, *Liquidamber*, *Populus*, *Diospyros*, *Alnus*, *Carpinus*, *Morus*) in a warm, moist climate. Most of these are raised for artificial cultivation of shiitake mushroom. *L. edodes* occurs naturally throughout Southeast Asia. It has been reported from China, Japan, Korea, Vietnam, Thailand, Burma, North Borneo, the Philippines, Taiwan, and Papua New Guinea (3,4).

Cultivation

The cultivation of this mushroom has been practiced for a thousand years, with its cultivation originating in China during the Sung Dynasty (AD 960–1127). Both history and legend credit Wu San Kwung as the originator of shiitake cultivation. Almost every mushroom-growing village in China has a temple in his honor (5). In 1313, Chinese author Wang Cheng recorded shiitake-growing techniques in “*Book of Agriculture*.” He described how to select a suitable site, choose appropriate tools, and cut down the trees on which one could cultivate the mushrooms. He outlined the basic methods, which are as follows: Cut the bark with a hatchet and cover the logs with soil. After 1 year, top the soil and water frequently. Beat the logs with a wooden club to induce mushroom production. The mushrooms will appear after a rain (5,6). Shiitake mushroom cultivation techniques were probably introduced to Japanese farmers by the Chinese between AD 1500 and 1600 (2).

At present, shiitake is one of the five most cultivated edible mushrooms in the world (7). Its production (3.5 million tons) is second only to button mushroom *Agaricus bisporus*. Grown mainly in East Asia (China is the world leader in shiitake production), shiitake is now arousing interest worldwide (3,4,7,8). Increasing markets have been spawned, partly by the exotic and well-appreciated taste of shiitake and partly by advances in research that has demonstrated its significant medicinal properties. Shiitake mushroom is becoming popular as nutritional and medicinal product throughout Asia, Europe, and North America.

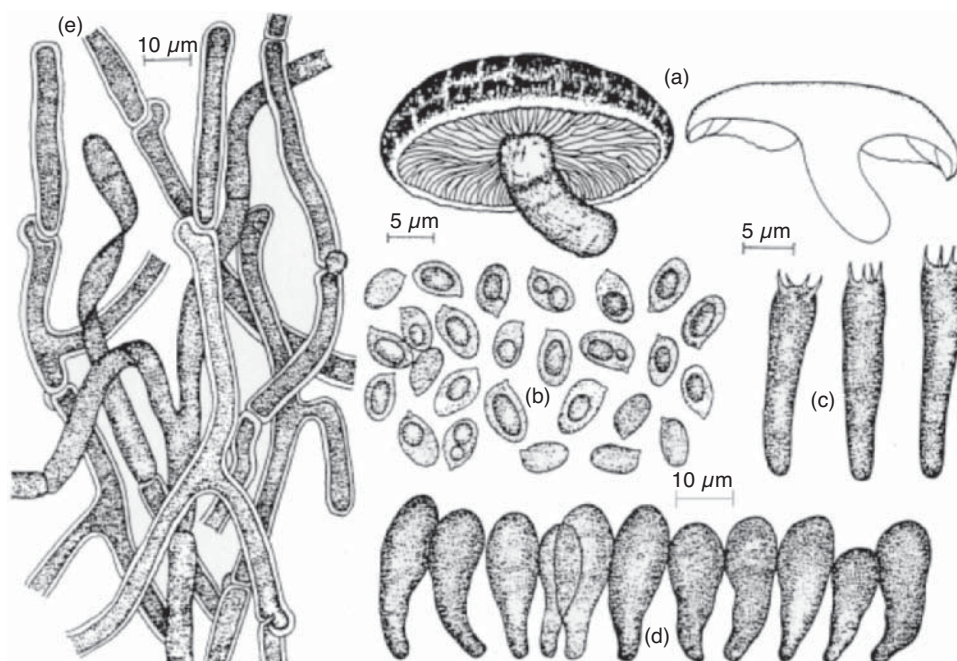


Figure 1 Shiitake mushroom [*Lentinus edodes* (Berk.) Singer]. (A) Fruit body, (B) spores, (C) basidia, (D) cheilocystidia, (E) elements of pileal cuticle.

Although specialists in medicinal mushrooms and their cultivation are familiar with shiitake mushrooms as *L. edodes*, some alternative taxonomic classifications are discussed in literature (9–13). Although the mushroom had been grown in Asian countries for centuries, the interest there, as well as in the Western countries, has increased rapidly since World War II, especially in the last 15 to 20 years. Shiitake mushroom cultivation is now a multimillion-dollar industry worldwide. World production of shiitake mushroom reached 3 million tons per year.

The process for producing shiitake mushroom fruiting bodies (Fig. 2) is the same as for other cultivated edible mushrooms and can be divided into two major stages. The first stage involves the preparation of the fruiting culture, stock culture, mother spawn, and planting spawn, and

the second stage entails the preparation of the growth substrates for cultivation. Currently, the methods most widely adopted for commercial production are wood log and synthetic sawdust bag (3,4,6,8,14). A discussion of the cultivation methods used is beyond the scope of this review. Interested readers may refer to the works already cited; growth parameters for cold- and warm-weather strains are given in Ref. 4.

Chemistry and Production of Products

Shiitake mushrooms are traditionally well-known edible mushrooms of high nutritional value. Raw or dried forms, used in Chinese curative powers of shiitake mushroom, are legendary. It was stated in *Ri Youg Ben Cao*, Vol. 3 (1620), written by Wu-Rui of the Ming Dynasty, “shiitake accelerates vital energy, wards off hunger, cures colds, and



[Enlarge Image]

Figure 2 Shiitake mushroom [*Lentinus edodes* (Berk.) Singer]: cultivated fruiting bodies.

defeats body fluid energy." In later years, it was found that the mushroom contained various important nutrients. Moreover, recent scientific investigations have isolated many compounds and have found evidence of their health-promotion activities (1,3,4,15,16).

Shiitake mushrooms have excellent nutritional value. Their raw fruit bodies include 88% to 92% water, protein, lipids, carbohydrates as well as vitamins and minerals. It should be noted that amounts of nutrients and biologically active compounds differ in various strains and are affected by substrate, fruiting conditions, and methods of cultivation. On a dry weight basis, they have a relatively high nutritional value when compared to commonly consumed vegetables.

Dried shiitake mushrooms are rich in carbohydrates and protein. They contain 58% to 60% carbohydrates, 20% to 23% protein (digestibility of 80–87%), 9% to 10% fiber, 3% to 4% lipids, and 4% to 5% ash. The mushroom is a good source of vitamins, especially provitamin D₂ (ergosterol), 325 mg%, which under ultraviolet light and heat yields calciferol (vitamin D₂). It also contains B vitamins, including B₁ (thiamine), B₂ (riboflavin), B₁₂ (niacin), and pantothenic acid (1,6,15,16). Minerals found in shiitake mushroom include Fe, Mn, K, Ca, Mg, Cd, Cu, P, and Zn. Analysis of dried cultured shiitake mycelium gives the following mineral concentrations (in mg/g of dry weight): K, 15.1; Ca, 22; Mg, 44–78; Mn, 1.2; Cd, 0.96; Fe, 2.36; Ni, 52.5; Cu, 89.1; P, 281; Zn, 282; Ge, 3; Br, 11.4; and Sr, 164.

Water-soluble polysaccharides amount to 1% to 5% of the dry weight of the shiitake mushroom. In addition to glycogen-like polysaccharides, (1–4)-, (1–6)- α -D-glucans and antitumor polysaccharides, lentinan, (1–3)-, (1–6)- β -bonded heteroglucans, heterogalactans, heteromannans, xyloglucans, etc., have been identified. The mushrooms' indigestible polysaccharides, which serve as dietary fiber, include heteroglycan, polyuronide, β -glucan, as well as chitin. Among the free sugars present are trehalose, glycerol, mannitol, arabinol, mannose, and arabinose (1,3,4,15,16).

In shiitake mushrooms, dietary fiber consists of water-soluble compounds such as β -glucan and protein and water-insoluble substances extractable only with salts, acids, and alkalies such as polyuronide (acidic polysaccharide), hemicellulose, β -glucan with heterosaccharide chains, lignin, and chitin present as cell-wall constituents (15).

The aroma components include alcohols, ketones, sulfides, alkanes, fatty acids, etc. The major volatile flavor contributors are *matsutakeol* (octen-1-ol-3) and ethyl-*n*-amyl ketone. The characteristic aroma of shiitake mushrooms was identified as 1,2,3,5,6-pentathiepane. According to Mizuno (15), the components responsible for the delicious flavor are monosodium glutamate, 5'-nucleotides, free amino acids, lower molecular weight peptides, organic acids, and sugars. Their relative ratios are responsible for the variation in flavor naturally seen in this mushroom. Organic acids contributing to the flavor of shiitake mushroom include malic acid, fumaric acid, α -keto-glutaric acid, oxalic acid, lactic acid, acetic acid, formic acid, and glycolic acid.

The fatty acids account for 3.38% of the total lipids (14,15). Their composition is as follows: linoleic acid (18:2), 72.8%; palmitic acid (16:0), 14.7%; oleic acid (18:1), 3.0%;

tetradecenoic acid (14:1), 1.6%; stearic acid (18:0), 0.9%; and myristic acid (14:0), 0.1%.

Dosage and Preparation of Products

Shiitake mushroom concentrate can be freeze-dried or spray-dried to form a granular powder. There are many products containing powdered shiitake mushroom extract, such as a mixture of this powder with vitamin C crystals or with medicinal plants such as ginseng. In Eastern countries, the mushroom is mainly used as a concentrate when extracted with boiling water. Residues from these processes still contain substantial amounts of useful polysaccharide substances, including those effective as antitumor compounds such as β -glucans, nucleic acids, dietary fiber, etc. An alcohol extraction product is obtained by preserving fresh or dried shiitake mushroom in alcohol, which has been mixed with sugar or molasses.

Shiitake mushroom is prescribed in various forms. It may be injected as a solution (1 mg/vial) or ingested as a sugar-coated tablet, capsule, concentrate, powdered extract, syrup, tea, wine, and/or as a medicinal dish. Tablets are usually made from a dried water extract of the mycelia or fruiting bodies because drying concentrates the lentinan and other active principles. Standardized extracts are also available, and they are preferred because the amount of lentinan present is certified and clearly stated on the bottle. Lentinan's anticancer effect is highly dose dependent. The standard dose of the dried fruiting body in tea or in mushroom dishes is given as 6–16 g, equivalent to approximately 60–160 g of fresh fruiting bodies. The dosage, usually in the form of 2 g tablet, is 2 to 4 tablets per day.

Commercial preparations can be found in many countries in health food stores and supermarkets. Although the fresh form can be a valuable dietary supplement, the quantities one would require for therapeutic doses are so great that its consumption could cause digestive upset. That is why *L. edodes* mycelium (LEM), which is concentrated and easily absorbed, is preferred for medicinal use (3,4,16).

Fresh and dried shiitake mushrooms are used in medicinal mushroom dishes ("Yakuzen"). These dishes can be prepared in many ways, limited only by one's ingenuity: boiled, grilled, skewered, or on aluminum foil with different types of seasoning. Concentrates, obtained by concentrating boiling water extracts of whole fruit bodies or powdered mushrooms, are used as drinks. They can also be consumed as canned "shiitake tea" or many other shiitake "healthy tea" products sold as mushroom-containing tea bags. Some products, including "healthy shiitake wine," are sold as a nightcap or as a tonic drink (4,15).

Preclinical Studies

This section mainly discusses preclinical in vitro and in vivo (animal) studies.

Shiitake is one of the best-known and best-characterized mushrooms used in medicine. It is the source of several well-studied preparations with proven pharmacological properties, especially polysaccharide lentinan, shiitake mushroom mycelium, and culture media extracts (LEM, glycoprotein (LAP) and KS-2) (3,14,15,17–19).

Anticarcinogenic and Antitumor Effects

By using methods of fractionation and purification of polysaccharides, Chihara et al. (20–22) isolated a water-soluble antitumor polysaccharide from fruiting bodies of shiitake, which was named “lentinan” after the genus *Lentinus* to which the shiitake mushroom belongs. Chihara was one of the first to report on the antitumor properties of the mushroom, stating that lentinan “was found to almost completely regress the solid type tumors of Sarcoma 180 and several kinds of tumors including methylchloranthrene-induced fibrosarcoma in synergic host-tumor system A” (21,22). The antitumor effect of lentinan was originally confirmed by using Sarcoma 180 transplanted in CD-1/ICD mice (20). Later, it showed prominent antitumor activity not only against allogenic tumors such as Sarcoma 180, but also against various synergic and autochthonous tumors, and it prevented chemical and viral oncogenesis (23). The molecular formula of β -D-glucan lentinan is $(C_6H_{10}O_5)_n$, and the mean molecular weight is about one million ($\sim 5 \times 10^5$ Da); $[\alpha]_D + 20$ – 22° (NaOH). Its structure was confirmed as β -(1–3)-D-glucopyranan with a branched chain of β -(1–6)-monoglycosyl (branching degree: 2.5°), showing a right-handed triple helix (3,15,17,18). It is water soluble, heat stable, and alkali labile. β -D-Glucan binds to lymphocyte surfaces or serum-specific proteins, which activate macrophage, T-helper cells, natural killer (NK) cells, and other effector cells. All these increase the production of antibodies as well as interleukins (IL-1, IL-2) and interferon (IFN- γ) released upon activation of effector cells (19,24). Thus, the carcinostatic effect of lentinan results from the activation of the host’s immune system. In animal testing of carcinostatic activity, IP administration is used, but oral administration (PO) is occasionally effective.

The purified polysaccharide has been shown in animal studies to produce strong tumor regression and even the disappearance of sarcoma tumors in 5 weeks, ascite hepatoma 134, (18,19,25), and Ehrlich carcinoma as well as a number of other experimentally induced cancers in allogenic, syngeneic, and autologous hosts. It also exhibits preventive activity against chemical carcinogenesis. Injections of lentinan into mice produced either an 80% reduction in tumor size or complete regression in most of the animals tested. Moreover, an intact immune system and a functioning thymus gland were found to be requisite for its anticancer effect (9,10). When immunosuppressive agents such as β -benzylthioguanosine or X-radiation were given with lentinan, the antitumor effect decreased. The polysaccharide has also been found to restore the enzyme activity of X-prolyl-dipeptidyl-aminopeptidase, which can be depressed in cancer patients and in mice with implanted tumors (26).

Laboratory tests seem to indicate a role for the adrenal-pituitary axis and central peripheral nervous system including serotonin, 5HT, histamine, and catecholamines in lentinan’s antitumor activity (1,16,17,24).

The oral administration of the polysaccharide to AKR mice exerted strong antitumor activity resulting in raised levels of lymphocytokines, such as IFN- γ , tumor necrosis factor (TNF- α), and IL-1 α . Tissue cultures of murine macrophages CRL-2019, B-lymphocytes HB-284, and T-lymphocytes DRL-8179, which were treated with lentinan, showed high levels of activation by using flow

cytometry. Lentinan-activated immunocytes, particularly the T-helper cells, might render the physiological constitutions of the host highly cancer- and infection resistant. Adoptive immunotherapy of the immunodeficient mice such as the nude (athymic) mice, B-cell deficient mice, and severe combined immunodeficient mice via the transfer of the lentinan-activated immunocytes resulted in the inhibition of tumor growth. Lentinan appeared to represent a unique class of host defense potentiators (HDP), protecting the hosts from the side effects of conventional therapeutic measures and improving various kinds of immunological parameters with no toxic side effects in animal models (19,24,27,28). Lentin, a novel protein isolated from shiitake mushroom, exerted an inhibitory activity on proliferation of leukemia cells (28).

Compounds that block the formation of carcinogenic *N*-nitroso compounds from nitrates (which occur in vegetables and meats) are produced in dried and heated mushrooms (3,4,16). The uncooked form contains no detectable amounts of the nitrite-scavenging compound thiazolidine-4-carboxylic acid, whereas the dried variety has 134 mg/100 g (dry weight basis) of this compound, and the boiled form holds 850 mg/100 g. Thus, shiitake mushrooms may have cancer-preventative properties and can be a beneficial dietary supplement.

LEM and LAP Extracts from Shiitake Mushroom Mycelium and Culture Media

LEM is prepared from an extract of the powdered mycelia of the shiitake mushroom. Its yield is approximately 6 to 7 g/kg of medium. The precipitate obtained from a water solution of the mycelium by adding four volumes of ethanol was named LAP. The yield of LAP is ≈ 0.3 g/g of LEM. LEM and LAP are glycoproteins containing glucose, galactose, xylose, arabinose, mannose, and fructose (15). The former also contains various nucleic acid derivatives, vitamin B compounds especially B₁ (thiamine), B₂ (riboflavin), and ergosterol (3,4).

In 1990, an immunoactive substance, EP3, was obtained by fractionation of LEM. EP3 is a lignin complex composed of approximately 80% lignin, 10% carbohydrates, and 10% protein. After removal of the last two components, biological activity was not affected, but when lignin is removed, activity was reduced. Therefore, the active substance is believed to be a water-soluble lignin containing numerous carboxyl groups (14,15).

Both LEM and LAP have demonstrated strong antitumor activities orally and by injection to animals and humans. They were shown to act by activating the host’s immune system and are also useful for the treatment of hepatitis B (12–15).

KS-2- α -Mannan Peptide

Polysaccharide KS-2 was obtained by extraction of cultured mycelia of shiitake mushroom (strain KSLE 007) with hot water, followed by precipitation with ethanol (14,15,29). The product is an α -mannan peptide containing the amino acids serine, threonine, alanine, and proline (as well as residual amounts of the other amino acids). The polysaccharide was shown (29) to be effective on Sarcoma 180 and Ehrlich’s carcinoma, either IP or PO, and to act via interferon-inducing activity. The acute LC₅₀

of KS-2 was found to be extremely low in mice, more than 12,500 mg/kg when administered orally.

The mechanism of action of KS-2 is not yet clear, but the results showed no direct KS-2 cytotoxic effect against the tumor cells *in vitro*. Its antitumor activity was observed to be higher at the lower inoculum size of tumor cells, regardless of the routes of KS-2 administration (60% survival rate at 5×10^3 tumor cells/mouse, 10% survival at 1×10^6 tumor cells/mouse). The results also showed that the antitumor activity of KS-2 in mice was always accompanied by the induction of interferon in the sera. Furthermore, preliminary findings indicated that macrophages obtained from KS-2-treated mice exhibited tumoricidal activity (14,16,30), and it was reported that macrophage activation became tumoricidal when incubated *in vitro* with interferon. Considering these findings, the antitumor activity of KS-2 may be explained by macrophage activation with or without interferon induced by KS-2.

Immune-Modulating Effects

As was stated earlier, lentinan and other polysaccharides from shiitake mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host. Lentinan, for example, appears to act as HDP, which is able to restore or augment the responsiveness of host cells to lymphokines, hormones, and other biologically active substances by stimulating maturation, differentiation, or proliferation of cells involved in host defense mechanisms (19,24). HDPs are functionally different from biological response modifiers. Thus, lentinan is able to increase host resistance against various kinds of cancer and infectious diseases, including acquired immunodeficiency syndrome (AIDS) (3,28).

The initial interactions of lentinan in the human body or animals are not presently known. However, there is a transitory but notable increase in several serum protein components in the α - and β -globulin region, namely, complement C3, hemopexin, and ceruloplasmin (3,16,19,24).

Lentinan stimulates various kinds of NK-cell-, T-cell-, B-cell-, and macrophage-dependent immune reactivities. Its antitumor effect is abolished by neonatal thymectomy and decreased by the administration of antilymphocyte serum, supporting the concept that the polysaccharide requires immunocompetent T-cell compartments. The effect of lentinan was also inhibited by antimacrophage agents, for example, carrageenan. Unlike other well-known immunostimulants, lentinan is in a unique class of distal tubular-cell-oriented assistant, in which macrophages play some part (3,16,19,24).

For example, lentinan can activate NK cells *in vitro* in the same concentrations that are achieved in the blood plasma of patients treated clinically with lentinan (14,16,24). NK-cell activity is involved in tumor suppression, and although these cells do not stimulate T-killer cell activity or do so only under certain conditions, they are strong T-helper cell stimulants both *in vitro* and *in vivo* (1,3,14,16,19,24). Using the blood of healthy donors and cancer patients, some authors have shown that the polysaccharide is able to stimulate peripheral blood lymphocytes *in vitro* to increase IL-2-mediated lymphokine-activated killer cell (LAK-cell) and NK cell activity at levels achievable *in vivo* by administration of clinical doses

of lentinan. It has been shown to inhibit suppressor T-cell activity *in vivo* and to increase the ratio of activated T-cells and cytotoxic T-cells in the spleen when administered to gastric cancer patients undergoing chemotherapy (3,16,24).

Many interesting biological activities of lentinan have been reported including (a) an increase in the activation of nonspecific inflammatory responses such as acute phase protein production, (b) vascular dilation and hemorrhage *in vivo*, (c) activation and generation of helper and cytotoxic T-cells, (d) augmentation of immune mediators like IL-1 and IL-3, colony-stimulating factor(s), and migration inhibitory factor, and (e) increasing the capacity of peripheral blood mononuclear cells of patients with gastric cancer and producing IL-1 α , IL-1 β , and a TNF- α (3,16,19,24,27).

In an *in vivo* study of rats with peritonitis, combined lentinan-gentamicin treatment had a significantly better survival rate than the controls. Lentinan activated the peritoneal macrophages' secretory activity of active oxygen and produced cytokines, thus enhancing the ability of polymorphonuclear leukocytes to produce active oxygen, which has a bactericidal effect (31). It also increases peritoneal macrophage cytotoxicity against metastatic tumor cells in mice, but not against a highly metastatic tumor type (32). Some patients treated with lentinan for carcinomatous pleuritis or carcinomatous peritonitis have improved with the disappearance of malignancy, whereas in another group their condition deteriorated or diminished (33). The polysaccharide can activate the normal and alternative pathways of the complement system and can split C3 into C3a and C3b enhancing macrophage activation (34).

Many biological reactions are accelerated and induced by lentinan, including the very important phenomenon of infiltration of eosinophils, neutrophils, and granulocytes around target tissues. Early responses initiated by lentinan and possible pathways for inflammatory reactions are discussed by Mizuno (14).

Lentinan's immune-activating ability may be linked with its modulation of hormonal factors, which are known to play a role in tumor growth. Aoki (34) showed that the antitumor activity of lentinan is strongly reduced by administration of thyroxine or hydrocortisone. It can also restore tumor-specific antigen-directed delayed-type hypersensitivity reaction.

Lentinan is not formally included among the nonspecific immunostimulants (RES stimulants), but it augments the induction of antigen-specific cytotoxic T-lymphocytes, macrophages, and other nonspecific immune responses. Possible immune system regulating actions of lentinan were summarized by Chihara et al. (23).

Cardiovascular Effects

It is known that shiitake mushroom is able to lower blood serum cholesterol (BSC) via a factor known as eritadenine (also called "lentinacin" or "lentysine"). Apparently, eritadenine reduces BSC in mice, not by the inhibition of cholesterol biosynthesis, but by the acceleration of the excretion of ingested cholesterol and its metabolic decomposition. It has been shown to lower blood levels of cholesterol and lipids in animals. When added to the diet of rats, eritadenine (0.005%) caused a 25% decrease in total cholesterol in as little as 1 week. The cholesterol-lowering

activity of this substance is more pronounced in rats fed a high-fat diet than in those on a low-fat diet. Although feeding studies with humans have indicated a similar effect, further research is needed. Hobbs (1,16) and Yang et al. (35) have shown that shiitake mushrooms lowered BSC levels. Various studies have confirmed (1,3,14,16) that the mushroom can lower blood pressure and free cholesterol in plasma, as well as accelerate the accumulation of lipids in the liver by removing them from circulation.

Hepatoprotective Effects

The injection of LEM slowed the growth of cancerous liver tumors in rats (14,18,36). A polysaccharide fraction from shiitake mushrooms demonstrated liver protection in animals as well as the ability to improve liver function and enhance the production of antibodies to hepatitis B (3,37). Lentinan improved serum glutamic pyruvic transaminase and completely restored glutamic pyruvic transaminase (GPT) levels in the livers of mice with toxic hepatitis. Crude extracts of shiitake mushroom cultures have demonstrated liver-protecting actions (14,16,18,37).

Antiviral, Antibacterial, and Antiparasitic Effects

Lentinan and its derivatives are effective against various kinds of bacterial, viral (including AIDS), and parasitic infections (3,16,18,28,38). An important area of this polysaccharide research deals with its ability to mobilize the body's humoral immunity to ward off bacterial infections resistant to antibiotics (3). Many cancer and AIDS patients die of opportunistic infections due to immunodysfunction (3,27). In vitro studies show that lentinan, when used in combination with azidothymidine (AZT), suppressed the surface expression of HIV on T-cells more so than did AZT alone (39). Lentinan and the sulfated form exhibited potent in vitro anti-HIV activity resulting in inhibition of viral replication and cell fusion. Among the various therapeutic approaches used, prevention of the development of AIDS symptoms in carriers should be stressed. Based on these in vitro studies, it is possible that such prevention may be realized by the use of HDPs such as lentinan or its related substances. For example, LEM may have potential in the treatment of AIDS. It has been shown to inhibit HIV infections of cultured human T-cells, and it potentiates the effects of AZT against viral replication in vitro. The mechanism of its action is not known for certain, but the extract was found to activate macrophages and stimulate the production of IL-1 (3,16,35,39). Lentin, a novel protein isolated from shiitake mushroom, exerted an inhibitory activity on HIV-1 reverse transcriptase (28).

Water-soluble lignins EP3 and EPS4 from shiitake mushroom mycelium have shown antiviral and immunomodulating effects (40). A water-soluble extract of mycelium known as JLS and JLS-18 has the ability to block the release of *herpes simplex* virus type 1 in animals JLS-18-consisting of 65% to 75% lignin, 15% to 30% polysaccharides, and 10 to 20% protein has inhibited the herpes virus both in vitro and in vivo (41).

In addition, lentinan has shown (a) antiviral activity in mice against vesicular stomatitis virus encephalitis virus, Abelson virus, and adenovirus type 12; (b) stimulated nonspecific resistance against respiratory viral infections in mice; (c) conferred complete protection against an LD75 challenge dose of virulent mouse influenza

A/SW15; (d) enhanced bronchoalveolar macrophage activity; (e) increased resistance against the parasites *Schistosoma japonicum* and *S. mansoni*; (f) exhibited activity against *Mycobacterium tuberculosis* bacilli resistant to anti-tuberculosis drugs, *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Candida albicans*, and *Saccharomyces cerevisiae*; and (h) increased host resistance to infections with the potentially lethal *Listeria monocytogenes*. Antibacterial polyacetylene compounds, centinamycin A and B, have also been identified in shiitake mushroom. Eritadenine, a compound that affects cholesterol metabolism, also possesses antiviral properties (3,16,37).

It should be noted that a protein fraction of shiitake mushroom fruiting bodies, labeled fruiting body protein (FBP), prevented the infection of plants with tobacco mosaic virus. The binding of the virus to the plant cells was inhibited by FBP (3,14,15).

Antifungal Activity

From the fruiting bodies of the shiitake mushroom, a novel protein designated lentin with potent antifungal activity was isolated in 2003 (28). It was unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and Mono S. The N-terminal sequence of the protein manifested similarity to endoglucanase. Lentin, which had a molecular mass of 27.5 kDa, inhibited mycelia growth in a variety of fungal species including *Physalosporia piricola*, *Botrytis cinerea*, and *Mycosphaerella arachidicola* (28).

Human Clinical Studies and Medicinal Uses

In the last 15 to 20 years (2), shiitake mushroom has been subject to various clinical studies in humans and is thought to be beneficial for a wide variety of disorders including different types of cancer, heart disease, hyperlipidemia (including high blood cholesterol), hypertension, infectious disease, and hepatitis. The mushroom is used medicinally for diseases involving depressed immune function (including AIDS), cancer, environmental allergies, fungal (especially *Candida*) infection, frequent flu and colds, bronchial inflammation, and regulating urinary incontinence. It was shown that the success of immune adjuvant in therapy depends on the type of cancer (location) being treated, the individual's general health, immunological and hormonal status as well as the individual's constitution.

Cancer

Lentinan was demonstrated to have antitumor activity as well as to increase the survival time of patients with inoperable gastric cancer (16) and women with recurrent breast cancer following surgical therapy (for details on protocols, see Refs. 3,16,18). When the polysaccharide is administered once or twice a week with chemotherapy to a patient with progressive cancer but with no serious liver, kidney, or bone marrow dysfunction, it produced a statistically significant improvement in immune and anticancer activity when compared to chemotherapy alone (42,43). Two hundred seventy-five patients with advanced or recurrent gastric cancer were given one of two kinds of chemotherapy (mitomycin C with 5-fluorouracil or tegafur) either alone or with lentinan injections. Statistically, the best results were obtained when lentinan was administered prior to chemotherapy and in patients with a basis

of prolongation of life, regression of tumors or lesions, and the improvement of immune responses.

Lentinan was administered into malignant peritoneal and/or pleural effusions of a group of 16 patients with advanced cancer (44). Eighty percent of the lesions showed probable clinical responses, with an improvement in performance status demonstrated in seven subjects. The survival time for those who responded immunologically to the treatment was 129 days and 45 days for those who did not respond.

Viral Diseases

LEM from shiitake mushroom has been shown boost the immune response in AIDS patients (3,16,18). When it was used to treat HIV-positive patients with AIDS symptoms, the T-cell count rose from a baseline of 1250/mm³ after 30 days up to 2550/mm³ after 60 days. An improvement in clinical symptoms was also noted. Although in vitro studies have indicated that lentinan and LEM from shiitake mushroom may be more effective than AZT in the treatment of AIDS (see discussions in the section on "Pre-clinical Studies"), it must be stressed that more clinical trials will be necessary to assess the long-term benefit of the these products for HIV and AIDS.

Lentinan has shown favorable results in treating chronic persistent hepatitis and viral hepatitis B (16). Forty patients with chronic viral hepatitis B and seropositive for Hbe antigenemia were given 6 g of LEM daily (orally) for 4 months. The study focused on the number of patients seroconverting from Hbe antigen positive to antiHbe positive, which was 25% after LEM therapy, and was higher in patients with chronic active hepatitis (36.8%). In addition, 17 patients (43%) became seronegative for Hbe antigen. Liver function tests improved even for patients who remained seropositive, and they had raised plasma albumin, and adjusted protein metabolism.

Cardiovascular Disease

Dried shiitake mushroom (9 g/day) decreased 7% to 10% serum cholesterol in patients suffering with hypercholesterolemia. For many patients 60 years of age or older with hyperlipidemia, consuming fresh shiitake mushroom (90 g/day in 7 days) led to a decrease in total cholesterol blood level by 9% to 12% and triglyceride level by 6% to 7% (16,35).

Toxicity and Side Effects

Shiitake mushroom is edible, but some individuals may experience minor side effects or allergic reactions. Literature describes (3,16,18,45) cases of shiitake-induced toxicodermia and shiitake dermatitis. Allergic reactions to the spores of shiitake mushrooms have been reported in workers picking mushrooms indoors, who are prone to an immune reaction to spores called "mushroom worker's lung." Symptoms include fever, headache, congestion, coughing, sneezing, nausea, and general malaise (46). A water extract of the fruiting body was found (47) to decrease the effectiveness of blood platelets in initiating coagulation. So people who bleed easily or who take blood thinners should be closely monitored when under long-term treatment with shiitake mushroom or its water-soluble fractions.

LEM has shown no evidence of being acutely toxic, even in massive doses of over 50 mg/day for 1 week, though mild side effects such as diarrhea and skin rash may occur. In this respect, the author does not consider these as massive doses. As a rule, symptoms disappear after a short period, when the body has adapted to the extract. Lentinan has no known serious side effects. However, in clinical trials of patients with advanced cancer, minor side reactions occurred such as a slight increase in glutamate-oxaloacetate transaminase and GPT liver enzymes and a feeling of mild pressure on the chest. But these changes disappeared after lentinan administration was stopped (34).

Drug Interactions

A watery extract of the whole fruiting body of *L. edodes* is reported to lessen the effectiveness of the blood platelets during the process of coagulation. People who bleed easily or who take blood thinners should use caution when chronically using *L. edodes* extracts in therapeutic amounts or in its water-soluble fractions (LEM) (16,47).

For cancer patients, smaller doses of intravenous and intramuscular lentinan are more effective than larger ones (e.g., 1 mg per injection is considered safe, whereas 10 mg may produce marked depression in the host immune response). Aoki (34) notes that what is considered an excessive dosage intravenously may be a favorable dosage when using oral administration.

For treating the initial stages of AIDS or chronic hepatitis, the best oral dose of LEM is between 2 and 6 g/day in two to three divided doses. If the disease is stable, the dosage may be decreased to 0.5 to 1 g/day (3,16).

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St. John's Wort

Jerry M. Cott

INTRODUCTION

St. John's wort (SJW), *Hypericum perforatum* L. (Hypericaceae) is a perennial plant and one of the best known and well researched of the western herbals. Though clearly a favorite medicinal among herbalists and European physicians, concerns have arisen because of reports of drug interactions and lack of efficacy. SJW (Fig. 1) has been used for millennia for its many medicinal properties, including wound healing, treatment for kidney and lung ailments, insomnia, and depression. Current uses are primarily in treating central nervous system (CNS) indications such as depression, anxiety, and insomnia, but formulations are also available for other uses as well. Although the identity of the phytochemical constituents responsible for biological activity in humans remains unknown, the chemistry of the plant has been well studied. Constituents identified to date belong to several chemical classes and include hypericin, hyperforin, and various flavonoid glycosides.

Background

SFW preparations have become increasingly popular in Germany where they are approved for use in the treatment of mild-to-moderate depression and have remained a first-line treatment for many years. The plant is named for its flowering time at the end of June, around the birthday of John the Baptist. Originally brought from Europe to North America, the plant can be found growing wild along roadsides and in fields and pastures where livestock poisoning, due to photosensitivity, was not uncommon. As already noted, current uses are primarily in treating CNS indications such as depression, anxiety, and insomnia. Oil-based preparations are used for stomach upsets and are also applied topically to treat bruises, muscle aches, and first-degree burns (1). A cream-based formulation was recently shown to be more effective than the vehicle for treating atopic dermatitis (2). In 2008, SJW was the eighth best-selling herbal supplement (at just over US\$8 million) in the food, drug, and mass market retailers category in the United States, up 1.5% from 2007 (3). These sales totals are generally lower than those of previous years. Causal factors for reduced sales may include published reports of poor quality control (4), herb-drug interactions (see Table 4), and concern that SJW may be ineffective due to the publication of the clinical trial data that failed to show an effect in the *Journal of the American Medical Association* (5,6)



Figure 1 St. John's wort. (Compliments of Peggy Duke.)

Chemistry and Preparation of Products

SJW has long been known to contain red pigments that have been postulated to be the primary active constituent(s) in this plant genus, though there is little or no evidence for this assumption, other than a weak inhibition of MAO in vitro. These compounds are the naphthodianthrone hypericin and pseudohypericin (Fig. 2), and other derivatives that make up approximately 0.1% to 0.15% by dry weight. The plant also contains flavonoid glycosides (hyperoside, quercitrin, isoquercitrin, rutin), free flavonoids (quercetin, biapigenin) (2–4%), the biflavonoid, amentoflavone, and the phloroglucinols, primarily hyperforin (2–4%) (Fig. 3). The latter constituent is the one most often invoked as the “active” principle. Other

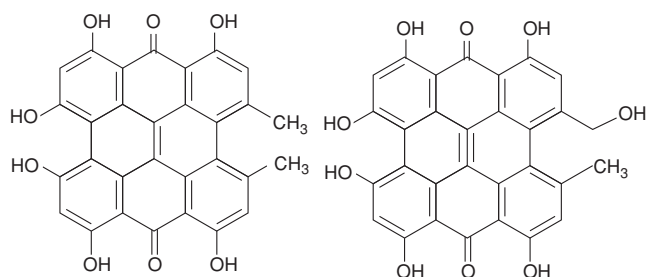


Figure 2 The naphthodianthrone hypericin and pseudohypericin.

constituents include volatile oils, tannins (6.5–15%), and caffeic acid derivatives (7). Although originally believed to be a necessary component for antidepressant activity, hypericin is now considered primarily as a marker compound for purposes of botanical identification. It is responsible for the photosensitivity side effects that have long been known (8).

The early SJW clinical studies from Germany examining antidepressant activity were based on extracts standardized to hypericin only (7). More recent research has suggested that hyperforin might also be important, but that this constituent may degrade (oxidize) under normal manufacturing conditions (9). Therefore, some companies began to stabilize their formulations (such as by the addition of ascorbic acid) to prevent oxidation and standardize the hyperforin content at 3% to 5% (9). The necessity for this was called into question when the relatively hyperforin-free (<0.2%) formulation, Ze117, showed clinical antidepressant efficacy in major depression (see later). Beginning in 1996, there have been several reports that SJW products failed to meet label claims for naphthodianthrone (4). However, most of the products marketed in the United States over the time frame covered by these publications were assessed using an ultraviolet (UV) spectrophotometric method for determination of total naphthodianthrone first promulgated in the 1985 *German Pharmaceutical Codex* (DAC). This outdated method yields considerably higher results than the more accurate and specific high-performance liquid chromatography methods now specified by the United States Pharmacopeia (USP). One result of this difference in analytical approach is that for some years, label claims on products seemed

high because they were generated using the outdated method, whereas results obtained by postmarket analysis using modern methods were lower. The therapeutic relevance of these method-based discrepancies in phytochemical content remains questionable, as these constituents, particularly hypericin and hyperforin, have not been definitively shown to be crucial for efficacy. However, herbal products are required to contain the constituents identified on their label in the declared amounts.

USP dissolution standards require that ingredients identified on the label must also be released either in the stomach or in small intestine within a defined time (which is generic and not product- or plant-specific). Specific tests of dissolution by using the USP method (with paddle stirring of simulated gastric fluid at 37°C), with or without the addition of bile components, have been carried out on several different preparations of SJW (10). Results suggest that the hydrophilic flavonoids are released by most formulations within the specified time, but the more lipophilic hypericins and hyperforin are poorly released within the specified time. Although Jarsin® 300 (LI160; Lichtwer Pharma AG, Berlin, Germany) has shown antidepressant activity in many trials, and was used in the two American studies, it released less than 25% of its hyperforin and 50% of its flavonoids within 4 hours. Other experiments indicate that the stability of SJW preparations—especially under conditions of high humidity—may be of concern (11).

Preclinical Studies

Receptor-binding studies with physiologically plausible concentrations of the crude extract have shown little affinity for any of the standard neurotransmitter receptors, with the exception of γ -aminobutyric acid (both types A and B) (12). Many individual components of SJW have been evaluated in binding assays, and a review of these data is presented in recent papers by Butterweck et al. (13,14). As these authors explain, the data are difficult to interpret because the constituents are present in commercial extracts at levels several orders of magnitude below their K_i ; there are occasional solubility problems; and there is a lack of information regarding functional activity.

SJW has been reported to share a mechanism (uptake inhibition) with synthetic antidepressants. But just as the initial research with hypericin suggested a monoamine oxidase (MAO) inhibition mechanism, the uptake inhibitor hypothesis is based more on conventional wisdom about antidepressant mechanisms rather than on data. Hyperforin is the constituent postulated as the primary uptake inhibitor because it has been reported to affect the serotonin 5-hydroxytryptamine (5HT) re-uptake process, the concentrations required are unrealistically high (requiring up to 1000 times higher concentrations than synthetic uptake inhibitors). In addition to 5HT, uptake of every other neurotransmitter that has been measured appears to be inhibited by either hyperforin or the whole extract as well. This lack of specificity is not consistent with the profile of an agent that has a true pharmacologic effect. In addition, the side effects of SJW are not at all similar to those of other 5HT uptake inhibitors (see clinical reviews). Other studies suggest that neither SJW nor hyperforin is a true uptake inhibitor as they do not bind to the 5HT uptake site (Table 1).

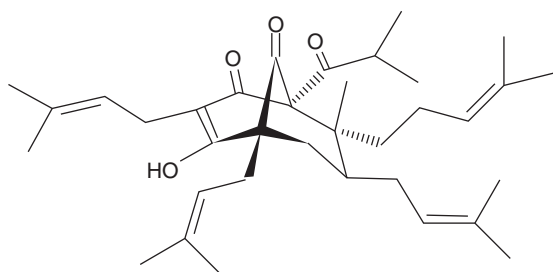


Figure 3 Hyperforin.

Table 1 Uptake Inhibition and Hyperforin

Hyperforin-claimed activity
Primary constituent linked to "uptake inhibition" (21,22)
Does not bind uptake site (23,24)
Causes monoamine release (25)
Release related to intracellular sodium concentration (23)
Release related to calcium mobilization (26)
Release related to ion channel modulation (27)
No uptake inhibition occurs in humans (28)
SJW side effects unlike uptake inhibitors (29)

Rather than inhibiting re-uptake, they appear to cause the release of monoamines and other neurotransmitters from synaptosomes, yielding the same net effect in the *in vitro* assay. This "pseudo uptake inhibition" may result in non-selective transmitter release that is related to increasing intracellular sodium concentrations, calcium mobilization, and ion channel modulation (Table 1). In addition to the *in vitro* evidence previously outlined, treatment with SJW also fails to inhibit uptake in depressed human patients, unlike the tricyclic antidepressants (TCAs) and selective serotonin re-uptake inhibitor (SSRI) antidepressants (Table 1). Finally, the relatively hyperforin-free formulation, Ze117 (15) (250 mg, twice daily), shows clinical antidepressant efficacy when compared with placebo (16) and has equivalence to 20 mg/day fluoxetine (17) and 150 mg/day imipramine (18) in major depression. Efficacy of this formulation is reported to be maintained for up to 1 year (19). These reports support the Butterweck hypothesis that flavonoids are at least partly responsible for the therapeutic efficacy of SJW extracts (20). Table 1 summarizes some of the controversies regarding the importance of hyperforin, whereas Table 2 describes various proposed mechanisms of the action of SJW.

Table 2 Other Proposed Mechanisms of Antidepressant Action

Antidepressant mechanism	Probable relevance	Reason
MAO inhibition	—	Very weak effect (12)
	—	No effect <i>in vivo</i> (30)
	—	No MAOI side effects (29)
Inhibition of IL-6	—	Requires high concentrations (31)
Inhibition of dopamine- β -hydroxylase	—	Requires 21 μ M (32)
Direct neurotransmitter release	—	No current examples (33)
β -receptor downregulation	—	Requires up to 1 mg/mL (34)
Alteration of neuronal membrane fluidity	?	Insufficient data (35)
Adenosine receptor antagonism	—	Requires 0.1–0.5 mg/mL (36)
CRF ₁ antagonism	—	Requires 30-fold higher plasma levels (37)
Inhibition of free radical production	?	Insufficient evidence; high concentrations? (38)
Combination of other mechanisms	—	No evidence (39)

Although SJW demonstrates very weak monoamine oxidase (MAO) inhibition *in vitro*, *in vivo* administration (IP) of SJW to rats shows no effect on MAO. This is supported by the relative lack of reported cases of potential MAO-inhibitor (MAOI)-associated hypertension in patients using SJW—only one could be found in the literature (40).

Pharmacokinetic studies have been performed with the standardized SJW extract, LI 160, in normal volunteers. The peak plasma concentrations of components analyzed thus far include hypericin and pseudohypericin at around 8 and 6 ng/mL (~16 and 12 nM), respectively, at steady state (41) and hyperforin at 150 ng/mL (280 nM) (42). However, intravenous (IV) doses of hypericin as high as 2 mg/kg in monkeys cannot be detected in the cerebrospinal fluid, suggesting that direct central effects are unlikely (43).

Surprisingly, rat atrial tissue preparations showed SJW extracts to have 5HT antagonist activity and negative chronotropic and inotropic actions (44). Amentoflavone has been acknowledged to bind with high affinity to benzodiazepine receptors, and efforts have been made to show that it may be able to cross the blood–brain barrier (45). However, IV administration to mice failed to affect ³H-flunitrazepam binding to brain benzodiazepine receptors (46), making it a doubtful candidate for therapeutic activity. Thus, the true mechanism(s) of antidepressant action for SJW is yet to be determined (47,48). It is possible that more sophisticated neuropharmacologic techniques of the future will reveal novel and subtle pharmacologic activities for herbal medicines that current research methods fail to detect.

Clinical Studies

Depression

The efficacy of SJW in the treatment of mild-to-moderate major depressive disorder is well established. With the notable exception of two American trials (5,6), other clinical studies and meta-analyses have found SJW to be better than placebo or comparable to standard synthetic antidepressants. In a recent meta-analysis by Linde et al (49), trials including a total of 5489 patients met the criteria for inclusion. Eighteen trials were placebo controlled and 17 were comparisons of SJW with standard antidepressants. In nine larger trials the combined response rate ratio (RR) for SJW compared with placebo was 1.28 [95% confidence interval (CI), 1.10–1.49] and from nine smaller trials was 1.87 (95% CI, 1.22–2.87). Results of trials comparing SJW and standard antidepressants were not statistically different. Compared with tri- or tetracyclic antidepressants and SSRIs, respectively, RRs were 1.02 (95% CI, 0.90–1.15; five trials) and 1.00 (95% CI, 0.90–1.11; 12 trials). In placebo-controlled trials and in comparisons with standard antidepressants, those conducted in German-speaking countries reported more favorable findings with SJW. Patients given SJW dropped out of trials less frequently due to adverse effects than those given first-generation antidepressants [odds ratio (OR) 0.24; 95% CI, 0.13–0.46] or SSRIs (OR 0.53, 95% CI, 0.34–0.83). The authors concluded that SJW is superior to placebo and similarly effective as standard antidepressants in patients with major depression. SJW

also had fewer side effects than standard antidepressants (49).

The low hyperforin/hypericin Swiss formulation, Ze 117, was tested in a chronic (1 year) safety and efficacy study in 440 outpatients (19). Mean HAM-D scores decreased steadily from 20.6 at baseline to 12.1 at week 26 and to 11.2 at week 52.

Schulz (50) reviewed 34 clinical trials conducted between 1990 and 2002. Eleven were placebo controlled, and in nine cases, the SJW extract was shown to be significantly superior to placebo. Comparative results with SJW were as good as or even better than with imipramine or fluoxetine, whereas amitriptyline was significantly superior to SJW after 6 weeks of therapy. There were no significant differences in treatment outcome between SJW and synthetic antidepressants (maprotiline and sertraline) or light therapy. The results of the trials conducted up to 2002 showed no major differences in efficacy among the various methanol or ethanol extracts. In the medically supervised treatment of mild-to-moderate depression, doses of approximately 500 to 1000 mg of extract per day of these preparations of SJW are of comparable efficacy to synthetic antidepressants.

Whiskey, Werneke, and Taylor (51) updated and expanded previous meta-analyses of SJW. As of 2000, 22 randomized controlled trials were identified. Entry criterion for severity was most often ≥ 16 or ≥ 17 . Two were ≥ 18 and one (5) was ≥ 20 . Information related to duration of illness was not provided. Meta-analysis showed that SJW was significantly more effective than placebo but not remarkably different in efficacy from active antidepressants. A subanalysis of six placebo-controlled trials and four active comparator trials that met stricter methodological criteria also showed SJW to be more effective than placebo and similar to standard antidepressants. There was no evidence of publication bias.

Kim, Shreltzer, and Goebert (52) performed a meta-analysis in 1999 using only well-controlled, double-blind studies with strictly defined depression criteria. There were six randomized double-blind trials, which included a total of 651 outpatients. As with the other meta-analyses, there was little mention of such factors as mean duration of depression, family history of affective disorders, previous episodes of depression, and exclusion of bipolar illness. The trials appear to have been performed in psychiatric and general practice sites. Five of the studies were carried out in Germany, and Hamilton scores ranged from 15 to 24. SJW was found to be 1.5 times more likely to result in an antidepressant response than placebo and was equivalent to TCAs.

Most of the clinical trials were performed with a standardized SJW extract such as LI160. This is one of the several officially recognized formulations in Germany for the treatment of depression. SJW remains a first-line treatment for mild-to-moderate depression in Europe (53). Two high-profile clinical trials failed to show efficacy in moderate to severe major depression (5,6). Critiques of these trials can be found as Letters to the Editor of JAMA, where the studies appeared.

There have been two encouraging open-label studies in children and adolescents. Findling et al. (54) treated 33 youths (aged 6–16 years) with major depression with up to 900 mg/day SJW for 8 weeks. Twenty-five of these

Table 3 Adverse Events

Adverse event rate <i>N</i> on SJW	Type of study
26% SJW; 45% standard drug	Meta-analysis (63)
4% SJW; 4.8% PBO 20% SJW; 36% standard drug	Meta-analysis (64)
31% SJW 29% PBO; 41% SJW 53% standard drug	Meta-analysis (49)
3% SJW	Review (65)
1–3% SJW; photosensitivity ~ 1 in 300,000	Review (66)
26% SJW; 47% TCAs	Meta-analysis (52)
18% SJW; 16% PBO 28% SJW, 47% standard drug	Meta-analysis (51)
Common ADRs: gastrointestinal symptoms, dizziness/confusion, tiredness/sedation	Review (67)
Insomnia, vivid dreams, restlessness, anxiety, agitation, irritability, dry mouth, headache	Review (7,66,68)

met criteria for response and was well tolerated. Simeon et al. (55) treated 26 adolescents aged 12 to 17 with 900 mg/day SJW for 8 weeks. Of 11 completers, 9 showed significant improvement. Seven patients were discontinued for continuing depression. Tolerability was reported to be good. Both groups felt that additional double-blind studies should be conducted. There is currently an ongoing study (ClinicalTrials.gov identifier: NCT00557427) of SJW and fluoxetine (randomized but open-label) in mild-to-moderate adolescent depression.

Anxiety Disorders

Studies of synthetic antidepressants often show that there may be a concomitant reduction in symptoms of anxiety within the depressive syndrome. Some antidepressants have demonstrated sufficient efficacy in generalized anxiety disorder (GAD) to gain US Food and Drug Administration approval for this indication. Thus, herbal antidepressants might also possess this added benefit. Although there are two case report papers, each with three patients with GAD who responded to treatment with SJW (56) and Kobak et al. (57) there have been no controlled trials to test this hypothesis.

A placebo-controlled pilot study in social phobia ($n = 20$ per group) suggested a trend toward improvement with SJW. Both groups improved, but the differences between groups were not significant (58). In addition, an initial open-label study in obsessive compulsive disorder was positive (59), but a later, controlled trial against placebo failed to show any significant differences (60). A small pilot study examining major depressive disorder with comorbid anxiety found no benefit from SJW on the anxiety component (61).

SJW has also shown efficacy in placebo-controlled trials in somatoform disorders (medical conditions that appear to be psychological in origin) (62). The authors found a pronounced and relatively rapid onset of activity and a notable lack of adverse effects when compared with other treatments of somatoform disorders. They noted that efficacy was not dependent on the presence of depressive symptoms, as patients without significant depression improved as much as those who did.

Adverse Effects

Tolerability of SJW has been found to be very good with few adverse drug reactions reported (Table 3). Extensive use in Germany has not resulted in reports of serious drug interactions or overdose toxicity. Although SJW is often

said to be contraindicated in severe depression, this may have more to do with the need for medical supervision and the inappropriateness of self-treatment rather than a true lack of efficacy.

In a long-term open-label study, the safety of the low hyperforin/hypericin Swiss formulation, Ze 117, was evaluated in 440 outpatients (19). Adverse events were reported by 217 (49%) of the patients with a total of 504 adverse reported. Of these, 30 (6%) were considered as possibly or probably related to treatment. Gastrointestinal and skin complaints were the most common events associated with treatment. No age-related differences were found. There were no changes in clinical chemistry parameters or ECG recordings. Body mass index did not change with treatment.

Table 3 summarizes adverse event results from a number of trials and meta-analyses.

In their meta-analysis of 22 trials, Whiskey, Werneke, and Taylor (51) reported that adverse effects occurred more frequently with standard antidepressants than with SJW. The meta-analysis by Kim, Streltzer, and Goebert (52) showed that there was a higher dropout rate due to side effects in the tricyclic groups and that these drugs were nearly twice as likely to cause side effects—including those that were more severe—than did SJW. Linde and Mulrow (63) report that extensive use of SJW in Germany has thus far not resulted in published cases of serious drug interactions or toxicity after overdose. A more recent meta-analysis of 5489 patients (49) reported that side effects of SJW are usually minor and uncommon; and that patients given SJW dropped out of trials due to adverse effects less frequently than those given older antidepressants (OR 0.24; 95% CI, 0.13–0.46) or SSRIs (OR 0.53, 95% CI, 0.34–0.83).

The trials published by Shelton et al. (5) and Hypericum Depression Trial Study Group (6) found SJW to be well tolerated. In the latter study, the number of withdrawals due to side effects was two for SJW, three for placebo, and five for sertraline. Patients reaching the maximum dose during the trial were 54% for SJW and placebo and 36% for sertraline ($P < 0.005$). The significant side effects of sertraline were diarrhea (38%), nausea (37%), anorgasmia (32%), forgetfulness (12%), frequent urination (21%), and sweating (29%). Those of SJW were anorgasmia (25%), frequent urination (27%), and swelling (19%). Although the adverse events reported for sertraline are very consistent with literature reports, those for SJW are not. Specifically, anorgasmia has not been reported to occur in any of the German studies. Finally, both the patients and the physicians correctly guessed the sertraline treatment in the majority of patients, whereas placebo and SJW were correctly guessed by no more than chance (6). A follow-up publication (69) reported that one-sixth patients randomized to placebo had hyperforin in their stored blood and one-sixth patients assigned to SJW had none (69). No blood levels were determined for hyperforin in the sertraline group or for sertraline in any of the groups.

In the Shelton study, headache was the only adverse event that occurred with greater frequency in the SJW group (41%) than with placebo (25%). Patients discontinuing due to adverse events comprised only 1% in both the placebo and SJW groups. There were no reports of anorgasmia (5).

As with any antidepressant, there is always the possibility of induction of mania in patients with bipolar disorder. Case reports of mania that appear to be due to SJW have been reported (70).

Photosensitivity

The potentially serious adverse effect, photosensitivity, occurs very rarely (Table 3). Photosensitivity from SJW preparations appears to be due to the naphthodianthrones, hypericin, and pseudohypericin. These compounds are photoactive quinones that produce singlet oxygen and free radicals when exposed to light. For most people receiving high doses of SJW, the extent of photosensitivity is a slight reduction in the minimum tanning dose. This was demonstrated in a randomized, placebo-controlled clinical trial in which fair-skinned subjects who burned easily were given metered doses of SJW extract (LI160) and exposed to UVA and UVB irradiation (71). Volunteers received placebo or 900, 1800, or 3600 mg SJW extract, containing 0, 2.8, 5.6, and 11.3 mg of total hypericin prior to testing. Sensitivity to selective UVA light was increased slightly (~20%) after the highest dose of SJW. Another group received multiple dosing at twice the recommended dose [600 mg three times a day (t.i.d.)]. In the SJW group, there was a slight increase in solar simulated irradiation (SSI) (both UVA and UVB) sensitivity (~9%), and a larger increase to UVA light (~21%). The authors concluded that photosensitization was without clinical relevance at the recommended dosages. Although the German Commission E notes that photosensitization is possible, especially in fair-skinned individuals, it concludes that animal and human research suggests that photosensitization is not likely to occur at recommended dosages (1). No reports of long-term use of SJW in areas of the country with higher sun exposure were found.

Reproduction

The potential cognitive effects of prenatal exposure of SJW were tested in mice. SJW or a placebo was given in food bars (estimated to deliver ~180 mg/kg/day) for 2 weeks before mating and throughout gestation. The SJW did not affect body weight, body length, or head circumference measurements; physical milestones (teeth eruptions, eye opening, external genitalia); reproductive capability, perinatal outcomes, or growth and development of the second-generation offspring (72). In an identically designed study (73), one offspring per gender from each litter (SJW: 13; placebo: 12) was tested with various learning and memory tests. In the majority of the tests, there were no differences between groups. Female offspring exposed to hypericum, required more time to learn the Morris maze task in two of five sessions ($P < 0.05$). Other occasional minor differences were found in favor of placebo-treated mice, but the authors questioned whether these were biologically significant.

Gregoret et al. (74) investigated SJW (100 and 1000 mg/kg) in pregnancy and lactation in Wistar rats (6/group; from 2 weeks before mating to 21 days after delivery). There were no differences in maternal or offspring weights or number of live pups. Microscopic analysis found signs of toxicity in the liver and kidneys of animals of both treated groups of both mothers and pups.

The meaning of these findings is not clear. No similar reports could be found in the literature. There did not appear to be adverse effects on reproduction, gestation, delivery, or lactation in this study.

In a study of embryotoxicity, Borges et al. (75) examined the effects of SJW during organogenesis. Rats were treated orally with 35 mg/kg powdered extract during days 9 to 15 of pregnancy. At day 21, the maternal reproductive tract was removed, the ovaries dissected and the corpora lutea recorded. The number of fetuses and resorptions in the uterine cornua were also recorded. The indices of implantation (number of implantations/number of corpora lutea) and resorption (number of resorptions/number of implantations) were calculated. No signs of maternal or fetal toxicity (as measured by these indices) were seen at this dose.

In a prospective study, Moretti et al. (76) followed subjects taking SJW compared with a matched group of pregnant women taking other pharmacologic therapy for depression and a third group of unexposed, healthy women. Follow-up information was obtained on 54 SJW exposed pregnancies and 108 pregnancies in the other two groups. Results indicated that the rates of major malformations were similar across the three groups, with 2/38 (one obstructed ureter and one hypospadias), 2/48 (one plagiocephaly and one esophageal atresia with tracheoesophageal fistula) and 0/51, in the SJW, depressed comparison group, and the healthy group, respectively ($P = 0.26$). This was not different from the 3% to 5% risk expected in the general population. The live birth and prematurity rates were also not different among the three groups. Although large-scale studies are still needed, this study provides some evidence of fetal safety.

The safety of SJW to nursing mothers and their infants was examined in a Canadian study (77). Thirty-three self-selected breastfeeding women who were taking SJW were followed between May 1999 and April 2001 and compared with matched controls. There were no statistically significant differences found in maternal or infant demographics or maternal adverse events. No changes were observed in the frequency of maternal reports of decreased milk production, or in infant weight over the first year of life.

Five mothers who were taking 300 mg of SJW three times daily (LI 160) and their breastfed infants were assessed (78). Samples of breast milk and mother and infant plasma samples were analyzed for hyperforin. Hyperforin was found to be excreted into breast milk at low levels and concentrations in infants' plasma were at the limit of quantification (0.1 ng/mL). Milk/plasma ratios for hyperforin ranged from 0.04 to 0.13. The relative infant doses of 0.9% to 2.5% indicate that infant exposure to hyperforin through milk is comparable to levels reported in most studies assessing antidepressants or neuroleptics. No side effects were seen in the mothers or infants.

Mutagenicity

The mutagenic potential of SJW was determined in an Ames test and the unscheduled DNA synthesis (UDS) assay. High concentrations of the extracts showed an increase in the number of revertants, both with and without metabolic activation. The authors ascribed the mutagenic effects to the flavonols found in SJW. Of the substances

present in SJW, quercetin has generated the most controversy with respect to mutagenic potential. The genotoxicity of a standardized aqueous ethanolic extract (Psychotonin M) was examined in different *in vivo* and *in vitro* test systems with mammalian cells. The *in vitro* investigations were performed with the hypoxanthine guanine phosphoribosyl transferase (HGPRT) test, UDS test, and the cell transformation test using Syrian hamster embryo (SHE) cells. In these studies, both the *in vitro* tests as well as the *in vivo* tests—fur spot test of the mouse and the chromosome aberration test with the Chinese hamster bone marrow cells—were negative. Thus, there was mixed evidence regarding the mutagenic potential of SJW (see Ref. 68 for review). No studies using purified hypericin or hyperforin using standard genotoxicity assays were found. One study using a proposed new test, the Comet Assay, was reported and showed that DNA strand breaks were increased at concentrations of up to 1 μ M hypericin (79). The authors felt that this type of damage (strand breaks) was not of great concern because it is normally repairable by healthy cells and in this assay occurred only under conditions that caused considerable cytotoxicity (meaning the cells could not replicate).

Drug Interactions

Studies have shown that SJW can reduce plasma levels of several drugs (48). Current knowledge regarding the metabolism of these drugs indicates that the liver cytochrome P450 drug metabolizing enzyme system cannot, by itself, account for these effects. There is fairly substantial evidence that SJW induces both intestinal CYP3A4 and the *P*-glycoprotein (Pgp) transmembrane pump after chronic administration (80,81). Although drugs that are substrates of only one of these systems may show a measurable decrease, these are not generally clinically relevant. However, medications with a narrow therapeutic index that are substrates of both CYP3A4 and Pgp (e.g., cyclosporine and indinavir) are of concern due to the possibility that SJW may result in substantial decreases in plasma concentrations (48).

Although case reports have suggested that other medications or enzyme systems may be affected, these studies must be carefully evaluated in light of the specific methodology and the total body of evidence. For example, Obach reported that crude SJW methanolic extracts showed inhibition of all cytochrome P450 (CYP) enzymes when tested at very high concentrations [50% enzyme inhibition (IC_{50}) ranged from 10 to 1000 μ g/mL] (82). Hyperforin inhibited 2D6, 2D9, and 3A4 with IC_{50} of 1.6, 4.4, and 2.3 μ M, respectively. The significance of these data is uncertain as the concentrations were higher than those attained clinically, for example, hyperforin maximum plasma level (C_{max}) was reported to be approximately 280 nM (150 ng/mL) (42). In addition, the activities of isolated chemical constituents may not be relevant to whole or crude plant extracts. However, within physiologically relevant concentrations, the SJW constituent, hyperforin, induces CYP3A4 in hepatocyte cells via the pregnane X nuclear receptor ($K_i = 27$ nM) (83) and the steroid X receptor (84).

Artificially high concentration of test substances in *in vitro* assays can result in false positives. Direct (*in vivo*) evidence of interaction with CYP450 is more useful for

Table 4 Drugs Reported to Interact with SJW Based on Solid (Pharmacokinetic) Data

Drug (subjects)	SJW treatment	Effect
Indinavir (<i>n</i> = 8)	300 mg t.i.d. 14 days	57% ↓ AUC (92)
Cyclosporine (<i>n</i> = 2)	300 mg t.i.d. 21 days Li160	~ 50% ↓ AUC (93)
Digoxin (<i>n</i> = 13)	900 mg/day 10 days Li160	25% ↓ AUC (89)
Digoxin (<i>n</i> = 8)	300 t.i.d. 14 days	18% ↓ AUC (80)
Digoxin (<i>n</i> ~ 8)	250 mg b.i.d. 14 days (Ze 117)	No effect (90)
Phenprocoumon (<i>n</i> = 10)	300 mg t.i.d. 11 days Li160	17% ↓ AUC (94)
3-Ketodesogestrel (<i>n</i> = 3/17)	600/900 mg/day 2 cycles Li160	42–44% ↓ AUC (95)
Norethindrone/ethinyl estradiol (<i>n</i> = 12)	300 mg t.i.d. two cycles (Rexall-Sundown)	14% ↑ clearance / 48% ↓ <i>t</i> _{1/2} (96)
3-Ketodesogestrel/ethinylestradiol (<i>n</i> = 16)	250 mg b.i.d. 14 days (Ze117)	No effect (97)
Carbamazepine (<i>n</i> = 5)	300 mg t.i.d. 14 days	No effect (98)
Amitriptyline (<i>n</i> = 12)	900 mg/day 14 days Li160	22% ↓ AUC (99)

predicting clinical interactions. Clinical trials designed specifically to test for CYP450 enzymes show induction of CYP3A4 but not of other major enzymes, CYP1A2 and 2D6 (85–89).

SJW has been reported to induce Pgp as well as CYP3A4. The administration of the extract to rats or humans for 14 days resulted in a 3.8-fold and 1.4-fold increase, respectively, of intestinal Pgp expression (80). This would explain the report that SJW reduces the plasma levels of digoxin (80,89), which is not a substrate of P450 enzymes, but rather of Pgp. Of possible importance to this discussion, the low-hyperforin, low-hypericin formulation, Ze117, lacks interaction potential with digoxin (Table 4) (90). Hypericin has been reported to induce Pgp, though not as much as the whole extract (91).

Reduced levels of phenprocoumon (an anticoagulant related to warfarin) in 10 subjects were possibly caused by an interaction between SJW and CYP2C9 (the primary liver enzyme associated with warfarin metabolism) although there is no direct evidence for CYP2C9 inhibition. Ibuprofen is eliminated via CYP2C9, and SJW was reported to have no effect on its pharmacokinetics (100). Another possible explanation for the interaction is reduced intestinal absorption due to induction of Pgp. In support of this possibility, rats treated orally with SJW did not show changes in liver enzyme activity but did exhibit reduced plasma levels of orally administered warfarin (101). These data also suggest (in rats at least) that the warfarin reduction associated with SJW takes place in the intestine, rather than in the liver.

Intermenstrual bleeding has been reported in women who had been taking long-term oral contraceptives and recently started taking SJW. Induction of 3A4 by SJW could be responsible, as steroids are known substrates of CYP3A4. A recent study (95) on the pharmacokinetics of ethinylestradiol and 3-ketodesogestrel found no evidence of ovulation during low-dose oral contraceptive and SJW combination therapy. There were no significant changes in follicle maturation, serum estradiol, or progesterone concentrations when compared with oral contraceptive treatment alone. There was, however, an increase in intracyclic bleeding episodes and a decrease in serum 3-ketodesogestrel concentrations, suggesting that SJW might increase the risk of unintended pregnancies. The hyperforin-poor formulation, Ze117, however, did not affect the pharmacokinetics of low-dose 3-ketodesogestrel and ethinylestradiol, in 16 healthy females given the extract for 14 days (97) (Table 4).

Another study examined the effects of SJW on the anticonvulsant, carbamazepine (98). There were no significant differences before and after the administration of SJW in carbamazepine concentrations at peak, trough, or area under curve (AUC). This suggests that SJW is either not a particularly powerful CYP3A4 inducer or it cannot induce carbamazepine metabolism beyond the extent to which it induces itself.

The concern about interactions of SJW with other antidepressants are hypothetical and probably stem from reports about its inhibition of MAO and 5HT uptake. The theory suggests that the combination of SJW (an antidepressant) and an MAOI could cause hypertensive crisis, and the combination of SJW with an uptake inhibitor could result in 5HT syndrome. There has been one case report suggesting that SJW may be associated with hypertensive crisis, but the individual was not known to be using MAO inhibitors. This is consistent with current evidence suggesting that MAO inhibition may be an *in vitro* artifact (12). There are a few case reports of "5HT syndrome" in the United States that implicate SJW. However, there have been none in Europe where SJW has been used extensively for many years (66). One case report concerned four elderly patients in New York City described as having "mild 5HT syndrome" but were consistent with exaggerated side effects of sertraline, namely nausea, vomiting, and restlessness (102). All of them were stable on sertraline and experienced these effects within 3 to 4 days of adding SJW. There are many conflicting literature references to drug metabolism, and sertraline is certainly an example. Although most references do not list sertraline as a substrate of CYP3A4, there is a case report of a 12-year-old boy on sertraline who experienced a 5HT syndrome when erythromycin, a known CYP3A4 inhibitor, was added (103). There is evidence that acute doses of SJW have an inhibiting action on CYP3A4 (103,104). As all these patients were stable on sertraline at the time they initiated SJW, this response can be explained by an increase in sertraline plasma levels—a pharmacokinetic effect, rather than a pharmacodynamic effect. A fifth elderly patient in the Lantz, Buchalter, and Giambanco (102) report was stable on nefazodone when she added SJW. A similar exaggerated serotonergic response resulted that is consistent with increased blood levels of nefazodone due to acute inhibition of CYP3A4 (103,104). The opposite effect could be predicted if the SJW had been started first, followed by the antidepressant. This is in fact the result of a clinical trial of amitriptyline and SJW (Table 4). In this

study, 12 depressed patients received 900 mg SJW extract along with 75 mg twice daily of amitriptyline for 14 days. Reductions in AUC of 21.7% were seen for amitriptyline and 40.6% for nortriptyline. Levels of amitriptyline and its metabolite continuously decreased over the 14-day period, consistent with enzyme induction. Amitriptyline is another drug for which considerable contradiction exists in the literature regarding its metabolism. David Flockhart (105) lists amitriptyline as a substrate for CYP1A2, 2C19, 2C9, and 2D6, whereas Feucht and Weissman (106) also describe it as a substrate for CYP3A4 and glucuronyl transferase.

Recent studies have included the previously unstudied enzyme, CYP2E1. It was found that chronic treatment with SJW in both mice (107) and humans (108) leads to induction of this enzyme. The most commonly associated substrate for CYP2E1 is ethanol, but anesthetic gases also use this route of elimination (105).

Thus, SJW is capable of inhibition of CYP3A4 acutely, and moderate induction of intestinal CYP3A4 and CYP2E1 activity after repeated dosing. Chronic administration of SJW also induces the drug transporter protein, Pgp. Drugs that are substrates of both systems (e.g., indinavir and cyclosporine) are of particular concern as they would be predicted to be doubly affected by SJW. Although the currently popular constituent, hyperforin, appears to be responsible for the enzyme induction, it may not be necessary for the therapeutic effect. This is evidenced by the low hyperforin, low hypericin formulation, Ze117, showing efficacy in major depression (109). Of particular interest in this context is that clinical pharmacokinetic studies have shown this formulation to lack interaction potential with either the CYP3A4 system or the Pgp transporter (90,97).

CONCLUSIONS

Safe use of this herb requires some familiarity with its potential effects on intestinal P450 enzymes and *p*-glycoprotein, due to the presence of hyperforin in most extracts. Understanding the efficacy data requires a critical analysis of depression trials that have been performed with this antidepressant and an appreciation of why clinical trials of psychoactive agent so often fail. SJW is a well-studied botanical extract that appears to have a clinically significant therapeutic activity in mild-to-moderate major depressive disorder. Although side effects are infrequent and mild, interactions with other medications may occur, particularly with those that are substrates of *both* intestinal cytochrome P450 3A4 (CYP3A4) and the *p*-glycoprotein transporter. Evidence suggesting that hyperforin is not critical for the antidepressant effect was reviewed. If true, the issue of medication interaction could perhaps be avoided with formulations containing significantly less hyperforin.

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Taurine

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INTRODUCTION

Taurine, a conditionally essential β -aminosulfonic acid, is a nutrient obtained primarily from meat and seafood. Recently, as we gain a better understanding of its roles in the human body not only for bile acid synthesis and fat digestion but also in the normal development and function of the nervous, sensory, cardiovascular, reproductive, and immune systems, we see taurine marketed as an active ingredient of therapeutic and dietary supplement products. This chapter provides an overview of taurine's common and scientific names, general description, biochemistry and physiological functions, biosynthesis, conditional essentiality, pharmacology including its potential therapeutic roles in heart disease, its antioxidant effects and how they relate to its modulation of inflammation and immunity, how these functions may be useful to people with serious conditions such as cystic fibrosis and diabetes, and the role of taurine compared with other ingredients in the effects of energy drinks. Given the widespread exposure of consumers to taurine supplementation via energy drinks and its therapeutic potential, the safety of taurine under these conditions of use and its international regulatory status are also reviewed.

COMMON AND SCIENTIFIC NAMES

Taurine has the systematic name 2-aminoethanesulfonic acid, synonym: 2-aminoethylsulfonic acid, with the molecular structure $\text{H}_2\text{NCH}_2\text{CH}_2\text{SO}_3\text{H}$ (formula $\text{C}_2\text{H}_7\text{NO}_3\text{S}$) and a molecular mass of 125.1427 g/mol. Its standardized abbreviation is Tau and its Chemical Abstracts Service registry number is 107-35-7 (1).

GENERAL DESCRIPTION

Taurine is a β -aminosulfonic acid, first isolated from the bile of the ox (*Bos taurus*, hence taurine) in 1827 and identified as a sulfur compound in 1846 (2). Taurine is ubiquitous in the animal kingdom and abundant in some algae, but present only in trace levels in a few plants, fungi, and bacteria. Mammals, primarily omnivores and carnivores including humans, have high concentrations of taurine: a 70-kg man has up to 70 g of taurine. It is not incorporated into proteins but is present abundantly in the free state (up to $\sim 60 \mu\text{mol/g}$ wet weight) in blood platelets, leukocytes (up to 50 mM), electrically excitable tissues, and secretory structures, and in low or variable concentrations in all other tissues (3,4).

BIOCHEMISTRY AND FUNCTIONS

Taurine is a colorless, tasteless, crystalline compound with melting point 325°C to 328°C , density 1.734 g/cm^3 , acid dissociation constant (pKa) 4.96, octanol–water partition coefficient ($\log P$) -3.36 , water solubility $8.07 \times 10^4 \text{ mg/L}$, and vapor pressure $1.72 \times 10^{-4} \text{ mm Hg}$ (1,5). Unlike the amino acids, a sulfonyl group replaces the carboxyl group and the amino group is at the β -C position rather than the α -C ($\text{H}_2\text{NC}^\beta\text{H}_2\text{C}^\alpha\text{H}_2\text{SO}_3\text{H}$). It has a zwitterionic nature ($^+\text{H}_3\text{NCH}_2\text{CH}_2\text{SO}_3^-$) at physiological pH conferring high water solubility, low lipophilicity, high dipole, and very slow diffusion through membranes, hence the need for active transport for uptake and the ready maintenance of very high-concentration gradients in certain tissues such as the retina and neurons (3). Unlike the sulfur-containing amino acids, taurine is not a source of inorganic sulfate or organic sulfur (3,6). Although not a component of proteins or nucleic acids, 5-taurinomethyluridine and 5-taurinomethyl-2-thiouridine have been found in human mitochondrial tRNAs, a potential lead for further understanding of taurine's biochemical functions (4).

The oldest known role of taurine is in the synthesis of bile acids, which are important for fat digestion (3,7,8). However, studies over the last 20 years indicate significant additional functions of taurine in lipid metabolism (8,9), calcium homeostasis (10–14), heart failure (10,13,15,16), protection against ischemic cardiac damage (17,18,19), cardiomyopathy (13), hypertension reduction (10,11,17,20, 21), osmoregulation (22), glucose metabolism regulation (23–26), immunity and inflammation regulation (4), as an antioxidant/free-radical scavenger (13,14,19,25,27–30), and as a membrane stabilizer (12,19). Some of the more important physiological functions and potential therapeutic effects are discussed further, below.

BIOSYNTHESIS

Taurine is one of the end products of sulfur metabolism in mammals. Ingestion of protein provides the essential amino acid L-methionine and also L-cysteine, normally nonessential as methionine is converted by trans-sulfuration via S-adenosylmethionine, S-adenosylhomocysteine, homocysteine, and cystathionine to L-cysteine. The liver is the primary site of taurine biosynthesis (12) but immunocytochemical studies have shown biosynthesis to occur in other tissues too, for example, cerebellar neurons (31) and glial cells, especially astrocytes, and the kidney, but not lymphoid tissue or the lung (4). The primary precursor for multiple pathways leading to taurine

(Fig. 1) is L-cysteine. The principal biosynthetic pathway (bold arrows in Fig. 1) involves cysteine dioxygenase-catalyzed conversion to cysteine sulfinic acid (cysteine sulfinate), from which carbon dioxide is removed by cysteine sulfinic acid decarboxylase to produce hypotaurine, the sulfur of which is then oxidized by hypotaurine dehydrogenase to produce taurine. Alternatively, the sulfur of cysteine sulfinic acid may first be oxidized by cysteine dioxygenase to cysteic acid (cysteine sulfonate) and then decarboxylated directly to taurine. L-Cysteine may also be (i) converted to α -aminoacrylic acid that can be combined with sulfite from cysteine sulfinic acid via β -sulfinylpyruvic acid to make cysteic acid that is decarboxylated to taurine; (ii) converted via the pantothonate pathway to cysteamine, the sulfur of which can undergo two oxidation steps to hypotaurine and then taurine; or (iii) converted to L-cystine, oxidized to cystine disulfoxide, decarboxylated to cystamine disulfoxide, and cleaved to produce hypotaurine that is oxidized to taurine (2,3,12,19). Several steps (indicated in Fig. 1), including the rate-limiting action of cysteine sulfinic acid decarboxylase, require pyridoxyl-5'-phosphate, so vitamin B₆ deficiency may lead to reduced production of taurine (19).

CONDITIONAL ESSENTIALITY

An "indispensable" or "essential" nutrient is one which cannot be synthesized *de novo* by the organism out of materials ordinarily available to the cells at a speed commensurate with the demands for normal growth, body maintenance, and other specific physiological functions (32). The precursors of taurine, methionine and cysteine, are ordinarily available to the cells and as shown in Figure 1, there are multiple pathways to *de novo* biosynthesis. However, the rate of synthesis can be constrained by its dependence not only on availability of its precursors, methionine and cysteine, and metabolic demand for these amino acids but also on normal levels of activity of the necessary biosynthetic enzymes. Enzymatic activities may be affected by genetic variability, developmental maturity, health of tissues at the site of metabolism, and availability of cofactors such as pyridoxyl-5'-phosphate for the rate-limiting enzyme in the case of taurine (see "Biosynthesis" section).

Studies in cats, rodents, pigs, chickens, and primates have determined that a deficiency of taurine results in abnormalities in development of the central nervous system, cardiovascular system, reproductive system, immune system, retina, and tapetum lucidum (4,33).

Most of the taurine required for biological function is obtained through the diet. Intake from animal sources, primarily meat and seafood, ranges from 40 to 400 mg/day of taurine (34). Taurine also occurs in human milk (35). For a healthy human, dietary supplementation of taurine is not required as dietary intake and biosynthesis are normally sufficient to meet functional needs (6,32). However, supplementation of taurine is recommended in cases of digestive malabsorption where precursor amino acid supply may be limited and there may be substantial losses of bile acids and taurine in the stools, for example, in children with cystic fibrosis, people with blind loop gut syndrome, and in choleric diarrhea (35,36). Pa-

tients on long-term taurine-free parenteral nutrition, for example, after trauma or intensive chemotherapy or radiation therapy, also exhibit low taurine concentrations in plasma, platelets, and urine. Low intracellular taurine concentrations in muscle are a typical feature in patients with chronic renal failure, probably due to impaired metabolic conversion of cysteine sulfinic acid to taurine. There is some evidence that taurine might be indispensable during episodes of catabolic stress as suggested by low extracellular and intracellular taurine concentrations after trauma and infection. Intracellular taurine depletion may be associated with the well-known muscle fatigue and arrhythmic episodes that occur in uremia (22).

Taurine has been characterized as a conditionally essential amine in pre-term infants and neonates. Pre-term and full-term human infants fed purified infant formulas with high protein quantity and quality but virtually no taurine showed lower plasma levels of taurine that decreased during the study, but higher plasma levels of free amino acids, compared with pre-term and full-term infants fed pooled taurine-rich human milk (33). This suggests a problem with taurine biosynthesis despite an abundant supply of precursor amino acids. There was no obvious clinical evidence of abnormalities in these infants despite this apparent deficiency. However, similar studies with human formula versus human milk in newborn rhesus monkeys that were sacrificed after 26 months for histological examination revealed significant problems in eye development in the formula-fed group that were absent in the milk-fed group. This led to further investigations in human infants that did detect abnormalities in the development of the eye, central nervous system and immune system plus lower linear growth and weight gain in groups fed taurine-deficient formula compared to human milk (4,33). In human children on parental nutrition lacking taurine, their reduced plasma concentration of taurine has been associated in some cases with ophthalmoscopically and electrophysiologically detected abnormalities that were corrected when taurine was added to the parenteral nutrition solution (33). Consequently, taurine is now incorporated into most neonatal dietary regimens. In these cases of deficiency, supplementation requirements may range from 10 to 50 mg/kg body weight/day (22).

PHARMACOLOGY

Pharmacokinetics

Taurine is readily bioavailable: human studies showed significant increases in plasma taurine 1 to 2 hours after consumption of a taurine-rich meal with levels declining to background within 3 to 7 hours (37). Absorption is via an active transport mechanism in the small intestine wall (25,38). Taurine shows selective distribution. Whereas taurine accounts for only 3% of the free amine-containing acids in the plasma (39), there is active uptake against a concentration gradient into many organs (25); for example, it accounts for 53% in muscle, 50% in kidney, 25% in liver, and 19% in brain (39). To maintain adequate levels in the tissues, taurine is tightly regulated by excretion and reabsorption by the kidney. The taurine transporter in the proximal tubule brush border membranes appears to be the primary target for adaptive regulation by

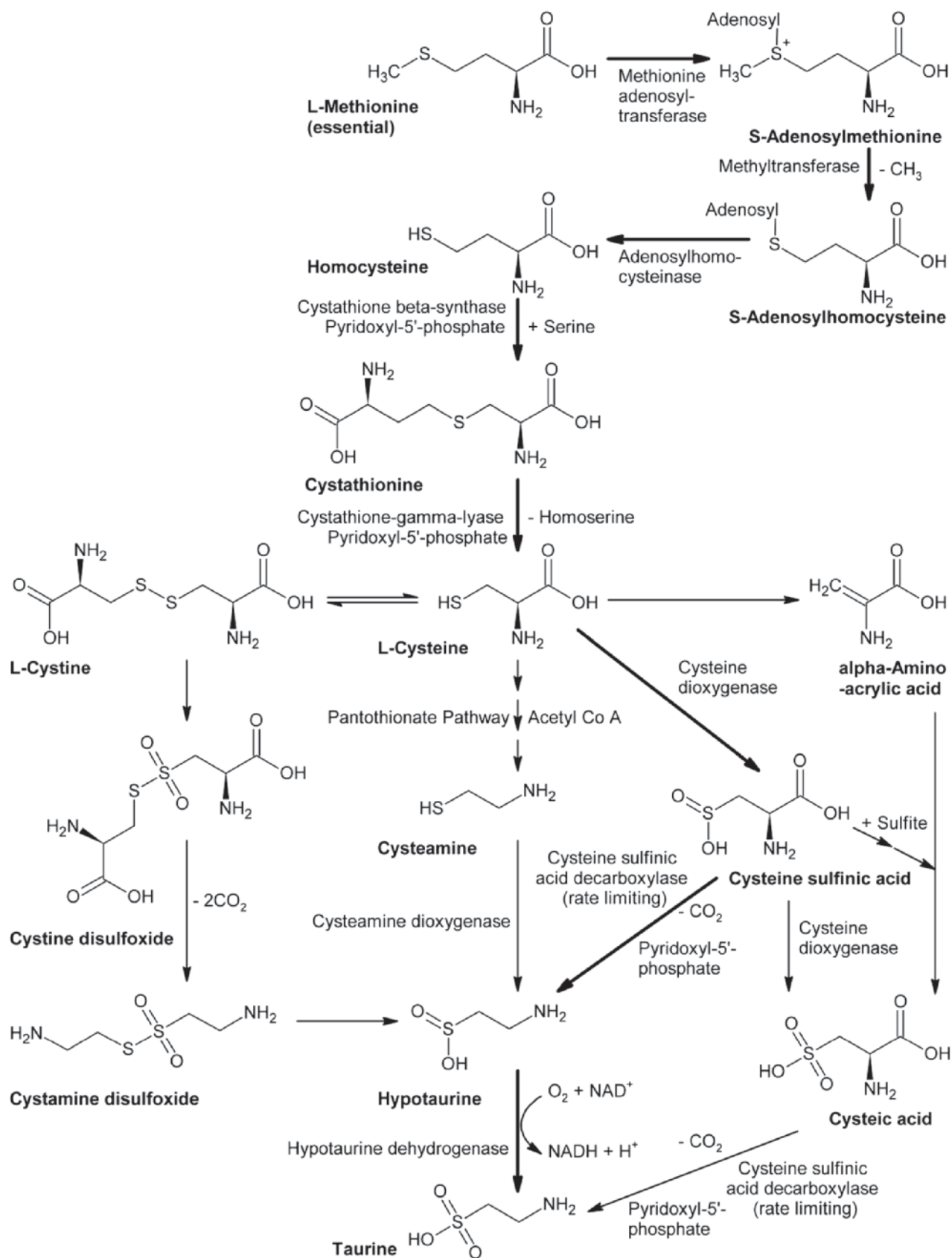


Figure 1 Pathways for taurine biosynthesis.

dietary availability of taurine (4). Taurine is excreted in the urine (95%), approximately 70% as taurine and 25% as the sulfate, which is derived from taurine-conjugated bile salts degraded by intestinal microorganisms and then absorbed; only 5% is excreted in the feces (40).

For interpretation of the studies that follow, taurine at doses of 3 g/day in humans is considered safe, as discussed in more detail in a later section.

Cardiovascular Disease

Taurine has potential roles for the prevention and treatment of congestive heart failure (CHF), hypertension, ischemic heart disease, atherosclerosis, and diabetic cardiomyopathy. However, longer-term randomized, double-blind, placebo-controlled clinical trials (RCTs) need to be conducted before taurine can be unequivocally recommended as part of micronutrient supplementation for the prevention or treatment of cardiovascular disease (CVD) (13,14,41).

Hypercholesterolemia

Taurine has been shown to decrease hypercholesterolemia in some experimental animal models fed with high-cholesterol or high-fat diets. Mochizuki et al. (42,43) report that the mechanism involves an increase in cholesterol catabolism and excretion of bile acids, with very-low-density lipoprotein and low-density lipoprotein being the components eliminated in greatest quantity. They demonstrated that taurine at 30 g/kg of diet for 15 days appears to increase serum high-density lipoprotein concentrations in both normocholesteremic and polychlorinated-biphenol-induced hypercholesterolemic rats.

However, Ebihara et al. (8) found that in ovariectomized rats fed with coconut oil, rich in saturated fatty acids, plasma levels of cholesterol, triglycerides and phospholipids did not change but apolipoprotein A-IV decreased and the levels of cholesterol 7 α -hydroxylase (CYP7A1) enzyme activity and fecal bile acid excretion increased at doses of 30 or 50 g taurine/kg of diet. As the diet did not contain cholesterol, the authors suggest that taurine altered endogenous cholesterol metabolism but could not explain why taurine did not prevent ovarian-hormone deficiency-induced hypercholesterolemia in this experimental animal population. To put these effects into a human therapeutic context, the rats ingested on average 13.6 g of diet/day, representing a taurine intake of 400 mg/day at 30 g/kg diet or 700 mg/day at 50 g/kg diet. Assuming typical adult Wistar rat body weights of approximately 200 to 400 g, this would be a dose of between 1 and 3.5 g of taurine/kg body weight/day. This would represent a dose of 70 to 245 g/day for an adult man weighing 70 kg. The safety of this dose is unknown, as it is approximately 1000 times a typical dietary intake and far greater than the highest dose, 10 g/day for 6 months, used in a human clinical trial (34).

Zhang et al. (9) conducted an RCT that evaluated the effects of 7 weeks of 3 g/day taurine on serum lipids in 30 young adults of body mass index ≥ 25.0 kg/m². In the taurine group, triglyceride levels, atherogenic index and body weight were significantly reduced. The authors concluded that taurine produces a beneficial effect on lipid metabolism and may have an important role in CVD prevention in overweight or obese subjects. Note that these

subjects were from Inner Mongolia, and the findings may be patient population-specific or may differ in a population with more access to fresh fish.

Fennessy et al. (44) found that flow-mediated endothelial-dependent vasodilatation was significantly impaired in 15 otherwise healthy young smokers (20 cigarettes/day for ≥ 2 years), as compared with 15 young lifelong nonsmokers. Pretreatment of smokers for 5 days with 1.5 g/day taurine reversed the existing vasoconstriction to the level of the healthy controls ($P < 0.0005$). The researchers hypothesized that flow-mediated endothelial-dependent vasodilatation, which is impaired in both young smokers and individuals who are at risk for developing atherosclerosis, is associated with a monocyte-mediated reduction in the endothelial expression of the endothelial nitric oxide synthase (eNOS) gene. This study demonstrated that taurine has a cytoprotective effect, as it appears to reverse the inhibition of endothelial nitric oxide (NO) synthesis and could potentially halt the progression of endothelial injury and subsequent atherosclerotic plaque formation. The authors recommended that the effects of taurine on altered endothelial function in hyperlipidemia be investigated. Note that this was a short-term study of 5 days and it is unclear whether tolerance would develop with longer treatment.

Hypertension

Militante and Lombardini (45) reported that adding 3% taurine to drinking water effectively reduced hypertension in rats on deoxycorticosterone acetate and a high-salt diet by normalizing the metabolism of epinephrine and norepinephrine. Taurine was also associated with increased β -endorphin-like immunoreactivity in the hypothalamus, which is thought to be important to its antihypertensive effect. In Dahl-S rats, susceptible to hypertension and sensitive to a high-salt diet, 3% taurine in drinking water given concomitantly with a high-salt diet significantly reduced development of hypertension. There was also an associated increase in urinary excretion of the hypotensive enzyme kallikrein, suggesting greater renal kallikrein gene expression and thus lower risk of developing essential hypertension in both rats and humans. In Wistar rats rendered hyperinsulinemic and consequently hypertensive by a high-fructose diet, 2% taurine in drinking water inhibited increases in blood pressure (BP) and plasma insulin.

McCarty (18) hypothesizes that taurine's usefulness in human ischemic heart disease may be due to its prevention of the endothelial damage that follows neutrophil activation and endothelial adhesion. Taurine might quench reactive oxygen species (ROS) produced by the hypochlorous acid (HOCl) released from activated neutrophils, thus counteracting the inhibition of endothelial NO synthesis, and downregulating the generation of endothelial platelet-activating factor. The end result could be prevention of endothelial damage that can cause atherosclerotic plaques and consequent blood vessel stenosis.

Militante and Lombardini (45) state that the interest in taurine as an antihypertensive was apparently due to a correlation between dietary intake of sulfur-containing amino acids and prevention of hypertension and stroke. Kohashi et al. (20) treated eight patients with essential hypertension with a restricted-salt diet and 6 g/day of

taurine for 6 weeks, resulting in significant decreases in BP. Fujita et al. (21) studied the effect of oral administration of 6 g/day of taurine for 7 days on BP and plasma catecholamines in 19 young patients with borderline hypertension. Taurine significantly reduced both systolic and diastolic BP in the taurine-treated group ($P < 0.05$) compared with placebo. Yamori et al. (17) treated 17 mild/borderline hypertensive Tibetan men with 3 g/day of taurine for 2 months. In 11 out of 17 subjects, there was a significant decrease in both systolic and diastolic BP. Although these studies suggest that taurine might be a useful treatment option in hypertension, they are limited by low patient numbers.

Satoh and Kang (46) administered 1000 to 2000 mg of taurine to 102 healthy students. The 2000 mg dose caused significant hemodynamic changes from pretreatment values: reduction in blood vessel wall tone, measured as the central aortic pulse augmentation index ($P < 0.05$); heart rate ($P < 0.05$); and BP ($P < 0.01$). Approximately 5% of the students were nonresponders, which may be due to the difficulty in measuring subtle hemodynamic changes in healthy persons. It would be expected that taurine would have a greater effect on the vascular functions of unhealthy individuals. In the clinical treatment of CHF, dilation of arterioles and veins and decreased cardiac output are necessary; taurine might be clinically useful in modulation of calcium ion channels in cardiac myocytes and consequent regulation of blood vessel tone and heart rate.

Heart Failure

Patients suffering from CHF exhibit impaired myocardial energy production, myocyte calcium overload, and increased oxidative stress. Taurine plays an important role in the prevention of these conditions and is known to be present at suboptimal levels in this patient population. Larger-scale studies examining micronutrient depletion in heart failure patients and the benefits of dietary replacement need to be performed (13).

Ahn (16) studied 22 healthy Korean women, aged 33 to 54 years, with high cardiovascular risk factors and above-average levels of thiobarbituric acid reactive substances (TBARS), an indicator of oxidative stress. Serum lipids, plasma taurine and plasma homocysteine (Hcy) levels were measured before and after supplying 3 g/day taurine for 4 weeks. After taurine supplementation, increased levels of plasma taurine decreased both the concentration of plasma homocysteine ($P < 0.05$) and TBARS levels ($P < 0.001$). The study suggests that taurine may reduce plasma homocysteine and oxidative stress indicator levels, perhaps reducing the risk of cardiovascular disorders like atherosclerosis and coronary heart disease.

In an RCT of 41 CHF patients (ejection fraction $\leq 40\%$) about to have aortocoronary artery bypass surgery, Jeejeebhoy et al. (15) reported that supplementation with CoQ10, taurine, and carnitine produced significantly higher myocardial levels of these compounds. Supplementation was associated with a significantly decreased left ventricular end-diastolic volume in the treatment group compared with the placebo group. This study suggests that supplementation with taurine and other modulators of mitochondrial function and cell calcium can mitigate the effects of cardiomyopathy and improve outcomes of surgical revascularization. Potential limitations

of this study are small sample size and short observation period.

Azuma et al. (11) conducted a small ($n = 14$), 4-week, crossover RCT with taurine at 6 g/day that showed an increase in left ventricular function in patients with heart failure without affecting BP or heart rate and with no adverse effects. Azuma et al. (10) performed a randomized, double-blind comparative clinical study with 3 g/day taurine or 30 mg/day coenzyme Q10, in 17 patients with heart failure secondary to ischemic or idiopathic dilated cardiomyopathy. The taurine group demonstrated increased left-ventricular function ($P < 0.01$), as well as a significant improvement in stroke volume and cardiac output, after 6 weeks ($P < 0.05$); the coenzyme Q10 group did not. The authors state that taurine plays a role in the regulation of intracellular calcium homeostasis through modulation of cation fluxes, therefore increasing the amount of calcium available for myocardial contraction while protecting against calcium overload injury.

Antioxidant Effects

Hu et al. (29) state that animal studies have indicated a cytoprotective role for dietary taurine in rat liver by powerful free-radical scavenging and consequent reduction of lipid peroxidation induced by acetaminophen, cadmium and copper, and oxidized fish oil. Chang et al. (27) cite trials demonstrating that taurine prevented both homocysteine-induced proliferation in rat vascular smooth muscle cells and injury in cultured human endothelial cells. They also reported that taurine halted the effects of homocysteine-induced endoplasmic reticulum stress by scavenging ROS and restored the secretion of extracellular superoxide dismutase (EC-SOD). Schuller-Levis and Park (4) described rodent experiments where pretreatment with supplemental taurine reduced oxidative lung tissue damage caused by exposure to NO₂, ozone, and bleomycin-induced increased NO production.

Taurine's ability to act as an antioxidant is not immediately obvious. It is not readily oxidizable to act directly as an antioxidant as mammals lack the enzymes needed to oxidize its sulfonate group to a sulfate. Chemically, taurine reacts poorly with superoxide, peroxide, and the hydroxyl radical. However, when neutrophils release the ROS, hydrogen peroxide, and myeloperoxidase, the peroxide and enzyme react with Cl⁻ to produce hypochlorite (HOCl), also an oxidant. Hypochlorite reacts with the primary amine of taurine, which is found in high concentrations in neutrophils and other types of leukocytes, to form taurine chloramine (Tau-Cl). If Tau-Cl can be transported rapidly and efficiently into erythrocytes and reduced by glutathione, regenerating the taurine, this serves as a protective mechanism by removing the toxic hypochlorite, but accumulation of high levels of Tau-Cl can result in cell death. In addition, taurine protects against peroxidative damage to membrane lipids by decreasing the rate of malondialdehyde formation from the action of ROS on polyunsaturated lipids (3). Taurine also increases mitochondrial Mn-superoxide dismutase and antagonizes the effects of homocysteine on the endoplasmic reticulum thus restoring the secretion and expression of EC-SOD (27).

Hu et al. (29) conducted an RCT involving 24 chronic hepatitis patients with two to five times higher than

normal levels of the liver enzymes alanine aminotransferase (ALT) or aspartate aminotransferase (AST). After administration of 2 g taurine three times a day for 3 months, plasma levels of ALT, AST, cholesterol, and triglycerides were significantly decreased ($P < 0.05$) compared with placebo, although this effect did not persist after patients stopped supplementation. After 2 months of taking taurine, the treatment group plasma TBARS level, an indicator of oxidative stress, was significantly decreased ($P < 0.05$) and remained so after cessation of treatment. Results of the present study suggest that dietary taurine could act as an antioxidant to halt the peroxidation of blood plasma in hepatitis patients.

Zhang et al. (28) state that exhaustive exercise is known to increase free radicals that can lead to lipid peroxidation and consequent oxidative stress-induced DNA damage. To investigate the potential of taurine as an antioxidant, the authors evaluated the effects of 6 g/day for 7 days on oxidative stress and exercise performance in 11 sedentary 18- to 20-year-old men in two identical bicycle ergometer exercises. Results demonstrated significantly decreased DNA migration out of white blood cells (WBC) 24-hour postexercise (9.3 ± 1.1 , $P < 0.01$), when compared with the same postexercise period in the presupplementation test (11 ± 2.2 mm, $P < 0.01$). There were significant increases in VO_{2max} , exercise time to exhaustion and maximal workload after 1 week of taurine supplementation ($P < 0.05$). These results demonstrate that taurine supplementation decreases exercise-induced DNA damage in WBC from untrained subjects, possibly due to direct scavenging of free radicals and indirect antioxidant effects. The authors hypothesize that the association of taurine with improved exercise performance may be due to the regulation of Ca^{2+} homeostasis and subsequent enhancement of myocardial and skeletal muscle contraction under exhaustive conditions.

Modulation of Inflammation and Immunity

Tau-Cl, produced by the binding of taurine to hypochlorous acid generated by leukocytes, has been demonstrated to be a powerful regulator of inflammation. Schuller-Levis and Park (4) reviewed the mechanisms by which Tau-Cl, as a stable oxidant, can be produced at the site of inflammation and downregulate the production of proinflammatory cytokine production leading to a significant reduction in the immune response. It inhibits in a dose-dependent manner the translation of tumor necrosis factor- α (TNF- α) mRNA and thus production of TNF- α , inhibits transcription of the inducible nitric oxide synthase (iNOS) gene and thus iNOS m-RNA, suppresses superoxide anion, reduces interleukin-2 (IL-2), suppresses production of proinflammatory IL-6 and IL-8 in activated human peripheral blood polymorphonuclear leukocytes, diminishes the activity of nuclear factor kappa-light-chain enhancer (NF- κ B) of activated immune cells by oxidizing its inhibitor I κ B- α , and diminishes activator protein-1 transcription factor (4). Gupta et al. (19) suggested that taurine acts as a cytoprotective agent in the secondary inflammatory process of spinal cord injury by interfering with the endothelial damage caused by this inflammatory response. Jang et al. (30) have further elucidated the molecular mechanisms of action in macrophages, showing that Tau-Cl increases expression of the cytoprotective

antioxidant enzymes peroxiredoxin-1, thioredoxin-1, and heme oxygenase-1, and increases nuclear translocation of nuclear factor erythroid 2-related factor 2 and its binding to the antioxidant response element. With respect to the converse action of Tau-Cl as an oxidant that can damage proteins and induce apoptosis, Shacter et al. recently reported that the actin-binding protein cofilin is a key target of oxidation by Tau-Cl (47).

Cystic Fibrosis

Colombo et al. (7) conducted a multicenter RCT to investigate the need for taurine supplementation during the administration of ursodeoxycholic acid (UDCA), given for treatment of liver disease associated with cystic fibrosis (CF). Taurine deficiency is frequently found in CF patients due to bile acid malabsorption and has been shown to cause a decrease in the lipolytic phase of fat digestion, which normally occurs when tri- and diglycerides are hydrolyzed to free fatty acids and 2-monoglycerides by lipase. Previous studies have shown that taurine can improve the rate of micellar solubilization of these lipolytic products, supporting its use as adjuvant therapy in CF patients with severe steatorrhea. This study evaluated the effects of taurine supplementation, combined with either a placebo or UDCA. In the taurine-placebo treatment group, there were no changes in clinical and laboratory measures of liver damage or on the deterioration of overall clinical conditions, as indicated by the Shwachman-Kulczycki score. However, there was a significant improvement in the level of serum prealbumin, an indicator of nutrition status, and a trend toward a reduction in the degree of fat malabsorption.

Diabetes

Franconi et al. (48) assayed plasma and platelet taurine concentrations in 39 patients with insulin-dependent diabetes mellitus (IDDM). Prior to taurine oral supplementation of 1.5 g/day for 90 days, the IDDM subjects had lower taurine levels in plasma and platelets than the matched control group: 65.6 and 93.3 μ mol/L, respectively. The levels for the IDDM group were equal to the control after the treatment period. In *in vitro* experiments, taurine reduced diabetic patients' platelet aggregation in a dose-dependent manner, whereas 10 mmol taurine/L did not modify aggregation in the 34 matched control subjects. Seghieri et al. (24) also showed that non-diabetic women with history of gestational diabetes, a condition that is prototypal for high risk of β -cell dysfunction, have lower levels of plasma taurine postpregnancy than women with normal glucose tolerance.

Hansen (23) reviewed the effect of altered taurine metabolism and the development of cellular dysfunctions that cause clinical complications observed in diabetes, for example, retinopathy, neuropathy, nephropathy, cardiomyopathy, platelet aggregation, endothelial dysfunction, and atherosclerosis. He recommended further studies that would include taurine, other osmolytes, and aldose reductase inhibitors concomitant with insulin therapy.

Brøns et al. (49) conducted a cross-over RCT on overweight men with a positive history of noninsulin dependent diabetes mellitus (NIDDM) to assess the effect of taurine supplementation of 1.5 g/day for 8 weeks.

Results showed no effect on insulin secretion or sensitivity, or on blood lipid levels. Thus, this study does not support previous studies suggesting that dietary supplementation with taurine can be used to prevent the development of NIDDM.

NIDDM is considered a coronary heart disease risk factor equivalent. Stevens (50) reviewed the effect of increased levels of blood glucose and insulin resistance to explain this link and proposed that one mechanism is oxidative stress, which may lead to endothelial dysfunction, and these conditions together have been shown to be predictive for the development of CVD. In subjects with hypertension, taurine lowered systolic BP via an antisympathetic mechanism. Taurine in combination with angiotensin-converting enzyme inhibitors potentiated reductions of BP and reverse ventricular hypertrophy and, in subjects with heart failure, taurine therapy improved left ventricular systolic function.

To explain the deficiency of taurine in diabetics, Merheb et al. (25) considered the pharmacokinetics of taurine. After a 10-hour fast, blood was drawn from 16 adults, who were then given a one-time dose of six 500-mg taurine tablets. Blood was drawn every hour for 6 hours after supplementation. The diabetic patients, of whom six had NIDDM and two had IDDM, excreted significantly more taurine than the nondiabetic matched control subjects. These researchers proposed to follow-up their pilot study to determine whether the diabetic patients had impaired renal re-absorption or decreased net intestinal absorption of taurine.

Energy Drinks

Energy drinks were developed for periods of increased mental and physical exertion and to help temporarily restore mental alertness or wakefulness when experiencing fatigue or drowsiness. Common ingredients are sugar, caffeine, taurine, and glucuronolactone. In human clinical trials, positive effects of energy drinks were reported on latency of response to auditory stimuli, attention capacity in a stressful situation and motor reaction time (51), rapid visual information attention, reaction time for verbal reasoning and alertness (52), and improvements in aerobic and anaerobic cycling performance, alertness, and mood (53). The cognitive benefits were attributed mainly to the effects of caffeine on adenosinergic receptors (51,53), whereas mood and physical performance benefits were attributed to sugar and glucuronolactone as energy sources and taurine modulation of glycinergic, GABAergic, cholinergic, and adrenergic neurotransmitter systems (51,53).

SAFETY

The European Food Safety Authority (EFSA) Panel on food additives and nutrient sources added to food (38) reported that toxicological studies did not reveal any evidence of teratogenic, genotoxic, or carcinogenic potential from taurine, but that there was no adequate chronic toxicity or carcinogenicity study for taurine. From a new rat study, the EFSA panel confirmed their previous no observed adverse effect level (NOAEL) of 1000 mg taurine/kg bw/day for pathological changes and a NOAEL of 1500 mg taurine/kg bw/day for behavioral effects. The panel also reviewed over 30 human studies of taurine in

large numbers of adults, children, and infants suffering from a variety of serious diseases. Taurine was administered mostly by oral ingestion daily for periods of up to 1 year, generally in the 3 to 6 g/day range, but also parenterally at 0.64 g/day for 20 months, 12 g/day for 15 days, and 18 g/day for 60 days. A secondary outcome from these studies was the conclusion that oral daily ingestion of taurine in the 3 to 6 g range for periods of up to 1 year does not produce adverse health effects, so the EFSA did not have safety concerns regarding taurine at the levels found in energy drinks.

Shao and Hathcock (34) provide the most recent comprehensive safety review for taurine. They concluded that the absence of a systematic pattern of adverse effects from oral administration in >30 human clinical trials precludes the selection of a NOAEL or LOAEL, so a tolerable upper level of intake cannot be established. Instead, they conducted an observed safe level risk analysis and concluded that the evidence for the absence of adverse effects is strong for taurine at supplemental intakes up to 3 g/day.

Taurine is a common ingredient in energy drinks in quantities ranging from 300 to 30,000 mg taurine/L (38,54,55) but the North American mean daily exposure is probably similar to the European exposure of 500 mg taurine/day from this source (38). With the increased consumption of energy drinks in the last two decades, there has been an associated increase in reports of adverse reactions such as cardiac dysrhythmia, seizure, kidney failure, and fatalities. Because most of the cases are associated with concomitant use of alcohol and/or physical activity, a causal relationship has not been proven (38,56). In reviewing adverse reaction reports, EFSA (38) considered caffeine as the causally linked ingredient because of its known effects, whereas a causal relationship with taurine intake lacks scientific evidence. Notably, BfR (German Federal Institute for Risk Assessment) (56) recommends a maximum taurine concentration of 4000 mg/L in caffeinated energy drinks, in which the maximum caffeine concentration is 320 mg/L, with cautionary risk statements for consumers with high BP or heart disease.

INTERNATIONAL REGULATORY STATUS

In the United States, taurine has been authorized by the Food and Drug Administration for addition to purified human infant formulas since 1984 (33). It is also available in prescription-only mixed amino acid injections for pediatric parenteral nutrition, for example, TrophAmine[®] 6% (taurine 15 mg/100 mL) and 10% (taurine 25 mg/100 mL) from B. Braun Medical Inc. (57), and Aminosyn[®]-PF 7% (taurine 50 mg/100 mL) and 10% (taurine 70 mg/100 mL) from Hospira Inc. (58). Taurine neither has generally regarded as safe (GRAS) status (59), nor is it an ingredient of approved drug products (60). The US FDA has taken compliance actions against dietary supplement manufacturers making therapeutic claims for products containing taurine (e.g. 61).

In Canada, professional-use only (but nonprescription) mixed amino acid injections for pediatric parenteral nutrition are available with taurine, for example, Aminosyn[®]-PF 7% and 10% from Hospira Healthcare Corp. and Primene[®] 10% (taurine 60 mg/100 mL) from Baxter Corp. (62). Taurine is also a medicinal ingredient of

at least 12 authorized natural health products, including capsules for supporting cardiovascular health, liver health and healthy blood glucose levels, powders for antioxidant and nutritional support, and liquid energy drinks with health claims (63).

In Australia, taurine is a constituent of pediatric mixed amino acid IV infusions, for example, Primene® 10% from Baxter Healthcare Pty Ltd. (64) and is listed as an acceptable active ingredient or excipient for use in non-prescription listed medicines (65) to support healthy liver function, gall bladder function, blood lipid levels, fat digestion, cardiovascular function, electrolyte function, eye function, and as a dietary antioxidant (66).

In the United Kingdom, the Medicines and Healthcare products Regulatory Agency (MHRA) has issued marketing authorizations for several prescription-only mixed amino acid injection products for parenteral nutrition, for example, Aminoven 3.5% (taurine 350 mg/L), 5% (taurine 500 mg/L), 10% (taurine 1000 mg/L), 15% (taurine 2000 mg/L), StructoKabiven (taurine 1000 mg/L), and Smofkabiven (taurine 1000 mg/L), all from Fresenius Kabi Ltd. (67).

In the European Community, taurine is authorized for use in infant formulas at a level of at least 10 $\mu\text{mol}/100\text{ kJ}$ (42 $\mu\text{mol}/100\text{ kcal}$) (68). Regarding taurine in energy drinks and other supplements, the EFSA Panel on Dietetic Products, Nutrition and Allergies (69) has reviewed and rejected evidence for health claims associated with taurine.

CONCLUSIONS

Taurine is a β -aminosulfonic acid obtained from dietary sources such as meat and seafood and by biosynthesis from L-methionine and L-cysteine. It can become a conditionally essential nutrient when the dietary and precursor amino acid supplies are limited, biosynthetic enzymes or their cofactors are deficient, or loss is excessive, such as in cases of long-term parenteral nutrition or digestive malabsorption. Taurine is important in bile acid synthesis for fat digestion, but more recently, physiological and potential therapeutic roles have been identified in calcium homeostasis, osmoregulation, membrane stabilization, glucose metabolism, development of the central nervous system, eyes, and reproductive system, as an antioxidant/free-radical scavenger, and in treatment or risk reduction for heart disease, hypertension, inflammation, and immune system problems. To mitigate risks to human development from taurine deficiency in cases where it may be conditionally essential, taurine is authorized as an ingredient of pediatric mixed amino acid parenteral nutrition products. With respect to its use in dietary supplements, there is a plausible mechanism for potential benefits of taurine in energy drinks with respect to improvements in mood and physical endurance. At the levels commonly present in energy drinks and other dietary supplements, that is, up to 3 g/day, taurine generally appears to be safe.

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Thiamin

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ABBREVIATIONS

RDA, recommended dietary allowances; THTR-1, thiamin transporter 1; THTR-2, thiamin transporter 2; TMP, thiamin monophosphate; TPP, thiamin pyrophosphate; TRMA, thiamin responsive megaloblastic anemia; TTP, thiamin triphosphate

INTRODUCTION

Thiamin (vitamin B₁) was the first member of the water-soluble family of vitamins to be described. Reference to beriberi (a thiamin-deficiency disease) in Chinese medical literatures dates back to as early as 2700 BC. Thiamin plays an essential role in normal cellular functions, growth, and development via its involvement in critical metabolic reactions related to energy metabolism. Furthermore, this vitamin also plays a role in reducing cellular oxidative stress (1,2). Thus, low intracellular levels of thiamin lead to impairment in energy metabolism and to a propensity for oxidative stress. In addition, deficiency in intracellular thiamin level leads to apoptosis (3,4). Clinically, thiamin deficiency and suboptimal levels (which represent a significant nutritional problem) lead to a variety of abnormalities including neurological and cardiovascular disorders. On the other hand, optimization of thiamin level may be of help in the treatment of diabetic nephropathy and retinopathy (5,6). It is also effective in the treatment of many of the clinical symptoms associated with congenital disorders of thiamin metabolism and physiology. Thus sufficient intake of thiamin (from dietary or supplemental sources) is important for maintaining proper health and well-being as well as in preventing clinical abnormalities.

Structure of Thiamin and Derivatives

The thiamin molecule is composed of a pyrimidine and a thiazole ring that are joined by a methylene bridge (Fig. 1). The alcohol group of the side chain of the thiamin molecule can be enzymatically phosphorylated with up to three phosphate moieties resulting in the formation of thiamin-monophosphate (TMP), -diphosphate (also called thiamin pyrophosphate; TPP), and -triphosphate (TTP) (Fig. 1). The original name used for thiamin was *aneurine* because of its function in preventing and curing polyneuritis in chicken that were deficient in this vitamin due to their feeding of polished rice. Following the discovery of its structure and synthesis, the name was changed to thiamin. The free base form of thiamin is unstable but its hydrochloride and mononitrate derivatives are both

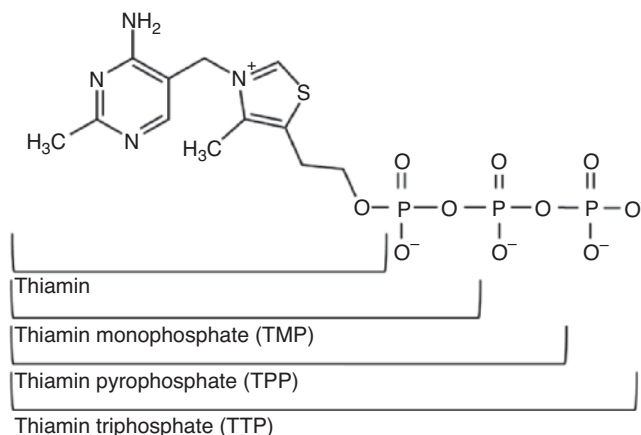


Figure 1 Structure of free thiamin (mol. wt. 337.27) and that of thiamin monophosphate. Abbreviations: TMP, thiamin diphosphate; TPP, thiamin pyrophosphate; TTP, thiamin triphosphate.

stable (with the latter derivative being less hygroscopic than the former) and are commonly used to enrich food products and in pharmaceutical preparations. Thiamin is susceptible to destruction by X-rays, gamma rays, and UV irradiation.

Allithiamins are thiamin derivatives that are produced by oxidative cleavage of the thiazole ring in alkaline solutions. These compounds are biologically active and spontaneously dehydrate as a result of reductive cleavage of their disulfide bridge to regenerate thiamin. A number of allithiamins exist in plants (e.g., in members of the genus *Allium*), and synthetic allithiamins have also been generated (e.g., thiamin propyldisulfide, thiamin tetrahydrofurfuryldisulfide, *O*-benzoylthiamin disulfide, and *S*-benzoylthiamin-*O*-monophosphate). Due to their lipid solubility (which allows them to cross the intestinal epithelium easily), these derivatives are used to treat thiamin deficiency.

THIAMIN ANTAGONISTS AND THIAMINASES

Sulfites, which are widely used as preservative in food, attack thiamin at the methylene bridge, especially at acidic pH, leading to cleavage of the molecule into pyrimidine and thiazole rings. Also a number of heat-stable polyhydroxyphenolic compounds that exist in food (like ferns,

tea leaves, blueberry, red chicory, red beetroot, black currant, red beetroot, Brussels sprouts, and red cabbage) can cleave the thiamin molecule. The latter reaction can be prevented by reducing agents such as ascorbate. Furthermore, a number of thiamin structural analogues (amprolium, oxythiamin, pyriethiamin, and chloroethylthiamin) have been chemically synthesized for experimental purposes to antagonize thiamin at the level of metabolism and/or transport. An inhibitor of thiamin synthesis has also been reported in germs (7).

Thiaminolytic enzymes (i.e., enzymes that degrade thiamin) are found in a variety of microorganisms and food. Two such enzymes are known: thiaminase I and II. Thiaminase I is relatively widely spread in a variety of microorganisms (e.g., *Bacillus thiaminolyticus*), plants [e.g., fern, fish (e.g., carp), and insects (e.g., African silkworm *Anaphe spp.*)]. It catalyzes a base-exchange reaction between the thiazole moiety of the thiamin molecule and a variety of bases. In addition to depleting thiamin, the by-products of the latter reaction may also act as thiamin antagonists (8). Thiaminase II, which hydrolyzes thiamin into thiazole and methoxypyrimidine, is relatively rare with existence being limited to a small number of microorganisms (mainly intestinal bacteria like *Bacillus thiaminolyticus* and *Clostridium thiaminolyticum*).

THIAMIN CONTENT AND METABOLIC FORMS

In adult humans, total thiamin content is estimated to be around 30 mg, and the biological half-life of the vitamin is between 10 and 20 days. Thiamin in tissues exists in the free form as well as in the form of TMP, TPP, and TTP. TPP is the predominant form of thiamin in mammalian tissues (approx. 80% of total thiamin), whereas free thiamin and TMP are the predominant forms in the plasma. TPP is synthesized from free thiamin by the action of pyrophosphokinase, whereas TMP arises mainly from sequential hydrolysis of TTP and TPP (Fig. 2). Total thiamin concentration in human whole blood is in the range of 0.1 to 0.2 μM (9) and it is unevenly distributed among different cell types/compartments (15% in leucocytes, 75% in erythrocytes, 10% in plasma). In the cerebrospinal fluid (CSF), thiamin exists in the free and TMP forms only (10,11). In the urine, free thiamin together with small amounts of TMP, TPP, and a number of thiamin catabolites have been found.

Metabolic Role of Thiamin

Thiamin plays an essential role in a variety of cellular functions. Thiamin pyrophosphate is the predominant metabolically active form of the vitamin, although recent studies have reported additional functions for thiamin's other derivatives. TPP acts as a coenzyme for five different enzymes involved in carbohydrate (energy) and lipid metabolism. Three of these enzymes are mitochondrial, one is cytoplasmic, and one is peroxisomal. The mitochondrial enzymes (dehydrogenases, which exist in multienzyme complexes) are involved in carbohydrate and lipid metabolism. The cytoplasmic (transketolase) enzyme is involved in the pentose phosphate cycle that supplies pentose phosphate to a variety of reactions including those

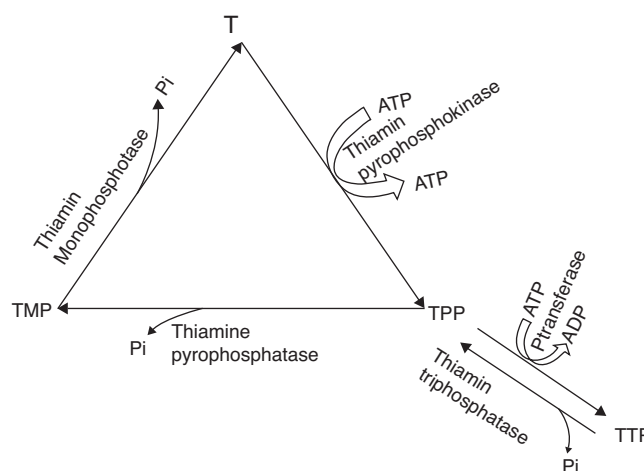


Figure 2 Diagrammatic representation of thiamin interconversion to its phosphate derivatives TMP, TPP, and TTP in mammalian cells.

involved in the synthesis of nucleotides and fatty acids as well as steroid hydroxylation. The peroxisomal enzyme (lyase) is involved in the metabolism of branched chain fatty acids. Because thiamin bridges the glycolytic and the pentose phosphate metabolic pathway, which is critical for creating chemical reducing power in cells, the vitamin is also considered to have an important role in reducing cellular oxidative stress (1,2).

In the nervous system, development of neurological abnormalities in thiamin deficiency do not follow the pattern of development of impairments in the function of thiamin-dependent enzymes (pyruvate and 2-oxoglutarate dehydrogenase and transketolase). Therefore, additional functions for thiamin in the nervous system besides its role as a coenzyme have also been suggested. Other studies have shown that TTP, which can be synthesized in nerve cells, plays a role in electrical conduction in these cells. More recent investigations have reported a role for TPP in regulating the function of membrane chloride channels in nerve cells (12,13) and as a phosphate group donor to other membrane proteins (14).

Physiology of Thiamin

Intestinal Absorption of Thiamin

Humans and all other mammals cannot synthesize thiamin, and thus, must obtain the vitamin from exogenous sources via intestinal absorption. The human intestine is exposed to two sources of thiamin: a dietary source, and a bacterial source (i.e., the normal microflora of the large intestine). Dietary thiamin exists mainly in the phosphorylated form, which is hydrolyzed by intestinal phosphatases to free thiamin prior to absorption (reviewed in Ref. 15). Absorption of free thiamin (which exists in the monocationic form at pH 5–7.4) then takes place predominantly in the proximal half of the small intestine and involves a specialized carrier-mediated process at both the apical and basolateral membrane domains of the polarized absorptive epithelial cells (reviewed in Ref. 15). It is believed that the positively charged thiamin crosses the

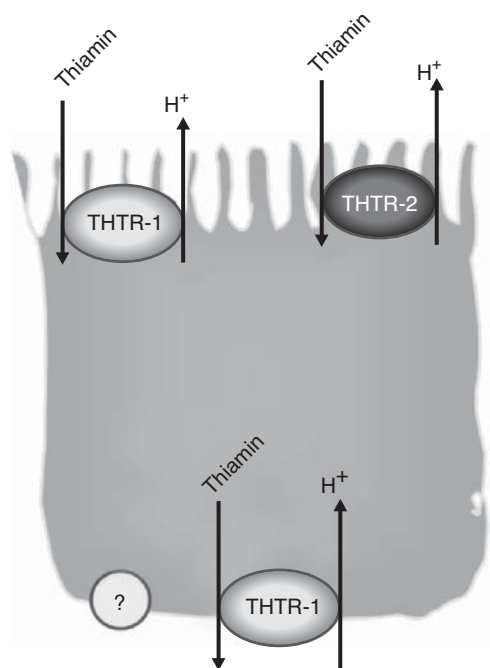


Figure 3 Diagrammatic representation of our current understanding of thiamin transport across mammalian intestinal (and possibly other) epithelial cells. There is a reason to speculate the existence of transport system that can carry thiamin out of the enterocyte in the absence of functional THTR-1. This suggestion is based on the fact that intestinal thiamin uptake and plasma thiamin level are normal in THTR-1 deficient mice (16). A similar scenario may exist in TRMA patients who lack functional THTR-1, yet display normal plasma thiamin level. The compensatory induction in THTR-2 expression in the intestine of THTR-1 deficient mice may explain why transport across the brush border membrane proceeds normally.

intestinal cell membrane in exchange for H^+ (Fig. 3). The diuretic amiloride was found to inhibit intestinal thiamin uptake (as well as thiamin uptake by a number of other epithelia). Some of the absorbed thiamin is converted into the phosphorylated forms of the vitamin (mainly to thiamin pyrophosphate) in intestinal epithelial cells, but only free thiamin exits the intestinal absorptive cells (reviewed in Ref. 15).

As to the bacterial source of thiamin, previous studies have shown that the normal microflora of the large intestine synthesize considerable amounts of thiamin and that up to 50% of this thiamin exists in the free, that is, absorbable form (17–19). In addition, the large intestine of humans and rats is capable of absorbing thiamin from their lumens (19,20). Studies using human-derived colonic epithelial cells have shown the existence of an efficient carrier-mediated process for thiamin uptake in the large intestine (21). The latter findings suggest that bacterially synthesized thiamin in the large intestine may contribute to thiamin nutrition of the host, especially toward cellular nutrition of the local colonocytes. Further studies are needed to determine the exact level of contribution of this source toward overall host nutrition and the effect of dietary and environmental factors on this process.

Two transport systems appear to be involved in intestinal absorption of physiologic levels (nano- and mi-

cromolar concentration) of thiamin: thiamin transporter-1 and -2 (THTR-1 and THTR-2). This was established recently in studies using THTR-1 and THTR-2 knockout mice and by studies with cultured human intestinal epithelial cells utilizing gene-silencing approach with gene-specific siRNA (16,22). Absorption of high (pharmacological; $>20 \mu M$) concentrations of thiamin occurs mainly by simple diffusion.

Studies using human and animal intestinal preparations have shown that the thiamin absorption process is adaptively regulated by the level of the vitamin in the diet, with significant up-regulation occurring in thiamin deficiency (23,24). The mechanism involved in this up-regulation (at least in mice) appears to be transcriptionally mediated and affects THTR-2 only (23). Thiamin uptake by human intestinal epithelial cells also appears to be regulated by an intracellular Ca^{2+} -calmodulin-mediated signaling pathway (reviewed in Ref. 15).

Transport of Thiamin in Renal and Other Epithelia

Normal levels of thiamin compounds in human serum averages 13, 7.1, and 3.8 nM for free thiamin, TMP, and TPP, respectively (9). Filtered thiamin in the renal glomeruli is reabsorbed by proximal renal tubular epithelial cells to prevent loss in the urine. Thus, the kidneys also play an important role in maintaining and regulating thiamin body homeostasis. Thiamin uptake by human and animal renal epithelial cells also occurs via a specialized carrier-mediated process that involves both THTR-1 and -2. Furthermore, studies with human renal epithelial cells and in mice have shown that the renal thiamin uptake process is adaptively upregulated in thiamin deficiency (to further minimize losses) via a transcriptionally mediated mechanism that involves both THTR-1 and THTR-2 (23,25).

In the placenta, thiamin transport is again carrier mediated. The vitamin travels preferentially from the mother to the fetus, and not in the other direction. In the umbilical cord, thiamin plasma level is some 2.5-fold higher than its level in maternal plasma, and its concentration in cord vein is significantly higher than its concentration in the umbilical artery indicating significant retention of the vitamin by the fetus.

Transport of thiamin across the blood–brain barrier is similarly carrier-mediated in nature.

Uptake of Thiamin by Pancreatic β -Cells and Islets

Thiamin is important for both the exocrine and endocrine functions of the pancreas (26–28). Thiamin deficiency in rats leads to a severe reduction in pancreatic content of digestive enzymes and to a marked impairment in insulin synthesis and secretion (26–28). Of relevance to the latter is the development of diabetes mellitus in patients with thiamin-responsive megaloblastic anemia (TRMA; Rogers syndrome) a condition caused by mutation in THTR-1 (see later) (29,30). Supplementing TRMA patients with high doses of thiamin brings about a marked improvement in the clinical symptoms of the disease including a reduction or cessation in the need for exogenous insulin (29,30). The molecular mechanisms that tie thiamin to insulin synthesis and secretion await further investigations. Recent studies have delineated the mechanism of thiamin uptake by human and mouse pancreatic islets and β -cells and established the involvement of a carrier-mediated process (31).

Both THTR-1 and THTR-2 are expressed in these cells with expression of the former being significantly higher than that of the latter (31). As pancreatic β -cells are a major pathological target of TRMA, the pattern of expression and functionality of clinically relevant mutants of hTHTR-1 were also investigated with results showing a spectrum of expression phenotypes. Certain mutants were found to be expressed at cell membrane, whereas others were either retained intracellularly or expressed at the cell membrane but with lower efficiency than wild-type hTHTR-1. However, all of the clinical mutants examined were dysfunctional in pancreatic β -cells (31).

The thiamin uptake process of pancreatic β -cells was again found to be adaptively regulated by the prevailing thiamin level with higher uptake occurring by cells maintained in the presence of low compared with high thiamin levels (31). This was associated with a markedly higher level of expression of THTR-1 and THTR-2 at the protein and mRNA level, as well as higher transcription rate of their respective genes. The response of THTR-1 to changes in thiamin level, however, was markedly more pronounced when compared with THTR-2, a finding that could explain why these cells are the pathological target in TRMA. This is most likely due to the fact that THTR-1 is the predominant thiamin transporter in these cells and that dysfunction in this transporter leads to impairment in the ability of these cells to acquire sufficient amount of thiamin. With the limited capability of the cells to up-regulate the other thiamin transporter, that is, THTR-2, this will lead to the development of a state of intracellular thiamin deficiency. This will in turn result in disturbances in intracellular metabolism, oxidative stress, and apoptosis.

Sources and Recommended Dietary Allowances of Thiamin

Thiamin is widely distributed in foods with rice bran, dried baker's yeast, whole grain cereal, nuts, and dried legumes being rich sources for this vitamin, whereas highly refined foods like polished rice, oils, refined sugar being poor (or very poor) sources. Thiamin of animal origin exists mostly in the phosphorylated form, whereas that of plant origin could be a mixture of free and phosphorylated forms.

The recommended daily allowances (RDAs) for thiamin are 1.4, 1.1, 1.5, 1.6 mg/day for men, women, and for women during pregnancy and lactation, respectively. Because requirement for thiamin relates to the total caloric intake (especially that from carbohydrate), consumption of an unbalanced (calorie rich) diet may change the RDAs.

Assessment of Thiamin Status

Three methods are available for the assessment of thiamin status in humans. The first involves determination of transketolase activity in hemolyzed erythrocytes. The second involves determination of urinary excretion before and after administration of a 5-mg dose of thiamin. The third method involves determination of thiamin concentration in whole blood and in erythrocytes. The latter method is probably the current method of choice.

Thiamin Deficiency

Thiamin deficiency and suboptimal levels represent significant nutritional problems in both underdevel-

oped/developing and developed countries. In underdeveloped and developing countries, the main cause of thiamin deficiency is poor dietary intake of the vitamin (i.e., consumption of thiamin deficient/depleted diets). In the developed countries, chronic alcoholism is probably the main cause of thiamin deficiency. However, thiamin deficiency and suboptimal levels also occur in other conditions as in patients with diabetes mellitus (32), inflammatory bowel disease, celiac disease, renal diseases, AIDS, cancer, and those with congestive heart failure, as well as in subjects on chronic diuretic therapy. In addition, thiamin deficiency and suboptimal levels have been reported in the elderly despite an average daily intake that exceeds their recommended requirement (33).

Chronic thiamin deficiency leads to two distinct types of conditions: Beriberi and Wernicke's encephalopathy. Beriberi is recognized in three different forms. The first form is dry beriberi, which is a symmetrical ascending peripheral neuritis that usually affects older individuals and is associated with wasting; also, it may or may not be associated with cardiac involvement. The second form is wet (or edematous) beriberi, which involves the heart and leads to edema of lower extremities resulting from the ensuing heart failure. The third form is the acute "fulminating" beriberi (which is also called shoshin beriberi) which occurs more frequently in infants and is associated with heart failure and metabolic abnormalities with little evidence of peripheral neuritis.

Deficiency of thiamin in the human central nervous system may lead to Wernicke's encephalopathy and Korsakoff's psychosis. These conditions are associated with chronic alcoholism and manifest as the Wernicke-Korsakoff syndrome. Some evidence, however, exists to suggest that thiamin deficiency alone is not sufficient to cause Wernicke-Korsakoff syndrome but that alcohol is a necessary factor for the induction of this abnormality (34). Korsakoff's psychosis is associated with confusion and loss of recent memory, although long-term memory may continue to be intact. Wernicke's encephalopathy develops later and is associated with clear neurological abnormalities (nystagmus, extraocular palsy, ataxia, confabulation, and coma) and anatomic lesions (hemorrhagic lesions in the thalamus pontine tegmentum, and mammillary body with severe damage to astrocytes, neuronal dendrites and myelin sheaths).

Congenital Disorders

Congenital defects in thiamin physiology and metabolism also occur in human. These defects include thiamin-responsive megaloblastic anemia (TRMA), maple syrup urine disease (branched-chain disease), Leigh's disease, and lactic acidosis.

TRMA (also known as Roger's syndrome) is an autosomal recessive disorder caused by mutations in thiamin transporter-1, THTR-1 (29,30). This rare disease of infancy and childhood is characterized by megaloblastic anemia, diabetes mellitus, and sensorineural deafness; optic and cardiac abnormalities may also occur. Oral pharmacological doses of thiamin are effective in resolving the anemia, decreasing or eliminating insulin requirements, and arresting hearing loss.

A recessively inherited syndrome similar to Wernicke's encephalopathy that develops in the second

decade of life has recently been described (35). The cause of this condition is mutations in thiamin transporter-2, THTR-2. The disease is associated with seizures, ophthalmoplegia, nystagmus, and ataxia and responds to treatment with pharmacological doses of thiamin (35).

Lack of the enzyme branched-chain α -oxoacid dehydrogenase complex is the cause of maple syrup urine disease. Urine of the affected patients (infants) smells like maple syrup, due to the presence of high concentration of α -oxoacid. The disease is characterized by acidosis and seizures in the early neonatal period with some patients responding to daily high pharmacological doses of thiamin.

Other diseases that develop during infancy and early childhood and respond to high pharmacological doses of thiamin are Leigh's disease (subacute necrotizing encephalomyelopathy) and congenital lactate acidosis. Leigh's disease is associated with weakness, anorexia, difficulties in speech and eye motion, as well as growth delay. Congenital lactic acidosis is characterized by lactic and pyruvate acidosis, neurological abnormalities, and development delay. The condition is believed to be due to a defect in the pyruvate dehydrogenase complex (35).

TOXICITY

Even very high oral doses of thiamin (up to 500 mg) are well tolerated in human with no toxic effect. Also large parenterally administered thiamin (single or repeat injections) are generally well tolerated. In very rare instances, however, symptoms resembling anaphylactic shock and minor allergy have been described after parenteral administration.

Despite lack of toxicity of high doses of thiamin, long-term use of such doses in normal individuals may potentially have an unintended negative effect. This relates to recent findings in mice and human epithelial cells showing downregulation of intestinal and renal thiamin uptake processes following long-term exposure to high levels of the vitamin (23,25). If the same happens in normal humans in vivo and such individuals experience serious acute illnesses that lead to abrupt cessation of food intake, this downregulation may lead to precipitous depletion of this important essential micronutrient at a time when its adequate supply is critical to meet the heightened metabolic demands. Notable among such cases are persons experiencing catastrophic accidents, stroke, acute gastrointestinal disorders (e.g., obstruction, infarction, severe gastroenteritis, acute abdominal events, etc), and fulminant infections. In such cases parenteral administration of thiamin may be considered to avoid precipitous development of a serious deficiency state. Further studies, however, are needed to examine whether such a scenario occurs and to determine the time frame required to restore normal intestinal and renal thiamin uptake in such individuals.

CONCLUSIONS

The water-soluble vitamin thiamin is essential for normal cellular function, growth, and development. Humans (and other mammals) obtain the vitamin from exogenous

sources (diet and normal microflora of the large intestine). Thiamin deficiency and suboptimal levels represent significant nutritional problems and can occur due to a variety of conditions. Significant progress has been made in recent years in understanding the molecular aspects of thiamin physiology and metabolism. However, much more work is needed to fully understand the details of thiamin molecular nutrition. Studies are needed to uncover the association between thiamin level, insulin synthesis and secretion, and diabetes mellitus. Further molecular studies are needed to delineate the role of thiamin transporters in regulating cellular homeostasis in different tissues, and uncovering the function of thiamin derivatives other than TPP in the nervous system and other organs in health and disease. Finally, the contribution of the normal microflora of the large intestine toward host thiamin nutrition, and strategies to further enhance this source need to be more fully investigated.

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Turmeric

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INTRODUCTION

The various names by which turmeric is known reflect both its characteristic golden color and its cultural and historical importance as a spice and medicine [“yellow root” or “Indian saffron”; “Curcuma”, a latinization of an Arabic term for saffron (al-kurkum); “turmeric,” derived from the old French appellation, terre merite (valuable soil or clay); and “herb of the sun”], so named 4000 years ago during the Vedic period in India due to its importance as a medicinal and religious herb (1). Traditionally, turmeric has been used as an anti-inflammatory treatment for ailments such as arthritis and minor infections, and as a digestive agent. During the last two decades, as our understanding of the importance of inflammation in the etiology of other diverse disease processes such as atherosclerosis and cancer has grown, so too has interest in the use of turmeric to prevent or treat various conditions now afflicting modern societies. Drawing upon a scientific literature related to turmeric and turmeric-derived compounds that has increased exponentially in recent years, the current state of our modern understanding of the biological effects and medicinal potential of this ancient plant is summarized here.

BACKGROUND

Source

Turmeric (*Curcuma longa* L.) belongs to the Zingiberaceae family, which includes other tropical medicinal plants, such as ginger. Turmeric is cultivated commercially in Southeast Asia, its area of geographic origin. India is by far the largest producer and consumer of turmeric, supplying 70% of the world's market while only exporting 10% of its crop (1). The plant, of which there are numerous varieties, is propagated for its rhizome, which is used as a culinary spice and traditional medicine. Seed rhizomes are sown in May to August; aerial shoots and leaves form over the next 5 months, followed by a period of accelerated rhizome growth, with rhizome harvest at 8–10 months (2). Bulbs and the more highly valued fingers of this “green turmeric” cannot be stored, as they are easily damaged by insects and so are cured [boiled in water for hours until soft; dried in the sun (1–2 weeks); polished in a turning drum; and colored by application of turmeric and other colorants, such as tamarind] to obtain a dry turmeric rhizome (~20% yield from green turmeric) (2).

Ethnobotanical Use

Having a rich and long history of culinary and medicinal use, turmeric remains one of the major spices traded worldwide (1). Its culinary and medicinal uses were first developed in India, which has been its primary site of cultivation for millennia. As with many spices, turmeric's culinary uses include food preservation, flavoring, and coloration. The average diet in India is estimated to contain 2.5 g of turmeric rhizome per day, of which 100 mg (3% by weight) are polyphenolic curcuminoids (3). In western countries, such as the United States and France, turmeric is primarily used in the food industry as a colorant (e.g., mustard), resulting in estimates of daily curcumin consumption 10-fold lower than those in India (4). Medicinal turmeric is central to many Indian systems of traditional medicine (Ayurveda, Siddha, Unani) and is part of Eastern Asian systems as well (Traditional Chinese Medicine, Japanese Kampo, Korean, Malay). Turmeric is traditionally used in India for medical disorders of (i) skin (wounds, urticaria); (ii) upper respiratory track (rhinitis, pharyngitis, and cough); (iii) joints (rheumatism); and (iv) the gastrointestinal system (digestive aid, biliary and liver disorders) (1,2). Modern scientific inquiry related to turmeric, and in particularly curcumin, has focused on its potential use in other disease states including cardiovascular disease (CVD) and cancer, which are the major causes of mortality in adults in western countries.

Chemistry

Turmeric rhizomes contain two main classes of secondary metabolites: curcuminoids and essential oils (Fig. 1), each comprising approximately 3% by weight of the dried rhizome and conferring protection to the plant through insecticidal and other properties (5). Dichloromethane-methanol extraction of the dried rhizome yields an extract (9% yield), of which curcuminoids and essential oils each comprise approximately one-third by weight (6–9). Essential oils are typically discarded as a byproduct of industrial curcuminoid isolation, but can be selectively isolated (3–4% yield) from dried rhizomes by hexane extraction (9,10). While factors such as geographic origin and plant variety alter their chemical composition, turmeric essential oils are primarily composed of sesquiterpenoids, with turmerones (ar and α - or β -turmerone) and curcumenes (e.g., zingiberene) being the major classes of compounds represented (Fig. 1) (5). Alternatively, the curcuminoids can be selectively isolated, yielding products that are primarily composed of three compounds, curcumin, demethoxycurcumin, and bisdemethoxycurcumin

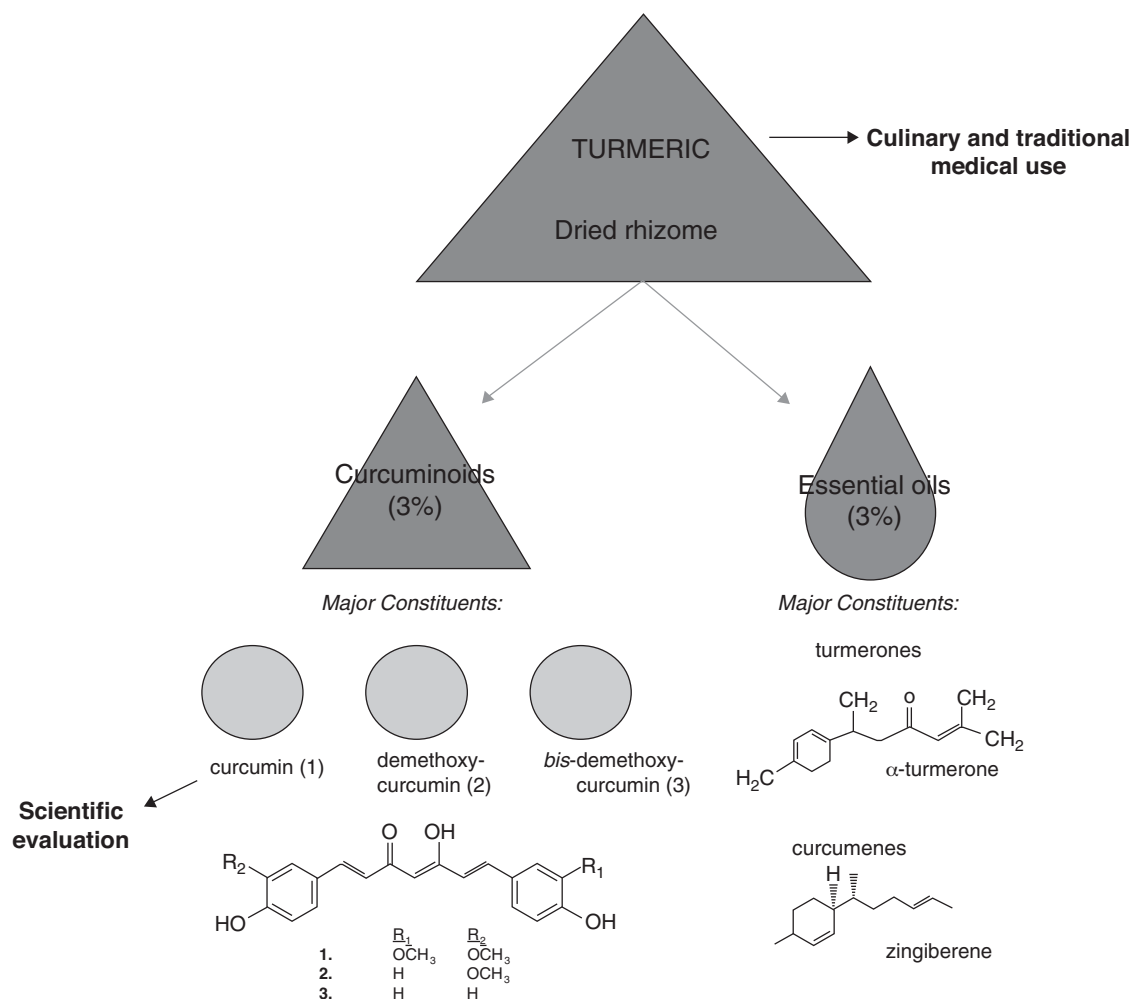


Figure 1 Schematic indicating major secondary metabolites and classes of chemical compounds derived from dried turmeric rhizome.

(Fig. 1) (5). Again, the relative ratios of these compounds vary with geographic origin and variety of the plant from which the turmeric rhizome is harvested; curcumin usually predominates (50–70%), but the relative amounts of the other two compounds can vary significantly [e.g., bis-demethoxycurcumin content can range from 3% to 33% of total (5,8)]. The curcuminoids appear to be the biosynthetic product of an enzyme(s) requiring as substrates, malonyl-CoA and hydroxycinnamoyl-CoA esters that are derived from the phenylpropanoid pathway (11). The enolate form of curcumin, which is favored in basic pH (pH 7.5), is more reddish in hue, accounting for the historic use of turmeric-treated paper as a pH indicator (12).

Traditional Preparation and Dietary Supplementation

During culinary use, turmeric rhizome is often heated and/or combined with oil, being responsible for the golden hue of curries (3). For topical use for wound treatment, an aqueous paste of turmeric is prepared from the whole rhizome (3). For other indications, turmeric rhizome is taken orally in various preparations (5). Regu-

latory bodies in Europe and Canada recommend the use of turmeric preparations made from the whole rhizome (rhizome itself, 1:1 aqueous infusion, or 1:5 to 1:10 tinctures utilizing 70% ethanol), with dosing usually normalized to 1–4 g equivalents of rhizome (13–15). Commercial turmeric dietary supplements sold in the United States do not contain pure curcumin but are composed of a mixture of the three major curcuminoids and are devoid of essential oils, being sold in capsules labeled to contain 400–500 g of curcumin(oids), corresponding to 13–17 g of dried rhizome (at 3% yield) (8). On average, only 50% of the curcuminoid content of randomly selected turmeric dietary supplements in the United States is composed of curcumin, the remainder being demethoxycurcumin and bisdemethoxycurcumin in varying ratios (8). The chemical composition of turmeric products used in clinical trials is often not documented (Table 1) (16–51). One exception to this is the evaluation in phase 1 and 2 trials of a commercial curcuminoid-enriched product composed of a mixture of the three major curcuminoids, whose curcumin content exceeds 70% (27–30, 32,34,35).

Table 1 Published Clinical Trials

Disease process	Material description	Product	Dosing		Study Design					Subjects <i>n</i> , (botanical treated)	Reference
		Chemical composition (reported/ independently verified)	Source [commercial (C) or non-C]	Dose (g)	Duration (weeks)	Randomized, <i>r</i>	Placebo- control (PC)	Blinded (single or double)	Other design		
Gastrointestinal (GI)											
Dyspepsia/peptic ulcer disease	Turmeric	-/-	NC	3	4	-	-	-	-	45	16
<i>Helicobacter pylori</i> with dyspepsia	Curcumin ^a	-/-	C	0.06	1	-	-	-	-	25	17
Irrit bowel syndrome (IBS)	Turmeric extract	-/-	C	0.144	8	r	-	-	-	107	18
Colitis (Crohn's + UC)	Curcumin	Yes/-	C	1.1–1.7	8	-	-	-	-	10	19
Ulcerative colitis (UC)	Curcumin	Yes/-	C	1	24	r	PC	DB	-	45	20
Diarrhea in HIV+	Curcumin	-/-	Various C	6–6	<64	-	-	-	-	8	21
Gall bladder (contraction)	Curcumin	-/-	C	(20 mg X1)	-	r	-	DB	CO	12	22
Gall bladder (contraction)	Curcumin	-/-	C	(20–80 mg X1)	-	r	-	SB	CO	12	23
Familial adenomatous polyps (FAP)	Curcumin ^b	-/-	C	8	12–36	-	-	-	-	5	24
Cancer, GI											
Colorectal carcinoma (CRC)	Curcuminoids + essential oils	Yes/yes	C	0.04–0.2 curc/ 0.4–2 EO	4	-	-	-	DE	15	25,26
CRC	Curcuminoids	Yes/yes	C	0.5–4	>1	-	-	-	DE	15	27
Liver metastases with CRC	Curcuminoids	Yes/yes	C	0.5–4	1	-	-	-	DE	12	28
CRC	Curcuminoids	Yes/yes	C	0.5–4	1	-	-	-	DE	12	29
Pancreatic adenocarcinoma	Curcuminoids	Yes/-	C	8	8	-	-	-	-	25	30
Precancerous, non-GI											
Precancerous lesions	Curcumin	Yes/-	C	0.5–8	-	-	-	-	-	25	31
Monoclonal gammopathy	Curcuminoids	Yes/-	C	4	12	-	-	-	-	26	32
ISkin/mucosa											
Oral submucous fibrosis	Turmeric extract ± essential oil or oleoresin	-/-	C, NC	3 g TE/0.6 g EO	12	-	-	-	-	39	33
Oral lichen planus	Curcuminoids	Yes/yes	C	2	7	r	PC	DB	-	33	34
Psoriasis	Curcuminoids	Yes/-	C	4.5	16	-	-	-	-	12	35

PRECLINICAL STUDIES

Preclinical analyses of potential health promoting or medicinal effects of turmeric and/or curcumin comprise the bulk of the more than 4000 reports on these agents in the scientific literature. Documented effects of turmeric, which have been the subject of numerous review articles (3,5,12,52–55), will be briefly summarized here for several of the major disease categories targeted for scientific exploration, which reflect both traditional use and the extrapolation of potential benefit to other diseases sharing a similar physiologic basis. This summary includes very little information regarding the essential oils, reflecting a relative lack of scientific investigation despite the traditional use of whole turmeric rhizome.

Cancer

Numerous *in vitro* studies demonstrate the ability of curcumin to arrest proliferation and/or induce apoptosis in multiple tumor cell lines, including those isolated from colon, liver, breast, prostate, and hematopoietic (lymphoid and myeloid) cells, with evidence supporting multiple potential mechanisms for these effects, including inhibition of NF- κ B (52). Arresting cells in the G2/M phase of the cell cycle may also enhance sensitivity to radiation and chemotherapy (12), although evidence that curcumin may render cancer cells more resistant to treatment with traditional chemotherapeutics also exists (53). *In vivo* studies suggest that curcumin may also limit tumor growth by inhibiting angiogenesis (54). In rodents, curcumin has been reported to prevent the *in vivo* growth of colon, liver, breast, prostate, and other tumors (12). In the case of breast and prostate cancer, as curcumin does not bind to the estrogen or androgen receptor (55), these beneficial effects appear to be hormone independent. In addition, turmeric may also play a role in cancer prevention, as high dose curcumin can inhibit the development of precancerous lesions, including those caused by chemical carcinogens (12). Turmeric essential oils have been much less studied for effects on any disease process, including cancer. Nonetheless, there are several reports in the literature documenting induction of apoptosis by turmeric oils and/or their components in cancer cell lines, particularly those of hematopoietic origin (56).

Arthritis

Turmeric has been proposed as an alternative to pharmaceutical cyclooxygenase (COX) inhibitors for arthritis treatment (7). *In vitro*, the curcuminoids and the essential oils of turmeric are equipotent inhibitors of PGE₂ production (6). *In vivo* testing of a library of chemically characterized turmeric extracts containing curcuminoids and/or essential oils in an animal model of rheumatoid arthritis has demonstrated that the curcuminoids and essential oils are each antiarthritic (7–9). However, when administered orally and in isolation, *in vivo* anti-inflammatory effects of curcuminoid-containing extracts are more pronounced than for the oils (7–9). Inhibition of nuclear translocation of the transcription factor, NF- κ B, appears to be central to the antiarthritic effect of the curcuminoids, preventing the subsequent activation of an inflammatory cascade that includes the articular production of inflammatory cell-attracting chemokines, the local induction of COX-2 gene

expression and prostaglandin production, and expression of inflammatory cytokines, such as interleukin-1 (8). Curcuminoid-containing turmeric extracts also blocked periarticular increases in bone-resorbing osteoclasts and resultant bone destruction (8), a finding consistent with previous *in vitro* reports that inhibition of NF- κ B by curcumin can block receptor activator of NF- κ B (RANK)-stimulated differentiation of bone-resorbing osteoclasts (57), the major vertebrate pathway regulating bone resorption. In addition, *in vivo*, curcuminoid-containing turmeric extracts normalized the high levels of RANK-stimulating ligand and low levels of protective RANK decoy receptor that favor bone resorption in arthritic joints (8). Evaluation of effects of turmeric or curcumin in animal models of osteoarthritis, a more common clinical disorder, is lacking, although *in vivo* studies suggest that curcumin may be chondroprotective, blocking the effects of inflammatory cytokines (58). Curcumin has also been reported to reduce carrageenin-induced paw edema, a model of acute inflammation rather than actual destructive arthritis, and paw swelling in adjuvant-induced arthritis (12).

Cardiovascular Disease

The importance of inflammation in cardiovascular disease (CVD) has increasingly been recognized and oxidative damage is also thought to be key to its pathogenesis. Curcumin has been identified as a compound of interest for CVD treatment because of its reported *in vitro* anti-inflammatory (as discussed earlier) and antioxidant effects (12,59,60). Tautomerization of curcumin to the enolate form creates a compound that, as with other phenolic antioxidants, can act as a scavenger of free radicals (12). In addition, curcumin's ability to chelate certain metal ions can also contribute to its antioxidant effect (12). *In vitro*, curcumin prevents the activation of vascular endothelial cells as well as vascular smooth muscle cell proliferation and intracellular cholesterol accumulation, processes central to the pathogenesis of atherosclerotic lesions (60–62). Low-density lipoprotein (LDL) uptake by LDL receptors on liver cells, the primary LDL clearance pathway, is stimulated *in vitro* by curcumin, suggesting one mechanism by which curcumin could lower cholesterol levels (63). *In vivo*, curcumin does limit diet-induced hypercholesterolemia and attenuate the development of atherosclerotic lesions in various murine models (60,64). While effects of turmeric essential oils on atherosclerosis are less well studied, low doses (1.6 mg/kg) of a complex turmeric extract containing both metabolites (only 10% curcuminoids by weight) are also reported to prevent atherosclerotic lesion development in rabbits fed a high-fat diet (64). In addition, in murine models of ischemic stroke, acute administration of either curcuminoids or the essential oils of turmeric can each limit neuronal damage, effects that have been attributed to antioxidant properties (65,66). Less is known about protective effects of turmeric in myocardial infarction, but preliminary evidence suggests that curcumin can limit ischemic myocardial injury (59).

Diabetes

As inflammation and oxidative stress are increasingly recognized as contributors to the pathogenesis of type 2

diabetes mellitus (DM), a disease with high and increasing prevalence, the use of turmeric, and curcumin in particular, in disease management is increasingly being studied (67,68). Both curcuminoids and the essential oils of turmeric have been identified as agonists for peroxisome proliferator-activated receptor- γ (PPAR- γ) (67,68), an adipocyte transcription factor targeted by pharmaceutical drugs whose activation lowers insulin resistance. Turmeric extracts, and/or both classes of secondary metabolites, have been reported to lower blood sugar in murine models of diabetes (9,67), normalize cholesterol levels, prevent increases in serum markers of inflammation, and/or reduce lipid peroxidation in animal models of diabetes (9,67). Moreover, in a murine model of type 2 DM, a complex essential oils- and curcuminoid-containing turmeric extract proved superior to curcuminoids alone, normalizing glucose and preventing the development of abdominal fat, effects attributed to synergistic stimulation of PPAR- γ by both classes of secondary metabolites (68). Inhibition of the hepatic steatosis associated with insulin resistance by curcumin has also been attributed to its activation of PPAR- γ in hepatic stellate cells in murine models. In vitro studies suggest that curcumin can also protect insulin-secreting pancreatic β cells from inflammatory or oxidative injury (67). Pathways specifically contributing to diabetic complications, such as aldose reductase, are reportedly blocked by curcumin (67). In vivo studies also suggest protective effects of curcumin in ameliorating renal, retinal, and neuropathic complications in rodent models of diabetes (12,67).

Alzheimer Disease

In a murine model of Alzheimer disease (AD), oral administration of curcumin reduces amyloid plaque formation, an effect that was initially attributed to curcumin's antioxidant properties (69). However, subsequent studies found that curcumin can cross the blood-brain barrier and specifically bind to amyloid, preventing its polymerization and subsequent plaque formation (70). In addition, curcumin can stimulate in vitro clearance and uptake of amyloid by macrophages, an effect that may be enhanced when combined with vitamin D (71).

Colitis

Numerous laboratories have documented a protective role of curcumin, when administered orally or via intraperitoneal injection, in the treatment of experimental colitis (72,73). Inhibition of NF- κ B activation and changes in the balance of Th1 and Th2 cytokines have been reported (72,73). Included in these studies are results suggesting that the efficacy of curcumin in colitis may be dependent on the nature of the underlying immune dysregulation, being more effective in Th2- than in T1-dependent colitis, whose clinical correlates are ulcerative colitis and Crohn's disease, respectively (72,73).

Cystic Fibrosis

In 2004, curcumin was reported to reverse the primary defect causing cystic fibrosis, allowing mutated cystic fibrosis transmembrane conductance regulators to be translocated out of the endoplasmic reticulum and into the plasma membrane (74). However, subsequent preclinical

studies have called this result into question, also identifying other potentially adverse effects of curcumin on the activity of these chloride channels (75).

PHARMACOKINETICS

As with the study of biological effects, pharmacokinetic analyses related to turmeric have almost solely focused on curcumin. When curcumin is administered orally to rodents, absorption has been reported to be 60%, with rapid glucuronidation and fecal excretion as a major route of elimination (76,77). Serum curcumin levels are in the μ g/mL range in response to daily dosing with more than 1 g/kg (76). In humans, curcumin is reported to peak in the serum 1–2 hours after oral administration and, as in rodents, undergoes extensive glucuronidation. Serum curcumin levels of 3–50 ng/mL have been reported in humans consuming curcumin in the range of 50–500 mg/kg/day (2–12 g/day) (77). This can be contrasted with the higher serum levels of 500–1000 ng/mL in rats treated with 1–2 g/kg, doses which correspond in humans to 150–300 mg/kg/day after correcting for body surface area (77). As curcumin products available for study are often not actually pure curcumin, but rather a mixture of the three major curcuminoids (7,25), it is unclear what effect, if any, the unknowing use of such a product in a published pharmacokinetic study would have on experimental outcomes.

Very little data are available on the pharmacokinetics of turmeric essential oils. However, one very recent study in rabbits suggests that the half-life of the major sesquiterpenes is on the order of 2 hours after intravenous administration of 50 mg/kg of essential oils (78). A report examining the ability of turmeric oils to enhance the oral bioavailability of curcuminoids in humans did not include concomitant assessment of the bioavailability of the oils (48).

Because the potential for curcuminoid bioactivity is high, but their bioavailability is low, considerable research has focused on either the synthesis of related compounds, which will not be reviewed here, or the design of delivery methods to enhance the bioavailability of naturally occurring curcuminoids (79). For example, curcumin readily degrades in aqueous solutions (12), a process that can be inhibited by binding to casein, the primary protein present in milk, which interestingly is used sometimes as a "vehicle" for turmeric delivery in traditional use (e.g., pharyngitis). Also, curcumin readily binds to phosphatidylcholine, forming micelles that efficiently chelate metals—and enhance the bioavailability of curcumin when administered orally to humans (76,79). Bioavailability of oral curcumin in humans and other vertebrates is reportedly increased up to 20-fold when comixed with oils, including the essential oils of turmeric (76). This then raises the question of whether curcumin bioavailability is greater during culinary versus dietary supplement use, as it is mixed with both turmeric essential oils and additional cooking oil when used as a culinary spice, while turmeric dietary supplements are often enriched for curcuminoid content and devoid of essential oils. Similarly, some commercial products combine curcumin with piperine, one component of a traditional blend of spices (Trikatu) used in Ayurvedic

medicine to enhance the bioavailability of botanicals (76). Other approaches to enhance curcumin bioavailability include the use of nanotechnology to formulate particles with higher solubility and bioavailability (79). In addition to possible matrix effects of essential oils in enhancing curcuminoid absorption, interactions between the curcuminoids have also been reported (e.g., demethoxycurcumin enhances the stability of curcumin), as have differences in their relative stability (e.g., demethoxycurcumin > curcumin in vitro) (76,80,81). Lastly, evidence that curcumin can accumulate in the gastrointestinal track even in the absence of high serum levels has led to suggestions that its low bioavailability is neither relevant nor problematic when targeting gastrointestinal diseases (12,77). At the same time, it must be noted that curcumin accumulation has been documented in other organs after oral delivery, including reports of a positive gradient between brain and blood in a murine model of Alzheimer disease (69).

CLINICAL EFFICACY

Clinical Trial Quality

Published turmeric clinical studies, the majority of which are summarized in Table 1, have examined turmeric effects in disease processes similar to those for which it is traditionally used, with half focusing on gastrointestinal disorders and the majority of the remainder evaluating anti-inflammatory effects. However, in contradistinction to traditional use, wherein turmeric preparations are made from the whole rhizome, half of the clinical trials report testing the isolated effects of curcumin (Table 1), the most abundant curcuminoid found in turmeric rhizomes. However, as pure curcumin is not easily isolated from the other curcuminoids and therefore difficult to obtain (82), it is unlikely that pure curcumin was the actual test product in many of these studies. Indeed, in the majority of turmeric clinical trials, extrapolation of results to clinical use is complicated by a lack of documentation of product composition (Table 1). In addition, study quality with respect to experimental design (e.g., lack of placebo controls, randomization, or blinding) is also problematic (Table 1). While these flaws, including the lack of control groups, severely limit the quality of this database and thus the conclusions to be drawn, available clinical evidence examining turmeric use for disease prevention and treatment is summarized below.

Gastrointestinal Disorders and Malignancies (Table 1)

Symptoms of dyspepsia and peptic ulcer disease, but not *Helicobacter pylori* colonization, improved after short-term (≤ 1 month) treatment with either uncharacterized turmeric or a very low dose of curcumin given in combination with *N*-acetylcysteine and lactoferrin (16,17). Irritable bowel syndrome (IBS) symptoms were also improved after 2 months of treatment with a low dose (< 200 mg) of an uncharacterized turmeric (18). Two colitis trials, including one well-designed study, have reported improvements, as measured by clinical symptomatology, endoscopic evaluation, and/or sedimentation rate, in patients with ulcerative colitis (UC) or Crohn's disease upon treatment with curcumin (≥ 1 g/day) (19,20). In familial adenomatous polyposis (FAP), polyp size and number decreased

after treatment with high-dose curcumin (8 g) in combination with quercetin (24). At the time of this writing, additional clinical trials examining curcumin in IBS, FAP, and UC are underway (83). Seven clinical trials evaluating turmeric for both the prevention and treatment of colorectal adenoma carcinoma are also ongoing (83) and follow the publication of several phase I studies (26–29) documenting the pharmacokinetics and tolerability of a commercial curcuminoid mixture (0.5–4 g) in this setting. Results of an open-label study evaluating the same commercial product in patients with pancreatic adenocarcinoma suggested a possible response in 2 of 25 patients (30). Three additional pancreatic adenocarcinoma trials are currently underway (83).

Mucocutaneous Disorders (Table 1)

Topical turmeric is used traditionally for wound treatment and cosmetics. Clinical trials assessing effects of oral treatment with curcuminoid-containing turmeric products on postoperative wound healing, oral lichen planus, and psoriasis have demonstrated no clear effect (34,35,38), while evaluation of a histological endpoint in patients with oral submucous fibrosis were suggestive of a positive response (33). A study assessing turmeric in the treatment of oral mucositis is currently underway (83).

Musculoskeletal Disease (Table 1)

While turmeric is traditionally used for arthritis treatment, only two clinical trials have directly examined this use, describing equivalency in the response of rheumatoid or osteoarthritis patients to short-term treatment with curcumin(oids) (1–2 g) versus nonsteroidal anti-inflammatories in trials lacking placebo controls (36,37). Additional clinical trials examining turmeric in rheumatoid, osteoarthritis, and gonococcal arthritis are currently underway. One published study of patients with monoclonal gammopathy, a potential precursor of multiple myeloma, reports a decrease in serum paraprotein and urinary *N*-telopeptide levels in response to daily treatment with 4 g of curcuminoids (32). Clinical trials evaluating curcumin in multiple myeloma and osteosarcoma are also in progress (83).

Neurologic Disorders (Table 1)

While not traditionally used for neurologic disorders, great interest in turmeric for Alzheimer's disease (AD) treatment has been engendered by preclinical data identifying curcumin's ability to cross the blood–brain barrier and specifically interact with amyloid to prevent plaque formation. The lower prevalence of AD in India (e.g., 2% vs. 10% in United States) has also been postulated to possibly reflect a protective effect of dietary turmeric (69,70), although clearly other factors may account for this difference, including the population age structures when considering a disease with typical onset after age 65. Results from only a single clinical trial have been published (50), with three additional trials currently underway in this area, and a fourth completed, but not published (83) (Table 1). Results from the published (randomized, placebo-controlled, double-blinded) trial examining the effects of 6 months of treatment with 1 or 4 g of an uncharacterized curcumin product in patients with AD were

inconclusive, as no cognitive decline was documented in controls during the study period (50).

Cardiovascular Disease and Diabetes Mellitus (Table 1)

Despite very interesting preclinical data, little clinical work has been done to date to examine turmeric effects on cardiovascular and metabolic function. Studies in healthy individuals suggest that turmeric products have little effect on metabolic parameters (44–48). Interestingly, a single clinical study in adults with type 2 DM suggests a role for curcuminoids in reducing inflammation and improving vascular function while having little effect on metabolic parameters (49).

MECHANISM OF ACTION

Public and scientific interest in turmeric use for certain diseases, such as arthritis and colon cancer prevention, has often centered on curcumin's potential to inhibit COX activity (12). However, as indicated here, a multitude of biological effects of turmeric products have been documented in isolated cells and intact animals. Some of these pleiotropic effects may be attributable to turmeric's ability to alter the activity of transcription factors and nuclear receptors that regulate the expression of large numbers of genes (e.g., inhibition of NF- κ B, a critical inducer of inflammatory gene products, and activation of PPAR- γ , a nuclear receptor that is a pharmaceutical target for diabetes treatment). However, the precise molecular target(s) initiating these responses continues to be elucidated. Existing evidence has identified the following biological moieties as direct targets of curcumin: (i) agonist binding to the vitamin D receptor (84); (ii) antagonist/inverse agonist for the cannabinoid CB1 receptor (85); (iii) inhibitor of HIV-1 integrase and the DNA repair polymerase- λ activity (86,87); (iv) chelator of iron and copper (12); and (v) inhibitor, by direct binding, of the polymerization of certain proteins (amyloid, transthyretin, and α -synuclein) associated with human CNS disease (Alzheimer and Parkinson diseases) (70,88). Curcumin alters arachidonic acid metabolism, blocking leukotriene synthesis by inhibition of lipoxygenase and prostaglandin synthesis by inhibition of COX-1 (>COX-2) enzymatic activity (8). In contrast to pharmacologic COX-2 inhibitors, curcumin primarily inhibits COX-2 effects by blocking its inducible gene expression (8,89). Curcumin is reported to block protein kinase C activity by competing with Ca^{2+} for binding to its regulatory subunit, a mechanism that has been postulated to explain curcumin's ability to alter the activity of other Ca^{2+} -regulated proteins (90). In addition, *in vitro*, curcumin has been reported to alter the fluidity of biological membranes (52). While curcumin has traditionally been the primary target of scientific inquiry related to turmeric, it is increasingly recognized that, as with turmeric dietary supplements, available reagent grade curcumin is usually a mixture of the three primary curcuminoids (7,25). Studies examining the relative potencies of these three related compounds have demonstrated significant differential effects, including biological effects of the less studied compounds (e.g., bisdemethoxycurcumin) that are not shared

by curcumin (81). As the essential oils of turmeric have been much less studied, their molecular targets are also not well characterized. However, data suggest that the essential oils and curcuminoids alter similar physiological processes, as the essential oils can (i) stimulate PPAR- γ (68), (ii) more potently inhibit PGE₂ production than the curcuminoids (6), (iii) interfere with platelet aggregation (8), and (iv) induce apoptosis of malignant cells (56).

REGULATORY STATUS

The 1997 success of India's Council for Scientific and Industrial Research in contesting a U.S. patent (No. 5401504) related to medicinal turmeric serves as an important example of the challenges faced by the international community in regulating intellectual property rights and biopiracy issues when traditional medicinal plants become a focus of biotechnology. In the United States, turmeric has a "generally recognized as safe" designation as a food additive and is sold and regulated as a dietary supplement as an extract or encapsulated dried rhizome (52). Despite turmeric's traditional use and clinical investigation for multiple disease processes, the German Commission E monograph, last revised in 1990, only lists turmeric root for use in dyspepsia, with recommended doses of 1.5–3 g of the dried rhizome per day when consumed as a powder, or prepared as a tincture or infusion, with gall stones and biliary obstruction being a contraindication (13). More recent evaluations of medicinal turmeric root use in 2008 by the European Medicines Agency and Health Canada reached essentially the same conclusions with respect to recommended usage (14,15). The highest traditionally used doses listed in these reports are for preparations equivalent to 14 g/day of dried rhizome, which at 3% yield by weight for both the curcuminoids and essential oils approximates a daily dose of 400 mg of curcumin(oids) or essential oils.

SAFETY

Interestingly, the U.S. National Toxicology Program was unable to perform a toxicology and carcinogenesis analysis of curcumin in rodents, as the pure compound was not available in sufficient quantities (82), a fact that underscores the need to consider that most scientific studies of "curcumin" probably do not actually utilize the pure compound. Studies in rodents using an organic extract of turmeric (79–85% curcumin) found evidence of tumorigenesis (hepatocellular adenoma, clitoral gland adenoma, thyroid follicular cell hyperplasia, and carcinoma of the small intestine) and toxicity (gastrointestinal inflammation and ulceration) at the highest dose tested (8.5 g/kg/day rodent body weight), with a no adverse effect level (NOAEL) of 4.3 g/kg/day (82). More recent reproductive toxicity studies using purified curcumin revealed a NOAEL of 300 mg/kg/day curcumin in rodents, which led WHO to set the level of acceptable daily intake at 0–3 mg/kg/day for humans (91), a dose exceeded approximately threefold by the typical turmeric dietary supplement capsule [400–500 mg curcumin(oids)] but analogous to the estimated daily dietary consumption in India.

Safety Evaluations in Clinical Trials

Discussions of turmeric's safety profile usually include mention of its long history of dietary and traditional medicinal use, as well as the apparent safety of purified curcumin when administered at a daily dose of up to 8 g, as reported, for example by Cheng et al. (31). It should be noted, however, that daily culinary intake of complex turmeric, even in countries of highest consumption, has been estimated to equivalent to 100 mg of curcuminoids, a dose much lower than that typically studied and recommended for clinical use or dietary supplementation (Table 1). Also, the chronic use of more than 4 g curcumin(oids) per day has been documented in the scientific literature for only a small number of patients (~100) for relatively short periods of time (<4 months) (Table 1). Adverse effects most commonly reported in these turmeric clinical trials (Table 1) were primarily associated with the gastrointestinal system and included, diarrhea, nausea, and elevations in liver enzymes.

Adverse Events (Clinical and Preclinical)

Gastrointestinal, Hepatic and Biliary Effects

Adverse side effects reported for curcumin and/or turmeric in vertebrates in preclinical trials include hepatotoxicity. In our own laboratories, hepatotoxicity in vertebrates was more evident in response to the essential oils of turmeric than the curcuminoids (7–9). Reports in 2009 of liver-related deaths and hepatotoxicity in humans associated with the use of one particular turmeric supplement manufactured in the United States and marketed abroad has been attributed to the addition of nimesulide, a nonsteroidal anti-inflammatory drug, to the products (92). As previously indicated, use of curcumin-containing products in the presence of biliary obstruction is contraindicated, based on the studies of Rasyid et al., which documented marked contraction of the gall bladder in response to 20 mg curcumin (23). Nausea and gastric irritation in clinical trials, and stomach ulceration in rodents upon oral administration of curcumin, is also reported (27,82).

Contact Dermatitis

Commonly used as a cosmetic in India, there are reports of skin irritation in response to products containing turmeric (93). However, as these products also contain other ingredients, the specific role of turmeric in this effect is not clear. Oral consumption of the essential oils of turmeric has also been associated with skin rash ($n = 1$ of 7 subjects) (48).

Immune Function

Animal studies suggest that more complex turmeric extracts, but not curcuminoid-only extracts, may inhibit granulomatous inflammation, a process that keeps tuberculous and certain other infectious agents quiescent (7–9). Interestingly, although clinical studies evaluating turmeric essential oils are rare, in one small ($n = 7$) phase 1 clinical trial of turmeric essential oils performed in India, one participant withdrew due to the diagnosis of lymphatic tuberculous shortly after the initiation of treatment (48).

Reproductive

Preclinical reproductive toxicity studies have documented a decrease in the birth weight of second-generation pups (91). There is also one report of curcumin directly inhibiting sperm mobility in vitro with altering viability, leading to its suggested use as a contraceptive (94).

Drug Interactions

Care should be taken when curcuminoid containing products are used in combination with anticoagulants, as there are multiple reports of curcumin inhibition of platelet aggregation, an effect attributed to its interference with arachidonic acid metabolism (89). Similarly, the essential oils of turmeric are also reported to interfere with platelet aggregation (8). Preclinical evidence suggests that turmeric may also have effects on the cytochrome P450 system, and thus alter the kinetics of other drugs (12).

Use by Children and Pregnant or Lactating Women

No safety data are currently available to formulate a recommendation for use in children or women who are pregnant or lactating (3). As noted in Table 1, very few clinical trials have enrolled children, and none report inclusion of pregnant or lactating women.

CONCLUSION

Turmeric has a long and rich history of traditional medicinal use for disease treatment. A large body of preclinical data documents beneficial effects of turmeric products in a myriad of disease processes, supporting turmeric's potential use in the prevention and management of common diseases. However, and in stark contrast, well-designed clinical trials validating the efficacy and safety of turmeric are lacking. Existing ethnobotanical and preclinical evidence suggests that research efforts to fill this gap are warranted.

Of particular concern, poor documentation of product composition in both preclinical and clinical trials makes assessment or extrapolation of turmeric research findings problematic. The essential oils of turmeric have been largely excluded from scientific assessment, despite being (i) included in traditional forms of use, (ii) bioactive in isolation, and (iii) potentially able to synergize with curcuminoids via their physiologic and/or matrix effects. The modern focus on the isolated use of curcuminoids in doses that far exceed culinary levels must also be differentiated from more traditional turmeric usage patterns involving preparations of whole rhizomes. In addition, as the bioavailability of curcumin(oids) is intentionally increased in new supplement formulations, given their pleiotropic actions, the possibility of untoward reactions may also increased. Even in studies focusing on "curcumin," the safety and efficacy profiles of the products used may vary widely, reflecting the different biological activity profiles of the three distinct compounds that comprise most curcumin products in varying proportions.

In the case of turmeric, as with all traditional medicinal plants, "ancient" should not be assumed to be a synonym for "safe," particularly when used in nontraditional ways, but rather as an indication that encourages the application of modern tools to understand and reveal the

full gamut of medicinal actions of this fascinating plant. Details from the rich legacy of turmeric's traditional use should continue to guide scientific inquiries giving careful consideration to both the botanical product used (composition and mode of administration) and the human disease targeted. In so doing, turmeric's full potential as a medicinal plant can be tapped and safely exploited, particularly when used as a dietary supplement.

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Valerian

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INTRODUCTION

Valerian or valerian root consists of the underground organs (root and rhizome, with or without stolons) of *Valeriana officinalis* L. and a number of other *Valeriana* species (family Valerianaceae). It has been used by people of different cultures since antiquity for a number of conditions, including insomnia, migraine, hysteria, neurasthenia, fatigue, and stomach cramps. In the West, valerian is best known for its use as a mild sedative and hypnotic for nervous unrest and sleep disturbances. It is a major component of dietary supplements and herbal remedies marketed directly to consumers for treating these conditions. Over the past several decades, valerian has undergone rather extensive chemical, pharmacological, and clinical studies. Its aqueous extract has been found to be mostly responsible for its sleep-inducing effect, while the volatile oil (mainly valerenic acid) and the valepotriates (through their degradation products) are major contributors to its sedative effects. Current data also indicate the valepotriates to be responsible for the antianxiety or tranquilizing effect of valerian. No serious toxic side effects associated with the clinical use of valerian have been reported.

HISTORY/ETYMOLOGY/TRADITIONAL USES

Valeriana species have been used medicinally since early Greek and Roman times and were termed "*Phu*" (*Fu*) by ancients, such as Pliny the Elder (AD 23–79), Pedanius Dioscorides (AD 40–80), and the Greek physician Galen (circa AD 131–208) who prescribed it for insomnia (1). *Phu*, retained as *V. phu* L., synonymous with *V. dioscorides* (2), apparently refers to the strong disagreeable odor associated with the dried roots or crushed plant. However, the scent in some Asian species was regarded as pleasant, and the roots in earlier times were used to perfume linen (3). The genus name *Valeriana* was introduced around the 10th century, from which time it was used synonymously with *Phu* or *Fu* and *Amantilla* (4). It is claimed that the ancient *Phu* related to species other than *V. officinalis*, which was introduced just over 1000 years ago (5). The name valerian is held to be derived either from the Latin *valere*, meaning "to be healthy," or to honor the Roman emperor Publius A.L. Valerianus (not Valerius) (AD 253–260) (4).

Use of valerian as a medicine is said to have been first documented by Hippocrates (460 to about 370 BC) (6). Theophrastus of Lesbos (370–286 BC) mentioned valerian use as a perfume, and Dioscorides apparently used several members of the valerian family for treating digestive

disorders, flatulence and nausea, as well as liver and urinary tract problems. The Greeks also used these plants as antiperspirant, poison antidote, emmenagogue, and for vaginal infections. The use of valerian for nervous disorders was not firmly established until the late 16th century, thereafter being widely used for hysteria and epilepsy. It is also recorded that preparations of the plant were used to treat soldiers suffering from shell shock during the First World War (7). European authorities subsequently indicated general application as antispasmodic, anthelmintic, diuretic, and diaphoretic (6).

The underground organs, that is, roots and rhizomes, with and without stolons, carefully dried under 40°C, constitute the pharmacopeial drug, which is the main focus of this entry. The therapeutic applications approved by the German Commission E and other European regulatory agencies are generally sedative, sleep aid, and antianxiety in nature (1).

TAXONOMY

Many of the approximately 250 species of *Valeriana* (family Valerianaceae) occurring worldwide (8), generally termed "valerian," have been used as traditional medicines in different cultures (6). Today, the best-known and most thoroughly researched species is *V. officinalis* L. s.l. (*sensu lato*). It consists of many morphologically diversified subspecies and varieties, including diploid, tetraploid, and octaploid, as well as transitional types (9). *V. officinalis*, common or "officinal" valerian, is native to Europe and Asia and naturalized in North America (10). Other prominent common names for this species are all-heal, amantilla, baldrian (Ger.), Belgian valerian (recognizing the premier commercial supplier of the cultivated plant), capon's tail, cat's love/valerian, Fragrant valerian, St. George's herb, set-wall, spikenard, and vandal root (10).

The most popular medicinal valerian species worldwide are as follows: Mexican valerian—*V. edulis* Nutt. ex Torr. and A. Gray ssp. *procera* (Kunth or H.B.K.) F.G. Meyer or *V. mexicana* DC [syn. *V. sorbifolia* H.B.K. var. *mexicana* (DC) F.G. Meyer]; Indian valerian—*V. wallichii* DC [syn. *V. jatamansi* Jones]; Chinese/Japanese (Kesso) valerian—*V. fauriei* Briquet, formerly *V. latifolia*, *V. officinalis* var. *latifolia* Miq., syn. *V. angustifolia* Tausch., formerly *V. officinalis* var. *angustifolia* Miq. (8,11). The American *Valeriana* species next in prominence to *V. edulis* is *V. sitchensis* Bong., particularly ssp. *scouleri* Rydb., or Pacific valerian, which is native to northwest America, and considered by the

Russians to be the most powerful of all species (4). This latter species should not be confused with American "valerian," an entirely different genus, *Cypripedium pubescens* Willd. or *C. parviflorum* Salisb. (4).

BOTANICAL DESCRIPTION OF VALERIANA OFFICINALIS

V. officinalis is a perennial herbaceous plant, which bears short, simple rhizomes, sometimes producing stolons (horizontal stems/runners), the buds of which lead to new plants. The usually robust, solitary, vertically grooved stalks, cylindrical and hollow, are simple or slightly branched, more or less hairy, especially at the base (4) and attain a height of up to 2 m. The leaves are arranged in pairs, with clasping petioles, united at their bases. Each leaf bears, as a rule, 6–10 pairs of more or less opposite (pinnate or pinnatisect) leaflets of varying breadth, broader when fewer. Leaflets are linear, lanceolate, or elliptical, entire, dentate-serrate (notched or toothed) (4,6,10,12). The compound inflorescence consists of cymes (broad, flattened clusters), with flowers ranging in color from white through flesh-colored to pink, and sometimes lavender or red (6). The flowers are hermaphrodite, with a tubular five-lobed corolla 2.5–5 mm long (13), often spurred at the base, with three stamens. The limb of the calyx at first inrolled expands to a feathery pappus crowning the inferior ovary (4). The fruit, 2–5 mm long, is a hairy or glabrous capsule containing one oblong compressed seed (4,13).

PHARMACOPEIAL STATUS

Valerian was official in the *United States Pharmacopeia* (USP) from 1820 to 1942, the *U.S. National Formulary* from 1888 to 1946, and the *British Pharmacopoeia* until 1980. It was reintroduced to the *U.S. National Formulary* by the USP Convention in 1998 (7). Today, valerian is official in the national pharmacopeias of Austria, France, Germany, Great Britain, Hungary, Russia, Switzerland, and in the *European Pharmacopoeia*, among others (1); according to the *European Pharmacopoeia*, the crude drug *Valerianae Radix* contains no less than 0.5% (vol/m) of essential oil.

Japanese valerian root (*V. fauriei*) is official in the national pharmacopoeia of Japan. Also, *V. edulis* has been incorporated into the *Mexican Pharmacopoeia*, but *V. wallichii*, included in the 2nd edition of the *Indian Pharmacopoeia* (1996), was excluded from the 3rd edition.

Dosage Forms

In Germany, where more than 100 over-the-counter (OTC) tranquilizers and sleep aids contain valerian, some of which are specifically formulated for children (14), preparations are available as crude dried root and rhizome, loose or encapsulated, for aqueous infusion, preparation of tinctures or for extraction with alcohol, hydroalcoholic or aqueous media, the latter often alkaline, for use in tableted formulations. Straight ethanol is generally used to maximize extraction of valepotriates and essential oil. "Standardized extracts" are usually standardized for valerenic

acid content, mostly to 0.8% (15). Commercial branded German and U.S. products are listed in a recent publication (16). A broad variety of combination valerian products are also available, including purported standardized extracts in combination with other sedative herbs, notably hops (*Humulus lupulus* L.), lemon balm (*Melissa officinalis* L.), passionflower (*Passiflora incarnata* L.), and lavender (*Lavandula angustifolia* Miller) (14).

Dosage

In the treatment of restlessness and sleep disorders, in 1995 the German Commission E recommended 1–3 g of dried root and rhizome daily (15), 450 mg of extract, taken at least 1 hour before bedtime, and for symptomatic treatment of anxiety, 200–300 mg of extract in the morning (17), tincture (1:5, 70% ethanol), 1–3 mL, taken once to several times daily (4), or an infusion of 2–3 g dried root and rhizome per cup, up to several times daily (14).

CHEMICAL CONSTITUENTS

Volatile Oil

Most of the research emphasis, both chemically and pharmacologically, has been placed on two major groups of valerian constituents, namely the volatile oil, composed of a mixture of monoterpenes and sesquiterpenes, and the iridoids or valepotriates (valerian epoxy triesters).

The content of volatile oil of *V. officinalis* is quite variable, dependent upon subspecies, and influenced notably by soil conditions, age, and time of harvesting. A number of percentage ranges have been recorded: 0.4–1.4 (18), 0.1–2 (19), 0.2–2.8 (10), and 0.5–2 (20). The literature reports that *V. officinalis* mainly contains valerenic acid and its derivatives (0.1–0.5%) as well as valepotriates (0.8–1.7%), whereas the other major species, *V. wallichii* and *V. edulis*, contain considerably higher percentages of valepotriates, 2.8–3.5 and 8–12, respectively (21). Fresh root of *V. thalictroides* Graebn. may contain as much as 14.5% valepotriates (Fig. 1) (22).

Stoll et al. (23) identified 12 monoterpenes and 17 sesquiterpenes in the essential oil of European *V. officinalis*. The monoterpenes are dominated by bornyl acetate and bornyl isovalerate. The major sesquiterpenes include valerenic acid and its derivatives, acetoxyvalerenic acid and hydroxyvalerenic acid, the structurally related valerenal, valeranone, and elemol (Fig. 1). Examination of the oils of a wide selection of *V. officinalis* plants grown in the Netherlands identified broadly three chemical races. Two of the three types contain no kessyl alcohols, one also devoid of valeranone, while the third has moderate quantities of the alcohols, along with elemol, valerenal, and relatively high levels of valeranone; the two nonkessyl types have, respectively, high levels of valerenal accompanied by moderate amounts of elemol and valeranone, and elevated levels of elemol and valerenal (24).

V. officinalis is the only valerian species in which valerenic acids have been identified. The parent valerenic acid is the major of the three acid constituents, and hydroxyvalerenic acid is generally either present in very low quantities or not present at all. Some regard

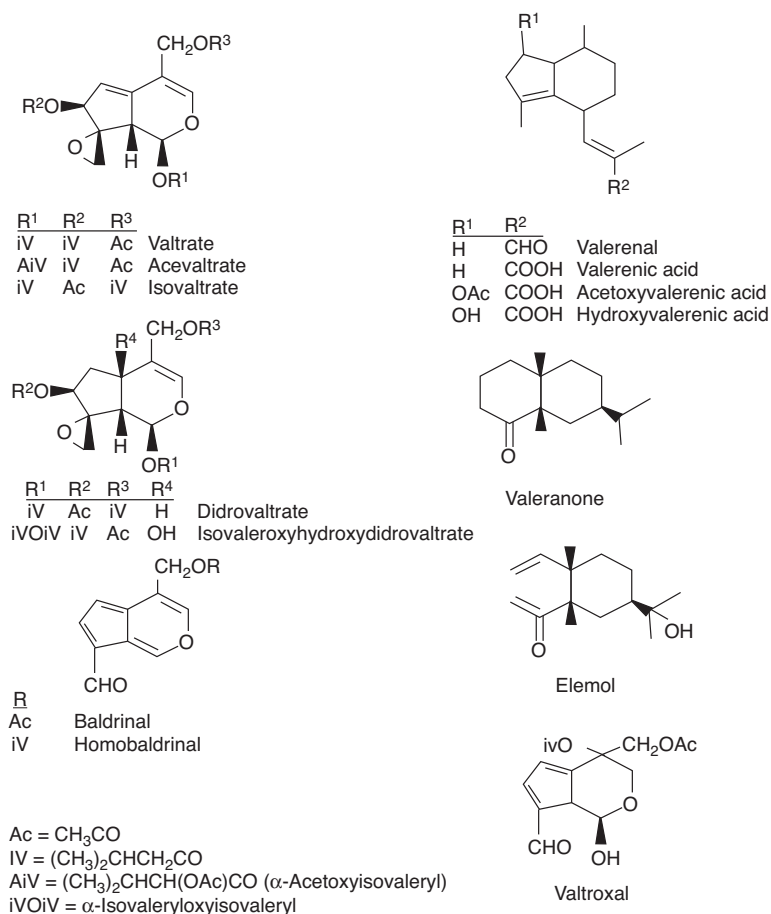


Figure 1 Major active constituents and degradation products of *Valeriana officinalis*.

hydroxyvaleronic acid as the artifactual product of hydrolysis of acetoxyvaleronic acid (25).

Valepotriates

Valepotriates are of two basic types, diene and monoene, the former, for example, valtrate, bearing an olefinic double bond in each of the two fused rings, while the latter, for example, didrovaltrate (dihydrovaltrate), has a single double bond in the six-membered ring.

Three nonglycosidic, water-insoluble iridoids, generally termed "valepotriates," were first isolated by Thies from *V. wallichii*. Two were the dienes, valtrate and acevaltrate, and the third, the monoene didrovaltrate (26). The full range of valepotriate variety has been catalogued by Houghton (19), including their chief decomposition products, baldrinal, homobaldrinal, valtroxal (13), and isovaltral (Fig. 2) (21).

A glucosylated valepotriate, valeroside, is also present (27) and chlorine-containing valepotriates, valechlorine and valeridine, have been reported (28). However, the latter pair is likely artifactual hydrogen chloride adducts to the valepotriate epoxy function, resulting from degraded chloroform used for extraction.

Valepotriates are highly unstable in aprotic solvents as well as in mineral acid and alkali, more so at higher

temperatures. In aqueous media, the diene valepotriates rapidly degrade to baldrinals: baldrinal from valtrate and acevaltrate; homobaldrinal and isovaltral from isovaltrate (21). Consequently, valepotriate formulations can only be produced effectively in solid dosage forms, preferably enteric coated (29), such as the German proprietary standardized extract, Valmane[®], consisting of 80% didrovaltrate, 15% valtrate, and 5% acevaltrate.

Valepotriates are only efficiently extracted using concentrations of alcohol greater than 70%. Bos et al. (21) investigated the stability of the monoene valepotriates, valtrate, and isovaltrate, in 70% ethanol, at 4, 20, and 36°C against a control of freshly prepared *V. officinalis* tincture at -20°C. Also analyzed were film-coated tablets prepared from *V. officinalis* and *V. wallichii*, as well as capsules of extracts prepared from *V. officinalis* and *V. edulis* ssp. *procera*. Neither valepotriates nor baldrinals could be detected in tinctures or in film-coated tablets of extracts. Baldrinals, which are not products of the dominant valepotriate, didrovaltrate, were also not detected in Valmane. The ethanol solution of the two monoene valepotriates gave no indication of decomposition, remaining colorless with no detectable levels of baldrinals. By contrast, in 70% ethanol, the concentration of valepotriates was reduced to 30% of the initial concentration after 2 weeks at 20°C and virtually nothing after 3–4 weeks; baldrinal content increased from

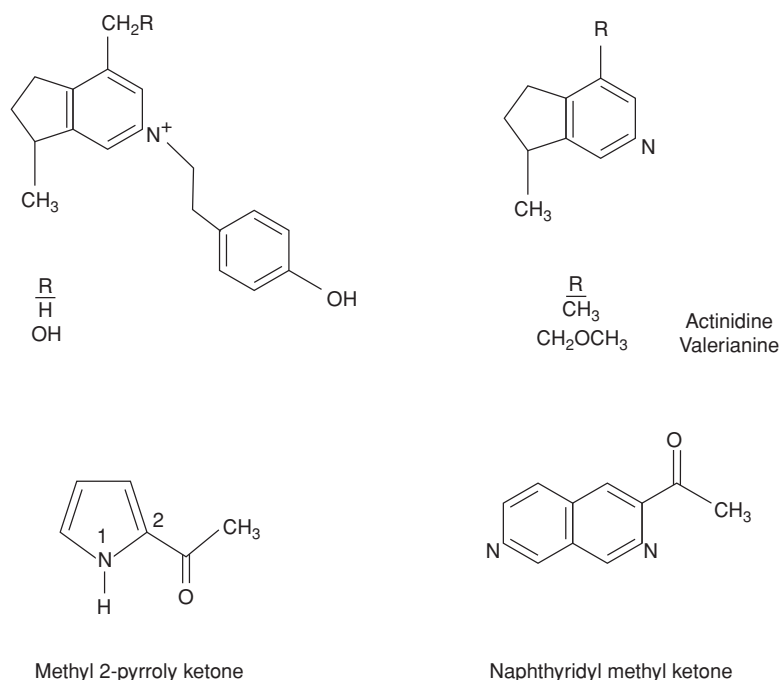


Figure 2 Alkaloids isolated from *Valeriana officinalis*.

5%, after 2 weeks, to 85%, after 3–4 weeks, the solution rapidly becoming yellow. At 36°C, the valepotriate content of the tincture also rapidly declined, but baldrinals were not detected, speculatively because of interaction with as yet unknown co-constituents. These observations indicate that water is necessary for decomposition of valepotriates to baldrinals and that the rate and course of decomposition are strongly temperature dependent.

In addition, both monoterpenic and valepotriate carboxylic esters are susceptible to hydrolysis, mainly enzymatic, yielding free carboxylic acids. Prominent among hydrolysis products is isovaleric acid, generated from bornyl isovalerate and isovaleryl valepotriates, and responsible for valerian's disagreeable odor.

Other Constituents

Small amounts of pyridine alkaloids have been reported to be present in *V. officinalis* (30–34). Also, substantial quantities of free amino acids have been found in aqueous extracts of valerian underground organs (15), notably γ -aminobutyric acid (GABA) along with glutamine, potentially metabolizable to GABA, both considered as possibly responsible for the sedative activity of such extracts (35). However, doubt has been expressed over the ability of orally ingested amino acids to reach the central nervous system (CNS) (36). Also, the amount of amino acids extracted is reduced by increasing alcoholic strength of hydroalcoholic solvents (36). A group of four closely related furanofuran lignans, derivatives of pinosresinol, have been reported (37). *V. officinalis* also contains small amounts of flavonoids and phenolic acids (38). Isovaleramide, a mild anxiolytic and sedative, has also been reported as a constituent of valerian (11,38).

PHARMACOLOGICAL ACTIVITY

So far, research into valerian constituents and their mechanisms of action strongly suggests a variety of activities associated with different chemical compounds. The sedative effects, characterized by relaxant and CNS-depressant actions, are likely due to the combined influence of the volatile oil (mainly valerenic acid), the valepotriates—through their degradation products, baldrinals and valtroxals—and possibly lignans. The valepotriates seem mainly responsible for the antianxiety or tranquilizing effect. The sleep-inducing effect, most pronounced in aqueous extracts of valerian, has not been conclusively associated with an obviously polar constituent, but GABA and glutamine are prominent candidates (36). 6-Methylapigenin, a benzodiazepine binding site ligand from *V. wallichii*, has been found to have anxiolytic properties and constituent 2S(–)-hesperidin to have sedative and sleep-enhancing properties, potentiated by 6-methylapigenin (39).

Total Extracts

The improvement of sleep characteristics by aqueous extracts of valerian (see later) suggests that the observed in vitro effects on GABA_A receptors are of therapeutic significance, involving an as yet unidentified water-soluble compound or compounds. An aqueous extract also displaced GABA from receptors in rat brain cortex tissue; a similar extract of valerian was also found to perform the same activity in rat brain cortex synaptosomes (40) and also to displace melatonin from such receptors (38), both actions consistent with sedative effect. However, the lipophilic fraction of a hydroalcoholic extract, as well as valerenic acid and valepotriate constituents,

showed no affinity with GABA_A receptors, but, rather, with barbiturate receptors—as did didrovaltrate—and to a lesser extent with the peripheral or mitochondrial benzodiazepine receptors (41). These observations strongly indicate that the sleep-inducing properties of valerian reside in the water-soluble element, while the more generalized sedative effects are likely due to the combined actions of different constituents, including valerenic acid derivatives and also possibly valepotriates, chiefly via activities at the GABA_A and barbiturate receptors in the CNS. A study compared the action of methanol, ethanol, and ethyl acetate extracts of *V. officinalis* roots on postsynaptic potentials in cortical neurons of rat brain preparations containing pyramidal cells of the cingulate cortex. The derived data suggested that activation of adenosine A(1) and GABA(A) receptors is mediated by different components of valerian extract, likely contributing independently to its sleep-inducing effect (42). Another recent rodent study, employing a 35% ethanolic extract, as well as a refined extract derived from it (Phytofin Valerian 368) proposed that not sedative, but anxiolytic and antidepressant activities considerably contribute to the sleep-enhancing properties of valerian (43).

Alkaloids

It is widely regarded that the content of valerian alkaloids is too low to contribute significantly to valerian's overall pharmacological effects (19). However, it has been postulated that actinidine is responsible for the animal attractant properties of valerian, which is claimed to stimulate cats as well as rodents and dogs (41). The structure of actinidine, also a constituent of cat-attractant *Actinidia*, is judged as similar to those of the closely related monoterpene lactones matatabilactone (30) and nepetalactone from catnip (*Nepeta cataria* L.) reported to have the same effect in cats (44,45). The latter lactone, interestingly, has been found to be a minor constituent of the essential oil of *V. celtica* L (46). It has also been noted that isovaleric acid, responsible for valerian's characteristic odor, is a component of the anal gland secretion of some members of the cat family and associated with mating behavior (47). Most of the research into valerian's pharmacological effects has been concentrated on the volatile oil and the nonvolatile valepotriates.

Volatile oil

Early 20th century investigation indicated that the sedative effect of valerian tinctures was due to its volatile oil content. However, it appeared from subsequent research that the volatile oil could be only partially responsible for the sedation observed in animal tests (19). Of the volatile oil constituents of *V. officinalis*, most attention has been focused on the sesquiterpenes, particularly valerenic acid and valeranone.

Valerenic acid affected a significant decrease in motility of mice (48) and increased pentobarbital-induced sleeping time somewhat less effectively than chlorpromazine and diazepam (49). A central rather than peripheral mechanism of action was indicated, and the compound was found to inhibit the enzyme system responsible for breakdown of GABA in the brain (50). The resultant increased level of GABA is expected to contribute to sedation and decreased CNS activity.

Valeranone is not regarded as important as valerenic acid (19). The ketone also prolongs barbitone-induced sleeping time and decreases motility in mice, attributed to reduced levels of 5-hydroxytryptamine (5-HT, serotonin) and noradrenaline in the brain (48). It has been noted, though, that the calculated corresponding effective doses of valeranone in humans would not likely be attained in standard valerian preparations (19). Valeranone has been shown to exert primarily a spasmolytic effect rather than acting on the CNS, as does valerenic acid (51).

Also reported to inhibit binding of 5-HT to its receptor is one of the four furanofuran lignans, 1-hydroxypinoresinol (37).

Valepotriates

Following the identification of valepotriates in valerian (25), much attention was devoted to their pharmacology. Their presence seemed to explain apparent discrepancies in activity of tinctures with low volatile oil content, which, nonetheless, displayed relatively high sedative/tranquilizing activity. Experiments with a high valepotriate content—as much as 8% total containing 80% didrovaltrate—preparation from Mexican valerian (*V. edulis* ssp. *procera*) showed lessened spontaneous motility in mice (52). Tests with cats revealed no decrease in reactivity but a reduction in aggressiveness, anxiety, and restlessness. In another experiment, Von Eickstedt (53) compared the effect of alcohol coadministered with chlorpromazine, diazepam, or an extract with a high smallcaps valepotriate content: Unlike the two prescription drugs, valepotriates diminished the effects of alcohol.

The valepotriates are readily susceptible to decomposition, and their major decomposition products, such as homobaldrinal from valtrate (54) and valtroxal from didrovaltrate (55), have been found to have more potent antimotility activity than their parent valepotriates. It has been proposed that such decomposition processes take place in the intestines under the agency of bacterial flora (54).

The mechanisms underlying the sedative/anxiolytic effects of the valepotriates remain unclear. A strong spasmolytic effect has been observed at doses well below those at which a direct CNS effect can be detected (56), and binding to dopamine receptors may inhibit the CNS stimulant effect of endogenous dopamine (57). Moreover, there still remains concern over the question of oral bioavailability of valepotriates and their degradation products such as valtroxal and baldrinals, which are more active intraperitoneally than orally, probably due to partial hydrolytic deactivation in the gut and/or poor absorption (19).

Toxicology of Valepotriates

The valepotriates have shown alkylating, mutagenic, and cytotoxic properties in vitro. Braun et al. (58) demonstrated the alkylating ability common to epoxides evinced by valtrate and didrovaltrate at the same rate as the epoxide epichlorohydrin, and the chlorinated tertiary amine, *N,N*-dimethyl-*N*-(2-chloroethyl) amine. An assessment of their effect on DNA synthesis in cultured hepatoma cells (59) indicated that the diene valtrate had a rapid and extensive inhibitory effect on incorporation of 3H-thymidine and 3H-leucine and that the monoenes, didrovaltrate, and

deoxydidrovaltrate, while being less active, nonetheless exhibited considerable cytotoxicity. Structure–activity considerations indicated that the C5–C6 (cyclopentenyl) double bond was important for cytotoxic activity but that the epoxide group is not essential.

A later comparative evaluation of valepotriate toxicity showed that the diene types were about two to three times more toxic than the monoenes. The decomposition products of diene valepotriates, baldrinal and homobaldrinal, were 10–30 times less toxic than their parent compounds, while isovaltral, like homobaldrinal, a product of isovaltrate, was more cytotoxic than its parent. Monoene valepotriates were found to be remarkably stable under storage, while dienes, as noted earlier, are very labile (60). However, in vitro observations of valepotriate cytotoxicity against mouse bone marrow cells (61) could not be replicated in vivo on the same type of cell, whether orally or intraperitoneally (62). These results indicate that human in vivo toxicity is likely considerably less than that observed in in vitro experiments, probably as a result of poor absorption and/or distribution and metabolism of these iridoids. It has been discovered that valepotriates and baldrinals, the latter likely produced in the gastrointestinal (GI) tract, are rapidly absorbed in the body, the latter more so. Baldrinal glucuronide esters have been isolated, which did not give positive reactions in both the Ames test and the SOS chromotest (63). At most, the administration of valepotriates would seem to pose only a slight but potential genotoxic risk to the GI tract and liver (29). However, until this is confirmed by long-term in vivo studies in humans, it would be prudent for children (14) as well as for adults not interested in the antianxiety effect to avoid consumption of valepotriates. Tinctures of *Valeriana* species stored at ambient room temperature for 2 months were found to have lost most of their valepotriate content, a recommended precautionary treatment for reducing valepotriate toxicity (64).

CLINICAL STUDIES

Most of the clinical studies with valerian relate to sleep disorders, mainly in treatment of insomnia. Those trials have generally employed valepotriate-free aqueous extracts of root/rhizome, although hydroalcoholic extracts with substantial sesquiterpene content and very low content in valepotriates have also been tested. Combinations of extracts of valerian root with extracts of hop flowers, lemon balm leaves, and passionflower aerial parts have also been evaluated.

A much smaller number of clinical trials have been conducted with valepotriate-rich extracts for treatment of generalized anxiety disorder and affective disorders.

Sleep Disorders

More than a dozen, double-blind, placebo-controlled trials of valerian have been conducted, most also randomized and/or of crossover design. The results of those studies have been summarized in great detail in the second edition of *Botanical Medicines* (15); a summary of salient details of the most recent, rigorously designed trials is presented in Table 1. In general, 400–450 mg of an aqueous extract taken at bedtime produced a significant decrease

in subjectively evaluated sleep-onset latency, as well as improved sleep quality of poor or irregular sleepers, without causing any hangover the following morning (64,65). The earlier of these two studies (64) compared the effects of an aqueous extract of valerian with those of a commercial preparation (Hova[®]) of combined valerian root and hop flower extract, against placebo. Hova affected neither sleep latency nor sleep quality but generated a marked increase in reports of feeling more sleepy than usual upon awakening. The reason for the inferiority of the commercial preparation cannot be speculated upon without knowledge of the details of its preparation. A much later study with Hova compared its effects with those of the benzodiazepine, bromazepam, in 37 middle-aged women and 9 men with nonpsychiatric/nonchronic sleep disorders (66); the randomized, double-blind, placebo-controlled, parallel-group designed trial found that the health of patients improved only after 2 weeks and that the valerian–hop combination product was equally effective as the benzodiazepine, thereby presenting a sensible alternative to the latter. A combination concentrated, standardized valerian–lemon balm extract was compared with triazolam, another benzodiazepine, and placebo in 20 healthy volunteers, poor sleepers aged 30–50 years (67). Both treatment groups showed a significant increase in sleep efficiency especially those in the subcategory of “bad” sleepers. In contrast to the triazolam group, however, no daytime sedation, impairment of concentration, or performance capabilities was observed in the valerian–lemon balm group.

Interestingly, a proprietary 70% alcohol extract of valerian root (Lichter AG, LI 156) produced significant improvement in sleep characteristics only after 2 weeks of treatment (68).

Kamm-Kohl et al. (69), in a randomized, placebo-controlled, double-blind study with 80 hospitalized elderly patients with sleep disturbances and/or rapid fatigue, investigated the efficacy of an aqueous extract of valerian root over 14 days (45 mg, three to nine times daily). Statistically significant improvements were observed in both subjective well-being and behavioral disturbances in the treated group, but not in the placebo group. Patients in the verum group reported improved ability to fall asleep and sleep through the night, as well as reduced feeling of fatigue.

More recently, three trials were conducted with the commercial preparation, Sedonium[®], in the form of coated tablets each containing 300 mg of a dry extract of valerian root (67–72). Each study involved 16 patients in a randomized, double-blind, placebo-controlled, crossover design. The first of these 70 examined the effects on electroencephalogram (EEG) in single (1.2 g) and multiple dose (600 mg/night) for 2 weeks; the observed changes in EEG approximated those produced by psychosedative anxiolytic drugs. The second (71) used polysomnographic tests to measure slow-wave sleep (SWS) and SWS latency, among other parameters. In objective measurements, valerian decreased SWS latency, compared with placebo, while increasing the duration of SWS compared with baseline; subjectively, the time to sleep onset was reduced by valerian as compared with placebo. The third (72) assessed the short-term (single dose of 2 × 300 mg tablets) and long-term (14 days with multiple dosage) treatment

Table 1 Selected Randomized Controlled Trials of *Valeriana officinalis* Root Extracts for Sleep Disorders

Treatment	Sample size	Results	Comments	References
<p>Single-night study assessed with questionnaires</p> <ul style="list-style-type: none"> 400 mg of a freeze-dried aqueous extract; 400 mg of combined aqueous valerian and hop flower extracts (in 2:1 ratio) in a proprietary preparation (Hova); placebo (brown sugar). Sealed in opaque capsules, the three coded samples were taken in a crossover design for 9 nonconsecutive nights. Each preparation was taken 1 hr before retiring and the postsleep questionnaire filled the following morning. 	128	<ul style="list-style-type: none"> The nonproprietary extract significantly reduced sleep latency, pronounced in the elderly ($P < 0.05$), as well as improving sleep quality, particularly in poor sleepers, especially women, the young, and smokers ($P < 0.01$). Dream recall, night awakening, and sleepiness the next morning were not significantly altered. Hova did not influence ratings for sleep latency and sleep quality but caused a marked increase in reports of feeling more sleepy than usual the next morning ($P < 0.01$), likely due to the hop extract. 	<ul style="list-style-type: none"> Total sleep time is not a reliable measure without baseline reference. It is doubtful whether the distinctive odor of valerian was effectively masked to prevent identification from the ground brown sugar placebo. Reliability of subject responses is difficult to assess. 	64
<p>Sleep laboratory evaluation of EEG parameters</p> <ul style="list-style-type: none"> 400 mg extract; placebo. Preparations taken in crossover design for 4 nights and questionnaires filled out the morning after each EEG night. 	10	<ul style="list-style-type: none"> The small-scale parallel sleep laboratory study revealed no significant changes in either EEG parameters or subjective measures. These results were likely influenced by the test population being exclusively young, good sleepers, found to be unaffected by valerian in the parallel study with a larger, more diverse population. 	<ul style="list-style-type: none"> The variability of the EEG likely masked any potential but small effects on sleep physiology. 	
<ul style="list-style-type: none"> 45 mg aqueous extract 3–9 times daily for 14 days. 	80 elderly	<ul style="list-style-type: none"> Statistically significant improvements were seen on both a subjective well-being (mood) scale (von Zarassen) and the Nurse's Objective Rating Scale for Inpatient Evaluations (NOISE) ($P < 0.01$): patients treated with the extract reported improved ability to fall asleep and to sleep throughout the night, as well as reduced feelings of fatigue. 	<ul style="list-style-type: none"> Feelings of rapid fatigue were assumed to be related to sleep disturbances. 	69
<ul style="list-style-type: none"> 450 and 900 mg aqueous extract, 30 min before retiring, on 4 nights during the week for 3 wk. 	7	<ul style="list-style-type: none"> The 450 mg treatment produced decreased sleep latency; there was a positive correlation between sleep quality measured by wrist-worn activity meters and questionnaires; no further improvements were noted with the 900 mg treatment, which made subjects feel more sleepy the next morning. No influence on total sleep time or total sleep time movement was observed. 	<ul style="list-style-type: none"> The use of activity meters to assess sleep quality is unconventional and of questionable reliability. As noted above, the use of brown sugar as placebo is not completely satisfactory on account of distinctive valerian odor. Very small study group. 	65
<ul style="list-style-type: none"> 400 mg of standardized valerian extract, virtually valepotriate free, combined with hops flowers and lemon balm extract. The placebo contained a full complement of hops and lemon balm but only 4 mg valerian extract. Preparations taken for 2 nights. 	27	<ul style="list-style-type: none"> 24 of 27 (89%) reported improved sleep with the test preparation; 21 of 27 (78%) rated the test preparation superior to control ($P < 0.0001$); 12 of 27 (44%) reported "perfect sleep" with the preparation. 	<ul style="list-style-type: none"> Very limited duration of study. Both valerian botanical accompaniments are known to have sedative properties. 	74
<ul style="list-style-type: none"> 600 mg of a 70% alcoholic extract (Sedonium, Lichtwer Pharma, Berlin). 	121	<p>Increase in clinical global impression, rating of sleep quality, and in subjective rating on the von Zarassen well-being scale. Physicians and patients judged the effect of the valerian treatment as "very good" or "good" in 61.0% and 66.1%, respectively. By contrast, placebo was judged as such in only 25.9% of cases.</p>	<p>Placebo, stated to be indistinguishable from verum, not described further.</p>	68

Table 1 Selected Randomized Controlled Trials of *Valeriana officinalis* Root Extracts for Sleep Disorders (Continued)

Treatment	Sample size	Results	Comments	References
Sedonium, 600 mg/night for 14 nights.	16	In objective measures, using polysomnographic tests, valerian decreased slow-wave sleep (SWS) latency (21.3 vs. 13.5 min) compared with placebo, as well as increasing the duration of SWS compared with baseline. By subjective assessment, the time to sleep onset was reduced (45 vs. 60 min) by valerian compared with placebo.	Relatively small patient population.	71
Sedonium: 600 mg extract in short term (single dose) or long term (14 days with multiple dosage). Patients underwent 9 polysomnographic nights: 1 control before and 8 nights during the study, the latter scheduled in two trial periods separated by a washout period of 13 days.	16	Single-dose valerian treatment exerted no observable effects on sleep structure and subjective sleep assessment. After multiple-dose treatment, sleep efficiency showed significant increase for both placebo and verum in comparison with baseline polysomnography. However, significant differences were noted in parameters describing SWS between valerian- and placebo-treated subjects.	The treatment chosen represents the high end of the recommended dose of natural valerian drug for treatment of sleep disturbances. While valerian is unlikely to be effective in the treatment of patients with acute, reactive sleep disturbances because of its slight and delayed influence, it can be recommended for patients with mild psychophysiological insomnia.	72
Sedonium, 600 mg/day or 10 mg/day oxazepam for 6 wk	202	After 6 wk, sleep quality, as judged by The Sleep Questionnaire B (SF-B; CIPS 1996) was markedly increased and the valerian treatment at least as efficacious as the oxazepam treatment, compared with baseline ($P < 0.01$). The other SF-B subscales, i.e., feeling of refreshment after sleep (GES), psychic stability in the evening (PSYA), psychic exhaustion in the evening (PSYE), psychosomatic symptoms in the sleep phase (PSS), dream recall (TRME), and duration of sleep, confirmed similar effects of the two treatments. Clinical Global Impressions Scale and Global Assessment of Efficacy, by investigator and patient, also showed similar effects for both treatments. Most patients rated their respective treatments as "very good" (82.8% in the valerian group, 73.4% in the oxazepam group).		73

being administered 1 hour before bedtime. No effects on sleep structure and subjective sleep assessment were observed after a single dose of valerian. After the multidose treatment, however, as in the second trial, significant differences were noted in SWS and SWS latency between verum and placebo groups. Interestingly, polysomnographic comparisons with baseline values indicated significant increases in sleep efficiency for *both* placebo and multiple-dose treatment groups. A fourth trial with Sedonium (LI 156) found efficacy with 600 mg/day of the valerian extract comparable with that of 10 mg/day oxazepam in the therapy of nonorganic insomnia according to ICD-10 (F 51.0) (73).

Lindahl and Lindwall (74) conducted a randomized, double-blinded, crossover comparison of two combination preparations. The first, a commercial product (Valeriana Natt) containing valerian extract equivalent to 400 mg of root, standardized to and containing primarily sesquiterpenes, and with only traces of valepotriates, combined with hop and lemon balm extracts. The second contained valerian extract equivalent to only 4 mg of root, combined with "a full dose of *Flores humuli* and *Lemon melissa*." Any observed differences would be attributed to the difference in valerian content. Eighty-nine percent of the 27 subjects reported improved sleep and 44% "perfect sleep" from Valeriana Natt. No side effects were reported,

nor nightmares, which had been experienced with customary sedatives.

A group at the Department of Family and Child Nursing of the University of Washington, Seattle, WA, conducted, with the support of the NIH, a systematic review of published studies to examine evidence on the efficacy of valerian as a sleep aid "with specific attention to the type of preparations tested and the characteristics of the subjects studied" (75). Of 592 publications initially identified, 36 describing 37 separate studies met the criteria for review: 29 controlled trials were evaluated for efficacy and safety, and 8 open-label trials for safety only. The authors of this review claim that most of the studies examined found no significant differences between valerian and placebo either in healthy individuals or in persons with general sleep disturbance or insomnia and "none of the more recent studies, which were also the most methodologically rigorous, found significant effects of valerian on sleep." A later study by the same group tested the effects of nightly administration of a "concentrated" extract (standardized by HPLC to contain 0.8% valerenic acid per 100 mg extract) of *V. officinalis* root on 16 older women (mean age 69.4 ± 8.1 years) with insomnia (76); no statistically significant differences were detected between valerian and placebo, after a single dose or 2 weeks of nightly dosing, on any measure of sleep latency, wake after sleep onset, sleep efficiency, and self-rated sleep quality.

Anxiety, Stress-Related, and Other Disorders, Restless Legs Syndrome

A recent review assessed the potential of clinical use of valerian for various anxiety disorders (77). While numerous case reports, open-label, and placebo-controlled trials have yielded some encouraging results, these findings are yet to be supported by further investigation in well-designed, large-scale, controlled studies.

In an open-label trial, 500 mg of an extract of *V. wallichii* was administered twice daily PO postprandial to 33 subjects (20 men, 13 women, of average age 34.2 years) (78). Patients were thoroughly investigated clinically and further evaluated using standard questionnaires based on different psychological rating scales, at baseline (day 0), midterm (day 30), and final (day 60). The treatment not only significantly ($P < 0.001$) attenuated stress and anxiety but also significantly ($P < 0.001$) improved depression and also enhanced willingness to adjustment. These results augur well for the potential of this valeriana species as a treatment for stress disorders and perhaps also as an antistress agent.

Ethanollic and aqueous extracts of *V. officinalis* root were investigated in anesthetized guinea pigs for anticoronaryspastic and antibronchospastic activities (79). The protective effects of orally administered doses of 50, 100, and 200 mg/kg were evaluated against pitressin-induced coronary spasm and pressor response, and compared with those of nifedipine; protective effects against histamine-induced and Oleaceae antigen challenge-induced bronchospasm were also evaluated. The two valerian extracts, analytically characterized by qualitative and quantitative chromatography, were shown to possess significant anticoronaryspastic, antihypertensive, and antibronchospastic activities, similar to nifedipine, supporting the design of studies to assess the clinical activity in human trials.

A prospective, triple-blinded, randomized, placebo-controlled, parallel study compared the efficacy of valerian with placebo on sleep quality and symptom severity in patients with restless legs syndrome (RLS) (80). Data were collected at baseline and 8 weeks on sleep disturbances (Pittsburgh Sleep Quality Index and Epworth Sleepiness Scale) and severity of RLS symptoms (International RLS Symptom Severity Scale) from 37 participants aged 36 to 65 years. Both treatment and placebo groups reported improvement in RLS symptom severity and sleep. However, significant differences were found in sleepiness ($P = 0.01$) and RLS symptoms ($P = 0.02$), when sleepy and nonsleepy participants were compared: a strong association between changes in sleepiness and RLS symptom severity was found ($P = 0.006$). It was concluded that use of 800 mg daily for 8 weeks improved symptoms of RLS and decreased daytime sleepiness in patients scoring 10 or greater on the Epworth Sleepiness Scale.

Safety

In all the clinical trials of valerian preparations conducted so far, only mild side effects such as stomach upset, headaches, and itching have occasionally been noted (81). No subchronic or chronic toxicity data are available. A recent case report, involving consumption of more than 20 times the recommended dose of powdered valerian root by an 18-year-old female college student, in an apparent suicide attempt, revealed only mild symptoms, all of which resolved within 24 hours (82). The symptoms noted were fatigue, abdominal cramping, chest tightness, lightheadedness, and foot and hand tremor. A study aimed at assessing possible delayed adverse effects of valerian overdose treated 10 men and 14 women after allegedly taking an overdose of a purported valerian-containing OTC product, Sleep-Qik, a valerian dry extract combined with hyoscine hydrobromide and cyproheptadine hydrochloride (83). No clinical evidence of acute hepatotoxicity or subclinical liver damage was detected, and delayed liver and other adverse effects were judged unlikely. Concern for valerian hepatotoxicity has been misdirected and misguidedly associated in its indictment, along with skullcap (*Scutellaria lateriflora*), in a case of hepatotoxicity (84), almost certainly due to germander (*Teucrium chamaedrys*) substituted for skullcap. The case of a high-output cardiac failure, in a 58-year-old man who had been consuming a plethora of medications including protracted high dosage valerian, following withdrawal of valerian was suspected to be a "benzodiazepine-like withdrawal syndrome" (85). However, while a causal association could not be established, similarities of mechanism of action between valerian and benzodiazepines strike a cautionary note.

Studies of valerian's influence on vigilance have revealed no effects that would impair coordination or decrease alertness (86). A randomized, controlled, double-blind trial with 102 male and female volunteers was conducted to determine whether reaction time, alertness, and concentration would be impaired by treatment with a proprietary valerian extract LI 156 (Sedonium) (87). The effect was examined first after a single evening dose of 600 mg of LI 156 versus 1 mg flunitrazepam and placebo (Section A), and then after 2 weeks of evening administration of LI 156 versus placebo (Section B). Ninety-nine subjects were analyzed in Section A and 91 subjects in Section B. The

primary criterion was the median of reaction time (MRT), measured with the Vienna Determination Test (VDT); secondary criteria were cognitrones (alertness test), tracking test (two-handed coordination), sleep quality (VIS-A, VIS-M), further VDT parameters, and safety criteria. No impairment of reaction abilities, concentration, or coordination was observed for Section A, and equivalence of LI 156 and placebo was proven by confirmative analysis concerning improvement of MRT ($P = 0.4481$) for Section B; evaluation of the secondary criteria was consistent with the results of the primary criterion, demonstrating no relevant negative impact on reaction time, alertness, and concentration, the morning following intake. However, while alcohol does not potentiate the effects of valerian (88), as do many synthetic tranquilizers, because of additive effect caution should be exercised with joint consumption during driving and operating heavy machinery.

Contraindications and Drug Interactions

European clinical monographs list no contraindications to its use in pregnancy or during lactation. Also, studies with pregnant rats indicate no deleterious effects of oral consumption of valepotriates (89). A study has been performed in which the fetuses of rats, orally dosed with a valerian extract in 45% ethanol, on either gestation days 1–8 or 8–15, were weighed and examined for external malformations (90). No signs of maternal toxicity were evident, indicating that consumption of 65 times the usual relative human dose of valerian had no adverse effect on fertility or fetal development. Nonetheless, erring on the side of caution, ingestion of appreciable quantities of valepotriates is generally disavowed, recommending either removal by extraction or sufficient length of storage to promote degradation of the iridoids (see earlier).

While no drug interactions have been reported in humans, studies in rodents indicate a potential for reaction with barbiturates and benzodiazepines (91). In mice, sleeping time induced by thiopental was extended and thiopental anesthesia prolonged, after treatment with both aqueous and alcoholic extracts of valerian, perhaps contraindicating valerian while undergoing treatment with barbiturates. On the other hand, the demonstrated affinity of valerian extracts and valepotriates for GABA and benzodiazepine receptor sites, as well as the diminution of diazepam withdrawal effects observed in rats treated intraperitoneally with valepotriates, suggests that valepotriate-rich preparations may be helpful in easing withdrawal from benzodiazepines.

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Vitamin A

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ABBREVIATIONS

AI, adequate intake; CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; DRI, dietary reference intake(s); EAR, estimated average requirement; IOM, Institute of Medicine; RA, retinoic acid, RAR, retinoic acid receptor; RBP, retinol-binding protein; RDA, Recommended Dietary Allowances; RE, retinyl ester(s); RXR, retinoid X receptor; UL, tolerable upper intake level.

INTRODUCTION

Vitamin A is a generic term for a family of small hydrophobic compounds with specific nutritional properties. Vitamin A molecules are essential for life and specifically for vision, the maintenance of epithelial tissues, immune function, and reproduction. All-*trans*-retinol (vitamin A alcohol) is considered the parent molecule of this family because all of the biochemically active forms of vitamin A can be generated from it through various metabolic processes. One of the oxidized forms of vitamin A, 11-*cis*-retinaldehyde, is an essential component of rhodopsin, the “visual pigment” of the retina that is specifically required for vision (1). A more oxidized form of vitamin A, retinoic acid (RA), is present at much lower concentrations in a broad range of tissues. RA functions in the maintenance of epithelial tissues (skin, immune system organs, gastrointestinal tract, reproductive organs, lungs, and others) and is necessary for well-regulated cell proliferation. RA has a major role in controlling the growth, cellular composition, and integrity of nearly all tissues. Biochemically, RA acts as a ligand for receptors of the retinoic acid receptor (RAR) family, which combine as dimers with receptors of the retinoid X receptor (RXR) family and together function as a transcription factor complex. The RAR-RXR pair, once activated by binding of ligand, generally cause activation, but sometimes cause repression, of the expression of numerous genes (2,3). Because of the many essential functions regulated by the metabolites of vitamin A, an adequate supply of the dietary precursor is essential throughout the life span, from early in embryonic development to old age (4). As will be discussed later in the physiology section, the liver and, to a lesser extent, other tissues can store vitamin A. Thus, the dietary requirement is not a day-to-day requirement but rather a requirement for enough vitamin A over time to allow for sufficient storage so that adequate retinol is continually available to tissues, regardless of immediate dietary intake. Once stores of vitamin A have accumulated, they can be drawn upon

to provide retinol for metabolic processes for a long period, which may be weeks, months, or longer. However, a regular, sufficient intake of vitamin A, neither lower than requirements nor far above, is still the optimal goal for good nutrition, as discussed under food sources, indications, and use section.

Principal Groups: Retinoids and Carotenoids

Humans ingest vitamin A in two forms: preformed vitamin A [retinol and its esterified form, retinyl ester (RE)] and provitamin A carotenoids. RE is the predominant form in foods of animal origin such as milk, cheese, butter, and meat and especially in liver and other organ meats. Retinol is most often esterified with a long-chain fatty acid, either palmitate, or stearate, or oleate, in tissues. Tissue RE is stored in a highly concentrated form in lipid droplets within several types of cells: retinal pigment epithelium (RPE) in the retina; stellate cells in liver and at lower levels in vitamin A-storing cells in the lungs, testes, and other organs. A synthetic RE, retinyl acetate, is sometimes used in food fortification and in the production of vitamin-mineral supplements. RE tends to be quite stable, with less sensitivity to oxygen than unesterified retinol, and therefore using RE in the supplement manufacturing process helps to increase the shelf life of the product. The second form of vitamin A in the diet is often referred to as provitamin A, which, biochemically, consists of several specific types of carotene. β -carotene is the major form in most diets. Carotenes are synthesized exclusively by plants, a few microorganisms and fungi. β -Carotene is added to some foods, such as margarine, as a source of vitamin A and as a yellow coloring agent, and is present in some nutritional supplements. Besides β -carotene, a few other dietary carotenoids have provitamin A activity, including α -carotene and β -cryptoxanthin. Food sources are discussed later in this chapter. The efficiency with which various carotenes are used as a source of vitamin A varies widely, because of a combination of structural differences inherent in the molecules themselves, differences in the foods in which the carotene is present, and differences in the ability of humans to digest and absorb β -carotene. Considerable variation in bioavailability may also be due to food preparation procedures. Once either preformed vitamin A or carotene is ingested and absorbed, both of them are transformed through various enzymatic processes within cells to form a number of metabolites, of which 11-*cis*-retinaldehyde and all-*trans*-RA are the major active forms. Therefore, either nutritional source of vitamin A, preformed vitamin A or provitamin A, is capable of

supporting all of the biochemical functions of the vitamin. Thus, humans and animals can consume a variety of diets ranging from strict vegetarian to carnivorous, and still consume an adequate amount of vitamin A. In the average U.S. diet, about one-third of total vitamin A is obtained from carotenoids and about two-thirds from retinol. However, in developing countries where milk, meat, and other animal products are costly and often scarce, the proportions are nearly reversed, with carotenoids as the major source of vitamin A for most people. In addition, the total amount of vitamin A consumed is generally lower in developing countries than in the United States and other industrialized countries with a high standard of living.

NAMES AND GENERAL DESCRIPTION

Retinol is a fat-soluble compound with the empirical formula $C_{20}H_{30}O$ and molecular weight 286.44. The retinol molecule contains a methyl-substituted cyclohexenyl ring (known as β -ionone), a side chain that has four conjugated double bonds and two methyl group substituents, and a terminal hydroxyl group (Fig. 1). The hydroxyl group is modified through oxidative metabolism to yield retinal and then, in an irreversible reaction, RA. When retinol is esterified with a long-chain fatty acid, RE is produced. RE is the major form in most tissues. Vitamin A₂ (3,4-didehydroretinol) is a minor variant form of retinol that is present in some fresh-water fish. The nutritional activity of vitamin A₂ is about half that of retinol (or vitamin A₁), although both are thought to be similar qualitatively (5). 3,4-Didehydroretinol is also present in the skin, but its function, if different from retinol itself, is not known.

The most important metabolites of retinol are 11-*cis*-retinaldehyde and all-*trans*-RA (Fig. 1). These are formed

metabolically through the sequential oxidation by various enzymes present in a number of tissues. Besides the forms mentioned so far, several dozen other retinoid compounds have been detected in tissues, usually at quite low concentrations, or transiently, and they may represent catabolic products. These forms include *cis-trans* isomers, ring oxidized derivatives, and conjugated forms. It is debatable whether these molecules possess any biological activity, or whether they are simply end products on their way to elimination. One of the quantitatively minor forms, retinoyl- β -glucuronide, is unusual in being soluble in aqueous solution. Retinoyl- β -glucuronide is formed through the conjugation of RA with glucuronic acid. It is present in plasma after a high dose of RA and is a normal excretion product of vitamin A found in the bile. When it has been synthesized and tested for activity, it has shown some of the biological activity of RA, which may be due to liberation of RA by slow hydrolysis. Because retinoyl- β -glucuronide is water soluble, its volume of distribution is likely to be quite different from the lipid-soluble retinoids.

In addition to the natural forms of vitamin A illustrated in Figure 1, a large number of analogues have been synthesized. The term retinoids is now used to refer to both the natural and synthetic forms. Synthetic retinoids have been developed based on the idea that they may be useful mimetics of natural retinoids, particularly with respect to inducing cell differentiation. In research conducted for over 30 years, several promising compounds have been discovered. Yet, interestingly, it has been the natural forms, although produced synthetically, that have become the most useful as drugs. Synthetic all-*trans*-RA (chemically identical to the natural form) and synthetic 13-*cis*-RA, identical to that found in plasma, are approved drugs for the treatment of acne and other dermatological conditions, and all-*trans*-RA is approved for the treatment of acute promyelocytic leukemia (APL), a particular form of cancer that has proved very responsive to RA treatment (6). Another isomer of RA, 9-*cis*-RA, is of uncertain physiological function in vivo, but still a potent ligand of the RXR in vitro and an agent that has shown promise in some clinical trials. Several synthetic retinoids with ring-modified or side chain-modified structure, which do not exist in nature, are also used therapeutically or are under investigation as drugs.

β -Carotene is a fat-soluble hydrocarbon with the empirical formula $C_{40}H_{56}$, molecular mass 536 g/mol. β -Carotene is a symmetrical molecule with two β -ionone rings (each identical to those in retinol) connected by a methyl-substituted polyene chain (Fig. 1B). During intestinal absorption, the β -carotene molecule is cleaved either at the central 15,15' bond (see Fig. 1), theoretically yielding two identical molecules of retinal, or it may be cleaved off-center in a process referred to as excentric cleavage, which can potentially lead to the generation of one molecule of retinal after further metabolism. The efficiency of central cleavage in vivo is not as efficient as the theoretical expectation of two molecules of retinal per β -carotene. On average, about 1–1.2 molecules are formed per β -carotene, as determined in human and animal bioassays. The efficiency of using β -carotene to produce vitamin A also depends on the physical form

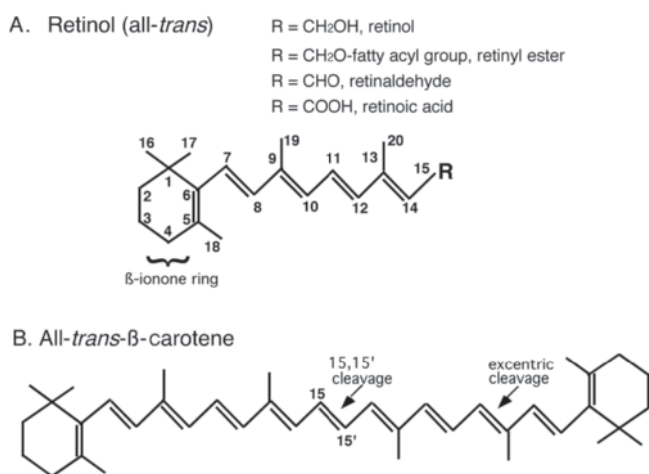


Figure 1 (A) Retinol. R indicates the various substituents at carbon 15 giving rise to retinol, retinyl esters, retinal, and retinoic acid. Some of these molecules also exist naturally in several isomeric forms such as 9-*cis*, 11-*cis*, and 13-*cis*-isomers. (B) β -Carotene. The position of central (symmetrical) cleavage at the 15,15' double bond is shown, as is that of excentric cleavage.

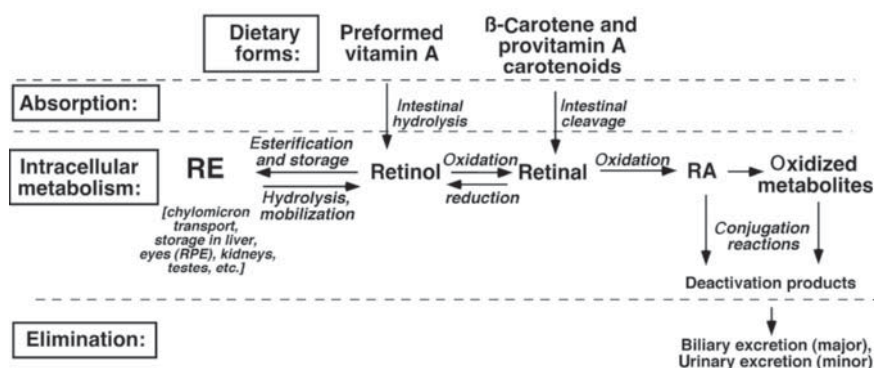


Figure 2 Schematic summary of the major forms of vitamin A in the diet, their conversion to active metabolites by cellular metabolism, and their elimination from the body. *Abbreviations:* RE, retinyl ester; RA, retinoic acid.

in which the carotene is present. In supplements or oily solutions, the carotene is “free” and does not require extensive digestion. In contrast, β -carotene in plants is most often a component of the “food matrices,” which consists of plant cell membranes, fiber, lignans, etc. These components are difficult for humans to digest, and thus not all of the β -carotene is liberated and made available for uptake into the intestine (see intestinal vitamin A absorption section). β -Carotene in fruits, which tend to be softer than vegetables, is more bioavailable than β -carotene in more fibrous plants such as green leafy vegetables. The other provitamin A carotenoids in the diet, α -carotene and β -cryptoxanthin, are found in carrots and in some other foods, and in oranges and orange juice, mangoes and other fruits, respectively. These molecules differ from β -carotene in that each has one β -ionone ring that is identical to that in β -carotene, but the other ring is modified, and that “half” of the carotene molecule cannot be converted to retinol. Thus, the biological activity of these carotenoids is about half that of β -carotene. Yet other carotenoids, such as lutein and zeaxanthin, have no vitamin A activity, although they are relatively abundant in foods and are absorbed, to some extent, in humans. But their structure precludes their conversion to vitamin A. Lycopene and phytofluene, which are precursors of β -carotene in plants and thus present in some of the same foods that are good sources of β -carotene, have no vitamin A activity. Lycopene, a carotenoid precursor in tomatoes, has been promoted as an antioxidant, although its efficiency as such *in vivo* has not been well demonstrated. In summary, a small number of the many carotenoids present in the diet provide vitamin A activity, but their nutritional activity is less on a molecule-for-molecule basis than that of either retinol or RE.

BIOCHEMISTRY AND FUNCTION

Metabolic Activation

RE, retinol, and β -carotene are precursors of functionally active vitamin A, but none possess bioactivity on their own. Each compound must be metabolized to become an

active form, retinal and RA. The principal pathway of vitamin A metabolism is illustrated in Figure 2. It should be noted that a number of additional side reactions also occur, which can in total generate up to a few dozen minor metabolites including various isomers and conjugated forms. The production of bioactive forms of vitamin A involves, first, the conversion of RE and β -carotene to retinol, and then the sequential oxidation of retinol to retinal and then to RA. Several reactions in retinoid metabolism are physiologically reversible, including the esterification of retinol to form RE and the hydrolysis of RE to yield retinol, although different enzymes catalyze the reactions in each direction. Several oxidation–reduction reactions also reversibly link retinol and retinal. Other reactions are irreversible: the cleavage of β -carotene, the formation of RA, and the oxidation of retinol and RA to more polar metabolites such as 4-hydroxy and 4-oxo-retinol and -RA. The physiological control of biosynthesis and catabolism is important for regulating the cellular levels of retinoids, especially for controlling the level of the receptor ligand RA. For several of the reactions shown in Figure 2, more than one enzyme or enzyme family has been implicated. Indeed, redundancy within the system of vitamin A metabolizing enzymes, binding proteins, and receptors is now well established.

Hormonal Functions

The hormone-like functions of vitamin A are mediated essentially entirely by RA. RA acts both as a classical endocrine hormone that is transported from sites of synthesis through the blood to target organs and as a paracrine or autocrine hormone that affects neighboring cells or acts in a cell-autonomous fashion. For tissues to maintain their normal status, they must receive in plasma or produce locally an appropriate amount of RA. In plasma, RA is present at low (10–50 nM) concentration bound to albumin. Because of its hydrophobicity, RA is readily taken up by most cells. No specific membrane transporter has yet been described for the uptake of RA, but a putative mechanism may involve intracellular binding proteins for RA, CRABP-I and -II, that may “draw” RA into cells from plasma. RA is essential for normal embryogenesis and for the

maintenance of well-differentiated epithelial cells. Proper cell differentiation, in turn, affects the overall health of the skin, immune system, reproductive organs, and other organ systems. The production of RA is thus a physiological requirement throughout the life cycle.

RA exists in at least three and possibly more isomeric forms: all-*trans*-RA, the major active metabolite; 9-*cis*-RA, a form whose function is still uncertain; 13-*cis*-RA, a form that appears to be a side product; and several di-*cis*-isomers that have been detected in human plasma. All-*trans*-RA is most potent in terms of cell differentiation assays and its mode of action is well characterized. All-*trans*-RA is a specific ligand for the RAR family of nuclear receptors (described later). While 9-*cis*-RA binds to both RAR and RXR receptors in vitro and has been shown to be able to activate the RXR receptors, there is some doubt about it being present at adequate levels in vivo to be important physiologically. 13-*cis*-RA remains an enigma. It is present in human plasma at concentrations near to or higher than that of all-*trans*-RA, it possesses some of the same bioactivity in some assays, and it is useful clinically, but it has not been shown to be a significant ligand for the nuclear retinoid receptors, and thus its mode of action is unknown. It is possible that it functions as a "prodrug" that can undergo slow isomerization to all-*trans*-RA such that the apparent effects of 13-*cis*-RA are actually mediated by all-*trans*-RA.

Nuclear Receptors

The RAR and RXR are members of the steroid/thyroid hormone receptor superfamily. The RAR and RXR subfamilies are similar in that both have three members, alpha, beta, and gamma, encoded each by a separate gene. The RAR and RXR proteins are made up of multiple domains, the major ones of which are a DNA-binding domain that interacts with specific DNA sequences, termed RA response elements (RARE) that are located in the regulatory regions of retinoid-responsive genes, and a ligand-binding domain that is shaped into an internal pocket where the all-*trans*-RA (for the RARs) or 9-*cis*-RA (mainly for the RXRs) binds (2,3).

Although the proteins share overall structural similarities, their DNA-binding domains and ligand-binding pockets are substantially different. The RAR and RXR genes and their protein products are expressed in tissue-specific patterns. RAR α and RXR α are most widely expressed, while RAR γ is more prominently expressed in certain tissues such as skin. Research is just beginning to reveal specific functions for the individual RARs, suggesting that while the three forms appear to be very similar and to function in a redundant manner, they play different preferred roles. Nearly all types of cells express at least one form of RAR and RXR.

Ligand binding serves as an activating signal for most RA-responsive genes. It is currently thought that most RAR-RXR protein complexes are bound to DNA in an inactive, repressed state in the absence of ligand, and that the binding of ligand triggers a conformational change in the receptor protein that, in turn, strengthens and/or modifies the RAR-RXR interaction and the binding of the complex to DNA. These changes then alter the interactions of the receptor proteins with coactivator or

corepressor molecules that play a major role in turning on the gene in question. Some genes are repressed by RA. Other processes such as active antagonism, where they oppose the binding of other transcription factors, such as activator protein-1, and transcriptional repression have also been described. It is still not known precisely how transactivation and repression work, although specific sequences in the gene's promoter region and/or the recruitment of other transcription factors and general coactivator or corepressor proteins to the RAR-RXR complex are involved (2,3,7,8). The mechanisms may vary slightly but significantly gene by gene, as each gene contains a unique set of regulatory sequences in its DNA.

Numerous synthetic retinoid analogues possess either agonistic or antagonistic actions on gene expression. Compounds that selectively bind to RXRs are now known as "rexinoids." These compounds can potentially affect multiple hormonal pathways because the RXR also forms a heterodimeric complex with other nuclear receptors including the vitamin D receptor, thyroid hormone receptor, peroxisome proliferator activator receptors, lipid-activated receptors (LXR, FXR), and xenobiotic-activated receptors (PXR, CAR). Retinoid signaling thus has both a retinoid-specific component mediated by RAR-RXR actions and a very broad influence on other signaling components due to the actions of the RXRs with other nuclear hormone receptors. Cell differentiation is often closely controlled by retinoid signaling through the RAR-RXR dimer. A large number of genes contain one or more RAREs. Retinoid-responsive genes are a part of nearly every biological pathway and are truly diverse in the functions they perform—some code for structural proteins, others for enzymes, receptors, growth factors and cytokines, and regulatory factors (2,3,7).

Development

During embryonic development, retinoid signaling is first observed soon after gastrulation. Appropriate timing, duration, and strength of RA signaling are critical for the establishment of the proper organization of the early embryo and the development of its anterior-posterior and dorsoventral axes. Retinoids are also critical during the period of organogenesis, for normal formation of the heart, lungs, renal system, gonads, sensory organs, limbs, and other structures. Several genes of the Hox family that are involved in establishing the body pattern are regulated directly or indirectly by RA. Retinoid signaling also interacts with the signaling by the fibroblast growth factor family. Both positive and negative signaling is required for proper development. Because of the role of RA in these processes, the development of many embryonic tissues is affected by either a nutritional deficiency of vitamin A or an excess of vitamin A, RA, or other acidic retinoids during critical periods of gestation (4). The types of birth defects seen in the offspring of women who have unintentionally consumed an excess of vitamin A or RA during early gestation are similar to those produced experimentally in retinoid-treated animals (9).

Studies of mouse and avian embryos during the early stages of development have shown that cellular retinoid-binding proteins, nuclear retinoid receptors, and certain enzymes involved in RA production (RALDH) and

catabolism (CYP26) are already present in the embryo. Indeed, CYP26A1 expression appears to precede that of RALDH2, suggesting that the embryo first must degrade or control the levels of RA, which are presumably derived from the mother at this stage, to prevent premature retinoid signaling, while shortly thereafter the embryo begins to express RALDH2 and produce RA, again in a very localized manner (10). This counterbalance of RA production and RA degradation in adjacent but nonidentical regions of the embryo is believed to be critical for establishing the boundaries that will define embryonic tissues and lead to organ development. Overall, RA levels must be closely regulated, and the embryo's own tissues appear to be involved in this process from very early stages in development.

It is, however, possible for these autonomous mechanisms to become overwhelmed by either too little or too much retinoid. Hypervitaminosis A and the direct administration of RA in animals produce similar birth defects, characterized by craniofacial malformations. Similar defects have occurred in the offspring of women who have become pregnant while using retinoids therapeutically. For this reason, strict regulations have been put into place to guard against retinoid use in women of childbearing age (11).

Differentiation

Natural retinoids, particularly all-*trans*-RA, and several of its synthetic analogues are potent agents of cell differentiation. They also often inhibit the proliferation of transformed cells and tumor cells. A blockage in cell cycle progression, reduced rate of proliferation, and a shift in the expression of genes and the appearance of cells are all characteristic of retinoid-treated cells, typically resulting in a more mature, differentiated cell phenotype. In certain types of cells, retinoids may also induce programmed cell death (apoptosis), and certain retinoids, such as CD437, a ligand for RAR γ , are known for promoting cell apoptosis (7). Because of the ability of retinoids to control the proliferation of certain cell types in culture and in animal models, a large number of clinical trials of retinoids in several cancers and other diseases have been conducted. A remarkable success has been achieved in the treatment of APL with all-*trans*-RA, and this agent together with

other therapies is now the standard of care for this disease (6). Retinoids are also very effective in diseases of the skin and are used extensively in the treatment of severe cystic acne and psoriasis. A number of novel synthetic retinoids have been synthesized in an effort to achieve greater efficacy and reduce the toxicity inherent to natural retinoids. All organ systems depend on retinoids for their integrity to some extent, but retinoids are particularly active in the maintenance of epithelial tissues including the skin, respiratory tract, immune system, and reproductive system and in the hematopoietic system including acting in the bone marrow, on blood cells, and on tissue-resident lymphocytes and macrophages. These types of tissues are particularly sensitive to a lack or excess of retinoids and thus are usually the first to exhibit abnormalities during the onset of vitamin A deficiency or in the situation of hypervitaminosis A (12).

Functions in the Eye

Vitamin A plays two distinct roles in the eye. In the retina (the inner lining of the eye), 11-*cis*-retinaldehyde is a critical component of the protein known as rhodopsin that absorbs light. When a photon of light strikes the 11-*cis*-retinal bound to rhodopsin, it induces the isomerization of the 11-*cis*-bond, forming all-*trans*-retinal. This first step, phototransduction (see later), leads to signaling to the visual cortex of the brain. In the front of the eye, the membranes that comprise the cornea and conjunctival overlying the iris and lens require vitamin A in the form of RA for their proper differentiation and maintenance. Mucous-secreting goblet cells in the conjunctiva membrane, which are responsible for lubricating the outer eye, are especially vulnerable to a deficiency in vitamin A. Dryness and inflammation of the conjunctiva and cornea are early signs of vitamin A deficiency, and both represent a state of inadequate RA levels, leading to a loss of the normal capacity for normal cell differentiation (see cornea and conjunctiva section later).

Retina

The RPE is a continuous single layer of epithelial cells that lines the retina. It is located between the blood supply, from which it obtains nutrients, and the photoreceptor cells, specifically the outer segment portion of the rod and cone cells responsible for phototransduction (Fig. 3).

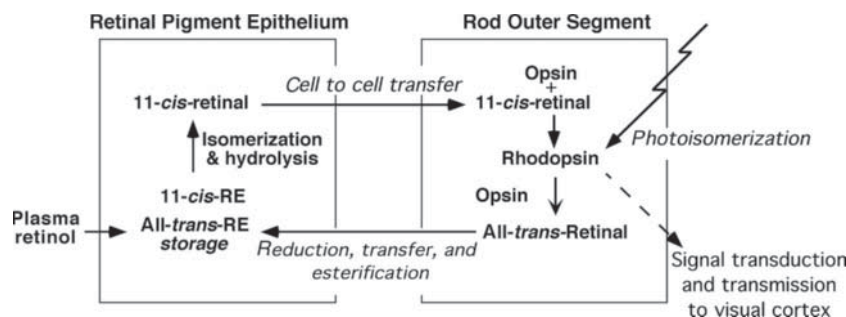


Figure 3 Processes and reactions of the visual cycle. The interplay of the retinal pigment epithelium (RPE) cell layer, where retinoids are regenerated in the visual cycle, and the adjacent photoreceptor cells (rods and cones), where light is absorbed and phototransduction occurs, is crucial to maintaining vision. Night blindness results when the visual cycle is impaired due to lack of storage of retinyl esters in the RPE cells.

The RPE plays multiple roles with respect to vitamin A metabolism including the uptake of retinol bound to retinol-binding protein (RBP) from the blood, the storage of vitamin A in the form of RE, and the conversion of retinol to 11-*cis*-retinal, which then is combined with rhodopsin in the outer segments of the rods, or with a similar opsin-like protein in each of the red, green, and blue-specific cone cells. These proteins when combined with 11-*cis*-retinal are the visual pigments that are necessary for light absorption. The RPE cells also play an essential role in maintaining the health of the rods and cones, which continuously produce new outer segment membrane and continuously shed old membrane from their tips. This shed membrane is taken up by RPE cells by phagocytosis and then degraded. The housekeeping function of the RPE helps to recycle retinal, which is reused for new rhodopsin synthesis after a series of regenerative reactions, and it prevents a detrimental build up of light-damaged rod and cone membranes. Rod cells, which are more numerous than cones in the human eye, are located throughout the retina and in its periphery and are ideally suited for the detection of motion and for vision in dim light. Cone cells (tuned to absorb light in the red, green, and blue regions of the visible spectrum) are less numerous but concentrated mostly in the fovea where vision is most acute. These cells function at higher levels of illumination and are essential for color vision (1).

For synthesis of rhodopsin and color visual pigments, molecules of 11-*cis*-retinaldehyde, formed from retinol in the RPE, must be transported to the rod and cone outer segments where they are covalently linked through a Schiff base to a specific lysine residue in the opsin protein specific for the cell type. The attached retinal molecule is conformationally restrained within a binding pocket. The process of vision is initiated when light strikes the 11-*cis*-retinal moiety of rhodopsin, catalyzing its photoisomerization (referred to as bleaching). This change in the molecular conformation of retinal triggers a neuronal signaling pathway to the visual cortex. Several rapid changes then quench the signaling, and thereafter the process of regenerating rhodopsin begins. For vision to continue, the all-*trans*-retinal molecules that were produced by photoisomerization must be converted back to 11-*cis*-retinal. This process occurs in the RPE, not in the photoreceptor cells themselves. A series of reactions known as the visual cycle is involved in the transport of all-*trans*-retinal from the photoreceptor cells to the RPE, where the molecules undergo enzymatic processing to restore the 11-*cis*-bond, followed by transfer back to the photoreceptor cells for combination again with opsin proteins. However, this process is relatively slow as compared with the light-induced phototransduction process. In the meantime, retinol that has been stored in the RPE as RE is hydrolyzed, isomerized, and oxidized to form new molecules of 11-*cis*-retinal, which then are exported to the rod cells to form the rhodopsin molecules necessary for continuous vision. Overall, the visual cycle has been likened to a conveyor belt (1). When the level of vitamin A stored as RE in the RPE becomes low (e.g., during the onset of vitamin A deficiency), the production of new rhodopsin molecules after exposure to bright light is slowed down, because the process of reforming rhodopsin from the recently bleached retinal is slower than the release of retinol from RE stores

and the formation of new 11-*cis*-retinal. A slowing down of the visual cycle occurs during vitamin A deficiency, often when the condition is still marginal. The resulting impairment in dark adaptation is known clinically as night blindness.

Cornea and Conjunctiva

Progressive changes in the membranes of the conjunctiva and cornea are referred to clinically as xerophthalmia. Xerophthalmia, including dryness (xerosis) of these membranes, was one of the first manifestations of vitamin A deficiency to be recognized and was known clinically before vitamin A was discovered. The cornea is an avascular tissue. Holo-RBP is present in tear secretions, and it seems that the retinol delivered by RBP must be oxidized locally to produce RA, which, in turn, results in the differentiation and maintenance of the corneal epithelium. However, details are not known. The mucous-secreting goblet cells of the conjunctiva become dysfunctional in vitamin A deficiency, which contributes to xerosis and the formation of Bitôt's spots, small foamy patches that are usually located on the outer quadrants of the eye, and which contain dead cells and bacteria. As vitamin A deficiency further progresses, the cornea may become soft, a condition known as keratomalacia. Remarkably, even at this late stage of vitamin A deficiency, this deterioration of the cornea can be halted and the person's vision rescued if a high dose of vitamin A is administered (13). However, without immediate treatment with vitamin A, the lens will ulcerate and this will cause irreversible blindness.

PHYSIOLOGY

Transport and Binding Proteins

Retinol-Binding Protein

RBP is encoded by a single gene on human chromosome 10q23-24; this gene is now referred to as RBP4 to distinguish it from intracellular RBPs numbered I, II, and III (1, 2, and 3 in gene nomenclature). The RBP is relatively small for a plasma protein, approximately 21 kDa, and it has a short half-life of less than a day in plasma. Thus, a continued synthesis of RBP is essential for normal transport of retinol. The RBP gene's open reading frame codes for a 199 amino acid pro-protein from which a 16 amino acid signal peptide is removed cotranslationally, forming the mature 183 amino acid RBP (14). The mRNA for RBP is one of the more abundant mRNAs present in liver, in hepatocytes, and the rate of RBP synthesis is very high even though the plasma level of RBP is relatively low. It has been thought that liver stellate cells might also synthesize RBP; however, it may be that this represented contamination of hepatic stellate cells with hepatocytes in which RBP is abundant. RBP mRNA is also present at lower levels (5–10% of liver levels) in the kidney and several other organs (14) including adipose tissue. The presence of mRNA strongly implies that RBP is also synthesized in these tissues. However, little is known of the synthesis, secretion, and even function of RBP4 produced outside of the liver. The extrahepatic synthesis of RBP4 is relevant because plasma retinol is known to recycle several times between plasma, liver, and extrahepatic tissues before undergoing irreversible degradation (15). If liver-synthesized RBP is

unlikely to play a role in the reverse transport of retinol from peripheral tissues, then a source of RBP for extrahepatic tissues is needed. The kinetic data as well as mRNA distribution data support a role of extrahepatic tissues in whole-body retinol and RBP metabolism.

The RBP contains a single high-affinity binding site for a single molecule of retinol. When newly synthesized apo-RBP binds retinol, holo-RBP is formed. In plasma, 90–95% of vitamin A is in the form of holo-RBP. The binding of retinol to RBP is further stabilized by the binding of RBP to another transport protein, transthyretin (TTR, once known as prealbumin) (16). Several studies have shown that the larger protein complex is subject to a reduced rate of glomerular filtration, and thus holo-RBP–TTR has a longer half-life in plasma than holo-RBP alone or apo-RBP, for which the half-life is very short, approximately 4 hours.

A major recent advance is the discovery of a plasma membrane receptor for RBP. The protein, known as STRA6, was known as a RA-induced gene in embryonic cells, but its function was not known. A biochemical investigation of the binding of RBP to RPE membranes first identified a membrane protein that, when sequenced, matched the previously identified STRA6 (17). This 7-membrane spanning protein, now known as a receptor for RBP, binds RBP–TTR or RBP alone and facilitates the uptake of retinol into the cell. STRA6 protein is abundant on the apical surface of RPE cells where it is proposed to take up retinol from plasma for esterification and further metabolism [see cellular retinoid-binding proteins section and discussion on lecithin:retinol acyltransferase (LRAT)]. It appears possible from cell culture studies that STRA6 also facilitates the export of retinol from cells, but this must be further tested.

A second interesting advance concerning RBP (also known as RBP4) is the concept that it may function as an adipokine, and that an elevated concentration of RBP4 may be related to insulin resistance and impaired glucose tolerance (18,19). Further research is needed to understand this role of RBP and to determine whether the relationship between RBP4 and glucose metabolism is causal or an association, and whether the retinol-binding function of RBP4 is related to the proposed role of RBP4 as an adipokine.

Cellular Retinoid-Binding Proteins

Several different forms of intracellular retinoid-binding proteins have been isolated. A number of them belong to the same gene superfamily as fatty acid-binding proteins and are similar in structure to each other. However, the cellular retinoid-binding proteins differ from one another in preferentially binding either retinol or RA and in their organ and cell-type distribution. Cellular retinoid-binding proteins have at least a dual function: first, they provide aqueous solubility to their lipid-soluble retinoids; second, they act as chaperones to direct their retinoid “cargo” to specific enzymes that catalyze processes such as retinol esterification or oxidation (20). In addition, cellular RA-binding proteins may regulate the distribution of RA between the cytoplasm and the nucleus of cells and thereby be a factor in retinoid-regulated gene transcription. Despite solid evidence that the cellular retinoid-binding proteins are important in retinoid metabolism, studies in mice

lacking one or more of these binding proteins have shown that the mice are viable and lack a serious phenotype, and thus it appears that these proteins are not essential. However, other studies have shown that some of these proteins improve the efficiency of retinoid metabolism. For example, mice lacking the cellular retinol-binding protein, CRBP-I, developed vitamin A deficiency very rapidly when they were fed a low vitamin A diet (21), while their visual cycle was somewhat slowed down as shown by electroretinogram testing (22). These results suggest that the cellular retinoid-binding proteins have evolved to facilitate the conservation of vitamin A and improve the efficiency of metabolism. Another member of the CRBP family, CRBP-II, is an abundant protein in the intestinal mucosal where it interacts with the enzyme LRAT (see later) to facilitate retinol absorption and RE formation, prior to incorporation of RE into chylomicra.

Metabolism

Intestinal Vitamin A Absorption

Preformed vitamin A. Dietary REs must be hydrolyzed in the lumen prior to absorption. Several retinyl ester hydrolases (REHs) have been characterized, and enzymes secreted in pancreatic juice or present on the brush border of enterocytes in the duodenum and jejunum are both implicated in the hydrolysis of dietary RE (Fig. 4). Bile salts and products of lipid digestion are necessary for micelle formation, which in turn is essential for the uptake of retinol into the enterocytes. Therefore, a sufficient amount of bile must be produced and the diet must contain an adequate quantity of fat (generally >5%) for maximal absorption of vitamin A. Any conditions that impair the luminal emulsification of fat, its hydrolysis, or micelle formation are likely to simultaneously reduce the absorption of vitamin A. In the healthy state, the efficiency of retinol absorption is quite high, about 70–90% (23). The absorption of retinol (preformed vitamin A) is not downregulated when the level of intake is elevated. This highly efficient absorption of retinol is likely to be part of the etiology of vitamin A toxicity, as absorption is not limited even when intake is excessive. Once retinol is absorbed into the enterocyte, about 95% of it is reesterified with long-chain fatty acids. CRBP-II binds retinol and delivers it to the enzyme LRAT, which forms new REs. Intestinal LRAT activity is high and remains high even in a state of vitamin A deficiency; thus, a person or animal that is treated with a bolus of vitamin A is able to absorb it right away. The newly formed intestinal REs are incorporated into the lipid core of nascent lipoproteins (chylomicrons) that transport newly absorbed fat. Chylomicrons are secreted into the lymphatic system and then enter the blood stream. They are first metabolized by lipoprotein lipase which releases their triglycerides as fatty acids into adipose and muscle tissues, and then the chylomicron remnant delivers the rest of their lipids including the majority of their RE to the liver, where the greatest proportion of the body's vitamin A is stored.

Carotenoids. Provitamin A carotenoids in fruits and vegetables are much less bioavailable because they are, to a significant extent, bound to the food matrices from which they must be liberated by digestion. Even pure β -carotene in oily solution, which is free from the food matrices, must be incorporated into micelles prior to its

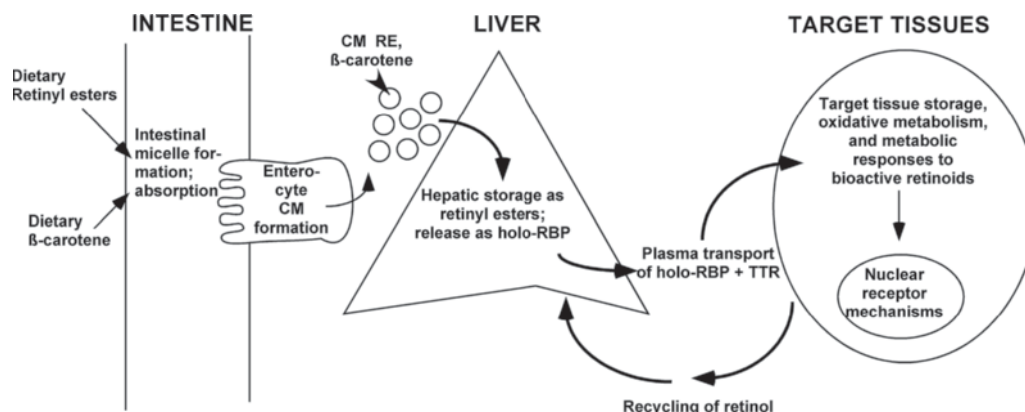


Figure 4 Physiology of interorgan vitamin A transfer. The role of the liver in the uptake of chylomicron (CM) vitamin A (VA) from the intestine, the storage of retinyl ester (RE) in the liver (mainly in stellate cells), and the recycling of retinol from extrahepatic tissues are emphasized. *Abbreviations:* RBP, retinol-binding protein; TTR, transthyretin.

uptake by enterocytes. The efficiency of utilization of β -carotene is substantially lower on a percentage basis and much more variable person to person than for retinol. The percentage of carotene absorbed tends to fall as the mass of carotene present in the lumen rises, so that, to some extent, β -carotene absorption is self-limiting (23). However, with excessive intake, enough is absorbed to accumulate in plasma and fatty tissues.

Of the relatively small fraction of provitamin A carotenoid that is actually absorbed and metabolized in enterocytes, most undergoes cleavage by carotenoid oxygenase enzymes (24). A recent advance occurred with the cloning of a β -carotene monooxygenase, an enzyme capable of cleaving the central 15,15' double bond, and a second cleavage enzyme with apparent actions as an asymmetric carotene cleavage enzyme (25). The products of carotene cleavage, both retinal and β -apocarotenals, must undergo further metabolism. Of the absorbed carotene that is further metabolized, most of it is converted to retinol, then esterified and absorbed into lymph as RE, as has been described for vitamin A. A minor portion of the retinal produced by β -carotene cleavage undergoes oxidation to RA and then is released into the portal vein. While other provitamin A carotenoids, principally α -carotene and β -cryptoxanthin, appear to be metabolized in a manner similar to β -carotene, they yield only half as much vitamin A activity, as described earlier, because these molecules have only one β -ionone ring and can be converted to only one molecule of retinol. Some foods (notably seaweed and certain algae) contain 9-*cis*- β -carotene. However, its fate is less well studied than that of all-*trans*- β -carotene. A portion of the 9-*cis*- β -carotene may undergo isomerization to form all-*trans*-metabolites or it may be used for the formation of 9-*cis*-retinoids (26). Several non-provitamin A carotenoids that are common in the diet, such as lycopene, lutein, and zeaxanthin, can be absorbed and are found in human plasma, but have no provitamin A activity.

A significant proportion ($\sim 1/3$) of the β -carotene absorbed by human enterocytes is incorporated into chylomicrons without undergoing cleavage and thus is absorbed intact. Most laboratory animals convert nearly all

of their absorbed β -carotene to retinol. In a clinical study in healthy older men and women, β -carotene metabolism continued to occur over an extended period of time after the initial phase of absorption, up to 53 days after feeding, which is thought to indicate the postabsorptive cleavage of β -carotene in tissues such as the liver (27).

Storage and Metabolism in Liver and Extrahepatic Tissues

The majority of chylomicron vitamin A is taken up by the liver. Smaller proportions are taken up by adipose and other tissues. Within hours after uptake, these newly assimilated REs are hydrolyzed and, thereafter, new molecules of RE are formed by esterification, mediated by CRBP-I and LRAT in the liver. In vitamin A-adequate conditions, more than 90% of total body vitamin A is present as RE stored in hepatic perisinusoidal stellate cells. β -Carotene is stored in liver and in fat, usually at relatively low concentrations.

As vitamin A is needed, retinol is released from storage by hydrolysis mediated by REHs, as noted earlier. A portion of the released retinol is bound to newly synthesized RBP, as shown in studies with cultured hepatocytes, and then secreted into plasma as holo-RBP, which then combines with TTR and circulates throughout the body. If a person's intake of vitamin A is inadequate, nearly all of the liver's vitamin A stores can be hydrolyzed and mobilized, resulting in a nearly complete depletion of vitamin A in the liver. Once the liver's reserves of vitamin A are exhausted, then plasma and tissue retinoid levels fall rapidly and symptoms of vitamin A deficiency begin to appear. Conversely, upon repletion, vitamin A is rapidly absorbed and taken up by the liver. Once plasma retinol levels have been restored to normal levels, the liver stores any excess of retinol as new RE.

Several enzymes in liver can oxidize retinol to RA. The liver also contains at least one and possibly more cytochrome P450 enzymes that are capable of forming 4-hydroxy and 4-oxo-RA. CYP26A1, a cytochrome P450 that is readily induced by RA, is relatively abundant in liver compared with other tissues but has a fairly wide tissue distribution. Numerous retinoid metabolites, which

Table 1 Plasma and Liver VA Levels Characteristic of Individuals with Deficient, Marginal, Adequate, and High VA Status

Stage	Plasma retinol	Liver stores	Clinical signs	Vulnerable groups/most common situations
Deficient	<0.35 $\mu\text{mol/L}$	Little, if any (<5 $\mu\text{g/g}$)	Night blindness; other ocular manifestations (xerosis); dry keratinized skin	Preschool-age children and pregnant or lactating women with low vitamin A intakes
Marginal	0.35–0.7 $\mu\text{mol/L}$	Low but measurable (5–20 $\mu\text{g/g}$)		
Adequate	1.05–3 $\mu\text{mol/L}$	~20–300 $\mu\text{g/g}$		
High ^a	Upper normal to >3 $\mu\text{mol/L}$. Possible presence of lipoprotein-associated RE in plasma	High (>300 $\mu\text{g/g}$)	Not apparent or very mild	Chronic supplement users; those with frequent intake of foods high in preformed VA (e.g., liver)

^aOvert vitamin A (VA) toxicity is very uncommon. When present, it is most likely due to an intake of foods or supplements with a high level of retinol that have been consumed regularly over a substantial period of time, although cases have been reported of acute toxicity due to an extremely high intake over a short time.

include oxidation products of RA in unconjugated form, as well as conjugates with glucuronic acid, such as retinoyl- β -glucuronide, are present in bile. In general, the oxidation of the retinoid ring at carbon-4 serves as an initial deactivating reaction, that is, a phase I hydroxylation reaction, and glucuronidation as a phase II conjugation reaction, which together result in the formation of water-soluble retinoids. How these processes are regulated by nutritional factors is not well understood. However, in a study in rats, an enzyme, LRAT, that catalyzes retinol esterification, and CYP26 were both elevated in vitamin A-supplemented animals compared with controls and reduced in animals fed a vitamin A marginal diet (28). These observations suggest that homeostatic mechanisms exist to keep the concentrations of retinol and RA within close bounds.

Many extrahepatic tissues including the eyes, kidneys, lungs, and endocrine organs also store vitamin A as REs, usually at levels approximately 5–10% of the concentration in liver. Many of these organs are also able to form bioactive retinoids, including RA. Organs that have formed RA may catabolize it locally or release RA into plasma, from which it can be taken up by the liver, oxidized by CYP26 or another CYP enzyme, conjugated with glucuronic acid, and excreted in bile. Overall, biliary excretion and loss of retinoids in the feces is the major route by which vitamin A is eliminated from the body. Some metabolites of vitamin A are also excreted in urine.

Plasma Transport

In plasma, vitamin A is present as REs in chylomicrons during the absorptive phase, and as holo-RBP at all times. Thus, plasma total retinol may be elevated in the postprandial period if the meal being absorbed was high in vitamin A. After ingestion, chylomicron vitamin A peaks in lymph and plasma at about 2–6 hours; the magnitude of the peak is directly related to the quantity of vitamin A ingested. Chylomicrons are cleared from plasma with a half-life of less than 20 minutes.

Except after meals, generally more than 95% of plasma vitamin A is in the form of retinol bound to RBP-TTR. Fasting retinol levels are quite constant with relatively low within and between person's variations. Mean concentrations are about 60 and 50 μg retinol/dL (~2 and 1.7 μM) in adult males and females, respectively. The con-

centration of RBP is slightly higher such that the saturation of RBP with retinol is about 90%. In the National Health and Nutrition Examination Surveys (NHANES), retinol levels increased slowly from childhood to adolescence, then stabilized. Retinol levels were higher in adult males than in premenopausal females (30–50 years). From age 51 years and older, retinol levels were nearly equal in males and females (29,30). Examples of plasma retinol levels and their associated levels of liver vitamin A stores, clinical signs, and most likely situations are listed in Table 1.

A significant aspect of retinol physiology is the recycling of retinol among organs. Each molecule of retinol is taken up by tissues, esterified and stored, hydrolyzed and mobilized, and then returned to plasma several times before it undergoes irreversible degradation. Using model-based compartmental analysis of plasma retinol in a healthy young man who had consumed 105 μmol of retinyl palmitate, it was calculated that 50 μmol of retinol passed through his plasma each day, although only 4 $\mu\text{mol/day}$ was degraded (31). Overall, the body's capacity for vitamin A storage is high, whereas its ability to degrade and eliminate vitamin A is quite limited. These features of metabolism help to explain the propensity for RE and retinol, or its oxidation products, to accumulate in tissues when vitamin A intake exceeds needs, and thus may help to explain the etiology of hypervitaminosis A.

The relationship between the concentrations of plasma retinol and liver vitamin A is far from linear; in fact, plasma retinol is maintained at a nearly constant level over a very wide range of liver vitamin A concentrations (32). Only when liver vitamin A stores are nearly exhausted (<20–30 $\mu\text{g/g}$) and the secretion of holo-RBP is compromised, does plasma retinol fall (32). Because of this, plasma retinol is not a good predictor of liver vitamin A reserves, except when it is obviously low. However, plasma retinol is still used as a provisional indicator of vitamin A status. Values of <0.35, <0.70, <1.05, and >1.05 μmol retinol/L are often interpreted as indicating severe deficiency, marginal deficiency, subclinical low status, and vitamin A adequacy, respectively. Plasma retinol is depressed in states of inflammation and fever, due to a reduction in RBP synthesis (33–35), which further confounds the assessment of vitamin A status during illness. However, as long as tissue vitamin A reserves are

adequate, plasma retinol will return to the normal range when inflammation subsides.

When liver vitamin A levels are very high (above ~300 µg total retinol/g liver), plasma total retinol may rise. However, this increase is not due to unesterified retinol but rather to the presence of unmetabolized RE in chylomicrons and other plasma lipoproteins. Elevated fasting RE is a sign of hypervitaminosis A.

Plasma β-carotene levels tend to reflect recent carotenoid intake. They may, however, vary considerably among similarly treated subjects due to intraindividual differences in absorptive efficiency and bioavailability of β-carotene (27,36).

Several acidic retinoids are present in plasma at nanomolar concentrations. These include all-*trans*- and 13-*cis*-RA, which circulate bound to albumin, not RBP. In pharmacokinetic studies of the clearance of RA from the plasma of non-human primates given high doses of all-*trans*-RA or 9-*cis*-RA, the half-lives were on the order of less than 1 to 2 hours, although clearance did not necessarily follow first-order kinetics (37).

Renal Filtration and Reuptake

Only a relatively small fraction of retinol and its metabolites is excreted in urine. Studies have shown that RBP is filtered in the glomerulus and that the complex of RBP-TTR (~75 kDa) is less susceptible to urinary loss than is the smaller apo-RBP (~21 kDa) (14). A multi-ligand membrane receptor, megalin, has been implicated in the recovery of RBP from the renal filtrate. Mice lacking megalin excreted more RBP and retinol in urine than wild-type mice (38). The kidneys are known to express RBP mRNA, and the production of new RBP by the kidneys may be important for the recovery of retinol from the renal filtrate and its recycling in plasma. While healthy adults are efficient in the reuptake of retinol, RBP and TTR, infants and especially very low-birth-weight infants have higher levels of retinol and its transport proteins in urine (24).

VITAMIN A STATUS AND NUTRITIONAL SOURCES

Vitamin A Deficiency

Vitamin A deficiency is still a public health problem in parts of developing world (13). Foods containing vitamin A may be scarce, too expensive, or they may be present but not considered appropriate for feeding to the most vulnerable groups. Young children after weaning and women of reproductive age, especially pregnant women, are most susceptible to becoming vitamin A deficient (39,40). Night blindness is most common in preschool-age children and pregnant women (see vision section discussed earlier).

Vitamin A deficiency also produces various systemic effects, including dryness of the skin (follicular hyperkeratosis), loss of mucous-secreting goblet cells in the trachea and respiratory tract, and a generalized metaplasia of epithelial tissues throughout the body. Functions of the immune system, especially those involving T cells and B cells, but also innate immunity, are often impaired in vitamin A-deficient animals and humans (35).

No specific deficiency of β-carotene is known, and no essential function has been described for β-carotene other than as a precursor of retinol (23). As long as the sum

of dietary β-carotene and retinol is adequate, the body's needs for vitamin A can be met. Nonetheless, carotenoids are widely thought of as lipid-soluble antioxidants and may have functions apart from their role as vitamin A.

FOOD SOURCES, INDICATIONS, AND USE

Food Sources

Vitamin A is contained in foods of both animal origin and plant origin, albeit in different chemical forms (Table 2). Individuals with widely varying dietary patterns (omnivorous, vegetarian, vegan) can obtain adequate vitamin A from their preferred type of diet. Preformed vitamin A is present in highest concentrations in liver and fish oils. Milk and egg yolks contain preformed vitamin A as well as some provitamin A. Provitamin A is the only form present in fruits and vegetables. Most provitamin A is consumed as leafy green and yellow vegetables; tomato products; colored fruits such as mangoes, oranges, and apricots; and in some vegetable oils, such as corn oil. In the United States, milk, margarine, cereals, and infant formulas may be fortified with vitamin A, as RE or β-carotene. Based on a U.S. dietary survey conducted in 1994–1996, the major contributors of vitamin A to the U.S. diet were vegetables and fruits (~55%) followed by dairy products and meats (~30%) (23). The median adult intake in the

Table 2 Food Sources of Vitamin A

Sources	% DV ^a
Animal sources of preformed vitamin A	
Liver, beef, cooked, 3 oz	610
Liver, chicken, cooked, 3 oz	280
Fat-free milk, fortified with vitamin A, 1 cup	10
Cheese pizza, 1/8 of a 12-in. diameter pie	8
Milk, whole, 3.25% fat, 1 cup	6
Cheddar cheese, 1 oz	6
Whole egg, 1 medium	6
Plant sources of β-carotene and other provitamin A carotenoids	
Carrot, 1 raw (7 1/2 in. long)	410
Carrots, boiled, 1/2 cup slices	380
Sweet potatoes, canned, drained solids, 1/2 cups	140
Spinach, frozen, boiled, 1/2 cups	150
Mango, raw, 1 cup sliced	130
Vegetable soup, canned, chunky, ready to serve, 1 cup	115
Cantaloupe, raw, 1 cup	100
Kale, frozen, boiled, 1/2 cup	80
Spinach, raw, 1 cup	40
Apricot nectar, canned, 1/2 cup	35
Tomato juice, canned, 6 oz	20
Apricots, with skin, juice pack, 2 halves	10
Pepper, sweet, red, raw, 1 ring, 3 in. diameter 1/4 in. thick	10
Peach, raw, 1 medium	10
Papaya, raw, 1 cup cubes	8

^a% DV, daily value. DVs are reference numbers based on the Recommended Dietary Allowance (RDA). DVs are set by the government and reflect current nutrition recommendations for a 2000-calorie reference diet (<http://www.fda.gov/Food/LabelingNutrition/ConsumerInformation/ucm078889.htm>).

The DV is not a unit of bioactivity. It is, however, a useful tool for quickly comparing the vitamin A contents of various foods. The DV for vitamin A is 5000 IU [1500 µg retinol = 1500 retinol activity equivalent (RAE)]. Percent DVs are based on a 2000-calorie diet.

Source: <http://www.nal.usda.gov/fnic/foodcomp>.

Table 3 Units of Vitamin A Activity Currently Used

Name of unit	Basis of definition	Equivalency (compounds in the all- <i>trans</i> -configuration)
International unit (IU)	Comparisons of retinol and β -carotene bioactivity, typically made in rat growth assay	1 IU = 0.3 μ g retinol 0.3 μ g RE (see next box) 0.0105 μ mol retinol 3 IU β -carotene 1.8 μ g β -carotene 3.6 μ g other provitamin A carotenoids
Retinol equivalent (RE)	Based on IU system but redefined and expanded to include equivalencies for provitamin A carotenoids in foods	1 RE = 1 μ g retinol 0.00349 μ mol retinol 2 μ g β -carotene (pure, in oil) 6 μ g β -carotene in foods 12 μ g other provitamin A carotenoids
Retinol activity equivalent (RAE) 2001, Institute of Medicine	Based on RE system but modified to reflect new information showing a lower bioavailability of carotenoids in most foods than estimated previously	1 RAE = 1 μ g retinol 2 μ g β -carotene (in oil) 12 μ g β -carotene in foods 24 μ g other provitamin A carotenoids in foods

U.S. NHANES III survey was equivalent to approximately 687 μ g of retinol/day (23).

The contents of bioactive retinoids such as retinal and RA in foods are inconsequential. These retinoids must be formed *in vivo* from vitamin A.

Units of Activity

The retinol activity equivalent (RAE) unit is currently in use to express the dietary reference intake (DRI) values for vitamin A (18) (23). Because of differences in the composition and bioavailability of vitamin A as RE and carotenoids as discussed earlier, and of differences in bioavailability among different carotenoids, equivalency factors are necessary for estimating the total amount (bioactivity) of vitamin A contained in foods. Over the years, several units have been defined. Table 3 summarizes the relationship between these units. The labels on most pharmaceutical preparations and the values in food tables before 2001 still express vitamin A in older units, either the international unit (IU) or retinol equivalent, RE. The change to RAE from the RE unit that was used in the 1989 Recommended Dietary Allowances (RDAs) was necessitated by a reevaluation of the nutritional equivalency of carotenoids in foods. Studies since 1989 had shown that the utilization of carotenoids in fruits and vegetables is lower and more variable than had previously been thought (27), and thus a new set of conversion values was needed (23). Significant interindividual variation has been observed in the response to oral doses of pure β -carotene in supplements and to carotenoid-containing foods (36), which is thought to reflect differences in the efficiency of carotene uptake into the mucosa, intracellular cleavage, postabsorptive clearance from plasma, or a combination of all of these factors (27).

Dietary Reference Intakes

The DRIs are a set of nutrient-based reference values established by expert committees of the U.S. Food and Nutrition Board of the Institute of Medicine (IOM) to provide guidance for planning diets. They extend and expand the concepts previously established for RDA. For vitamin A, there are four DRI categories: estimated average require-

ment (EAR); RDA, derived from the EAR; AI; and tolerable upper intake level (upper level, UL). In practice, the EAR is used to calculate the RDA, which is defined as the average daily dietary intake level that is sufficient to meet the nutrient requirements of nearly all healthy individuals. RDAs are set for life stage and gender groups.

DRI values for vitamin A were established by the IOM in 2001 (23). To calculate EAR values, data for adult men and women were used in a computational method that took into account the amount of vitamin A lost per day, minimum acceptable liver vitamin A reserves, organ and body weight factors, and efficiency of tissue storage. The RDA was then calculated as the EAR + 20%. Values for adolescents and children were based on adult values, scaled down on the basis of body weight. Reference values are given in Table 4. The EAR and 2001 RDA are expressed in RAE (see previous section). For infants, an AI was set based on the average amount of vitamin A in human breast milk that is consumed by infants in these age groups. The AI is equal to 400 μ g RAE/day for infants aged 0–6 months and 7–12 months. UL recommendations are discussed later in adverse effects section.

Table 4 Recommended Dietary Allowances (RDAs; 2001, United States Food and Nutrition Board of the Institute of Medicine) for Vitamin A Consumption (μ g RAE/day)

Children	Boys	Girls
1–3 yr	300	300
4–8 yr	400	400
9–13 yr	600	600
14–18 yr	900	700
Adults	Men	Women
10 yr and older	900	700
Pregnancy		
14–18 yr		750
19–50 yr		770
Lactation		
14–18 yr		1200
19–50 yr		1300

Abbreviation: RAE, retinol activity equivalent.

Use in Disease

In economically developed countries with an abundant food supply, there are few, if any, indications for vitamin A supplementation. However, if supplements that contain vitamin A are used, care should be taken that the total amount of *preformed* retinol in food, fortified foods, and supplements does not exceed the UL. The majority of nutritional supplements contain either all or at least a part of their vitamin A as retinol or RE. The amount of vitamin A in various supplements differs widely, from less than the U.S. RDA [$<100\%$ daily value (DV)] to 100% DV or higher. (See Table 2 for representative % DV in foods.)

In parts of the developing world, where vitamin A deficiency is still considered a public health problem, vitamin A is administered as a prophylactic measure to reduce the risk of vitamin A deficiency. In children 6 months to 5 years of age who received 200,000 IU (60 mg RAE) of retinol at 4–6-month intervals, or an RDA-level supplement weekly, morbidity and mortality were reduced significantly (41). Vitamin A is also recommended in the treatment of severe measles (42,43). Because vitamin A can be efficiently absorbed and stored in the liver and other tissues, then release retinol slowly over time, a single large oral dose, 100,000–200,000 IU depending on age, administered at the interval above, is generally effective over this period of time. However, the study using weekly doses with an RDA level of vitamin A showed the larger effect size in terms of reduced mortality (41). The safety of high-dose vitamin A was considered as a concern, but a number of studies have shown that bolus doses of 100,000–200,000 IU can safely be given to children 1 year and older (13), and 50,000 IU has been studied in newborns (44). Vitamin A supplementation of very low-birth-weight infants using a lower dose given by intramuscular injection has a small (7% reduction) but significant effect in reducing mortality or the requirement for supplemental oxygen at 1 month of age (45).

β -Carotene functions mainly as provitamin A. However, β -carotene is sometimes used for other purposes in persons with specific conditions, such as in the light-sensitive skin disease erythropoietic protoporphyria (46). Certain cosmetic changes (e.g., yellow skin complexion, carotenoderma) and carotenemia are associated with consumption of large amounts of β -carotene. These conditions are considered benign and the discoloration disappears over time after the intake of excessive β -carotene is stopped. Although neither a high intake of carotenoids in the diet nor the use of β -carotene supplements is known to produce toxicity, a “safe range” of intracellular β -carotene has yet to be determined (46). Problems with high-dose β -carotene encountered in randomized clinical trials of β -carotene supplementation in smokers, former smokers, and asbestos workers have significantly dampened the previous enthusiasm for β -carotene as a nutritional supplement (47).

Several natural and synthetic analogues of RA are approved for use as drugs; these should be used only under the supervision of physician and are not considered to be dietary supplements. Most synthetic retinoids have been designed to retain the hormonal activity of RA, or to have receptor-selective hormonal effects. However, no effective retinoid has yet been shown to be without any side effects, and most are potentially teratogenic.

Among the retinoids used in dermatology and cancer chemoprevention (48) are all-*trans*-RA (tretinoin), 13-*cis*-RA (isotretinoin, Accutane®), and Acitretin®. All-*trans*-RA is used in the treatment of APL (6).

ADVERSE EFFECTS

Hypervitaminosis A

An excessive intake of *preformed* vitamin A is associated with elevated plasma RE and saturation of vitamin A storage pools in tissues. This condition is generally referred to as hypervitaminosis A. Although hypervitaminosis A may be due to excess consumption of foods high in RE (such as liver), it most often is due to the use of high-dose nutritional supplements containing preformed vitamin A, unusual dietary practices, or an inappropriate use of therapeutic retinoids. Depending on the length of exposure and dosage, the resulting toxicity may be tolerable, severe, or lethal. The hallmarks of mild to moderate vitamin A toxicity include dizziness and nausea, changes in cerebrospinal fluid pressure, abnormal liver functions, and pain in weight-bearing bones and joints. These effects may be due to membrane lysis and/or to inappropriate gene regulation. An excess of vitamin A or retinoid drugs during pregnancy can be teratogenic, resulting in craniofacial abnormalities and other defects, including fetal death (9).

Treatment

There is no antidote for excess tissue vitamin A, which has a propensity to be retained in fatty tissues. It takes a very long time for tissue vitamin A levels to fall after intake is discontinued, and the liver damage resulting from hypervitaminosis A may be irreversible.

Upper Levels

Because of the serious and potentially irreversible effects of an excess intake of vitamin A, the IOM report of 2001 established a UL for preformed vitamin A (23). The UL is defined as the highest intake that is likely to pose *no risk* of adverse health effects in nearly all healthy individuals; it is meant to be a guideline for safe levels of consumption. Because of the body's capacity to store vitamin A, fluctuations in the day-to-day intake of vitamin A are usually not a cause for concern, but intakes that are elevated over an extended time are potentially harmful. The UL applies specifically and only to *preformed* vitamin A, obtained from foods, fortified foods and supplements combined. The UL is not meant to apply to individuals taking vitamin A under medical supervision. The critical adverse effects used to calculate the UL were risk of birth defects (teratogenesis) in women of reproductive age and liver abnormalities for men and women older than 50 years. Based on this, a UL of 2800–3000 μg of retinol/day was set for adults. UL values for children were scaled down based on body weight. Table 5 provides UL values for vitamin A by age groups (23). It is noteworthy that the UL values for vitamin A for some age–sex groups are less than fourfold above the RDA (see Table 4). A person's intake of carotenoids from foods and supplements is *not* included in calculating the UL because, as noted earlier, an accumulation of carotenoids in tissues is not known to be harmful.

Table 5 Tolerable Upper Intake Levels (ULs; 2001, United States Food and Nutrition Board of the Institute of Medicine) for Preformed Vitamin A Consumption (μg retinol/day)

Infants		
0–6 mo	600	
7–12 mo	600	
Children		
	Boys	Girls
1–3 yr	600	600
4–8 yr	900	900
9–13 yr	1700	1700
14–18 yr	2800	2800
Adults		
	Men	Women
10 yr and older	3000	3000
Pregnancy		
14–18 yr		2800
19–50 yr		3000
Lactation		
14–18 yr		2800
19–50 yr		3000

Users of vitamin–mineral supplements containing retinol or an ester of retinol should calculate their average *combined* intake from diet (liver, milk, and dairy products), fortified foods (e.g., breakfast cereals), and supplements to assure that it does not exceed the UL. Supplements for children should be checked to assure they are suitable for the child's age. For example, an adult supplement containing 5000 IU as retinol (equal to 1500 μg) contains more than the UL for children 1–3 and 4–8 years of age (UL of 600 and 900 μg , respectively; Table 5).

Contraindications

The teratogenic potential of preformed vitamin A is a significant concern (9,23). Women who could be pregnant should not take high-dose vitamin A supplements and should avoid total intakes of preformed vitamin A that exceed the UL. Several synthetic retinoids that are prescribed for therapeutic use are well known to be teratogenic to the fetus, and effective contraception is essential when they are used by women capable of becoming pregnant. The Food and Drug Administration has recently increased the regulatory requirements for retinoid prescriptions in women of childbearing age, to reduce the risk of birth defects (11).

Interactions

There are relatively few nutritionally significant interactions between vitamin A and other nutrients. Iron deficiency may impair the body's ability to mobilize vitamin A from storage, and vitamin A deficiency may have a similar effect on iron (49). Some drug–nutrient interactions are probable because vitamin A itself is metabolized by enzymes of the cytochrome P450 family, some of which also metabolize drugs and xenobiotics, including ethanol. The RXR receptor interacts with some receptors involved in drug metabolism, suggesting additional possible interactions. Chronic alcoholism and cirrhosis are associated with markedly reduced levels of liver vitamin A, which are likely of nutritional and metabolic origin. Retinol or β -carotene has been reported to exacerbate ethanol-induced liver damage, and therefore caution should be exercised regarding the use of vitamin A or β -carotene supplements by alcoholics.

COMPENDIAL/REGULATORY ISSUES

None.

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Vitamin B₆

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ABBREVIATIONS

4-PA, 4-pyridoxic acid; PL, pyridoxal; PLP, pyridoxal-5'-phosphate; PN, pyridoxine; PNP, pyridoxine-5'-phosphate; PM, pyridoxamine; PMP, pyridoxamine-5'-phosphate.

INTRODUCTION

Vitamin B₆ is unique among the water-soluble vitamins with respect to the numerous functions it serves and its metabolism and chemistry. Within the past few years, the attention this vitamin has received has increased dramatically (1–7).

This chapter provides an overview of vitamin B₆ as it relates to human nutrition. Both qualitative and quantitative information is provided in an attempt to indicate the importance of this vitamin within the context of health and disease in humans. The exhaustive literature on the intriguing chemistry of the vitamin is not dealt with in any detail, but readers are encouraged to refer to the citations for further information.

NAME AND GENERAL DESCRIPTION

As we leave the 20th century behind, there may be a tendency to lose the sense of excitement of discovery that Gyorgy and colleagues experienced when they began to unravel the mystery of the vitamin B complex. Some of the major highlights of the early years of vitamin B₆ research are presented in Table 1. Paul Gyorgy was the first to use the term vitamin B₆ (8). The term was used to distinguish this factor from other hypothetical growth factors B₃, B₄, B₅ (and Y).

Since Gyorgy first coined the term, there has been confusion in the terminology of the multiple forms of the vitamin. "Vitamin B₆" is the recommended generic descriptor for all 3-hydroxy-2-methylpyridine derivatives (9). Figure 1 depicts the various forms of vitamin B₆, including the phosphorylated ones. Pyridoxine (once referred to as pyridoxal) is the alcohol form and should not be used as a generic name for vitamin B₆. The trivial names and abbreviations commonly used for the three principal forms of vitamin B₆, their phosphoric esters, and analogs are as follows: pyridoxine, PN; pyridoxine-5'-phosphate, PNP; pyridoxamine, PM; pyridoxamine-5'-phosphate, PMP; pyridoxal, PL; pyridoxal-5'-phosphate,

Table 1 Historical Highlights of Vitamin B₆ Research

1932	A compound with the formula of C ₃ H ₁₁ O ₃ N is isolated from rice polishings.
1934	Gyorgy shows that there is a difference between the rat pellagra preventive factor and vitamin B ₂ . He calls this vitamin B ₆ .
1938	Lepkovsky reports isolation of pure crystalline vitamin B ₆ . Keresztesky and Stevens, Gyorgy, Kuhn, and Wendt, and Ichibad and Michi also report isolation of vitamin B ₆ .
1939	Chemical structure is determined and vitamin B ₆ synthesized by Kuhn and associates and by Harris and Folkers.
1942	Snell and coworkers recognize existence of other forms of pyridoxine.
1953	Snyderman and associates observe convulsions in an infant and anemia in an older child fed a vitamin B ₆ -deficient diet.

PLP; 4-pyridoxic acid, 4-PA. Other forms of vitamin B₆ exist, particularly bound forms.

The various physical and chemical properties of the phosphorylated and nonphosphorylated forms of vitamin B₆ are given in Table 2. Detailed data on fluorescence (12) and ultraviolet (13) absorption characteristics of B₆ vitamers are available. Of importance to researchers as well as to food producers and consumers is the relative stability of the forms of vitamin B₆. Generally, as a group, B₆ vitamers are labile, but the degree to which each is degraded varies. In solution, the forms are light sensitive (14,15), but this sensitivity is influenced by pH. Pyridoxine, pyridoxal, and pyridoxamine are relatively heat stable in an acid medium, but they are heat labile in an alkaline medium. The hydrochloride and base forms are readily soluble in water, but they are minimally soluble in organic solvents.

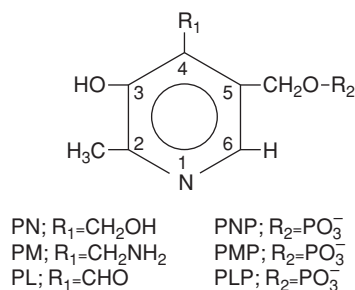


Figure 1 Structure of B₆ vitamers. Source: From Ref. (10).

Table 2 Physical Properties of B₆ Vitamers

0.1 N HCl	pH 7.0	Stability to white light ^a (pH 4.5)		pK		Fluorescence maxima ^b		Ultraviolet absorption spectra ^c			
		8 hr	15 hr	pK ₁	pK ₂	Activation (λ)	Emission (λ)	λ _{max}	ε _{max}	λ _{max}	ε _{max}
Vitamer	Molecular weight										
Pyridoxine	169.1	97%	90%	5.0	8.9	325	400	291	8900	254	3760
										324	7100
Pyridoxamine	168.1	81%	57%	3.4	8.1	325	405	293	8500	253	4600
										325	7700
Pyridoxal	167.2	97%	68%	4.2	8.7	320	385	288	9100	317	8800
Pyridoxine-5'-phosphate	249.2	—	—	—	—	322	394	290	8700	253	3700
										325	7400
Pyridoxamine-5'-phosphate	248.2	—	—	2.5	3.5	330	400	293	900	253	4700
										325	8300
Pyridoxal-5'-phosphate	247.2	—	—	2.5	4.1	330	375	293	7200	388	5500
										334	1300
4-Pyridoxic acid	183.2	—	—	—	—	325 ^d	425 ^d	—	—	—	—
						355 ^e	445 ^e				

^aPercentage stability compared to solution in dark (9). 8 hr, 15 hr = length of time exposed to light.

^bFrom Ref. 10; pH = 7.0.

^cData are for PN-HCl, PL-HCl, PM-2HCl, PLP monohydrate, PMP dehydrate (11).

^dpH 3.4, 0.01 N acetic acid.

^epH 10.5, 0.1 N NH₄OH, lactone of 4-PA.

The coenzyme form of vitamin B₆, PLP, is found covalently bound to enzymes via a Schiff base with an ε-amino group of lysine in the enzyme. While nonenzymatic reactions with PLP or PL and metal ions can occur (15), in enzymatic reactions, the amino group of the substrate for the given enzyme forms a Schiff base via a transamination reaction. PLP has been reported to be a coenzyme for more than 100 enzymatic reactions (16,17). Of these, nearly half involve transamination-type reactions. Transamination reactions are but one type of reaction that occur as a result of Schiff base formation. Ames et al. (18) have reviewed several PLP-dependent enzymes that are associated with various genetic and metabolic disorders, several of which have been treated with high doses of pyridoxine. A B₆ database is available online (19) that provides biochemical and molecular information on 121 PLP-dependent activities (20).

BIOCHEMISTRY AND FUNCTIONS

Before describing the functions of B₆ in human health in greater detail, it is worth noting that the measurement of B₆ vitamers and metabolites is complicated. Not only are there numerous methods but also various matrices are used. Reviews of the methods commonly used are available (21,22).

ASSESSMENT OF STATUS

The assessment of vitamin B₆ status is central to an understanding of its nutrition in humans. Various methods have been utilized for this purpose. These are given in Table 3 and are divided into direct, indirect, and dietary methods (23–25). Direct indices of vitamin B₆ status are those in which one or more of the B₆ vitamers or the metabolite 4-PA is measured. These are usually measured in plasma,

Table 3 Methods for Assessing Vitamin B₆ Status and Suggested Values for Adequate Status

Index	Suggested value for adequate status
Direct	
Blood	
Plasma pyridoxal-5'-phosphate ^a	30 nmol/L ^a
Plasma pyridoxal	NV
Plasma total vitamin B ₆	40 nmol/L
Erythrocyte pyridoxal-5'-phosphate	NV
Urine	
4-Pyridoxic acid	3.0 μmol/day
Total vitamin B ₆	0.5 μmol/day
Indirect	
Blood	
Erythrocyte alanine aminotransferase	1.25 ^b
Erythrocyte aspartate aminotransferase	1.80 ^b
Urine	
2 g tryptophan load test; xanthurenic acid	<65 μmol/day
3 g methionine load test; cystathionine	<351 μmol/day
Oxalate excretion	NV
Dietary intake	
Vitamin B ₆ intake, weekly average	1.2–1.5 mg/day
Vitamin B ₆ : protein ratio	0.02
Pyridoxine-β-glucoside	NV
Other	
EEG pattern	NV

NV, no value established; limited data available, each laboratory should establish its own reference with an appropriate healthy control population.

^aReference values in this table are dependent on sex, age, and protein intake and represent lower limits (25).

^bFor each aminotransferase measure, the activity coefficient represents the ratio of the activity with added PLP to the activity without PLP added.

erythrocytes, or urine samples because tissue samples are not normally available. Indirect measures are those in which metabolites of metabolic pathways in which PLP is required for specific enzymes are measured, or in which

activities of PLP-dependent enzymes are determined. In this latter case, an activity coefficient is often determined by measuring the enzyme activity in the presence and absence of excess PLP.

Dietary intake of vitamin B₆ itself is not sufficient to assess vitamin B₆ status, especially if only a few days of data are obtained. In addition to the inherent problems in obtaining accurate dietary intake information, the nutrient databases used in determining the vitamin B₆ content of diets are often incomplete with respect to values for this vitamin. Thus, reports of vitamin B₆ status on the basis of only nutrient intake must be viewed with caution. Some of the suggested values for the evaluation of status given in Table 3 are based on the relationship of vitamin B₆ and tryptophan metabolism (24). Plasma PLP concentration is considered one of the better indicators of vitamin B₆ status (26). Lumeng, Li, and Lui (10) have shown that plasma PLP concentration is a good indicator of tissue PLP levels in rats. In humans, plasma PLP concentration is significantly correlated with dietary vitamin B₆ intake (27). The means reported range was found to be from 27 to 75 nmol/L for males and from 26 to 93 μ mol/L for females (see Table 9 in Ref. 28.) These ranges should not necessarily be considered as normal, since the values reflect studies in which dietary intake was controlled and other studies in which dietary intake was not assessed. As discussed by Shultz and Leklem (27), dietary intake of both vitamin B₆ and protein influences the fasting plasma PLP concentration. Miller et al. (29) have shown that plasma PLP and total vitamin B₆ concentrations in males are inversely related to protein intake in males whose protein intake ranges from 0.5 to 2 g/kg/day. Similar results from metabolic studies in women support these findings in men (29).

Other factors that may influence plasma PLP and should be considered when using this index as a measure of vitamin B₆ status include the physiological variables of age (30–32), exercise (33), and pregnancy (34). Rose et al. determined the plasma PLP concentration in men ranging in age from 18 to 90 years (30). They observed a decrease in plasma PLP with age, especially after 40 years of age. However, one must keep in mind that the PLP concentration was determined 1 to 2 hours after a meal. The intake of vitamin B₆ may have influenced the data. Also, the carbohydrate intake could have resulted in a depressed plasma PLP concentration (33). Hamfelt and Soderhjelm reviewed the effect of age on plasma PLP concentration and observed that investigators in several countries (32) have seen decreased vitamin B₆ status with increasing age. The mechanism of this decrease remains to be determined. There is one controlled metabolic study that has evaluated vitamin B₆ status in different age groups. Lee and Leklem (31) studied five women aged 20 to 27 years and eight women aged 51 to 59 years under conditions in which the women received a constant daily vitamin B₆ intake of 2.3 mg for four weeks, followed by 10.3 mg/day for three weeks. Compared with the younger women, the older women had a lower mean plasma PLP and plasma and urinary total vitamin B₆ and slightly higher urinary 4-PA excretion with the 2.3-mg intake. Interestingly, there was no difference in urinary excretion of xanthurenic or kynurenic acid following a 2-g L-tryptophan load. Thus, while there may be age-related differences in vitamin B₆

metabolism, there is no significant age effect on functional activity of vitamin B₆ when intake is adequate. The metabolism of vitamin B₆ has been studied in elderly men and women older than 60 years. While younger individuals were not examined in the same study, the researchers concluded that the elderly had an increased vitamin B₆ requirement, indicative of increased metabolism. Kant et al. (35) observed no age-related impairment in the absorption or phosphorylation of vitamin B₆. However, there was an increase in plasma alkaline phosphatase activity with age that would increase hydrolysis of PLP.

The use of plasma PLP as a status indicator has been questioned (36) and the determination of plasma PL recommended. Others have also suggested that plasma PL may be an important indicator of status. When Barnard et al. (37) studied the vitamin B₆ status in pregnant women and nonpregnant controls, they found that plasma PLP concentration was 50% lower in the pregnant women but that the concentration of the total of PLP and PL was only slightly lower. When concentrations of PLP and PL were expressed on a per-gram-albumin basis, there was no difference between groups. In contrast, in pregnant rats, both plasma PLP and PL decreased, as did liver PLP, in comparison with nonpregnant control rats (38). These studies are in direct opposition to each other but do provide support for the need to determine several indices of vitamin B₆ status (25,26,36).

Urinary 4-PA excretion is considered a short-term indicator of vitamin B₆ status. In deficiency studies in males (39) and females (40), the decrease in urinary 4-PA paralleled the decrease in plasma PLP concentration. As reflected in the studies in which dietary intake was assessed or known, 4-PA excretion accounts for about 40% to 60% of the intake (see Table 10 in Ref. 28.) Because of the design of most studies and the limited number of studies done with females compared with males, it is not possible to determine whether there is a significant difference between males and females. However, males consistently had higher plasma PLP and total vitamin B₆ concentrations as well as higher excretion of 4-PA and total vitamin B₆. Urinary total vitamin (all forms, including phosphorylated and glycosylated) excretion is not a sensitive indicator of the vitamin, except in situations where intake is very low (28,39).

Erythrocyte transaminase activity (alanine and aspartate) has been used to assess vitamin B₆ status in a variety of populations (39–47), including oral-contraceptive users (25,44,45). Transaminase activity is considered a long-term indicator of vitamin B₆ status. Most often, it has been measured in the presence and absence of excess PLP (42). While this index is used to assess status, there is no unanimous agreement, and some consider it to be less reliable than other indicators (25,47). The long life of the erythrocyte and tight binding of PLP to hemoglobin may explain the lack of a consistent significant correlation between plasma PLP and transaminase activity or activity coefficient. An additional consideration that complicates the use of aminotransferases is the finding of genetic polymorphism of erythrocyte alanine aminotransferase (EALT) (48).

Urinary excretion of tryptophan metabolites following a tryptophan load, especially excretion of xanthurenic

acid, has been one of the most widely used tests for assessing vitamin B₆ status (49,50). The use of this test has, however, been questioned (51,52), especially in disease states or in situations in which hormones may alter tryptophan metabolism independent of a direct effect of vitamin B₆ metabolism (53).

Other tests for status include the methionine load (54), oxalate excretion, electroencephalographic tracings (55), and lymphocyte proliferation (56). These tests are used less often but under appropriate circumstances provide useful information. The review by Reynolds (36) provides an excellent critique of methods currently in use for assessment of vitamin B₆ status. A recent review by Spinneker et al. (57) provides an addition perspective on vitamin B₆ status assessment.

In the past 10 years, there have been several studies detailing assessment of vitamin B₆ status of a variety of populations. In most of these the concentration of plasma PLP is used as the prime indicator of status. A cutoff value of PLP of either 20 nmol/L (58) or 30 nmol/L (25) was used in some studies. The studies included the following populations: young people aged 8 to 14 years and elderly people older than 65 years in Britain (59), men and women aged 19 to 50 in the United States (60), overweight and obese rural adults in the United States (61), pre- and postmenopausal women living in urban areas of Canada (62), German vegans (63), elderly Taiwanese (64), over 6000 U.S. men and women more than one year of age and representative of the U.S. population (65), and over 10,000 Norwegian men and women aged 50 to 64 years (66).

Because variables that can affect plasma PLP [protein intake, coffee intake (67), vitamin B₆ intake, smoking, alkaline phosphatase activity, and age] were not included in all studies, comparison between studies was difficult. As an example, plasma PLP concentration in smokers has been found to be decreased by 33% in two separate studies (68,69).

FUNCTIONS

Immune System Functions

The involvement of PLP in a multiplicity of enzymatic reactions (70) suggests that it serves many functions in the body. PLP acts as a coenzyme for serine transhydroxymethylase (71), one of the key enzymes involved in one-carbon metabolism. Alteration in one-carbon metabolism can then lead to changes in nucleic acid synthesis. Such changes may be one of the keys to the effect of vitamin B₆ on immune function (72,73). Studies in animals have shown that vitamin B₆ deficiency adversely affects lymphocyte production (72) and antibody response to antigens (73). Additional studies in animals support an effect of vitamin B₆ on cell-mediated immunity. In otherwise healthy elderly men and women, 40% of men and 59% of women had vitamin B₆ deficiency on the basis of low PLP concentration (74). The natural killer cell immune function of these subjects was not significantly correlated with plasma PLP concentration. Kwak et al. (56) found that in controlled diet setting, improved vitamins B₆ status enhanced lymphocyte proliferation when comparing 1.5 mg B₆/day intake with a 2.1-mg B₆/day intake. No further en-

hancement in proliferation was seen with a 2.7-mg B₆/day intake. Use of a high dose of pyridoxine (600 mg/day) for seven days was found to increase prostacyclin production and inhibit thromboxane and leukotriene synthesis (75). Kwak and Leklem (76) found that in young women an intake of 50 mg B₆/day for 20 days did not result in a further increase in lymphocyte proliferation compared to a 2.0-mg B₆/day supplement for seven days. Interleukin-2 production from lymphocytes was not affected by a 25-mg B₆/day supplement for three months in patients with Sjögren's syndrome (77). Plasma PLP concentration was found to be low in mechanically ventilated critically ill patients and significantly associated with various immune function parameters (78). An additional study (79) of critically ill patients found that a daily injection of 50 mg B₆ or 100 mg B₆ increased several measures of immune response (T-suppressor and T-helper cell numbers). A supplement of 50 mg B₆/day for three months did not relieve humoral immunodeficiency in patients with common variable immunodeficiency (80).

Gluconeogenesis

Gluconeogenesis is key to maintaining an adequate supply of glucose during caloric deficit. PLP is involved in gluconeogenesis via its role as a coenzyme for transamination reactions (70) and for glycogen phosphorylase (81). In animals, a deficiency of vitamin B₆ results in decreased activities of liver alanine and aspartate aminotransferase (82). However, in humans (females), a low intake of vitamin B₆ (0.2 mg/day), as compared with its adequate intake (1.8 mg/day), did not significantly influence fasting plasma glucose concentrations (83). Interestingly, the low vitamin B₆ intake was associated with impaired glucose tolerance in this study. A study by Hagiwara et al. (84) found that in mice fed high levels of pyridoxamine and either a low or high fat diet improved blood glucose levels after glucose injection and fasting hyperinsulinemia. The level of pyridoxamine fed (300 mg/kg/day) is considered by the author of this chapter to be pharmacologic, and thus application to humans may be limited in an everyday nutritional sense.

Glycogen phosphorylase is also involved in maintaining adequate glucose supplies within liver and muscle and, in the case of liver, is a source of glucose for adequate blood glucose levels. In rats, a deficiency of vitamin B₆ has been shown to result in decreased activities of both liver (85) and muscle glycogen phosphorylase (81,85,86). Muscle appears to serve as a reservoir for vitamin B₆ (81,86,87), but a deficiency of the vitamin does not result in mobilization of these stores. However, Black et al. (86) have shown that a caloric deficit does lead to decreased muscle phosphorylase content. These results suggest that the reservoir of vitamin B₆ (as PLP) is only utilized when there is a need for enhanced gluconeogenesis. In male mice, the half-life of muscle glycogen phosphorylase has been shown to be approximately 12 days (88). In contrast to rats with a low intake of vitamin B₆, those given an injection of a high dose of PN, PL, or PM (300 mg/kg) show a decrease in liver glycogen and an increase in serum glucose (89). This effect is mediated via increased secretion of adrenal catecholamines. The extent to which lower intake of B₆

vitaminers has this effect or whether this occurs in humans remains to be determined.

Erythrocyte Function

Vitamin B₆ has an additional role in erythrocyte function and metabolism. The function of PLP as a coenzyme for transaminases in erythrocytes has been mentioned. In addition, both PL and PLP bind to hemoglobin (90,91). The binding of PL to the α -chain of hemoglobin (92) increases the O₂ binding affinity (93), whereas binding to the β -chain of hemoglobin S or A lowers it (94). The effect of PLP and PL on O₂ binding may be important in sickle cell anemia (95). Studies of Sickle disease and vitamin B₆ are discussed in more detail in a subsequent section.

PLP serves as a cofactor for δ -aminolevulinic acid synthetase (96), the enzyme that catalyzes the condensation of glycine and succinyl-CoA to form δ -aminolevulinic acid. This latter compound is the initial precursor in heme synthesis (97). Therefore, vitamin B₆ plays a central role in erythropoiesis. A deficiency of the vitamin in animals can lead to hypochromic microcytic anemia. Furthermore, in humans, there are several reports of patients with pyridoxine-responsive anemia (98). However, not all patients with sideroblastic anemia (in which there is a defect in 5-aminolevulinic acid synthetase) respond to pyridoxine therapy (99).

Niacin Formation

One of the more extensive functions of vitamin B₆ that has been researched is its involvement in the conversion of tryptophan to niacin (50). This research is in part related to the use of the tryptophan load in evaluating vitamin B₆ status. While PLP functions in at least four enzymatic reactions in the complex tryptophan–niacin pathway, there is only one PLP-requiring reaction in the *direct* conversion of tryptophan to niacin. This step is the transformation of 3-hydroxykynurenine to 3-hydroxyanthranilic acid and is catalyzed by kynureninase. Leklem et al. have examined the effect of vitamin B₆ deficiency on the conversion of tryptophan to niacin (100). In this study, the urinary excretion of *N'*-methylnicotinamide and *N'*-methyl-2-pyridone-5-carboxamide, two metabolites of niacin, was evaluated in women. After four weeks of a low-vitamin B₆ diet, the total excretion of these two metabolites following a 2-g L-tryptophan load was approximately half that when subjects received 0.8 to 1.8 mg vitamin B₆ per day. This suggests that low vitamin B₆ has a moderate negative effect on niacin formation from tryptophan.

Nervous System Functions

In addition to the effect of vitamin B₆ on tryptophan-to-niacin conversion, there is another tryptophan pathway that is vitamin B₆ dependent. The conversion of 5-hydroxytryptophan to 5-hydroxytryptamine is catalyzed by the PLP-dependent enzyme 5-hydroxytryptophan decarboxylase (101). Other neurotransmitters, such as taurine, dopamine, norepinephrine, histamine, and γ -aminobutyric acid, are also synthesized by PLP-dependent enzymes (101). The involvement of PLP in neurotransmitter formation and the observation that there are neurological abnormalities in human infants (102,103) and animals (104) deficient in vitamin B₆ provide support

for a role of vitamin B₆ in nervous system function. Recent reviews on the relationship between nervous system function and vitamin B₆ are available (105,106).

In infants fed a formula in which the vitamin B₆ was lost during processing, convulsions and abnormal electroencephalograms (EEGs) were observed (102). Treatment of the infants with 100 mg of pyridoxine produced a rapid improvement in the EEGs. In these studies reported by Coursin, the protein content of the diet appeared to be correlated with the vitamin B₆ deficiency and the severity of symptoms. Other evidence for a role of vitamin B₆ comes from studies of pyridoxine-dependent seizures, an autosomal recessive disorder. Vitamin B₆ dependency, though a rare cause of convulsions, has been reported by several investigators (107,108). The convulsions occur during the neonatal period, and administration of 30 to 100 mg of pyridoxine is usually sufficient to prevent them and correct an abnormal EEG (108,109). However, there are atypical patients who present a slightly different clinical picture and course but are responsive to pyridoxine (110).

Vitamin B₆ deficiency in adults has also been reported to result in abnormal EEGs (55,111), especially in individuals on a high-protein (100 g/day) intake. In one study (112), subjects received a diet essentially devoid of vitamin B₆ (0.06 mg). Grabow and Linkswiler fed to 11 men a high-protein diet (150 g) and 0.16 mg of vitamin B₆ for 21 days (112). No abnormalities in EEGs were observed nor were these changes seen in motor nerve-conduction times in five subjects who had this measurement. Kretsch et al. (55) observed abnormal EEG patterns in two of eight women after 12 days of a low (0.05 mg/day) vitamin B₆ diet. Feeding 0.5 mg/day corrected the abnormal pattern. While there were differences in the length of the period of deficiency in these studies, which may explain the differences observed, it appears that long-term very low vitamin B₆ intakes are necessary before abnormal EEGs are observed in humans.

Another aspect of the relationship of vitamin B₆ (as PLP) to the nervous system is the development of the brain under conditions of varying intakes of the vitamin. Aycock and Kirksey have conducted numerous well-designed studies in this area. These studies have utilized the rat model to examine the development of the brain, especially during the critical period when cells undergo rapid mitosis. Early experiments showed that dietary restriction of vitamin B₆ in the dams was associated with a decrease in alanine aminotransferase and glutamic acid decarboxylase activity and low brain weights of progeny (113).

Alterations in fatty acid levels, especially those involved in myelination (114), decreases in cerebral sphingolipids and in the area of the neocortex and cerebellum, as well as reduced molecular and granular layers of the cerebellum have all been noted (115).

One of the more intriguing and controversial aspects of vitamin B₆ is its role in lipid metabolism (116). Studies conducted more than 70 years ago suggested a link between fat metabolism and vitamin B₆ (117). Subsequent research showed that liver lipid levels were significantly lower in vitamin B₆-deficient versus pair-fed rats (118). The changes were due mainly to lower triglyceride levels, whereas cholesterol levels were not different. In

contrast, Abe and Kishino showed that rats fed a high-protein (70%), vitamin B₆-deficient diet developed fatty livers and suggested that this was due to impaired lysosomal degradation of lipid (119). The synthesis of fat in vitamin B₆-deficient rats has been reported to be greater (120), normal (121), or depressed (122). The observed differences may be related to the meal pattern of the animals (123).

The effect of vitamin B₆ deprivation on fatty acid metabolism has also received attention. A pyridoxine deficiency may impair the conversion of linoleic acid to arachidonic acid (102,124). Cunnane and coworkers (124) found that phospholipid levels of both linoleic and γ -linolenic acid were increased in vitamin B₆-deficient rats, but the level of arachidonic acid was decreased as compared with that of control levels in plasma, liver, and skin. They suggested that both linoleic desaturation and γ -linoleic acid elongation may be impaired by a vitamin B₆ deficiency. She et al. (125) have observed decreased activity of terminal Δ^6 -desaturase in the linoleic acid desaturation system in rats fed a vitamin B₆-deficient diet and a positive correlation between phosphatidylcholine (PC) content and Δ^6 -desaturase activity in liver microsomes. Subsequent work by She et al. suggests that alteration of (S)-adenosylmethionine (SAM) to (S)-adenosylhomocysteine is involved in these changes (126). In one of the few studies of vitamin B₆ and fatty acid metabolism in humans, deoxypyridoxine was utilized to induce a vitamin B₆ deficiency (127). Xanthurenic acid excretion following a 10-g D,L-tryptophan load indicated a moderate vitamin B₆-deficient state. Only minor changes in fatty acid levels in plasma and erythrocytes were observed as a result of the deficiency produced. The pattern of fatty acids observed was interpreted by the authors to support the findings of Witten and Holman (123). The work of She et al. (126) supports this. This provides a plausible mechanism, because the primary metabolic steps in fatty acid metabolism do not involve nitrogen-containing substrates, a feature common to most PLP-dependent enzymatic reactions.

The change observed in arachidonic acid levels and the role it plays in cholesterol metabolism may have clinical implications. The effect, if any, of vitamin B₆ on cholesterol metabolism remains controversial. Studies by Delmore and Lupien have shown that the rate of incorporation of [¹⁴C]-acetate into cholesterol was increased in vitamin B₆-deficient rats as compared to controls (128). However, the amount of cholesterol in plasma and liver of rats and other species has been reported to be increased, not changed, or even decreased (128). Significant positive correlation between plasma PLP and high-density-lipoprotein (HDL) cholesterol and negative correlations between total cholesterol and low-density-lipoprotein (LDL) cholesterol have been reported in monkeys fed atherogenic Western diets and a "prudent" Western diet (129). However, the diets fed to the monkeys contained distinctly different amounts of vitamin B₆. The use of supplemental vitamin B₆ in reduction of blood cholesterol has not been definitively tested. Serfontein and Ubbink reported decreased serum cholesterol (0.8 mmol/L) in 34 subjects given a multivitamin containing 10 mg of pyridoxine (130). The reduction was mainly as LDL cholesterol. In another study, pyridoxine (50 mg/day) administration prevented the increase in serum cholesterol

seen when disulfiram was administered (131). Controlled trials of pyridoxine are needed to resolve the role of vitamin B₆ in modifying serum cholesterol levels.

The role of vitamin B₆ in lipid metabolism remains unclear. Evidence to date suggests a role in modifying methionine metabolism and thus an indirect effect on phospholipid and fatty acid metabolism. This effect and one on carnitine synthesis (132) appear to be the primary effects of vitamin B₆ on lipid/fatty acid metabolism.

Hormone Modulation/Gene Expression

One of the more intriguing functions of PLP is as a modulator of steroid action (133,134). Reviews of this interaction are available (135,136). PLP can be used as an effective tool in extracting steroid receptors from the nuclei of tissues on which the steroid acts (137). Under conditions of physiological concentration of PLP, reversible reactions occur with receptors for estrogen (138), androgen (139), progesterone (140), and glucocorticoids (141). PLP reacts with a lysine residue on the steroid receptor. As a result of the formation of a Schiff base, there is inhibition of the binding of the steroid-receptor complex to DNA (133). Holley et al. found that when female rats were made vitamin B₆ deficient and injected with [³H]-estradiol, a greater amount of the isotope accumulated in the uterine tissues of the deficient animal than in the tissues of controls (142). Bunce and Vessal studied the dual effect of zinc and vitamin B₆ deficiency on estrogen uptake by the uterus (143). They found that there was an increased uptake of the hormone in both the vitamin B₆- and the zinc-deficient animals. A combined deficiency of the two nutrients resulted in even greater retention of estrogen. The number of estrogen receptors was not altered by the deficiency of vitamin B₆. This study suggests that there might be increased sensitivity of the uterus (or other end-target tissues) to steroids when vitamin B₆ status is abnormal.

Sturman and Kremzner found enhanced activity of ornithine decarboxylase in testosterone-treated vitamin B₆-deficient animals as compared to control animals (144). DiSorbo and Litwack observed increased tyrosine aminotransferase activity in hepatoma cells raised on a pyridoxine-deficient medium and treated with triamcinolone acetonide as compared to pyridoxine-sufficient cells treated with the same steroid (145). Allgood and Cidlowski (146) used a variety of cell lines and a range of intracellular PLP concentrations to show that vitamin B₆ modulates transcriptional activation by several (androgen, progesterone, and estrogen) steroid hormone receptors. This supports the role of vitamin B₆ as a physiological modulator of steroid hormone action.

Oka et al. (147) found that in vitamin B₆-deficient rats, the level of albumin mRNA was sevenfold that of control rats. They suggest that PLP modulates albumin gene expression by inactivation of tissue-specific transcription factors. Oka and coworkers also observed a sevenfold increase in the level of mRNA for cytosolic aminotransferase in vitamin B₆-deficient rats as compared with that of vitamin B₆-sufficient rats (148). Subsequent work by Oka et al. (149) shows an inverse relationship between intracellular PLP concentration and albumin mRNA in rats given amino acid loads. In an *in vitro* system, vitamin B₆ was found to not interfere with glucocorticoid

action in immune cells but did inhibit glucocorticoid receptor-dependent transactivation in nonimmune cells (150). In a study aimed at understanding how vitamin B₆ modulates steroid hormone receptors, Hug et al. (151) found that gene regulation occurred by PLP conjugation to a transcriptional coregulator. Thus, PLP may be a modulator of gene expression in animals, especially under conditions of altered amino acid supply. Given the intimate relationship of vitamin B₆ and amino acid metabolism, these investigations open up for study a new area of metabolic regulation via altered intracellular nutrient (PLP) concentration.

PLP has been shown to be a strong inhibitor of polymerase alpha and epsilon (152) and DNA topoisomerase (153). PLP is also a noncompetitive inhibitor for group I intron splicing (154). In humans, feeding a marginal vitamin B₆ diet for 28 days did not significantly affect DNA-uracil concentration in lymphocytes (155). Tumor cells have genome aberrations termed gross chromosomal rearrangements (GCR). Kanellis et al. (156) have found that pyridoxal kinase, and hence PLP, suppresses GCR events by preventing DNA lesions.

VITAMIN B₆ REQUIREMENTS

Considering the numerous functions in which vitamin B₆ is involved, assessment of the requirement for this vitamin becomes important. Reviews of vitamin B₆ requirements are available (157–159).

Several relevant studies have been conducted. These have been carried out in both young and elderly adults and in males and females (28,160–162). While some of them are similar to previous ones in that they employed depletion/repletion design (160–162) and diets with high B₆ bioavailability, others have used diets more representative of the usual U.S. diet (28).

What is also different about some of these studies is that they have included additional measurements that may be indicative of intercellular function of PLP. Meydani et al. (163) examined the effect of different levels of vitamin B₆ (pyridoxine added to a low-B₆ food diet) on immune function. They observed that adequate immune function in elderly women was not achieved until 1.9 mg/day of vitamin B₆ was fed. Men required 2.88 mg/day to return function to baseline levels. In addition, several indices of vitamin B₆ status were measured. On the basis of when these values for these indices returned to predepletion levels, the requirement for vitamin B₆ was estimated to be 1.96 and 1.90 mg/day for men and women, respectively.

Kretsch et al. (162) fed four graded doses of vitamin B₆ to eight young women following a depletion diet (for 11–28 days). On the basis of this and other studies, it was found that less than 0.5 mg/day is needed to observe clinical signs of vitamin B₆ deficiency. Functional signs, such as abnormal EEGs, were only seen with an intake lower than 0.5 mg/day. Various biochemical measures, including the functional tests of tryptophan metabolite excretion (xanthurenic acid) and erythrocyte aspartate transaminase (EAST) stimulation, were normalized at the 1.5 and 2.0 mg/day level, respectively. The authors stated that if all currently used biochemical measures were to be nor-

malized, then more than 0.020 mg of vitamin B₆ per gram of protein is required.

Hansen et al. (41) used a different approach in evaluating the effect of graded doses of vitamin B₆ on status. First, rather than feeding a diet deficient in vitamin B₆, a diet containing a level that was low but within the realm of what individuals might normally consume was fed. Various levels of pyridoxine (as an oral solution) were then added to the basal diet (range 0.8–2.35 mg B₆/day). Based on both direct and indirect measures (including tryptophan metabolite excretion), it was concluded that a B₆/protein ratio greater than 0.20 was required to normalize all vitamin B₆ status indices. Ribaya-Mercado et al. (160) evaluated the vitamin B₆ requirements of elderly men and women in a depletion/repletion study. The authors concluded that the vitamin B₆ requirements of elderly men and women are about 1.96 and 1.90 mg/day, respectively. The vitamin B₆ (pyridoxine) fed to these subjects was in a highly bioavailable form.

A metabolic study in young women evaluated the requirement for vitamin B₆ (159). Again, a depletion/repletion design was used and several indices of vitamin B₆ status were measured. These included urinary 4-PA excretion, plasma PLP, erythrocyte PLP, and EAST and EAST activity coefficients. Using predepletion baseline levels (after nine days of feeding 1.60 mg/day) of these indices as a basis for comparison in determining adequacy, the amount of vitamin B₆ required to normalize these indices was found to be 1.94 mg/day (B₆ to protein ratio of 0.019).

An important consideration relative to many of these metabolic studies that have been used in establishing the adult vitamin B₆ recommended dietary allowance (RDA) is the composition of the diets used. Most were ones in which the amount of vitamin B₆ from food was low and of relatively high bioavailability. Vitamin B₆ was added back to the diets in the form of pyridoxine hydrochloride and thus is considered 100% bioavailable. Therefore, the total vitamin B₆ in the diets is probably 95% to 100% bioavailable. Taken together, these four recent metabolic studies support a higher vitamin B₆ requirement for women and men than is currently employed. A value of 1.9 mg/day for women and 2.2 mg/day for men is recommended by the author of this chapter. Because the vitamin B₆ in these studies was highly available, the inclusion of a factor for bioavailability would further increase the RDA (164).

The above discussion has focused on the vitamin B₆ requirement for adults aged 18 to 70 years. There has been little well-designed research to support a statement of recommendations for children (aged 1–10) or adolescents (aged 11–18).

Food Sources

There are various forms of vitamin B₆ in foods. In general, these forms are a derivative of pyridoxal, pyridoxine, and pyridoxamine. Pyridoxine and pyridoxamine (or their respective phosphorylated forms) are the predominant forms in plant foods such as lima beans, spinach, broccoli, avocados, white beans, lentils, nuts, and brown rice. Although there are exceptions, pyridoxal, as the phosphorylated form, is the predominant form in foods.

Table 4 Drug-Vitamin B₆ Interactions

Drug	Examples	Mechanism of interaction
Hydrazines	Iproniazid, isoniazid, hydralazine	React with pyridoxal and PLP to form a hydrazone
Antibiotic	Cycloserine	Reacts with PLP to form an oxime
L-DOPA	L-3,4-Dihydroxyphenylalanine	Reacts with PLP to form tetrahydroquinoline derivatives
Chelator	Penicillamine	Reacts with PLP to form thiazolidine
Oral contraceptives		Ethinyl estradiol, mestranol, increased enzyme levels in liver and other tissues; retention of PLP
Alcohol	Ethanol	Increased catabolism of PLP; low plasma levels

(Data on the amount of each of the three forms are listed in Table 4 of Ref. (28))

DISEASE AND TOXICITY

Several books (2,3,6,7) and reviews (5) have examined the relationship between specific diseases and vitamin B₆ nutrition in detail. There are numerous diseases or pathological conditions in which vitamin B₆ metabolism is altered. The primary indicator of an alteration in vitamin B₆ metabolism has been an evaluation of tryptophan metabolism or the plasma PLP concentration. The first of these is an indirect measure of status, and the second is a direct measure. Conditions in which tryptophan metabolism has been shown to be altered and in which vitamin B₆ (pyridoxine) administration was used include asthma (165), diabetes (166,167), certain cancers (52,168), pellagra (169), and rheumatoid arthritis (140). Diseases and pathological conditions in which plasma PLP levels have been shown to be depressed include asthma (170), diabetes (171), renal disorders (172), alcoholism (173), heart disease (174), pregnancy (35,175,176), breast cancer (177), Hodgkin's disease (178), and sickle cell anemia (95). Relatively few of these studies have exhaustively evaluated vitamin B₆ metabolism. An update on studies in physiologic and disease conditions follows.

Coronary Heart Disease

The relationship between vitamin B₆ and coronary heart disease can be viewed from both an etiological perspective and that of the effect of the disease state on vitamin B₆ metabolism. With respect to an etiological role, altered sulfur amino acid metabolism has been suggested to result in vascular damage. A poor vitamin B₆ status can result in an increased circulating concentration of homocysteine (179). In the trans-sulfuration pathway, serine and homocysteine condense to produce cystathionine. This reaction is catalyzed by the PLP-dependent enzyme cystathionine β -synthase (180). In genetic disorders of this enzyme, homocysteine accumulates in the plasma (181). An increased incidence of arteriosclerosis has been associated with this enzyme defect (182). In addition, elevated levels of homocysteine in the plasma have been observed in people with ischemic heart disease (183,184).

There has been an exponential growth in the number of studies on homocysteine. In many of these studies, vitamin B₆ supplements either with supplements of folic acid and vitamin B₁₂ (a majority) or as the only supplement have been utilized to assess their impact on blood levels of homocysteine.

The relationship between homocysteine and vitamin B₆ status has focused primarily on plasma PLP concentration. Several studies involving a large populations have found an inverse relationship between plasma PLP concentration and plasma homocysteine concentration (65,185–189). However, not all studies have found such an inverse relationship (190–192). While a combination of B vitamins (folate, B₁₂, and B₆) are effective in lowering plasma homocysteine (193,194), daily pyridoxine supplements of 10 mg (195), 25 mg (196), and 40 mg (197) did not significantly change plasma homocysteine concentration. Under strict dietary vitamin B₆ restriction in humans of either 0.3 mg B₆/day for four weeks (198) or 0.5 mg B₆/day for four weeks (199), no change in plasma homocysteine was observed. Several reviews of homocysteine are available (200–202). One of these (H-18) (200) was a meta-analysis and concluded that beyond the significant effect that folic acid and vitamin B₁₂ supplements have on homocysteine, vitamin B₆ supplementation has no significant effect.

While some animal experiments have shown that rhesus monkeys made vitamin B₆ deficient develop atherosclerotic lesions (203), other studies do not reveal any pathological lesions (204). In humans at risk for coronary heart disease, a negative correlation between dietary vitamin B₆ and bound homocysteine has been observed (205). For some people with homocysteinuria, treatment with high doses of vitamin B₆ reduces the plasma concentration of homocysteine in certain patients but does not totally correct methionine metabolism (206), especially when there is an increased methionine intake. Thus, if vitamin B₆ therapy is to be successful in reducing vascular lesions, diet modification with a lowered methionine intake may be necessary.

Because of the relationship between coronary artery disease (CAD) and homocysteine (and vitamin B₆) recent research has focused on blood levels of PLP in CAD and related diseases as well as a possible relation to risk of these diseases. Several studies have reported a low concentration of plasma PLP in patients with heart disease (207–212) and stroke (213).

Several studies have reported that a low plasma PLP concentration is associated with increased risk for CAD (211,214–217). In a nested case-cohort study, a high plasma PLP was associated with a significantly reduced risk of myocardial infarction, but this association was abolished when adjusted for high specific c-reactive protein (hsCRP) and smoking (212). Others have reported that PLP and hsCRP are independent risk factors for CVD (217). Two studies reported an inverse relationship between vitamin B₆ intake and risk for CAD (218,219).

Trials designed to evaluate the effect of folic acid, vitamin B₆, and, in some cases, vitamin B₁₂ have found beneficial vascular effects (220,221) and in one study a reduction in the progression of subclinical atherosclerosis (222). However, two recent meta analysis studies found no evidence of a protective effect of homocysteine lowering supplements on progression or prevention of cardiovascular events (223,224). Over the past five years, clinical trials have conducted testing whether treatment with PLP [referred to as MC-1 in the studies in Ref. (225) is beneficial for patients undergoing coronary artery bypass graft surgery (Ref. 225 and references therein)]. A dose of 250 mg/day of PLP was used for 30 days after surgery. PLP supplementation was found to be not beneficial.

HIV/AIDS

Vitamin B₆ status (226,227) and, to a limited extent, metabolism (228) have been examined in persons with human immunodeficiency virus (HIV). Because of the link between immune function and vitamin B₆, one would expect that maintaining an adequate vitamin B₆ status is critical for HIV patients. Several studies have evaluated vitamin B₆ intake, (227,229,230) and the progression of the disease as related to intake of nutrients, including vitamin B₆ (230). These studies generally found low intakes of vitamin B₆, and one study (231) reported an inverse relationship between vitamin B₆ intake and progression.

Biochemical assessment of vitamin B₆ status has been done in several studies (226,227,230) and has revealed it to be poor. In most of these studies (166,167) α -EAST (this is the activity coefficient as defined by the ratio of the activity of erythrocyte aspartate aminotransferase with and without added PLP in the assay) was used as an index of status. In the studies, samples were frozen, which may have compromised the data and subsequent evaluation. Although other researchers have measured and reported low levels of "serum vitamin B₆," they failed to specify what form was being measured (230,231). Therefore, given the complexities of nutritional well-being in HIV/AIDS patients and methodological problems in these studies, it is difficult to assess the role of vitamin B₆ in this syndrome.

In vitro studies suggest that PLP may play a role in HIV/AIDS. Salhany and Schopfer (232) found that PLP binds to the CD4 receptors at a site that is competitive with a known antiviral agent (4,4'-diisothiocyanato-2,2'-stilbenedisulfonate). Other investigators have found that PLP is a noncompetitive inhibitor of HIV-1 reverse transcriptase (233,234). On the basis of these in vitro studies, studies in nonhuman primates with vitamin B₆ appear warranted.

Premenstrual Syndrome

Premenstrual syndrome (PMS) is another clinical situation for which vitamin B₆ supplementation has been suggested (235). Estimates indicate that 40% of women are affected by this syndrome (236). Using a wide variety of parameters, no difference in vitamin B₆ status was observed in women with PMS compared to those not reporting symptoms (51,237). Nevertheless, beneficial effects of B₆ ad-

ministration on at least some aspects of PMS have been reported.

Treatment of PMS with vitamin B₆ has been based in part on the studies of Adams et al. (238), in which PN was used to manage the depression observed in some women taking oral contraceptives. Of the several studies in which PN was used to treat PMS, there have been open-type studies and studies that were double-blind and placebo-controlled. Open studies are prone to a placebo effect error, often as high as 40%. Of the well-controlled type, one study showed no effect of pyridoxine therapy (239), whereas three studies reported significant improvement of at least some of the symptoms associated with PMS. In one study, 21 of 25 patients improved (240). Another study found that approximately 60% of 48 women showed improvement with pyridoxine (200 mg/day) and 20% showed improvement with placebo (241). The fourth study (242) reported improvement in some symptoms in 55 women treated daily with 150 mg of pyridoxine. Brush (175) reported results of studies he has conducted using vitamin B₆ alone and vitamin B₆ plus magnesium. His data suggest that doses of 150 to 200 mg of vitamin B₆ are necessary before a significant positive effect is observed. In addition, the combination of vitamin B₆ plus magnesium appears to be beneficial. The complexity of PMS and the subjective nature of symptom reporting continue to result in contradictions and controversy in the lay and scientific literature. Kleijen et al. (243) have reviewed 12 controlled trials in which vitamin B₆ was used to treat PMS. They concluded that there is only weak evidence of a positive effect of vitamin B₆. There may be a decrease in the availability of vitamin B₆ during PMS, possibly due to cell transport competition, from fluctuating hormone concentrations. An increase in vitamin B₆ concentration could overcome competition and may explain the relief of symptoms seen in some women following high-dose vitamin B₆ supplementation.

A review of nine published studies by Wyatt et al. (244) suggested that vitamin B₆ is more effective than placebo, but the quality of the research does not warrant recommending vitamin B₆ supplements. Bendich (245), in her review, also concludes that the evidence for vitamin B₆ supplements being effective is not convincing. A recent study utilizing 50 mg of vitamin B₆ (PN-HC) and 200 mg of magnesium found only a modest effect on PMS symptoms (246).

Sickle Cell Anemia

Low levels (18 μ mol/L) of plasma PLP have been reported in 16 persons with sickle cell anemia (95). Treatment of five of these patients with 100 mg of pyridoxine hydrochloride per day for two months resulted in a reduction of severity, frequency, and duration of painful crises in these persons. The mechanism by which vitamin B₆ acts is not known, but it may be related to pyridoxal and PLP binding to hemoglobin. Nelson et al. (247) observed low serum PLP concentration in 109 children (mean of 15.6 nmol/L) with sickle cell disease (SCD). 4-Pyridoxic measurement indicated that excess urinary vitamin B₆ losses did not contribute to their poor vitamin B₆ status. Balasa et al. (248) also observed a significantly lower concentration of

plasma PLP in children with SCD compared with healthy control children.

Asthma

Depressed levels of plasma and erythrocyte PLP have also been reported in persons with asthma (170). Of significance was the fact that all persons were receiving bronchodilators. Treatment of seven asthmatics with 100 mg of pyridoxine hydrochloride per day resulted in a reduction in the duration, occurrence, and severity of their asthmatic attacks. Subsequent work by one of these authors has not fully supported the earlier findings (249). Treatment of 15 asthmatics with vitamin B₆ did not result in a significant difference in symptom scores, medication usage, or pulmonary function tests as compared to placebo treatment. Ubbink et al. (250) have shown that theophylline lowers plasma and erythrocyte PLP. Pyridoxal kinase is inhibited by theophylline and was responsible for the decreased PLP level in the plasma and presumably intracellularly.

Carpal Tunnel Syndrome

At least five placebo-controlled trials from four different laboratories have shown that administration of PN relieved the symptoms of carpal tunnel syndrome (pain and/or numbness in hands) (251,252). In one study, no significant improvement was observed (253). Because supplementation with vitamin B₆ well in excess of the RDA was required for improvement (generally 50–150 mg), it would seem that individuals with this disorder have a high metabolic demand or that the vitamin is active in some non-coenzyme roles. Two recent studies examined the relationship between plasma PLP and carpal tunnel syndrome. One study (254) found no relationship between symptoms of carpal tunnel syndrome and plasma PLP, but a study by Keniston et al. (255) found a significant inverse univariate relationship between plasma PLP concentration and the prevalence of pain, the frequency of tingling, and nocturnal awakening. The use of vitamin B₆ supplements to treat carpal tunnel syndrome remains controversial. Two reviews (256,257) concluded that the use of vitamin B₆ is not supported. A review by Aufiero et al. (258) offered mixed support for use of vitamin B₆ in large part because of the poor quality of most studies.

Arthritis

Low plasma PLP concentration in patients with rheumatoid arthritis has been observed in several studies (199,259,260). In an animal model of inflammation, a low PLP level in blood and liver was observed, but not in muscle. Pyridoxine supplementation (50 mg/day for 30 days) of arthritis patients was observed to correct their low vitamin B₆ status but did not improve their inflammation measures such as hs-c-reactive protein (261).

Hypertension

In Dahl salt sensitive rats, pyridoxine oxidase and catecholamine-O-methyltransferase showed a highly significant association with blood pressure (262). Vitamin B₆ supplementation (20× normal diet) prevented ethanol-induced hypertension in rats (263). In humans, low plasma PLP was found to be significantly associated with in-

creased systolic blood pressure (264). The numerous factors that can affect plasma PLP concentration (see above in section, assessment of status) suggest that the interpretation of these findings should be viewed with caution. Also, neither vitamin B₆ nor protein or sodium intake was assessed. Feeding a vitamin B₆-deficient or a low-calcium diet to rats results in increased systolic blood pressure (SBP), and correcting the vitamin B₆ deficiency reduced SBP (265). Use of vitamin B₆ supplements (including near physiologic levels) in treating hypertension in humans awaits placebo-controlled trials.

Diabetes

Plasma PLP concentration has been found to be decreased in some diabetic patients (266) but normal in type 1 diabetes mellitus (267) and noninsulin-dependent diabetes (268). C-reactive protein, thought to be a predictor of cardiovascular disease, was found to be inversely associated with plasma PLP concentration in patients with diabetic nephropathy (269). Advanced glycation end products (AGEs) contribute to pathological complications in diabetes. PM has been shown to inhibit advanced glycation reactions and advanced lipoxidation reactions in animal models (270,271). Phase II clinical trials with PM (50 mg and 250 mg, bid) in type 1 and type 2 diabetic patients with nephropathy found mixed efficacy and a favorable safety profile (272,273). In addition to PM inhibiting formation of AGEs, PLP has been shown to inhibit aminophospholipid glycation in vitro (274) and prevent progression of diabetic nephropathy in diabetic rats (275). A high intake of PM in mice fed a high-fat diet has an antioxidative effect and is associated with improvements in glucose intolerance and obesity (84).

Cancer

Vitamin B₆ status and intake have been studied in relation to risk for several cancers given the role of vitamin B₆ in DNA synthesis and steroid hormone action.

Breast

Wu et al. (276) found no evidence between folate, B₆, and homocysteine and breast cancer. In the Nurses's Health Study, plasma PLP concentration was weakly associated with decreased risk of breast cancer (277). In the Women's Health Study, plasma concentration of PLP was not associated with overall risk of breast cancer (278). Dietary intake of folate, B₆, and B₁₂ were not associated with overall breast cancer risk (279,280). In a randomized trial, a combination of a daily intake of folate (2.5 mg), vitamin B₆ (50 mg), and 1 mg of B₁₂ for 7.3 years had no significant effect on overall risk of breast cancer (281).

Prostate

In a group of male smokers, serum PLP concentration was not associated with prostate cancer risk (282). In two separate studies, vitamin B₆ intake was found to be inversely associated with prostate cancer risk (283,284). A prospective study found that plasma PLP concentration was not associated with prostate cancer risk (285).

Colon

Of five studies which have evaluated the intake of vitamin B₆ with colorectal cancer risk, three found no significant association (286–288). Two studies found a significant inverse association (289,290). Consistent with the two latter studies plasma PLP concentration was significantly inversely associated with risk of colorectal cancer (288,291,292). A large case control study (2028 cases; 2722 controls) identified a moderately strong inverse and dose-dependent association between colorectal cancer risk and intake of dietary and total vitamin B₆ (293).

Pancreatic

While a significant inverse relationship between plasma PLP concentration and pancreatic cancer risk has been observed in male smokers (294), no significant association between dietary vitamin B₆ intake and risk of pancreatic cancer was seen in two other studies (295–297).

Lung/Bladder

Hartman et al. (298) found that in men a higher plasma PLP concentration was associated with a lower risk of lung cancer. Vitamin B₆ levels in red blood cells were low in patients with non-small cell lung cancer (299). Compared with controls, bladder cancer patients had a significantly lower intake of vitamin B₆ (300).

Kidney Diseases

Patients with end-stage renal disease and chronic renal insufficiency have low plasma levels of 4-PA (301). Patients undergoing hemodialysis have a decreased plasma PLP concentration (302,303) and may be related to the loss of vitamin B₆ metabolites during dialysis (304). Others have reported significantly elevated plasma PLP, PL, and 4-PA in patients undergoing hemodialysis (304). While idiopathic calcium stone formers have a significantly lower serum level of PLP, no significant correlation was observed between urinary oxalate and urinary 4-PA excretion or serum PLP concentration (305).

Bone

High blood homocysteine concentrations are a risk factor for osteoporosis, and as such, use of vitamin B₆ to lower homocysteine may affect risk for osteoporosis. However, Green et al. (306) found that supplementation with folate, vitamins B₆, and B₁₂, while lowering plasma homocysteine, had no beneficial effect on bone turnover. In contrast, Yazdanpanah (307) found a reduction in risk of fracture in relation to dietary vitamin B₆ intake. Recently, Holstein et al. (308) found that a low serum PLP concentration was associated with an altered morphology of human bone.

Neurologic Conditions

Cognition

Aging is associated with a decline in B vitamin status and cognition (309). Low plasma concentrations of B vitamins were found to predict cognitive decline (310). However, a review of studies on vitamin B₆ and cognition concluded that vitamin B₆ supplementation provided no benefit for improving cognitive function (311).

Depression

Two studies have reported that a low plasma PLP concentration was significantly associated with depression scores (312,313). However, no association between dietary intake of vitamin B₆ and depression has been found (314,315). A review of studies using vitamin B₆ supplementation for treating depression concluded there was no benefit (316).

Seizures

Pyridoxine-dependent seizures are a rare autosomal recessive disorder. Four genetic variants have been identified (317). In one of these, use of PLP supplements was more effective than that of pyridoxine (318).

Alzheimer's Disease

Plasma homocysteine is considered a risk factor for Alzheimer's disease (AD), and thus B-vitamins (including vitamin B₆) may play a role (319). Low vitamin B₆ status (low PLP) has been observed in AD (320,321), but PLP concentration has not been related to risk of AD (319). Low PLP was associated with worse cognitive function (321). Two studies have found that vitamin B₆ intake was not associated with risk of AD (322,323). Low dose (5 mg/day) (324) and high dose (25–50 mg) (325) vitamin B₆ supplementation do not slow cognitive decline. The finding of low PLP concentration and cognitive decline but lack of effect of vitamin B₆ supplements to prevent decline needs further study.

Autism

A combination of high-dose PN and magnesium has been used in children with autism. In a 10-week double-blind placebo-controlled trial, 639 mg PN and 216 mg of magnesium oxide was ineffective in ameliorating autistic behaviors (326). In a study of 33 autistic children (327), improvement in behavior was seen in 15/33 children when given vitamin B₆ (0.6 mg/kg/day or 6 mg/kg/day). A recent Cochrane Database System Review (328) of B₆ magnesium treatment for autism concluded based on the quality of the studies that use of this treatment could not be recommended.

Drug-Vitamin B₆ Interaction

Treatment of persons with various drugs may also compromise vitamin B₆ status and hence result in an increased need for the vitamin. Table 4 lists several drugs and their effect on vitamin B₆ status. Bhagavan has reviewed these interactions in detail (329). A common feature of these drug interactions is their adverse effect on central nervous system function. In addition, many of these drugs react with PLP via Schiff base formation. This reaction can result in decreased levels of PLP in tissues, such as the brain, leading to a functional deficiency. In most cases, supplemental vitamin B₆ reverses the adverse consequences of the drug. Oral contraceptives do not react directly with PLP but do induce enzyme synthesis. Some of these enzymes are PLP dependent, and as a result, PLP is metabolically trapped in tissues. This may then lead to a depressed plasma PLP concentration (330). In addition, the synthetic estrogens specifically affect enzymes of the tryptophan–niacin pathway, resulting in abnormal tryptophan metabolism (100). There may be a need for extra vitamin B₆ above the current RDA in a small proportion

Table 5 Toxicity Symptoms Reported to Be Associated with Chronic Use of High-Dose Pyridoxine

References	Symptoms
Coleman et al. (334)	Motor and sensory neuropathy; vesicular dermatosis on regions of the skin exposed to sunshine
Schaumburg et al. (331)	Peripheral neuropathy; loss of limb reflexes; impaired touch sensation in limbs; unsteady gait; impaired or absent tendon reflexes; sensation of tingling that proceeds down neck and legs
Brush (235)	Dizziness; nausea; breast discomfort or tenderness
Bernstein and Lobitz (335)	Photosensitivity on exposure to sun

of women using oral contraceptives and consuming low levels of the vitamin. Any drug that interacts with the reactive molecule PLP in a Schiff base reaction should be considered an instigator of resultant adverse effects on vitamin B₆ status and a subsequent negative influence on central nervous system function.

Hazards of High Doses

With the therapeutic use of pyridoxine for various disorders and self-medication has come the potential problem of toxicity. Schaumburg et al. have identified several individuals who developed a peripheral neuropathy associated with chronic high-dose use of pyridoxine (331). Subsequent to this, other reports of toxicity related to pyridoxine ingestion have been published (332). The minimal dose at which toxicity develops remains to be determined. Other toxicity symptoms have been identified. These symptoms and those reported by Schaumburg et al. are listed in Table 5. They are relatively rare, and the use of pyridoxine doses of 2 to 250 mg/day for extended periods of time appears to be safe (333).

In rats given high doses of pyridoxine hydrochloride for six weeks, there was a decrease in testis, epididymis, and prostate gland weight at the 500- and 1000-mg/kg dose (336). There was also a decrease in mature spermatid counts. This high intake would be equivalent to 1.5 to 2.0 g of vitamin B₆ for a human (337). Thus, the application of these data to human nutrition is not clear. Additional safety evaluation is found in the DRI guidelines (158). Understanding the mechanism by which a high intake of pyridoxine causes sensory neuropathy has been studied in animal models. Perry et al. (338) found that in rats pyridoxine intoxication causes necrosis of dorsal root ganglion sensory neurons and degeneration of peripheral and central sensory projections. Another study in rats found that a 5-mg/kg injection for 10 to 20 days damaged the cerebral cortex due to decreased synaptic density (339). In a study of patients in a neuromuscular clinic, 26 patients had highly elevated serum PLP levels (mean of 282 nmol/L) (340). A review of toxicology of pyridoxine and other micronutrients is available (341).

CONCLUSIONS

Since vitamin B₆ was first described, a great deal of information about its functional and metabolic characteristics has been gathered. The involvement of the active form, PLP, in such a wide spectrum of enzymatic reactions is an indication of the importance of this vitamin. In addition to

the involvement of PLP in amino acid metabolism and carbohydrate metabolism, its reactivity with proteins points to the diversity of action of this vitamin. Further research is needed on the factors controlling the metabolism of vitamin B₆ and determination of vitamin B₆ needs of specific populations. With knowledge of the functional properties of vitamin B₆ and quantitation of its metabolism under various physiological and nutritional conditions, the health and well-being of individuals can be improved.

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Vitamin B₁₂

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ABBREVIATIONS

AdoCbl, 5'-deoxyadenosylcobalamin; AI, Adequate Intake; ASF, animal source foods; Cbl, cobalamin; CNCbl, cyanocobalamin; CNS, central nervous system; DRI, Dietary Recommended Intake; Hc, haptocorrin; Hcy, homocysteine; IF, intrinsic factor; MCV, mean cell volume; MeCbl, methylcobalamin; MMA, methylmalonic acid; NK, natural killer; OHCbl, hydroxycobalamin; PA, pernicious anemia; RDA, Recommended Dietary Allowance; SC, spinal cord; SCD, subacute combined degeneration; SAM, S-adenosylmethionine; tHcy, total homocysteine; TC, transcobalamin; THF, tetrahydrofolate

INTRODUCTION

Because vitamin B₁₂ is found only in animal source foods (ASF), strict vegetarianism has long been associated with a greater risk of deficiency of this vitamin. The elderly, many of whom lose their ability to absorb vitamin B₁₂, and the small proportion of the population with pernicious anemia (PA) due to lack of intrinsic factor (IF) are also established high-risk groups for this vitamin deficiency. It is generally assumed that clinical symptoms of B₁₂ deficiency take many years to appear after intake or absorption becomes inadequate. However, in recent years, it has become apparent that this deficiency is much more prevalent than previously assumed, affecting a high proportion of people in developing countries and even many lacto-ovo-vegetarians. Considering the number and size of population groups at risk of deficiency, it is important that we determine the most sensitive and specific methods of diagnosing vitamin B₁₂ deficiency, understand its potential adverse functional consequences across the lifespan, and design and evaluate appropriate interventions to improve status.

VITAMIN B₁₂ STRUCTURE AND FUNCTION

Structure

Vitamin B₁₂, or cobalamin (Cbl), is a water-soluble vitamin with a molecular weight of 1355. Symptoms of vitamin B₁₂ deficiency were first described in the early eighteenth century, but the vitamin was not isolated until 1948. The molecule contains a corrinoid ring with a cobalt molecule at its center, beneath which is linked a phosphoribo-5,

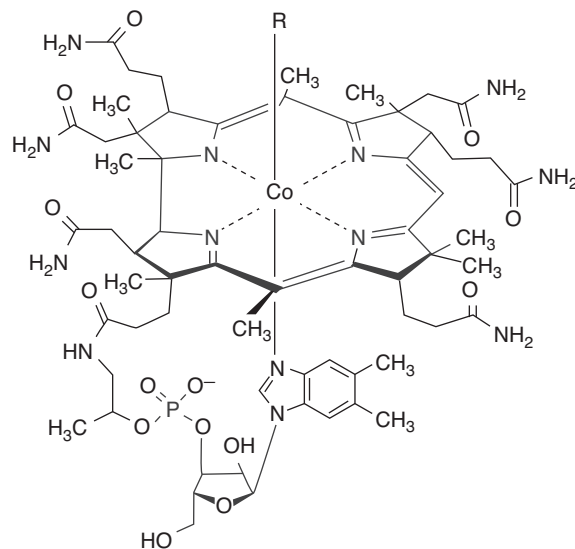


Figure 1 The chemical structure of vitamin B₁₂. Source: From <http://www.engr.psu.edu/wep/EngCompSp98/Aclausi/HodgkinD7.html>.

6-dimethylbenzimidazole side group (Fig. 1). Analogs of Cbl have different structures of the nucleotide and do not retain its active properties. However, the ligands linked to the cobalt atom above the plane of the ring account for the various forms of active Cbl (Table 1).

The two forms of vitamin B₁₂ with metabolic activity are 5'-deoxyadenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl). Hydroxycobalamin (OHCbl) and cyanocobalamin (CNCbl) are also biologically active after conversion to AdoCbl or MeCbl. CNCbl is rare in

Table 1 Cobalamins with Vitamin B₁₂ Activity

R-group	Biological role of cobalamin
-5'-Deoxyadenosyl	Adenosylcobalamin—coenzyme for methylmalonyl CoA mutase
-CH ₃	Methylcobalamin—coenzyme for methionine synthase
-CN	Cyanocobalamin—biologically active upon conversion to AdoCbl or MeCbl
-OH	Hydroxycobalamin—biologically active upon conversion to AdoCbl or MeCbl

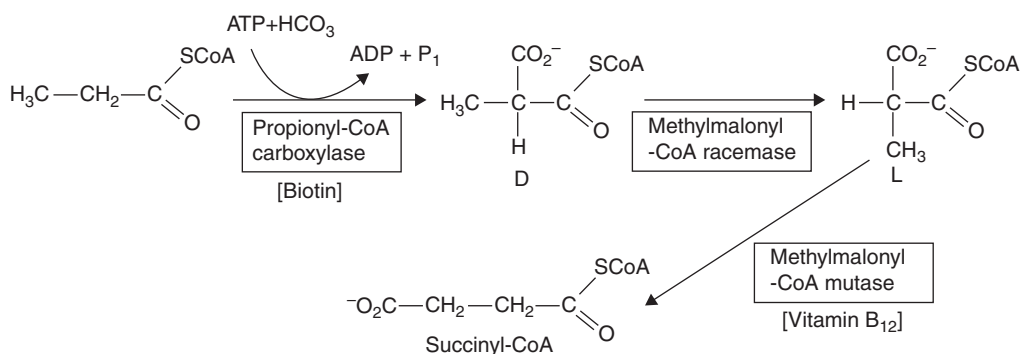


Figure 2 5'-Deoxyadenosylcobalamin is a coenzyme for methylmalonyl CoA mutase, which converts methylmalonyl CoA to succinyl CoA. Source: From: http://heibeck.freeshell.org/NESA/Biochem_Fall_2001/Lipid_Metab.pdf.

nature, but after isolation, it is used in the laboratory and is also the form used in vitamin B₁₂ supplements and fortification. AdoCbl and MeCbl are light sensitive, but CNCbl is relatively stable.

Coenzyme Function

In humans, vitamin B₁₂ functions as a coenzyme for only two reactions in the body, catalyzed by methylmalonyl CoA mutase and methionine synthase. AdoCbl transfers a hydrogen atom in the methylmalonyl CoA mutase reaction, which is required for the conversion of propionyl CoA to succinyl CoA, an integral step in odd-chain fatty acid breakdown (Fig. 2). Propionyl CoA is first converted to methylmalonyl CoA via a carboxylase, after which

AdoCbl-dependent methylmalonyl CoA mutase converts methylmalonyl CoA to succinyl CoA.

Methylcobalamin accepts and donates a methyl group in the second vitamin B₁₂-dependent reaction, in which methionine synthase converts methyltetrahydrofolate (CH₃-THF) and homocysteine (Hcy) to tetrahydrofolate (THF) and methionine (Fig. 3). Methionine is then metabolized to S-adenosylmethionine, a universal methyl donor. THF is further metabolized to methylenetetrahydrofolate (CH₂-THF), which is a cofactor for thymidylate synthetase, the enzyme that converts uracil (dUMP) to thymidine (dTMP). As a cofactor for methionine synthase, vitamin B₁₂ plays an important role in the synthesis of purines, pyrimidines, and amino acids, and in the transfer of methyl groups.

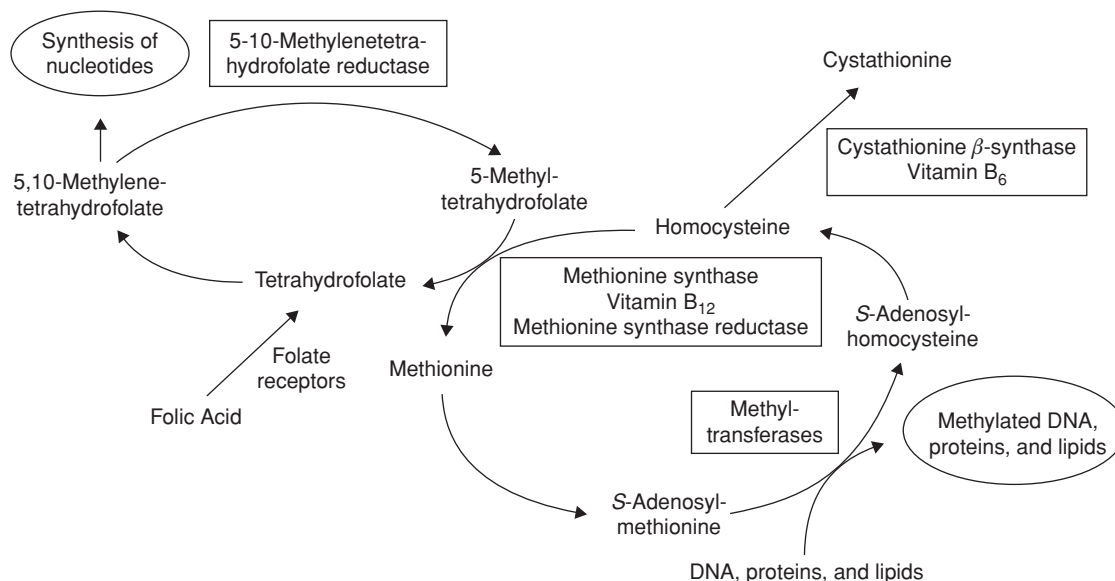


Figure 3 Methylcobalamin is a coenzyme for methionine synthase, which transfers methyl-tetrahydrofolate and homocysteine to tetrahydrofolate and methionine. Source: From Botto LD, Moore CA, Khoury MJ. et al. Neural tube defects. *N Engl J Med* 1999; 341:1515.

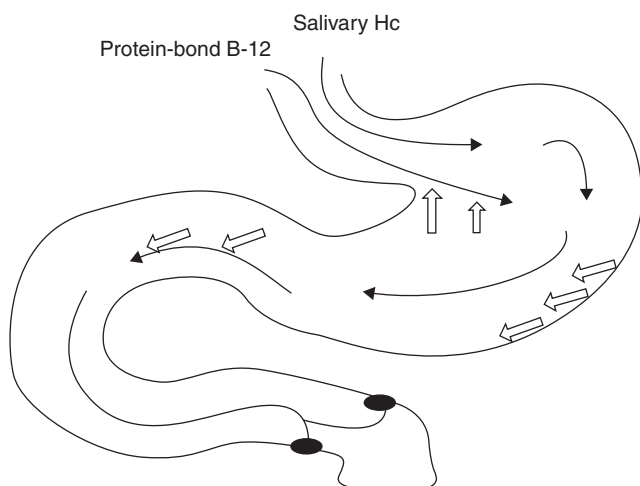


Figure 4 Vitamin B₁₂ digestion requires adequate stomach acidity, and secretion of proteolytic enzymes, haptocorrin and intrinsic factor before receptor-mediated absorption can occur in the ileum.

VITAMIN B₁₂ METABOLISM

Digestion and Absorption

The digestion of vitamin B₁₂ is unique in its complexity (Fig. 4). When Cbl is released from the proteins to which it is attached in food, haptocorrin (Hc), a B₁₂ binder found in the salivary and esophageal glands binds the vitamin. In the stomach, Cbl remains bound to Hc, whereas IF, a second B₁₂-binding protein, is secreted by the parietal cells. Haptocorrin has a higher affinity for Cbl than IF, and must be degraded by proteolytic enzymes from the small intestine before IF can bind the vitamin. In the ileum, Cbl-IF-specific receptors bind the complex, and absorption occurs by endocytosis into endothelial cells, in a calcium-dependent but energy-independent process that takes approximately 3 to 4 hours, with serum concentrations peaking at 7 to 10 hours after consumption. Percentage absorption decreases with the size of the dose as the ileal receptors become saturated. Absorption is ≈50% from a 1 μg dose, 15% from 10 μg, 3% from 25 μg, and 1% from higher doses, due to passive diffusion (1,3). Passive absorption can be important because sufficient amounts of the vitamin can be absorbed passively from large doses (e.g., 500 μg/day) to restore and maintain B₁₂ status in individuals lacking IF. The recommended dietary intake values for the United States/Canada assume that vitamin B₁₂ in food is 50% bioavailable, which is a reasonable assumption about absorption from unfortified meals.

The complexity of the digestive process means that abnormalities can occur at several points. An inability to effectively degrade proteins in food, such as that occurring in achlorhydria, or the atrophic gastritis, which is relatively common in elderly, often prevents release of vitamin B₁₂ from food. Transfer of vitamin B₁₂ from food to Hc, which is dependent on pH and pepsin secretion, may also be compromised. Finally, lack of IF, due to the autoimmune condition PA, will prevent the uptake of the vitamin by the ileal endothelial cells.

Transport Proteins

Transcobalamin

After absorption into the endothelium, IF is degraded, and free vitamin B₁₂ is bound to transcobalamin II (usually known as transcobalamin, TC), which then transports B₁₂ through the plasma. TC is produced locally by many cells and plays a role in cellular export of vitamin B₁₂, as well as plasma transport. It is the only one of the three plasma B₁₂-binding proteins (transcobalamins I, II, and III), which is responsible for receptor-mediated uptake of B₁₂ into cells and constitutes approximately 10% to 20% of the total plasma B₁₂. Transcobalamin is cleared from the plasma by the kidney, liver, heart, lung, spleen, and intestine.

Haptocorrin

Approximately 75% of plasma vitamin B₁₂ is bound to haptocorrin (Hc, transcobalamins I + III), which has a half-life of 9 to 10 days and can be thought of as a circulating store of the vitamin. Haptocorrin is produced in red blood cell precursors, hepatoma cells, salivary glands, and granulocytes, and is largely unsaturated. An absence of Hc protein does not alter vitamin B₁₂ metabolism detrimentally, but an inability to produce TC results in symptoms of vitamin B₁₂ deficiency, including megaloblastic anemia and neurological abnormalities. Several genetic polymorphisms have been identified in proteins involved in the metabolism of vitamin B₁₂, which may lead to reduced plasma concentrations of vitamin B₁₂ (4).

Excretion and Storage

Vitamin B₁₂ is excreted through the urine, bile, and feces. Enterohepatic recirculation of vitamin B₁₂ is efficient, with 65% to 75% reabsorbed (1), and therefore plays an important role in maintaining adequate Cbl status. When vitamin B₁₂ is radiolabeled in the dimethylbenzimidazole ring, but not when its cobalt is labeled, large amounts of radioactivity appear in the urine. This difference suggests that the dimethylbenzimidazole group is split from the corrinoid ring in any unabsorbed vitamin, perhaps by intestinal bacteria (5). Vitamin B₁₂ is stored in the liver, which in an adult human may contain 1 to 2 mg of a 2 to 5 mg total body pool.

VITAMIN B₁₂–NUTRIENT INTERACTIONS

Vitamin B₁₂ and Folate

Both vitamin B₁₂ and folate are involved in the methionine synthase pathway (Fig. 3). According to the “folate trap” hypothesis, CH₃-THF builds up in excess when vitamin B₁₂ deficiency prevents methionine synthesis from proceeding. Although it was originally thought that vitamin B₁₂ deficiency trapped CH₃-THF intracellularly, it is possible that Cbl-deficient cells fail to retain intracellular CH₃-THF. This theory is supported by data showing a rise in plasma folate concentration in vitamin B₁₂-deficient animals and humans (6). Inadequate availability of folate coenzyme (CH₂-folate) for DNA synthesis, due to folate or B₁₂ deficiency, can produce megaloblastic anemia. However, folate deficiency does not produce the neuropathy that accompanies strict vitamin B₁₂ deficiency. There is considerable debate about whether high doses of folic acid, consumed through supplements or high levels used

Table 2 Animal Source Foods Rich in Vitamin B₁₂

ASF	B ₁₂ (μg/100 g)
Beef liver, fried	83.0
Chicken liver, fried	21.0
Beef, cooked	1.3–4.0
Pork, cooked	0.7
Turkey, cooked	0.4
Chicken, cooked	0.3
Tuna, canned in oil	2.2
Egg, fried	1.4
Cheese	0.4–1.7
Milk, whole	0.5

Source: From USDA National Nutrient Database <http://www.nal.usda.gov/finic/cgi-bin/nut-search.pl>.

in food fortification, exacerbate symptoms of vitamin B₁₂ deficiency (7,8).

DIETARY REQUIREMENTS AND SOURCES

Food Sources

Vitamin B₁₂ is synthesized by microorganisms, but not plants and animals, and humans depend on ASF, fortified foods, and supplements for dietary B₁₂. Organ meats, beef, pork, poultry, fish, shellfish, eggs, and dairy products are rich sources of vitamin B₁₂ (Table 2). Cbls from other sources including algae and yeast are probably not biologically active (1). Although dietary requirements are minimal when compared with those of other micronutrients, the facts that vitamin B₁₂ is present in a limited number of food sources and that the digestive process is complex make deficiency a risk for certain populations. This certainly includes strict vegetarians, who lack sources of vitamin B₁₂ in their diet (9), and the elderly, who may have an impaired ability to absorb the vitamin from food sources due to gastric atrophy. There are also multiple reports of lower plasma vitamin B₁₂ concentrations in lacto-ovo-vegetarians than in omnivores (3,10). Populations in developing countries are also at risk for vitamin B₁₂ deficiency, due to the high cost and low availability of ASF, and a lack of fortified foods and supplements. (See the section on "Vitamin B₁₂ Deficiency.")

Dietary Reference Intakes

Daily loss of vitamin B₁₂ is estimated to be 0.1% of the total body pool (1). Assuming a total body pool of 2 to 5 mg, the daily losses for adults would be 2 to 5 μg. Due to the low ratio of daily losses to the total body pool, vitamin B₁₂ deficiency may take several years to develop after the removal of ASF, vitamin B₁₂ fortified foods, and supplements from the diet. This time could be substantially shorter in people who reabsorb less of the vitamin by enterohepatic recirculation, due to lower output in bile as a result of depleted liver stores or malabsorptive disorders. It has been estimated that with a daily turnover rate of 0.1% per day, it can take from 1.5 to 11.6 years to see signs of B₁₂ deficiency depending on initial liver B₁₂ stores (1).

The adult recommended dietary allowance (RDA) of 2.4 μg is based on the intake levels required to maintain hematological status and normal vitamin B₁₂ plasma concentrations (1). The RDA in pregnancy increases to 2.6 μg

Table 3 Dietary Recommended Intakes of Vitamin B₁₂

Age group	DRI B ₁₂ (μg/day)	Data used to determine DRI
Infants, 0–6 mo	0.4	AI of breastfed infants
Infants, 7–12 mo	0.5	AI extrapolated from AI of infants, 0–6 mo
Children, 1–3 yr	0.9	RDA extrapolated from RDA for adults
Children, 4–8 yr	1.2	RDA extrapolated from RDA for adults
Children, 9–13 yr	1.8	RDA extrapolated from RDA for adults
Adolescents, 14–18 yr	2.4	RDA extrapolated from RDA for adults
Adults, 19–50 yr	2.4	RDA based on intake required to maintain hematological status and plasma B ₁₂ concentration
Adults, over 50 yr	2.4	RDA based on intake required to maintain hematological status and plasma B ₁₂ concentration
Pregnant women	2.6	RDA based on adult RDA plus the amount of B ₁₂ deposited into the fetus daily
Lactating women	2.8	RDA based on adult RDA plus the amount of B ₁₂ secreted into breast milk daily

Abbreviations: DRI, dietary recommended intake; AI, adequate intake; RDA, recommended dietary allowance.

due to transfer of newly absorbed B₁₂ to the fetus, and to 2.8 μg during lactation to cover secretion of B₁₂ into breast milk. There is no tolerable upper level of intake as no negative consequences have been associated with excessive vitamin B₁₂ consumption.

The recommended intake of 0.4 μg for infants is based on the average intake of infants fed principally with breast milk (1). The intake estimates assume that breast milk concentration averages 0.42 μg/L milk, based on a review of B₁₂ concentrations in the milk of well-nourished women. However, vitamin B₁₂ is tightly bound to haptocorrin in human milk, and a newer method developed to achieve its full release from this binding indicates that concentrations in milk from well-nourished women need to be revised (11). The AI of infants aged 7 to 12 months is extrapolated from the requirement of 0- to 6-month-old infants. The remaining RDAs for children and adolescents are extrapolated down from adult values, as sufficient data on intake within these groups are lacking. Table 3 summarizes the current daily recommended intakes for all age groups.

VITAMIN B₁₂ STATUS THROUGHOUT THE LIFE CYCLE

The Pregnant Woman

Intestinal absorption of vitamin B₁₂ is increased during pregnancy (12), although an overall fall in maternal plasma vitamin B₁₂ is observed and accompanied by a fall in Cbl binders. As many as 15% to 30% of women may have low plasma vitamin B₁₂ during the third trimester of pregnancy, but concentrations rise sharply postpartum; therefore, hemodilution, which increases plasma volume by approximately 50%, may account for some of this transient decrease. However, the fact that users of oral contraceptives also have lower plasma B₁₂ concentrations (13) suggests that hemodilution alone may not be entirely

responsible. Low plasma B₁₂ during pregnancy is less likely to reflect a true deficiency in women with diets containing adequate ASF, and most pregnant women with low plasma B₁₂ concentrations do not exhibit other signs of deficiency (14).

Insufficient research has been conducted on the relationship between maternal vitamin B₁₂ status and pregnancy outcome. Importantly, low-serum vitamin B₁₂ (15) or TC (16) are associated with increased risk of neural tube defects. However, cord blood vitamin B₁₂ (but not maternal plasma vitamin B₁₂) was correlated with birth-weight in a study of 188 pregnant women (17). Moreover, homocysteinemia is a risk factor for numerous adverse pregnancy outcomes including birth defects (18) and preeclampsia (19); it is thus reasonable to assume, but not yet proven, that maternal vitamin B₁₂ deficiency could have adverse effects on pregnancy outcome. In rural Nepal, where 65% of a group of pregnant women had low plasma B₁₂ concentrations, homocysteinemia and low plasma B₁₂ were associated with a doubling of preeclampsia and preterm delivery (20). At 5 years of age, Indian children born to mothers in the lowest tertile of serum B₁₂ and the highest tertile of serum folate had about twice the risk of insulin resistance of those from mothers in the lowest tertile of serum folate and the highest tertile of serum B₁₂, raising the question of potential risk due to B₁₂-folate imbalance during pregnancy (21).

The Neonate and Infant

Up to 60% of the vitamin B₁₂ absorbed in pregnancy is concentrated in the fetus, and the rate of transfer increases throughout pregnancy (22). At birth, the total body content of vitamin B₁₂ is approximately 50 µg, about half of which is stored in the liver. Plasma vitamin B₁₂ concentration is usually twice that of the mother, but may be more than fourfold higher. When mothers suffer from vitamin B₁₂ deficiency, which may be highly prevalent in developing countries, their infants are also likely to have low vitamin stores, a phenomenon that can be improved by maternal supplementation with the vitamin.

After the neonatal period, the plasma vitamin B₁₂ concentration of the infant begins to decline (23). Based on a requirement of 0.1 µg vitamin B₁₂ per day for tissue synthesis, the neonatal body stores can last approximately 8 mo even if vitamin B₁₂ is completely absent from the diet. However, this assumes that the infant is born with adequate stores, which may not be the case for infants born to vitamin B₁₂ deficient mothers.

Breast Milk as a Source of Vitamin B₁₂

Human milk has an impressive unbound Cbl-binding capacity, approximately 1000 times greater than that of plasma, due to its high concentration of Hc. The large amount of Hc in milk may suppress Cbl-dependent intestinal microorganisms, such as *Escherichia coli*, because Hc-bound Cbl is unavailable to microorganisms.

Colostrum contains vitamin B₁₂ in excess, after which breast milk Cbl content decreases. Breast milk may contain 100 to 2000 pmol B₁₂/L, but on average, reported values for well-nourished women's milk are 300 to 600 pmol B₁₂/L throughout the lactational period. Recent improvements in measurement of the vitamin in breast milk

suggest that these values may be incorrect (see the section on "Dietary Reference Intakes"). In women with a low B₁₂ intake, concentrations are lower and often correlated with maternal plasma B₁₂ values. About half of depleted Guatemalan women had no detectable vitamin B₁₂ in their breast milk at 12 months postpartum (24). There are many case reports of breastfed neonates diagnosed with clinical signs of vitamin B₁₂ deficiency as a result of maternal veganism; both low infant stores at birth and low breast milk B₁₂ would contribute to this serious situation. The adverse effects of more severe vitamin B₁₂ deficiency on child development are not completely reversible by supplementation, in about half the cases (25).

VITAMIN B₁₂ DEFICIENCY

Methods for Evaluating Vitamin B₁₂ Status

The accepted cutoff point for a plasma (or serum) vitamin B₁₂ concentration that defines deficiency has been typically set as 148 pmol/L (200 pg/mL), and individuals with values below this point may show symptoms of deficiency. A plasma B₁₂ concentration between 148 and 220 pmol/L (200–300 pg/mL) is often used to designate marginal deficiency (1). In a study of infants, plasma methylmalonic acid (MMA) was markedly elevated (indicating vitamin B₁₂ deficiency) when plasma vitamin B₁₂ was less than 220 pmol/L (26), and in groups ranging from elderly adults (27) to Guatemalan schoolers (28), MMA increases when plasma B₁₂ falls below about 265 pmol/L (350 pg/mL). By using a cutoff of 300 pmol/L plasma vitamin B₁₂ to identify potential cases of B₁₂ deficiency, plasma B₁₂ had a diagnostic sensitivity of 0.40 and specificity of 0.98, based on elevated plasma MMA (>0.34 µmol/L) to confirm diagnosis (29). Thus, plasma B₁₂ is a reasonable, but not perfect, indicator of risk of vitamin B₁₂ deficiency, and low concentrations should generate concern.

Diagnosis of B₁₂ status by using plasma vitamin B₁₂ can be supplemented with additional assays for the metabolites MMA and Hcy, which become elevated in deficiency. MMA increases in plasma and urine due to an inability to convert methylmalonyl CoA to succinyl CoA via methylmalonyl CoA mutase. Normal plasma MMA concentrations are in the nanomole range, but in vitamin B₁₂ deficiency they may be in the micromole range. Impaired renal function also increases plasma MMA so measures of serum creatinine should be included to check for this condition, especially in the elderly. Total homocysteine (tHcy) may also be elevated in B₁₂ deficiency due to the inability of methionine synthesis to proceed (Fig. 3). However, although elevated MMA is specific to vitamin B₁₂ deficiency, it is not analyzed routinely due to the need for specialized equipment and its high cost. Elevated Hcy may be a product of folate deficiency, or disease, and its use as a tool to specifically diagnose vitamin B₁₂ deficiency is limited.

Causes of Deficiency

Poor absorption and inadequate ingestion are the chief causes of vitamin B₁₂ deficiency (3,30). For individuals in affluent settings, inadequate ingestion is less likely than poor absorption when consumption of ASF is relatively frequent. When ASF intake is limited, however, the risk

Table 4 Commonly Used indicators of Vitamin B₁₂ Deficiency

Marker	Cut-off	Specificity
Plasma vitamin B ₁₂	Deficient: < 148 pmol/L Marginal: < 220 pmol/L	Specific to B ₁₂ status
Plasma MMA	>0.28 μmol/L	Specific to B ₁₂ status
Plasma tHcy	>12 μmol/L	Nonspecific, also elevated in folate, riboflavin and B ₆ deficiency
MCV	>95 fL	Nonspecific, also elevated in folate deficiency
Neutrophil hypersegmentation	>5 five-lobed neutrophils	Nonspecific, also elevated in folate deficiency

Abbreviations: MMA, methylmalonic acid; tHcy, homocysteine.

of deficiency derives from low intake of the vitamin, although in the elderly the main cause of deficiency is usually poor absorption.

Inadequate Absorption

Inadequate absorption of vitamin B₁₂ is widely accepted to be the principal cause of vitamin B₁₂ deficiency in affluent countries and may occur for several reasons. First, in PA, the lack of IF leads to an inability to absorb the vitamin through the IF-Cbl receptor process. However, only approximately 2% to 4% of the elderly, for example, have PA (31). Second, conditions that alter intestinal function, such as achlorhydria and lack of enzymes such as pepsin, can lead to inefficient absorption of B₁₂ from food. Medications that reduce gastric acid secretion and gastrectomy will also reduce absorption of the vitamin. Third, overgrowth of intestinal bacteria competing for the vitamin may lead to deficiency, a cause that is more important in conditions such as tropical sprue and after some types of intestinal resection. One study found that patients with bacterial overgrowth due to atrophic gastritis absorbed significantly less protein-bound B₁₂ than control subjects, but that antibiotic therapy rapidly normalized absorption (32).

In the elderly, infection with *Helicobacter pylori*, in particular, is thought to contribute to B₁₂ deficiency by causing atrophic gastritis, lack of gastric acid, and, in its final stages, a lack of IF. Subsequently, there is impaired absorption of food-bound B₁₂ (33). Vitamin B₁₂ deficiency associated with malabsorption is common in elderly populations and prevalence is often high in this group. In the National Health and Nutrition Education Survey of the United States, deficiency (serum B₁₂ < 148 pmol/L) was present in ≈6%, and marginal status (serum B₁₂ 148–221 pmol/L) in >20%, of persons over 60 years of age (34).

Inadequate Ingestion

Dietary vitamin B₁₂ deficiency has been described in affluent populations, traditionally in exceptional communities that practice religious dietary restrictions, or adhere to strict dietary guidelines, such as macrobiotic or vegan diets. Hindus, for example, often restrict intake of meat, or meat and eggs, and have a higher prevalence of deficiency. More recent examination of the evidence indicates that plasma vitamin B₁₂ concentrations are actually related to intake over a wide range but plateau at an intake >10 μg (3). Thus lacto-ovo- or lacto-vegetarians (who consume animal products, but not meat) are also at risk for developing deficiency. In a study of German vegetarians, 60% had evidence of elevated plasma Hcy and MMA (35). Elevated

MMA was found in 5% of the omnivores, 77% of the lacto-ovo-vegetarians, and 83% of the vegans. Mean plasma vitamin B₁₂ concentrations of lacto-vegetarians were substantially lower than those of nonvegetarians in studies in India (36). Case reports of diet-induced vitamin B₁₂ deficiency have been made in teenagers and incidence of severe infant deficiency associated with maternal dietary restriction has also been reported. In a study of macrobiotic children (mean age 6.4 years) who had followed a strict macrobiotic diet in early childhood but had been omnivorous since that time, MMA and Hcy were elevated and cognitive function was altered (37).

In developing countries, diet-induced vitamin B₁₂ deficiency is much more common, especially in low socioeconomic status groups where ASF intake is limited by income (38). Widespread vitamin B₁₂ deficiency and depletion have been reported in many countries in Latin America, Africa, and Southeast Asia, where a predominantly plant-based diet is consumed (39). The reported prevalence of low plasma B₁₂ values in various countries in Latin America was approximately 40% across the lifespan, and in both sexes. More than one-half of pregnant Nepali women had elevated Hcy and MMA (20). In a group of vegetarian and nonvegetarian adults living in Pune, India, B₁₂ deficiency was detected in 47%, based on low plasma B₁₂, and in 73%, based on elevated MMA (36).

Consequences of Deficiency

Clinical symptoms of deficiency in adults are often nonspecific, and include fatigue, numbness, apathy, listlessness, diarrhea, and anorexia. Some patients experience oral discomfort, such as soreness of the tongue or ulceration. Although initial clinical symptoms may be vague, nevertheless, dramatic hematological, neurological, and immunological changes may occur (Table 4).

Hematological Changes

The hematological consequences of vitamin B₁₂ deficiency include megaloblastic anemia due to a reduced capacity to synthesize DNA rapidly, caused by alterations in the methionine synthase pathway. Hemoglobin develops at a normal pace but mitosis lags behind. As a result, RBC production is deranged and an abnormally large nucleus is extruded, leaving behind a large cytoplasm-filled cell. A mean corpuscular volume >115 fL defines megaloblastic cells, which may be as large as 130 to 150 fL. Total erythrocyte B₁₂ does not change as the blood cell matures because the nucleus of the red cell is extruded and B₁₂ is largely present in this organelle. Although megaloblastic anemia

is recognized as a classic symptom of vitamin B₁₂ deficiency, many individuals may not experience measurable hematological change. In addition, megaloblastic anemia is a nonspecific outcome of B₁₂ deficiency, as folate deficiency induces the same hematological changes through the same pathway. Although the presence of megaloblastic anemia may alert clinicians to the need to assess folate and B₁₂ status, it is important to recognize that the anemia occurs at a much later and more severe stage of B₁₂ depletion. There is little evidence that supplementation of depleted, nonvegan populations improves hemoglobin synthesis although there are few data on this question (40). Indicators of status, such as MMA and plasma vitamin B₁₂, are more sensitive to improved B₁₂ status.

Neuropathies and Cognitive Performance

In the elderly, vitamin B₁₂ deficiency produces subacute combined degeneration, a syndrome of irregular spongiform demyelination of spinal cord (SC) white matter and astrogliosis. Deficiency in the elderly primarily affects change in the SC, whereas in infants the central nervous system is damaged. In fact, vitamin B₁₂ deficiency in this age group can produce severe brain damage that may not be completely reversible upon therapy (25).

Common domains of neural symptoms in vitamin B₁₂-deficient elderly are (i) sensory (paresthesias, diminished proprioception, diminished vibratory sensation); (ii) motor (weakness); (iii) reflex disorder related; (iv) autonomic (incontinence, impotence); (v) gait-related (ataxia); (vi) mental (intellectual/behavioral impairment); and (vii) visual (impaired visual acuity) (41). Deficiency is often resolved, and symptoms reversed, with vitamin B₁₂ therapy. Either megadoses of the vitamin can be injected IM or large doses (500–1000 µg/day) of crystalline B₁₂ can be taken orally. Because 1% of the vitamin can be passively absorbed without the need for IF, oral treatment is effective for many patients with PA or deficiency caused by gastric atrophy, and consumption of foods fortified with B₁₂ predicts higher plasma B₁₂ in the elderly (42). Demyelination of nerves associated with SCD is likely responsible for the majority of symptoms experienced by the elderly.

A systematic review addressed the question of whether vitamin B₁₂ supplementation improves cognitive function in adults (43). From the relatively small number of trials conducted, only some of which were randomized and controlled, the conclusion was drawn that oral vitamin B₁₂ is not effective for improving cognitive function, although efficacy of IM supplements could not be ruled out. However, the studies included participants over a range of ages, cognitive function, and vitamin B₁₂ status at baseline and confounding factors such as disease duration were not often considered. In contrast, another review concluded that on the basis of more sensitive markers of status (plasma TC and MMA), cognitive function in elderly was associated with B₁₂ status across the normal range (44). Deficiency of the vitamin could cause brain atrophy or white matter damage. Additional studies of this important topic are ongoing.

There are numerous case reports of severe B₁₂ deficiency in infants of mothers with PA or mothers practicing a vegan/lacto-vegetarian diet (25). Symptoms include regression of mental development, abnormal pigmentation, hypotonia of muscles, enlarged liver and spleen, sparse

hair, tremors, irritability, anorexia, failure to thrive, poor brain growth, refusal of solid foods, and diarrhea. Marked cerebral atrophy and ventricular enlargement may also be present. Onset of deficiency in infants occurs within a few months of dietary absence and patients are often responsive to high-dose treatment. Vitamin B₁₂ deficiency in infancy may have long-term consequences. Although there are virtually no systematic follow-up studies, it is estimated that about half of the cases with clinical symptoms do not achieve full recovery. In the Netherlands, infants aged 4 to 18 months who were born to macrobiotic mothers developed psychomotor skills later than omnivorous controls (45).

Immune Function

In developing countries, both immunomodulation associated with malnutrition and repeated exposure to infection promote high rates of morbidity and mortality. An immunomodulating role specific to vitamin B₁₂ has been reported. Markers that may be influenced by vitamin B₁₂ status include (i) complement component C3; (ii) CD4 and CD8 T cell counts, and CD4/CD8 ratio; (iii) natural killer (NK) cell activity; and (iv) TNF-α concentration. Low lymphocyte counts, elevated CD4 cells, decreased CD8 cells, elevated CD4/CD8 ratio, and suppressed NK cell activity have been observed in B₁₂ deficiency. Therapy restored several of these abnormal values (46). Vegans in the United States have signs of compromised immune function, including lower leukocyte counts and C3, even when micronutrient levels appear normal (47). The extent to which vitamin B₁₂ deficiency is responsible for such changes in immune function requires further exploration.

Bone Health

A number of studies report an association between low plasma B₁₂ and/or elevated tHcy concentrations with markers of bone mineral loss during aging. In the third National Health and Nutrition Examination Survey in the United States, mean age 68 years, at each higher quartile of serum MMA bone mineral density was lower and osteoporosis increased (48). Those in the highest MMA quartile had a 7.2-fold greater risk of osteoporosis compared with the lowest MMA quartile. Serum B₁₂ was related to bone mineral density up to ≈200 pmol/L, and those with tHcy >20 µmol/L had lower bone mineral than those with values <10 µmol/L. Poor bone mineralization also occurs in untreated pernicious anemia but is preventable by supplementation. Together these data suggest that vitamin B₁₂ deficiency leads to poor bone mineralization but randomized controlled trials are needed for confirmation.

CONCLUSIONS

This review has highlighted several new aspects of our knowledge of vitamin B₁₂. The more important issues include the much higher global prevalence of this deficiency than is generally recognized and the fact that even those who avoid meat but eat other ASF are at higher risk of depletion. Deficiency also occurs more rapidly than was formerly believed, especially in people whose stores are relatively depleted or who malabsorb the vitamin. At the same time, our understanding of the mechanisms that cause the

adverse functional consequences of deficiency is at a relatively primitive stage, and previously unknown adverse consequences are being identified. Because vitamin B₁₂ can be safely added as a fortificant to food, or taken orally in high doses, greater attention should be paid to ensuring that this nutrient deficiency is prevented, detected and treated in at-risk groups. There is considerable interest in the potential value of adding vitamin B₁₂ as a fortificant to flour in populations with a high prevalence of inadequate intakes and low plasma concentrations (39), but further research is needed to confirm the effectiveness of this strategy (49).

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Vitamin C

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ABBREVIATIONS

AI, adequate intake; AUC, area under curve; DHA, dehydroascorbic acid; DRI, dietary reference intake; EAR, estimated average requirement; GLUT, facilitated glucose transporter; HIF, hypoxia inducible factor; IV, intravenous; LDL, low density lipoprotein; RDA recommended dietary allowance; SVCT, sodium-dependent vitamin C transporter; UL, upper limit.

INTRODUCTION

Vitamin C (L-ascorbic acid, ascorbate) is a water-soluble micronutrient essential for human health. It is a six-carbon lactone with a molecular weight of 176 (Fig. 1) (1). Humans and other primates cannot synthesize ascorbate because of multiple mutations in the gene encoding gulonolactone oxidase, the terminal enzyme in the biosynthetic pathway of the vitamin. Thus, human survival is dependent on obtaining vitamin C from foods.

BIOCHEMISTRY AND FUNCTIONS

Vitamin C is an electron donor, and this property accounts for its known and postulated functions. As an antioxidant, or reducing agent, the vitamin sequentially donates two electrons from the C2–C3 double bond. The first intermediate, formed by the loss of one electron, is the ascorbate free radical. Stability of the radical is dependent on availability of electron acceptors, especially iron or copper. In the absence of such acceptors, ascorbate radical is relatively unreactive and does not interact with other compounds to form potentially harmful free radicals, and can be reversibly reduced to ascorbate. In the presence of electron acceptors, ascorbate radical undergoes further oxidation to form dehydroascorbic acid (DHA) (Fig. 1), which can be reduced back to ascorbate by either glutathione or three distinct enzymatic reduction reactions (2,3). If not reduced, DHA undergoes ring rupture and is irreversibly hydrolyzed to 2,3-diketogulonic acid. The latter is further metabolized to xylose, xylonate, lyxonate, and oxalate. Oxalate is a potentially clinically significant end product of vitamin C metabolism, which under aberrant conditions may accumulate as a component of kidney stones.

Enzymatic Functions

Vitamin C is a cofactor for enzymes in mammals that use molecular oxygen (Table 1). Ascorbate facilitates enzy-

matic hydroxylation reactions necessary for the biosynthesis of collagen, carnitine, and a noradrenergic neurotransmitter. In addition, ascorbate is a cofactor for monooxygenase enzymes that catalyze the amidation of peptide hormones. Ascorbate is a cofactor, via hydroxylation of proline, for hypoxia-inducible factor isoenzymes, which are master regulators of oxygen sensing and metabolism. It is assumed that scurvy, the disease caused by vitamin C deficiency, is due to impaired functioning of at least some of these enzymes, although direct experimental proof is lacking. It is unclear whether other electron donors can replace vitamin C as the reducing agent in some of these catalytic reactions in vivo.

Reducing (Nonenzymatic) Functions

Vitamin C as an Antioxidant in Vitro

Vitamin C may have nonenzymatic functions due to its reduction–oxidation (redox) potential (Fig. 1, Table 1). When a substance provides an electron in a chemical reaction, it is referred to as a reductant (or reducing agent). Ascorbate in this role is therefore often referred to as an antioxidant. This has been demonstrated in many test tube and in vitro cell culture experiments (20). Such data suggest that ascorbate might protect cellular proteins from oxidation and act as the primary antioxidant in plasma for quenching aqueous peroxy radicals and lipid peroxidation products. In vitro, vitamin C is preferentially oxidized before other plasma antioxidants such as uric acid, tocopherols, and bilirubin (21). However, these oxidation–reduction reactions may not specifically require ascorbate in vivo. Vitamin C may quench oxidants generated by activated leukocytes, such as neutrophils and macrophages that, in turn, may damage supporting tissues. Many antioxidant effects demonstrated in vitro have uncertain importance in the intact organism (20).

Effects on Atherogenesis

Vitamin C can theoretically reduce atherogenesis by protecting low-density lipoprotein (LDL) from metal-catalyzed oxidation and by affecting monocyte adhesion and platelet aggregation. In vitro experiments have shown that ascorbate inhibits metal-catalyzed oxidation of LDL at concentrations above 40 to 50 μ M (22). However, the actual contribution of metal catalysts (such as copper and iron) to oxidative pathology in vivo is difficult to simulate experimentally. In contrast to the test tube, multiple safeguards operate in the intact organism to control metal-catalyzed oxidation reactions, so that the contribution of ascorbate relative to other systems is difficult to ascertain in vivo. In depletion–repletion studies, no significant

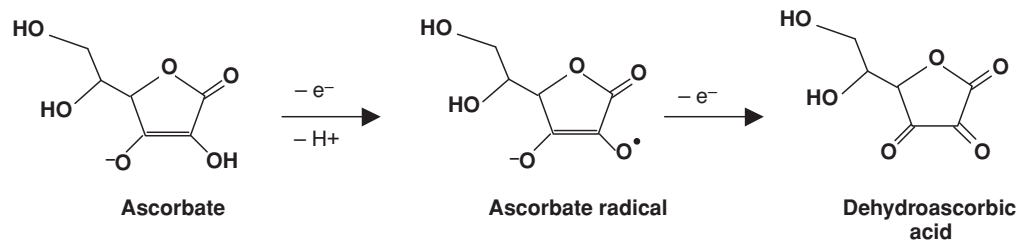


Figure 1 Reaction scheme for the oxidation of vitamin C. The predominant form in biology is the fully reduced ascorbate molecule (left). Oxidation of ascorbate begins with the loss of one electron (e^-) and a proton (H^+) to yield the ascorbate radical (middle). As with all radicals, the ascorbate radical has an unpaired electron (\bullet). Although the radical is depicted at the C2 oxygen, in fact is delocalized in resonance between the oxygen atoms at the C1, C2, and C3 positions. Loss of a second electron results in the formation of the fully oxidized molecule dehydroascorbic acid (right). Ascorbate is present in great abundance relative to either ascorbate radical or dehydroascorbic acid. Therefore the equilibrium favors oxidation, so that reactions proceed from left to right. Dehydroascorbic acid is an inherently unstable molecule subject to degradation. Dehydroascorbic acid exists in multiple forms, and only one is shown for simplicity.

relationship was found between vitamin C dose and plasma concentrations of F_2 -isoprostanes, which are considered as biomarkers of endogenous lipid peroxidation (23). Another potential protective mechanism is indirect, as vitamin C can regenerate oxidized α -tocopherol (vitamin E) in LDL in vitro. Additional effects of extracellular vitamin C in atherosclerosis could be due to its action

on adhesion of monocytes to endothelium or aggregation of platelets and leukocytes. Again, these effects have not been shown in vivo, and their clinical relevance is unclear. Although laboratory data show a possible protective role for vitamin C in atherosclerotic heart disease, epidemiologic data are inconsistent. Diets rich in fruits and vegetables, and therefore rich in vitamin C, are associated with

Table 1 Putative Enzymatic and Nonenzymatic Effects of Vitamin C in Humans are Listed in the Table

Cofactor for enzymes	
Enzyme	Function of enzyme
Dopamine β -monooxygenase	Norepinephrine biosynthesis (4)
Peptidyl-glycine α -amidating monooxygenase	Amidation of peptide hormones (5)
Prolyl 4-hydroxylase	Collagen hydroxylation (6) Hypoxia-inducible factor (HIF) hydroxylation (7)
Three collagen isoenzymes	
Four hypoxia inducible factor (HIF) isoenzymes	
Prolyl 3-hydroxylase	
Lysyl hydroxylase	
Trimethyllysine hydroxylase	Carnitine biosynthesis (8)
γ -Butyrobetaine hydroxylase	
4-hydroxyphenylpyruvate dioxygenase	Tyrosine metabolism (9)
Reducing agent	
Site	Action
Small intestine	Promote iron absorption (10)
Antioxidant	
Site	Action
Cells	Regulate gene expression and mRNA translation, prevent oxidant damage to DNA and intracellular proteins (11,12,13)
Plasma	Increase endothelium dependent vasodilatation, reduce extracellular oxidants from neutrophils, reduce low density lipoprotein oxidation, quench aqueous peroxy radicals and lipid peroxidation products (14)
Stomach	Prevent formation of N-nitroso compounds (15)
Prooxidant	
Target	Effect
DNA	DNA damage (16)
Lipid hydroperoxidase	Decomposition of lipid peroxidase leading to DNA damage (17)
Ascorbyl radical targets	Damage to some cancer cells (18,19)

Note: Three enzymes (prolyl 4-hydroxylase, prolyl 3-hydroxylase, and lysyl hydroxylase) take part in collagen biosynthesis. Prolyl 4-hydroxylase has several isoenzymes, three of which hydroxylate proline residues in pro-collagen, and another four hydroxylate proline residues in hypoxia-inducible Factor (HIF). Enzymatic functions of vitamin C have been shown in vitro. How the specific role of vitamin C in these enzyme reactions contribute to the signs and symptoms of the vitamin C deficiency state scurvy is not known. In clinical experiments, iron absorption is increased by vitamin C but its effect on hemoglobin concentration is uncertain. Vitamin C is a reducing agent and may exert beneficial effects as a water-soluble antioxidant. Under certain conditions vitamin C may be a prooxidant, especially when it is present in pharmacologic concentrations. Some antioxidant functions have been demonstrated in vitro but have not been proven in humans.

protection from atherosclerosis and other diseases (24,25). However, it is not known whether the observed associations are due to the high vitamin C content of such diets or to other reasons.

Effects on Blood Flow and Endothelial Function

In some, but not all, patients with coronary artery disease, administration of large doses of oral vitamin C (2–4 g for acute administration and 500 mg/day for chronic treatment) resulted in improved endothelium-dependent vasodilation (26). Such a vasodilatory effect may be compatible with enhanced bioactivity of endothelium-derived nitric oxide. Vitamin C did not alter blood flow in healthy subjects and did not reverse endothelial dysfunction in hypertensive patients. Some studies have shown that the vitamin may ameliorate endothelial vasomotor dysfunction when administered intra-arterially in patients with chronic heart failure, type 2 diabetes, and coronary spastic angina, or when given IV to smokers. However, intra-arterial concentrations in these studies were far higher than can be achieved under physiological conditions. More data are needed to determine whether endothelium-dependent vasodilation mediated by vitamin C has clinical relevance.

Effect on Nitrate Tolerance

Due to its redox properties, ascorbic acid is a candidate to prevent nitrate tolerance (27). Tolerance to nitrates, used to treat heart disease, develops within the first day of continuous exposure and makes treatment less effective. Vitamin C given orally at doses of 3 to 6 g/day prevented the development of tolerance in some healthy subjects and in patients with ischemic heart disease or heart failure. Because these studies were short term and involved small numbers of patients, the clinical utility of vitamin C treatment in the prevention of nitrate tolerance is not yet clear.

Effects in Stomach and Duodenum

Vitamin C can quench reactive oxygen metabolites in the stomach and duodenum, and prevent the formation of mutagenic *N*-nitroso compounds. As its concentration in gastric juice is approximately three times higher than that in plasma, vitamin C appears to be an attractive candidate for the prevention of gastric cancer. Whether this suggested antioxidant action has significance *in vivo* is uncertain. Although high vitamin C dietary intake correlates with reduced risk of gastric cancer, it is unknown whether the vitamin itself, or other components in plant-derived foods, or lifestyle choices contribute to the protective effect. Recent data in animals indicate that vitamin C is not protective against gastric premalignant lesions induced by *Helicobacter pylori* (28).

Iron Absorption

Vitamin C promotes iron absorption in the small intestine by maintaining the element in the reduced ferrous (Fe^{2+}) form. It can increase soluble nonorganic iron absorption 1.5- to 10-fold depending on iron status, the vitamin dose, and the type of test meal. Amounts necessary for enhancing iron absorption (20–60 mg) (10) are found in foods that are good sources of the vitamin. However, the effect of vitamin C on hemoglobin concentration is modest at best, at least in small, short-term studies.

PHYSIOLOGY

Tissue Distribution

Vitamin C is widely distributed in the human body, and many organs contain millimolar concentrations of the vitamin. The highest concentrations are found in adrenal and pituitary glands, at 30 to 50 mg/100 g of tissue. Liver, spleen, pancreas, kidney, brain, and lens contain 5 to 30 mg/100 g (29). The choroid plexus actively secretes the vitamin into the cerebrospinal fluid. Ascorbate is concentrated by many parts of the brain. It is unknown why many of these tissues concentrate vitamin C.

High ascorbate concentrations (10–50-fold higher than that in plasma) are also found in white blood cells such as neutrophils, lymphocytes, and monocytes. When activated by exposure to bacterial or fungal pathogens, human neutrophils rapidly accumulate additional vitamin C, with intracellular concentrations increasing approximately 10-fold. This accumulation is mediated by a process termed ascorbate recycling, which is of unknown function (30). As ascorbate recycling does not occur in pathogens, and as neutrophils are the primary host-defense cells in human blood, the process may represent a eukaryotic defense mechanism against pathogens.

Tissue Accumulation

Vitamin C is accumulated in tissues by two distinct pathways. One pathway is sodium-dependent transport. The other is termed ascorbate recycling (30), and DHA (oxidized vitamin C) is transported independent of sodium and reduced intracellularly to ascorbate.

Sodium-Dependent Vitamin C Transport

Two sodium-dependent vitamin C transporters, SVCT1 and SVCT2, have been identified (31,32). Both of these carrier proteins couple the transport of $2 \text{ Na}^+ : 1 \text{ vitamin C}$. SVCT1 is a low-affinity, high-velocity transporter (32). SVCT2 has a 10-fold higher affinity for vitamin C but exhibits a lower rate of uptake. SVCT1 is found in kidney, liver, small intestine, thymus, and prostate. In the small intestine and kidney, SVCT1 is primarily localized in the epithelium, consistent with a role in intestinal absorption and renal reabsorption of vitamin C. SVCT2 has a more general distribution, with mRNA found in most tissues, including the brain, retina, placenta, spleen, small intestine, and gonads (31). Neither of these proteins transport DHA (32). DHA is transported by the facilitative glucose transporters GLUT1, GLUT3 (33), and GLUT4, which do not transport vitamin C. The gene for human SVCT1 (hSVCT1) has been mapped to chromosome 5q23, and that for human SVCT2 (hSVCT2) to chromosome 20p12.3. The essential nature of the SVCT2 transporter was demonstrated by laboratory studies of SVCT2 gene knockout mice, which die at birth (34). Thus, DHA transported by glucose transporters cannot compensate for the lack of ascorbic acid transport by SVCT2 in the intact organism.

Sodium-Independent Transport of DHA (Ascorbate Recycling)

Ascorbate recycling is a process in which extracellular ascorbate is oxidized to DHA, which, in turn, is transported into cells through glucose transporters and reduced back to ascorbate (Fig. 2) (30). Ascorbate recycling

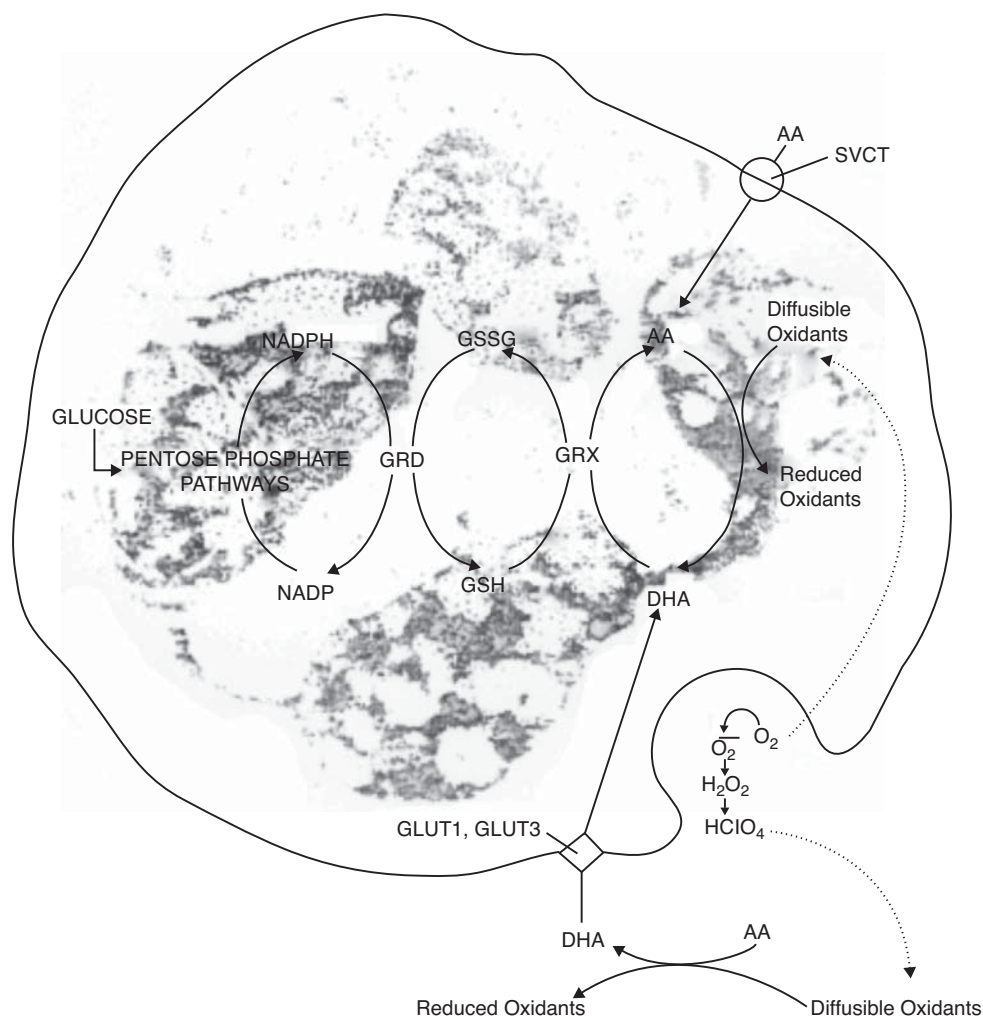


Figure 2 Mechanisms of vitamin C accumulation in human neutrophils. Vitamin C accumulation in neutrophils occurs by ascorbic acid transport and recycling. Ascorbic acid (AA) is transported by sodium-dependent vitamin C transporter 2 (SVCT2) that maintains millimolar concentrations inside resting neutrophils. Recycling occurs when bacteria, yeast, or pharmacologic agents activate neutrophils. Activated neutrophils secrete reactive oxygen species that oxidize extracellular AA to dehydroascorbic acid (DHA). DHA is rapidly transported into neutrophils by glucose transporters (GLUT1, GLUT3) and is then immediately reduced to AA by the glutathione-dependent protein glutaredoxin (GRX). Glutathione (GSH) used during DHA reduction is regenerated from glutathione disulfide (GSSG) by glutathione reductase (GRD) and NADPH. NADPH is a product of glucose metabolism through the pentose phosphate pathway. *Source:* From Padayatty SJ, Levine M. New insights into the physiology and pharmacology of vitamin C. *Can Med Assoc J* 2001; 164(3):353-355, with permission.

enables rapid accumulation of vitamin C in activated neutrophils and is induced by bacteria and *Candida albicans*. Neutrophils from patients with chronic granulomatous disease do not make oxidants due to defective superoxide generation, and these neutrophils cannot recycle vitamin C. The clinical importance of ascorbate recycling is not known. Possibilities include protection of neutrophil and surrounding tissues from oxidative damage, enhanced phagocytosis or bacterial killing, and activation of programmed cell death. Recycling does not occur in bacteria or in other pathogens, suggesting that it may be a host-specific protective mechanism.

Plasma and Cell Concentrations

Steady-State Plasma Concentrations in Relation to Dose

Steady-state plasma concentration data as a function of dose were obtained in 7 healthy men and 15 healthy

women aged 19 to 26 years, each of whom was hospitalized for approximately 5 to 6 months (23,35). A depletion-repletion design was used. Inpatient subjects consumed a diet containing less than 5 mg of vitamin C per day, and all other nutrients in adequate amounts. After depletion of the vitamin (plasma concentrations of $6.9 \pm 1 \mu\text{M}$ for men and $8 \pm 1 \mu\text{M}$ for women), steady-state plasma concentrations were obtained for daily vitamin C doses of 30, 60, 100, 200, 400, 1000, and 2500 mg. The vitamin was measured using high-performance liquid chromatography with coulometric electrochemical detection. The relationship between plasma steady-state concentration and dose was sigmoidal. At a dose of 30 mg, there was only a small increase in plasma vitamin C concentration compared to nadir. At the dose range of 30 to 100 mg, small changes in daily vitamin C intake resulted in large changes in steady-state plasma concentrations (Figs. 3 and 4). Although both curves for men (Fig. 3) (35) and women

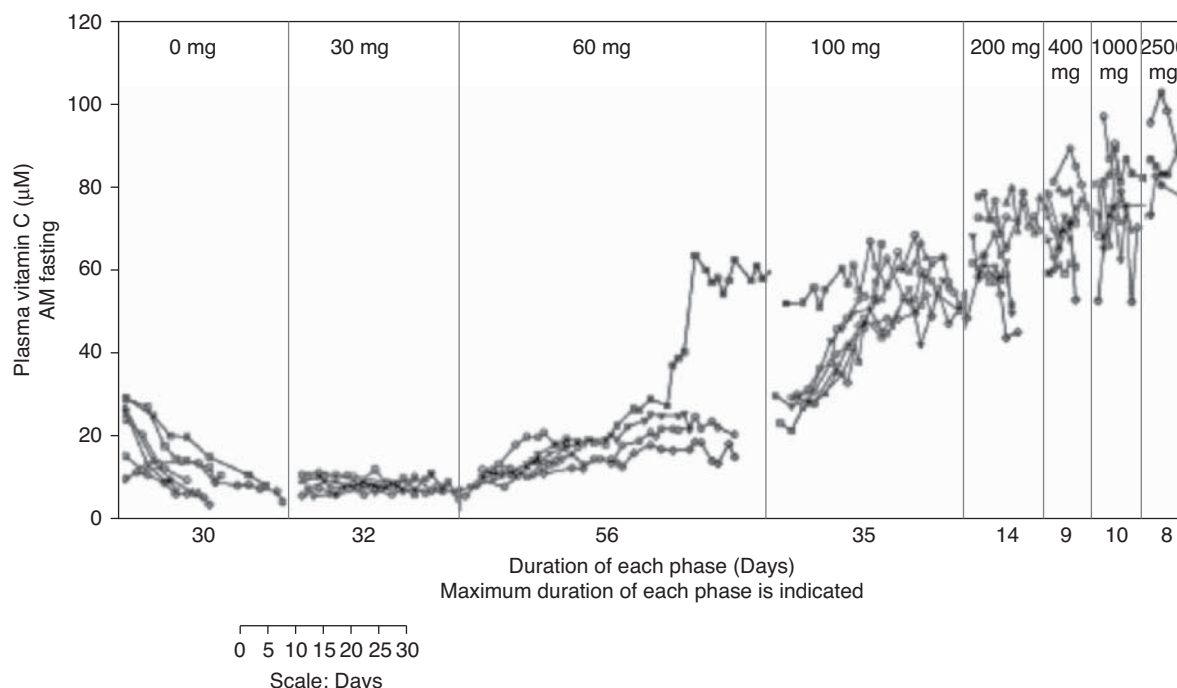


Figure 3 Vitamin C plasma concentration as a function of dose in men. Seven men were hospitalized for 4 to 6 months as described in the text. The duration in days for each subject for the depletion phase (0 mg) and for receipt of seven different vitamin C doses is shown on the X axis. The maximum duration of each phase is indicated numerically. Fasting vitamin C concentrations for all phases of the study are shown on the Y axis. Vitamin C doses for each phase are indicated at the top of the figure. Each subject is indicated by a different symbol. There was variation between subjects in the time taken to reach nadir and in the number of days required to reach steady state for each dose. Source: From Levine M, Wang Y, Katz A, et al. Ideal vitamin C intake. *Biofactors* 2001; 15:71-74, with permission.

(Fig. 4) (23) were sigmoidal, the steep portion of the curve for women was shifted to the left compared with men. Women had higher steady-state plasma vitamin C concentrations than men in the dose range of 30 to 100 mg daily. These differences disappeared at doses of 200 mg/day and higher. At a dose of 200 mg, the curves for both men and women were near plateau. At doses of 400 mg daily and higher, plasma was saturated with a vitamin C concentration of approximately 70 to 80 μM . At these large doses, ingestion of the vitamin resulted in decreased absorption (i.e., decreased bioavailability) and increased urinary excretion as described later.

Steady-State Circulating Blood cell Concentrations in Relation to Dose

Vitamin C was measured in circulating neutrophils, monocytes, and lymphocytes at steady state, at the same daily doses as for plasma. By using active transport, these cells accumulated vitamin C 10- to 50-fold compared to plasma concentrations. Intracellular concentrations increased substantially between 30 and 100 mg daily doses (23,35). Cells saturated before plasma, at daily doses of 100 to 200 mg. This is probably because the maximal transport velocity (apparent V_{max}) of the tissue vitamin C active transporter SVCT2 is approximately 70 μM .

Bioavailability

Bioavailability of oral vitamin C was determined in depletion-repletion studies by comparison of plasma concentrations of the vitamin after oral and after IV adminis-

tration. The studies were done with doses of 15, 30, 50, 100, 200, 500, and 1250 mg. The experiments were performed at steady state, because bioavailability is best studied at equilibrium for plasma and tissue (23,35). At steady state for any given dose of vitamin C, its concentrations in plasma and in tissues are in equilibrium. When oral or IV doses of vitamin C are given acutely at this stage, plasma concentrations rise and then return to baseline for that steady state. This rise and return to baseline forms an area under the curve (AUC), which can be used to determine bioavailability of the vitamin.

To measure bioavailability, the same doses of vitamin C were given orally and IV at different times during steady state, usually a day apart. After administration of the vitamin, its plasma concentrations were measured at intervals of minutes to hours. Bioavailability was calculated as the AUC for the oral dose (AUC_{po}) divided by the AUC for the IV dose (AUC_{IV}). For a single dose of vitamin C, it was calculated as (approximately) 100% for 200 mg, 73% for 500 mg, and 49% for 1250 mg.

The AUC method could not be used for vitamin C doses of 200 mg and lower. This method is only accurate if volume of distribution and rate of clearance are constant. Vitamin C distribution differs between plasma, circulating blood cells, and other tissues. The differences in distribution are less pronounced at higher doses, when plasma and circulating cells are saturated with the vitamin. Moreover, renal excretion is not linear, as it only starts above the renal threshold, as described later. Therefore, a mathematical model was developed to account for these factors.

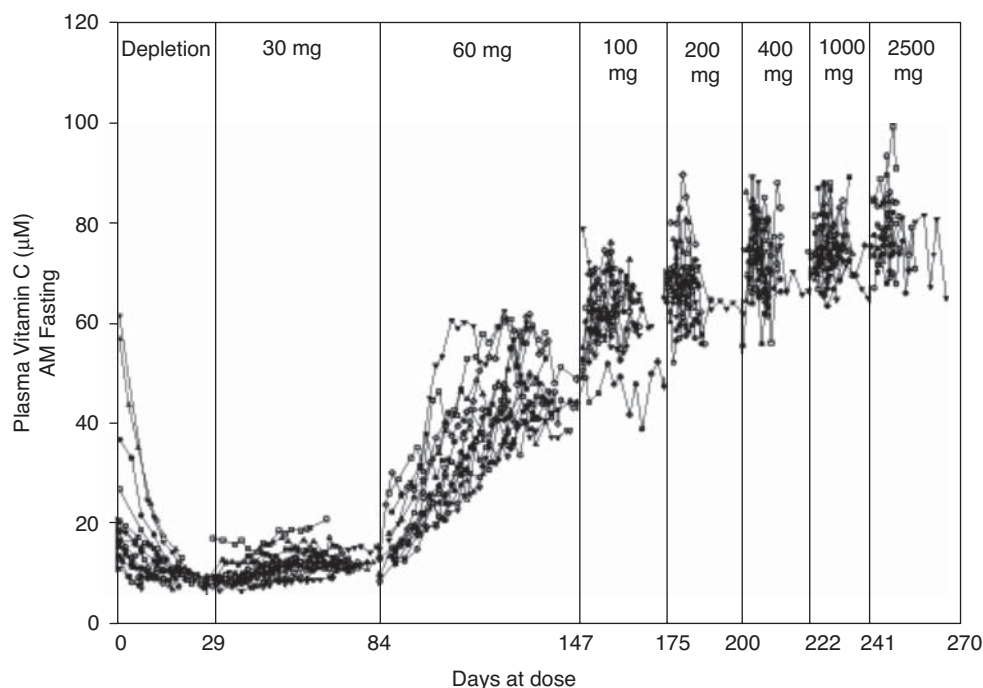


Figure 4 Vitamin C plasma concentration as a function of dose in women. Fifteen women were hospitalized for 4 to 6 months as described in the text. Vitamin C concentrations are shown as a function of days at dose. Doses are indicated at the top of the figure. Each symbol represents a different subject. There is a 1-day gap between all doses for bioavailability sampling. Doses through 200 mg daily were received by 15 subjects, through 1000 mg daily by 13 subjects, and through 2500 mg by 10 subjects. *Source:* From Ref. 23.

Using this, bioavailability was found to be 80% for 100 mg and 46% for 1250 mg.

The values calculated by the two methods mentioned earlier are for vitamin C administered as a chemically pure substance in an aqueous solution. When given as a supplement, bioavailability depends on the preparation and may be substantially diminished by factors such as supplement binders and supplement dissolution time in the gastrointestinal tract. The bioavailability of vitamin C when consumed in the natural matrix of our diet is currently unknown and might be altered by other substances in foods.

Renal Excretion

Urinary excretion of vitamin C was measured at steady state for doses of 30 to 1250 mg daily. At oral doses below 60 mg/day, no vitamin C appeared. At an oral dose of 100 mg/day, corresponding to a plasma concentration of approximately 60 µM in both men and women, approximately 25 mg of vitamin C in men and 50 mg in women was excreted in the urine (23,35). As oral doses increased, the vitamin appeared in the urine in increasing quantity. Although a larger quantity of vitamin C was absorbed as doses increased, the percentage of the absorbed dose decreased. This percentage decrease in absorption is decreased bioavailability. For example, when 1250 mg of vitamin C was given orally, approximately 600 mg was absorbed and subsequently excreted in the urine. Upon IV administration, without the confounding effects of intestinal absorption, virtually the entire administered dose

was excreted at 500 and 1250 mg. Because vitamin C is not protein bound, it is presumably filtered at the glomeruli and reabsorbed in the renal tubules. When the ability of the kidney to reabsorb vitamin C is overwhelmed (the transport mechanism for reabsorption is saturated), the vitamin appears in the urine, analogous to glycosuria in patients with uncontrolled diabetes. In patients with end-stage renal disease, excess vitamin C cannot be excreted. In such patients, vitamin C doses above 200 mg can accumulate and produce hyperoxalemia. On the other hand, many patients with end-stage renal disease on dialysis lose vitamin C during dialysis and have chronically low plasma concentrations.

Potential Variable Factors

Healthy subjects were studied for 5 to 6 months to determine plasma and cell concentrations, bioavailability, and renal excretion of ascorbate in relation to dose (pharmacokinetics). It is possible that the pharmacokinetics findings will be different in subjects with acute and chronic diseases and in the elderly. Prolonged hospitalization for these subjects is not possible for the purpose of obtaining pharmacokinetics data, and to do so will require the development of new methods.

Pharmacologic Ascorbate

Consumption of vitamin C in total amounts above those found in foods (i.e., 400–500 mg, Fig. 5) can be considered pharmacologic use. Such use can be achieved by ingestion of vitamin C supplements. Vitamin C is widely

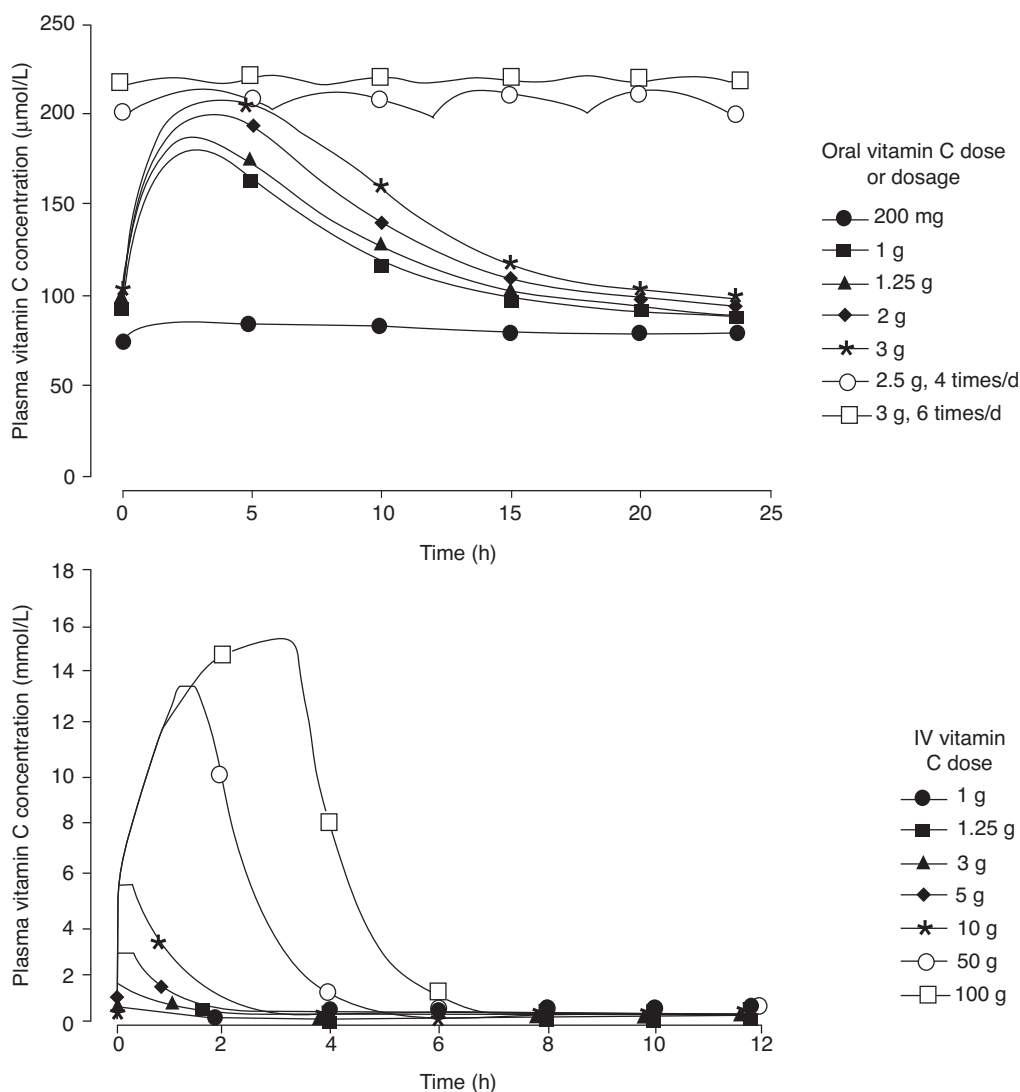


Figure 5 Predicted plasma vitamin C concentrations in healthy persons after oral (top) or IV (bottom) administration of vitamin C. Note that the units of plasma vitamin C concentrations after oral dosing are $\mu\text{M/L}$ whereas the units following IV administration are in mM/L (1000-fold higher). Amounts of vitamin C greater than 400 to 500 mg daily are difficult to obtain from foods alone. Source: From Ref. 36.

used by the public to prevent or treat many conditions, especially the common cold. In the United States, it is the most widely sold single vitamin. In addition, many multivitamin supplements contain vitamin C at or above its RDA. Oral intake of pharmacologic amounts of ascorbate can elevate plasma concentrations to a maximum of approximately 220 μM . In contrast to deficiency, there is no conclusive evidence of benefit from ingestion of pharmacologic amounts of ascorbate in humans. Ascorbate is also administered IV, as a drug, by complementary and alternate medicine practitioners to treat disease. IV-administered pharmacologic doses of vitamin C achieve 200- to 300-fold higher plasma concentrations than those that can be obtained from foods and approximately 100-fold higher than those from maximal oral dosing (36,9). Emerging evidence indicates potential mechanisms of action of pharmacologic ascorbate, with focus on cancer treatment (16,17,37,38). With appropriate patient

screening, IV ascorbate at pharmacologic appears to be safe on the basis of a phase I clinical trial (39). Targeted phase II clinical trials are warranted to determine whether pharmacologic ascorbate has benefit, or confers harm, in cancer treatment (40).

VITAMIN C DEFICIENCY AND SCURVY

The earliest recorded descriptions of scurvy are in Egyptian hieroglyphics ca. 3000 BC James Lind published his *A Treatise of the Scurvy* in 1753, providing evidence that fruits could prevent the disease (41). As noted by Lind and later confirmed by others, the early symptoms of scurvy are weakness, fatigue, listlessness, and lassitude. The physical signs that follow include petechial hemorrhage; perifollicular hyperkeratosis; erythema and purpura; bleeding into the skin, subcutaneous

tissues, muscles, and joints; coiled hairs; breakdown of wounds; arthralgias and joint effusions; swollen and friable gums; hypochondriasis and depression; Sjögren syndrome; fever; shortness of breath; and confusion. In severe scurvy, irreversible changes may occur, including dental loss, bone damage, and sequelae of internal hemorrhage and infection. Untreated, the disease is uniformly fatal. The signs related to wound dehiscence and friable gums may reflect impaired collagen synthesis. As already noted, however, there is no experimental evidence that directly links these signs and low vitamin C concentrations to diminished enzyme actions. Frank scurvy is now rare and is seen in the United States primarily among malnourished populations, including patients with cancer cachexia and malabsorption, poor and elderly people, alcoholics, drug addicts, and some individuals consuming idiosyncratic diets. Scurvy can occur in war torn areas and in refugee camps, usually in conjunction with malnutrition and multiple vitamin deficiencies. Subclinical vitamin C deficiency may be much more common than overt disease, and is difficult to recognize, because the early symptoms of deficiency are unremarkable and nonspecific.

INDICATIONS AND USAGE

Food Sources

Vitamin C is widely distributed in fruits and vegetables. Fruits rich in vitamin C include strawberry, papaya, orange, kiwifruit, cantaloupe, grapefruit, mango, and honeydew melon. Vegetables with a high content of the vitamin are broccoli, Brussels sprout, cabbage, potato, sweet potato, cauliflower, red and green pepper, tomato, snow pea, and kale (1). Fruit juices such as orange juice, tomato juice, grapefruit juice, and fortified juices are also sources of the vitamin. Vitamin C is labile, and its content in plant foods may vary to some extent depending on season, transportation, shelf time, storage, and cooking practices. Five servings of a variety of fruits and vegetables per day, as recommended by the U.S. Department of Agriculture (USDA) and the U.S. National Cancer Institute (NCI), provide 210 to 280 mg of vitamin C. Fruit and vegetable consumption restricted to a narrow selection could provide smaller amounts.

Functions in Relation to Concentration

Other than to prevent scurvy, there is no direct evidence that a particular vitamin C plasma or tissue concentration is more beneficial than others. It is uncertain whether the various biochemical roles of vitamin C, either known or postulated, are related to the concentration of ascorbate in vivo. There are, however, hints that the optimum plasma concentration is higher than the minimum required to prevent clinical scurvy (approximately 10 mg/day). For example, in his *Treatise*, James Lind mentions that the most prominent sign of impending scurvy is fatigue, or "lassitude." (41) In vitamin C depletion-repletion studies fatigue was also noted, and in one study, it appeared at vitamin C plasma concentrations of approximately 20 μ M. In the NIH studies, daily vitamin C intakes of 30 mg resulted in plasma concentrations of only 10 to 20 μ M. There are also indirect findings suggesting that a higher plasma vitamin C concentration may be beneficial:

vitamin C transporter SVCT2 achieves close to apparent V_{\max} at a concentration of 70 μ M; LDL oxidation in vitro is inhibited by vitamin C at 40 to 50 μ M; plasma concentration is tightly controlled at 70 to 80 μ M, and circulating blood cells saturate at approximately these concentrations. An optimal plasma concentration for vitamin C is yet to be determined by clinical studies.

Possible Benefits of Vitamin C Consumption

Diets with 200 mg or more of vitamin C from fruits and vegetables are associated with lower risk of cancer (especially cancers of the oral cavity, esophagus, stomach, colon, and lung) (42) and stroke, and with reduced overall mortality. Higher fruit and vegetable consumption and plasma vitamin C concentrations are inversely related to risk of ischemic heart disease, diabetic complications, and blood pressure in hypertensive patients. The USDA and NCI recommendations of consuming five fruit and vegetable servings daily are based on this extensive evidence. These studies, however, are correlational. Whether vitamin C in fruits and vegetables confers these benefits is not known. It may be a surrogate marker for fruit and vegetable consumption and, perhaps, for other healthy lifestyle practices (43) In the United States, approximately 30% of adults consume less than 2.5 servings of fruits and vegetables per day, and the estimated vitamin C intake is even lower among some groups, including children.

Vitamin C as a food supplement was tested for primary prevention of cancer, cardiovascular disease, stroke, and age-related eye diseases. In epidemiological studies and in some large-scale interventional studies, vitamin C was consumed in combination with other food supplements such as vitamins and antioxidants, and was partially obtained from foods. Under these conditions, it did not provide the health benefits seen with consumption of fruits and vegetables: Vitamin C supplements did not prevent cancer, heart disease, stroke, or cataract. To date, no large-scale interventional studies have been reported where vitamin C was administered as a sole supplement.

Vitamin C as a food supplement has also been tested for its effects on disease outcome for hypertension, diabetes, infectious diseases, and age-related eye diseases. Some small, short-term studies suggest that consumption of vitamin C supplements might lower blood pressure in hypertensive and diabetic patients. No large-scale studies are available to confirm these findings. Some experiments show an improvement in lipid profile and insulin sensitivity in diabetic patients who consumed vitamin C supplements, but others fail to do so. Contrary to what has been suggested by some, daily vitamin C supplementation did not decrease common cold incidence in most studies. Similarly, data are insufficient to conclude that vitamin C supplements have an effect on reducing severity of illness due to common cold, except, perhaps, in some people who are vitamin C deficient. Although some small studies suggested that supplementation might prevent cataract, larger studies showed no effect of vitamin C supplementation on the development or progression of cataracts.

Prevention of Deficiency

Steady-state plasma concentrations achieved by a vitamin C dose of 60 mg/day can prevent deficiency for 10 to

14 days, and those achieved by 100 mg/day can probably prevent deficiency for approximately 1 month. The above-mentioned only applies to healthy people who, under otherwise normal conditions, are depleted of vitamin C alone. There is currently little knowledge of vitamin C metabolism in disease states. Deficiency might occur more rapidly in various clinical circumstances where low concentrations were reported, such as in smokers, and in patients with diabetes, myocardial infarction, pancreatitis, end-stage renal disease, and in critical illness requiring intensive care unit support.

Treatment of Scurvy

Upon diagnosis of scurvy, based primarily on clinical findings and confirmed by plasma concentrations of vitamin C, treatment can be initiated with doses of 100 mg given three times a day. An initial IV dose of 100 mg may be administered. If diagnosis and treatment are prompt, permanent damage can be prevented.

Dietary Reference Intakes

Dietary reference intakes (DRIs) are a set of nutrient-based reference values that can be used for planning diets and that are meant to expand the concept of recommended daily allowances (RDAs) in the United States (44). DRIs have several categories: estimated average requirement (EAR), RDA, adequate intake (AI), and upper limit (UL). The EAR is the median usual intake value that is estimated to meet the requirements of half the healthy individuals in a life stage and gender group. This value is used to calculate the RDA, which is the average daily dietary intake level that is sufficient to meet the nutrient requirements of nearly all healthy individuals in a life stage and gender group. The RDA is calculated as the EAR plus two standard deviations of the EAR measurement. AI is derived when data to establish EARs are insufficient and, therefore, an RDA cannot be set. It is based on experimentally derived intake levels or approximations of observed mean nutrient intake by groups of apparently healthy people. UL is the highest level of continuing daily nutrient intake that is likely to pose no risk of adverse health effects in almost all individuals in a life stage and gender group. It should be noted that in the absence of rigorous scientific evidence, these definitions can only be considered interim measures that are useful for planning and for nutritional advice to the public. Hence, these values are provisional and subject to change when suitable data becomes available.

DRI Values for Vitamin C

DRI values were published for ascorbic acid by the Food and Nutrition Board of the U.S. National Academy of Sciences in a report released in April 2000 (44). EARs were calculated based on data on neutrophil saturation and urinary excretion that were obtained in a depletion-repletion study in men as described earlier. The EAR for men 19 years and older was established as 75 mg/day. The requirement for women in the same age group was extrapolated based on body weight differences, and the EAR was set at 60 mg/day. The RDAs for vitamin C in the United States were calculated from these EAR values. In this report from 2000, the RDAs were increased from

Table 2 Recommended Dietary Allowances (RDAs)^a for Vitamin C Consumption

Group	RDA (mg/day)	
	Boys	Girls
Infants^b		
0–6 mo	40	40
7–12 mo	50	50
Children	Boys	Girls
1–3 yr	15	15
4–8 yr	25	25
9–13 yr	45	45
14–18 yr	75	65
Adults	Men	Women
19 yr and older	90	75
Pregnancy		
14–18 yr		80
19–50 yr		85
Lactation		
14–18 yr		115
19–50 yr		120

^aU.S. Food and Nutrition Board of the Institute of Medicine, 2000.

^bValues for infants are given as adequate intake (AI), since RDAs are unavailable. Note that AI values may be higher than RDAs due to the different methods of estimation. The data used for infant AIs are milk composition and amount of milk consumed. RDAs for children are based on assumed differences in body weight from adults, for whom data are available.

60 to 90 mg/day for men and to 75 mg/day for women (Table 2). By using Food and Nutrition Board criteria, data published after the DRI recommendations were released suggest that the RDA for healthy young women should be increased to 90 mg/day, and that the RDA for men may have been underestimated and should be increased to 105 mg/day. UL recommendations are discussed below under adverse effects.

Use in Pregnancy

The RDA for vitamin C during pregnancy is 80 mg/day for women aged 14 to 18 years and 85 mg/day for women 19 years and older (Table 1). This increase, compared to the recommendations in nonpregnancy, is based on the assumption that additional vitamin C is required to provide adequate transfer to the fetus. Plasma vitamin C concentrations decrease during pregnancy, perhaps secondary to hemodilution or active transfer to the fetus, but this decrease has not been shown to have clinical significance. Vitamin C deficiency during pregnancy is associated with increased risk of infection, premature rupture of membranes, premature delivery, and eclampsia. However, it is unknown whether vitamin C deficiency contributes to these conditions or is simply a marker of poor nutritional status. Precise data are lacking regarding fetal requirements and quantity of maternal vitamin C transferred to the fetus. Therefore, an increase of 10 mg/day for pregnancy was recommended on the basis of data that intakes of 7 mg/day of vitamin C prevent young infants from developing scurvy.

Use in Disease

Some studies, as previously discussed, suggest that vitamin C administration may have health benefits in those

with endothelial dysfunction, such as patients with ischemic heart disease, diabetes, or hypertension, and that it may reduce tolerance to nitrates. However, there are currently not enough data to support specific vitamin C intake recommendations in such patients other than the RDA and the general recommendation for fruit and vegetable intake.

Optimum Vitamin C Intake

Recommendations for optimum intake should be based on its dietary availability, steady-state concentrations in plasma and in tissue in relation to dose, bioavailability, urinary excretion, adverse effects, biochemical and molecular function in relation to concentration, beneficial effects in relation to dose (direct effects and epidemiological observations), and prevention of deficiency. Although recent studies provided valuable data on some of these aspects, additional clinical reports are needed to provide definitive recommendations for optimal intake in health and disease. Some recommendations can still be made now by using available data. Five or more varied servings of fruits and vegetables daily will provide approximately 200 mg of vitamin C and might offer protection against cardiovascular diseases and stroke. It is recommended that healthy people strive to meet this ingestion amount by using fruits and vegetables, not supplements.

By virtue of its antioxidant properties, ascorbate can prevent browning and extend the shelf life of certain foods. Lipid-soluble forms of ascorbic acid can also reduce oxidation of fats and oils. Vitamin C is commercially added to many foods as a preservative and some vitamin C may be obtained by the consumption of such foods.

ADVERSE EFFECTS

The toxic effects of vitamin C are few and are dose related. Ingestion of 3 to 5 g at once can cause diarrhea and bloating. The vitamin enhances iron absorption from the small intestine and may, in large doses, increase the risk of iron overload in patients who are prone to that condition (such as patients with hemochromatosis, thalassemia major, or sideroblastic anemia, or patients who require multiple, frequent red blood cell transfusions). In healthy individuals, vitamin C most probably does not induce iron over absorption in doses as high as 2 g. In patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency or paroxysmal nocturnal hemoglobinuria (PNH), hemolysis can be induced by large oral or IV doses of vitamin C. Doses of 3 g may cause transient hyperuricosuria, but this does not occur at doses of less than 1 g. Likewise, oxalate excretion may be increased by ingestion of 1 g or more daily of the vitamin in some individuals, although the clinical importance of this is unknown. Large-scale studies in healthy individuals with no prior history of kidney stones did not show an increased risk of formation of renal calculi with increased vitamin C consumption from food and supplements. However, vitamin C at doses of 1 g daily and higher may precipitate this problem in some individuals with occult hyperoxaluria. In patients receiving dialysis treatment, hyperoxalemia has been induced by repeated IV administration of 1 g, and it may also be promoted by daily doses of 500 mg. Although adequate vitamin C in-

take for patients with end-stage renal disease on dialysis is not known, based on current evidence, it probably should not exceed 200 mg daily (1).

In its latest published recommendations, the Food and Nutrition Board set the tolerable UL for vitamin C at 2 g daily, based on gastrointestinal adverse effects at higher doses (44). Of note, there are no clinical indications at this time for such doses, although some patients ingest these amounts for possible benefit despite little or inconclusive evidence.

Vitamin C, at doses of 250 mg and above, may cause false-negative results for stool occult blood with guaiac-based tests. Intake of the vitamin should be reduced to less than 250 mg for several days prior to such testing. Several harmful effects have erroneously been attributed to the vitamin, including hypoglycemia, rebound scurvy, infertility, mutagenesis, and destruction of vitamin B₁₂. None of these effects are caused by vitamin C.

COMPENDIAL/REGULATORY STATUS

Not applicable.

CONCLUSION

Vitamin C is essential for human health. Human survival is dependent upon obtaining vitamin C from foods, and its deficiency results in the fatal deficiency disease scurvy. As an electron donor, it is a cofactor for 14 enzymes and is a principal water-soluble antioxidant in the body. Its only proven role in human health is to prevent scurvy, for which only a small intake is required. Ideal vitamin C intakes required for optimal health are as yet unknown. Fruits and vegetables are rich in vitamin C and five servings daily will provide ample amounts of the vitamin.

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Vitamin D

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INTRODUCTION

Vitamin D, a compound long known to be essential for bone health, holds a unique position in the realm of nutrients—that is, although this compound is clearly a nutrient, it is also a prohormone. Although vitamin D is normally produced by ultraviolet (UV) light acting on 7-dehydrocholesterol in skin, it can also be absorbed from food and supplements. There are a number of situations in which dietary consumption of vitamin D is required. In this chapter, we review the biochemistry, metabolism, and homeostasis of vitamin D as well as biological and environmental factors that influence its nutritional essentiality. We also discuss its many functions in the body. Dietary sources, methods and controversies in nutritional status assessment, and recommendations for intake across the lifecycle are then presented.

BIOCHEMISTRY, TRANSPORT, METABOLISM, AND STORAGE OF VITAMIN D

Although there are both endogenous and exogenous sources of vitamin D, their biochemistry and metabolism within the body share common pathways.

Structure and Metabolites

Structurally, vitamin D is considered a “seco-steroid” because it is derived from a sterol (either ergosterol in plants or cholesterol in animals) and because one of its four organic ring structures is broken between carbons 9 and 10 (Fig. 1). In plants, ergosterol can be activated by irradiation to ergocalciferol (vitamin D₂); this is a form commonly sold commercially. In animals, 5,7-cholestradienol (commonly called 7-dehydrocholesterol), found in the skin’s epidermis, is initially converted by ultraviolet B (UVB) light to precholecalciferol (previtamin D₃). Previtamin D₃ then slowly equilibrates to vitamin D₃ (cholecalciferol or calcitriol). Cholecalciferol diffuses from the skin cell into the blood where it is transported bound to the liver-derived α -globulin vitamin D-binding protein (DBP), also called transcalfiferin, to the liver.

Photobiology

Most people meet their vitamin D needs through exposure to sunlight (1). More specifically, UVB radiation with a wavelength of 282 to 310 nm penetrates uncovered skin and converts 7-dehydrocholesterol to previtamin D₃, which in turn becomes vitamin D₃ (Fig. 2) (2). Season, geographic latitude, time of day, cloud cover, smog, skin

melanin content, and sunscreen are among the factors that affect UVB radiation exposure and vitamin D synthesis (2). The amount of UVB radiation available to individuals living above the 42° north latitude line is insufficient for cutaneous vitamin D synthesis from November through February (1); in far northern latitudes, this reduced intensity lasts for up to 6 months; latitudes below 34° north allow for sufficient cutaneous vitamin D production throughout the year (3).

Complete cloud cover reduces UVB energy by 50%; shade (including that produced by severe pollution) reduces it by 60% (4). UVB radiation does not penetrate glass, so exposure to sunshine indoors through a window does not result in vitamin D synthesis. Sunscreens with a sun protection factor (SPF) of 8 or more appear to block vitamin D-producing UV rays, although in practice people generally do not apply sufficient amounts, cover all sun-exposed skin, or reapply sunscreen regularly. As such, skin likely synthesizes some vitamin D even when it is protected by sunscreen as typically applied.

The factors that affect UVB radiation exposure and a relative dearth of research on the amount of sun exposure needed to maintain adequate vitamin D levels make it difficult to provide general guidelines concerning the amount of sunlight needed for optimal vitamin D production. Nonetheless, it has been suggested by some vitamin D researchers that approximately 5 to 30 minutes of sun exposure between 10 a.m. and 3 p.m. at least twice weekly to the face, arms, legs, or back without sunscreen usually leads to sufficient vitamin D synthesis; and that moderate use of commercial tanning beds that emit 2% to 6% UVB radiation is also effective (2). Clearly, individuals with limited sun exposure need to include good sources of vitamin D in their diet or take a supplement.

Despite the importance of the sun to vitamin D synthesis, it is prudent to limit exposure of skin to sunlight and UV radiation from tanning beds. UV radiation is a carcinogen responsible for most of the estimated 1.5 million skin cancers and 8000 deaths due to metastatic melanoma that occur annually in the United States (5). Consequently, the American Academy of Dermatology advises that photoprotective measures be taken, including the use of sunscreen, whenever one is exposed to the sun (6).

Absorption and Circulation of Dietary Vitamin D

Dietary vitamin D is absorbed by passive diffusion into the small intestine enterocytes. This process requires the synthesis of micelles, the presence of dietary fat, and emulsification by bile salts. Approximately 80% of dietary vitamin D is absorbed, most in the distal small intestine. Once inside the enterocyte, vitamin D is incorporated within

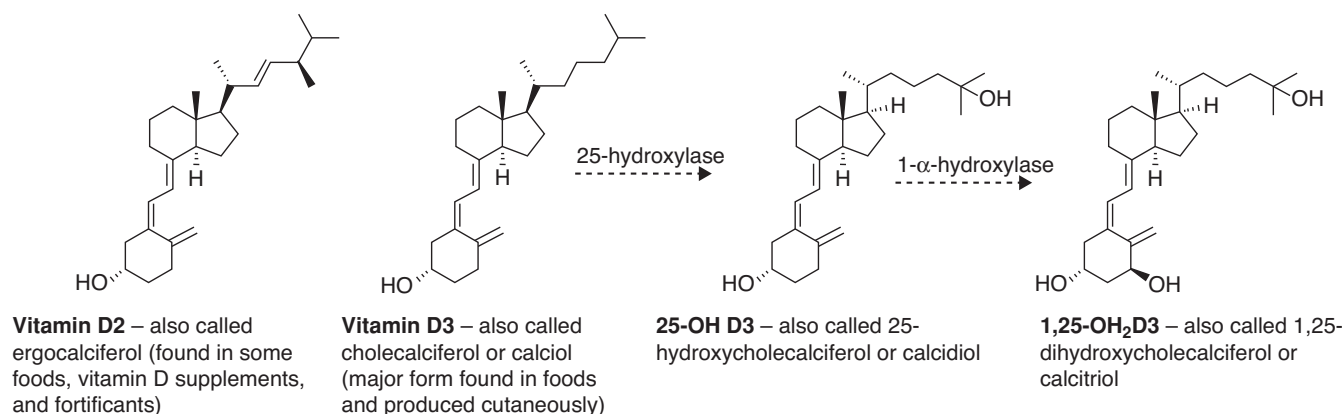


Figure 1 Simplified chemical structures of ergocalciferol (vitamin D2), cholecalciferol (vitamin D3), 25-hydroxycholecalciferol (25-OH D3), and 1,25-dihydroxycholecalciferol (1,25-OH₂ D3). Note that both vitamin D2 and vitamin D3 can be metabolized by the respective enzymes, but only structures for 25-OH D3 and 1,25-OH₂ D3 are shown here.

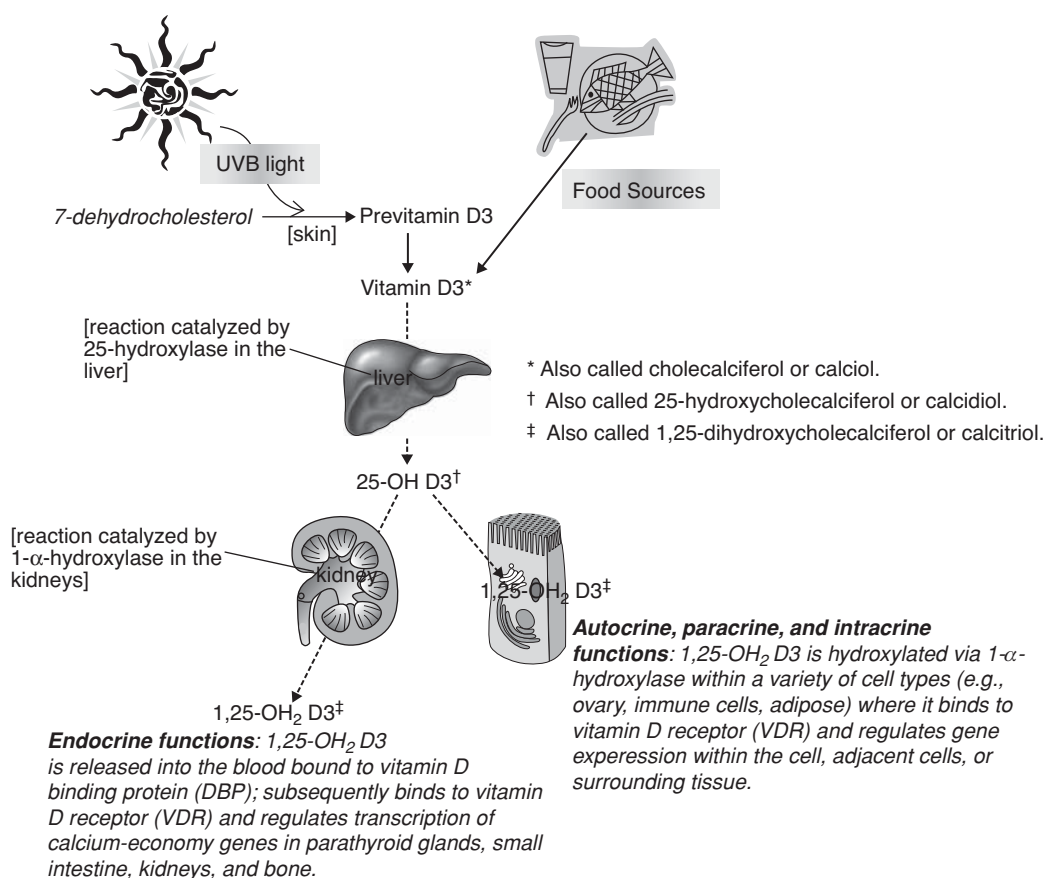


Figure 2 Vitamin D metabolism and function. Extrarenal 1- α -hydroxylase has been shown in many tissues and demonstrated in vitro to activate 25-OH D₃ to 1,25-OH₂ D₃, which has also been shown in vitro to affect gene expression. Source: From Prentice A, Goldberg GR, Schoenmakers. Vitamin D across the lifecycle: physiology and biomarkers. Am J Clin Nutr 2008; 88S:500S–506S.

chylomicra, which are subsequently transported into the lymphatic system and eventually enter the blood.

General Circulation, Hydroxylation, Activation, and Degradation in the Body

After it is taken up by the liver, both endogenously produced and dietary cholecalciferol are then metabolized by cytochrome P₄₅₀ hydroxylases to ultimately generate the active form of the vitamin: 1,25-dihydroxycholecalciferol (1,25-OH₂ cholecalciferol, called calcitriol). This is described here.

Hepatic 25-Hydroxylases

In the liver, mitochondrial 25-hydroxylase adds a hydroxyl group to cholecalciferol at carbon 25 to form 25-hydroxycholecalciferol (25-OH cholecalciferol, called 25-OH D3 or calcidiol). This enzyme appears to be more efficient during periods of vitamin D deprivation. Note that although the liver is the major source of this enzyme, it is also found in other organs such as the lungs, kidneys, and intestines.

1- α -Hydroxylase Activates Vitamin D in the Kidneys

Following hepatic hydroxylation, 25-OH D3 is released into the blood bound to DBP and taken up by the kidneys where it is further hydroxylated. Specifically, another mitochondrial enzyme 1- α -hydroxylase adds a second hydroxyl group at carbon 1 to form 1,25-dihydroxycholecalciferol (1,25-OH₂ cholecalciferol, called 1,25-OH₂ D3 or calcitriol). Like its hepatic counterpart, activity of 1- α -hydroxylase is increased when vitamin D availability in the cell is low. High intake of phosphorus has also been associated with decreased activity of this enzyme (7). Once synthesized in the kidneys, calcitriol is released bound to DBP and acts in an entirely endocrine fashion. It is noteworthy that DBP only weakly binds calcitriol; it binds more tightly to its less hydroxylated form, 25-OH D3. This difference in binding affinity results in greater release of calcitriol (as compared with 25-OH D3) to the vitamin D receptor (VDR) found on many target tissues.

1- α -Hydroxylase in Extrarenal Tissues

1- α -Hydroxylase has also been found in a wide variety of extrarenal tissues. In vitro studies suggest that, in these extrarenal tissues, this enzyme activates 25-OH D3 to 1,25-OH₂ D3. In vitro, this locally produced calcitriol has been shown to affect gene expression suggesting that extrarenal 1,25-OH₂ D3 may act locally via intracrine, autocrine, or paracrine mechanisms. However, to date, neither extrarenal activation of 1,25-OH₂ D3 nor its local action has been demonstrated in vivo. Such evidence is essential to demonstrate the role and function of the extrarenal 1- α -hydroxylase.

Metabolism (Degradation) of Vitamin D by 24-Hydroxylase

When sufficient amounts of calcitriol are available, not only are the activities of the aforementioned hydroxylases decreased, but the activity of another enzyme called 24-hydroxylase is also increased. This enzyme that is made in all target tissues adds a hydroxyl group, forming 24R,

25-OH₂ D3 and 1,24,25-OH₃ D3 from 25-OH D3 and 1,25-OH₂ D3, respectively. The same enzyme oxidizes 1,24,25-OH₃ D3 to 1,25-OH₂ 24-oxo D3, and ultimately calcitroic acid. These and other vitamin D metabolites (some are conjugates) are then excreted in the feces via the bile. Very little (1–4%) is excreted in the urine.

Storage of Excess Vitamin D

Relatively large amounts of vitamin A can be stored in the liver, but vitamin D is stored in significant amounts in adipose tissue. Its metabolites are not stored.

FUNCTIONS OF VITAMIN D

The initial discovery of a fat-soluble substance that cured rickets was made by Sir Edward Mellanby (1919) but he considered it to be a property of vitamin A. In 1922, E.V. McCollum destroyed vitamin A in cod liver oil by oxidation, but found that the ability of the oil to cure rickets remained. He correctly concluded this to be a new factor, which he called vitamin D. Since that time, scientists have learned much about the myriad functions of vitamin D including many of the molecular mechanisms by which they occur.

Overview of Vitamin D's Role in Regulation of Transcription

Integral to many of the roles vitamin D plays in the body is its function as a steroid hormone, binding to nuclear VDRs that selectively regulate gene expression. Calcitriol itself clearly binds to nuclear VDRs, resulting in a cascade of transcription-related events. In many cases, the vitamin D–VDR complex binds with retinoid X or retinoic acid receptors (RXR or RAR), which then interacts with additional nuclear receptors (vitamin D response elements or VDRE), influencing gene expression.

Modulation of Calcium Economy

One of vitamin D's main functions is to modulate calcium homeostasis. This is done in concert with parathyroid hormone (PTH), involving many tissues including the absorptive surface of the small intestine.

Calcium Homeostasis Via Regulation of PTH and Renal 1- α -Hydroxylase

Regulation of blood calcium is initially orchestrated by release of PTH in response to hypocalcemia. In turn, PTH stimulates 1- α -hydroxylase in renal cells and its resulting conversion of 25-OH D3 to calcitriol. This active form of vitamin D then works independently and coordinately with PTH to raise blood calcium. Factors such as race, geographical location, body composition, and physical activity can affect steady states of blood calcium and/or vitamin D concentration, suggesting possible homeostatic regulation in addition to day-to-day homeostatic control. For instance, circulating calcium concentrations decrease during long distance running events (8); and serum vitamin D tends to be lower in obese than nonobese subjects (9). Further, there is some evidence that vitamin D

economy may be enhanced in some racial groups such as African-Americans, but this finding is not consistent (10).

Regulation of Intestinal Calcium Absorption

Perhaps the best-understood target tissue for calcitriol is the small intestine, where 1,25-OH₂ D₃ increases absorption of both calcium and phosphorus. For this to occur, 1,25-OH₂ D₃ binds with a high-affinity nuclear VDR; this complex then upregulates expression of several genes that encode for proteins important for dietary calcium absorption. For example, synthesis of calbindin and TRPV6 (proteins important for the transport of calcium via brush border calcium channels) is stimulated by 1,25-OH₂ D₃ but is not necessary for the action of vitamin D on calcium absorption (11).

Regulation of Calcium Reabsorption in the Kidneys and Mobilization from the Bone

When blood calcium is low, 1,25-OH₂ D₃ also works together with PTH to stimulate calcium reabsorption in the kidneys by mechanisms similar to those simultaneously occurring in bone resorption. In addition, 1,25-OH₂ D₃ appears to function with PTH to stimulate production, maturation, and function of osteoclasts which, in turn, mobilize minerals (including calcium) from bone.

Vitamin D, Cellular Proliferation, and Cellular Differentiation

Emerging data also suggest that vitamin D may be involved in specific processes involved in cellular growth and differentiation. In fact, there appear to be numerous cell types that synthesize VDRs and hundreds of genes with VDREs directly or indirectly that may affect cell cycling and proliferation, differentiation, and apoptosis (12).

Cell Cycle Regulation

For instance, studies with VDR-knockout mice suggest that vitamin D and its associated VDRs are critical in controlling the growth of many normal tissue types such as those of mammary, colonic, epidermal, and hematopoietic lineages. In general, but not always, vitamin D appears to suppress hyperplastic growth in vitro. An example is the finding that calcitriol inhibits hyperplastic growth of parathyroid cells by diminishing expression of both transforming growth factor- α (TGF- α) and its receptor (epidermal growth factor receptor, or EGFR) (13). Calcitriol also appears to slow cell cycle progression by inhibiting the advancement from G1 to the S phases possibly via regulating p21waf1, p27kip1, cyclin D1, and other cell cycle regulators (12).

Cell Differentiation

Calcitriol also stimulates differentiation of immature to mature cells in a variety of tissues. For instance, 1,25-OH₂ D₃ prompts leukemia cells to differentiate into cells exhibiting a monocyte-macrophage phenotype in vitro likely via p21waf1; cultured colon cancer cells to express brush border membrane enzymes; and squamous carcinoma cells to express genes associated with epithelial differentiation (e.g., cystatin M1). The mechanisms by which these processes occur are likely redundant and overlapping. For example, calcitriol-induced differentiation of

keratinocytes may involve induction of phospholipase C as well as stimulation of phosphoinositide turnover (12).

Immunoregulatory Effects of Vitamin D

Considerable attention has also been directed toward several purported roles for vitamin D in modulating immune function. Both the adaptive and innate immune systems are affected by 1,25-OH₂ D₃ and VDRs, and the cells involved express not only the VDRs but also, in some cases, 1- α -hydroxylase. During an immune response, some activated cells induce the production of VDRs and in some cases the expression of 1- α -hydroxylase, providing feedback loops that help regulate the immune response in situ. The effect of 1,25-OH₂ D₃ on the immune system is complicated but, in general, cell-mediated immunity is suppressed by 1,25-OH₂ D₃ whereas other aspects of the immune system including induction of antibacterial peptides by the innate system are increased by 1,25-OH₂ D₃.

Studies also show that macrophages (cells of the innate immune system) produce calcitriol locally following activation through toll-like receptors (TLR) on their cell membranes. The locally produced 1,25-OH₂ D₃ then modulates cytokine production, lymphocyte and macrophage activity, and monocyte maturation. For instance, 1,25-OH₂ D₃ downregulates production of some inflammatory cytokines such as interleukin-12 (IL-12) and tumor necrosis factor (TNF)- α . In addition, antigen presentation is suppressed and the ability of the innate cells to stimulate an acquired immune response is altered so that T cells have decreased production of Th1 cytokines like interferon- γ (INF- γ) and increased regulatory function (e.g., production of IL-10 and TGF- β 1). The investigation of the effects of vitamin D on the innate immune response continues to be a fertile area of research.

The adaptive immune system can also be affected by vitamin D. As noted, some of the effects on adaptive immunity are indirect effects of vitamin D on the innate immune response. There are also well-documented direct effects of vitamin D on T cells. In particular, 1,25-OH₂ D₃ appears to regulate T helper (Th) cell function, skewing the Th1/Th2 balance toward Th2 responses. Experimentally, when vitamin D is low many Th1 cells develop at the expense of the Th2 cells. In addition, important regulatory cells whose function is to shut off an immune response become lacking. Vitamin D also directly and indirectly inhibits Th1 cells while inducing Th2 and other regulatory cells. This vitamin-D-mediated regulation has been shown to be critical for the control of experimental Th1-mediated autoimmune diseases. Paradoxically, infectious diseases such as *Listeria monocytogenes* and *Candida albicans* that require Th1 cells for clearance are not affected by 1,25-OH₂ D₃ or VDR deficiency. One would expect that if 1,25-OH₂ D₃ suppresses Th1 immune responses that *L. monocytogenes* and *C. albicans* infections would be more severe with 1,25-OH₂ D₃ treatment and less severe in the VDR-knockout mouse. This paradox points to significant gaps in our knowledge of the key vitamin-D-regulated targets in the immune response. Most likely, the effect of vitamin D on the immune system is dependent upon local expression of the VDR and 1- α -hydroxylase and the nature of the protective immune response for that disease.

Table 1 Vitamin D Contents of Selected Food Sources

Food type (serving size)	Vitamin D content (IU/serving)
Salmon, smoked (3 oz)	583
Canned tuna, packed in oil (3 oz)	229
Sardines, packed in oil (3 oz)	164
Fortified whole milk (1 cup)	124
Fortified bran cereal (1 cup)	104
Pork shoulder (3 oz)	68
Shiitake mushrooms, cooked (1 cup)	45
Egg, hard-boiled (1 large)	29
Beef frankfurter (1 frank)	16
Turkey (1 cup)	11
Ice cream ($\frac{1}{2}$ cup)	9
Butter (1 Tbsp)	9
Cheddar cheese (1 oz)	7

Source: USDA National Nutrient Database for Standard Reference, Release 22 (15).

FOOD SOURCES

Very few foods in nature contain vitamin D, although the flesh of fish (such as salmon, tuna, and mackerel) and fish liver oils are among the best sources (Table 1). Small amounts of vitamin D are also found in beef, cheese, poultry, and egg yolks. Vitamin D in these foods is primarily in the form of cholecalciferol and its metabolite 25-OH D₃. Some mushrooms provide vitamin D₂ (ergocalciferol) in variable amounts, and mushrooms with enhanced levels of vitamin D₂ from being exposed to UV light are now available.

In the 1930s, the United States implemented a milk fortification program to combat rickets, then a major public health problem. This program virtually eliminated the disorder at that time. Foods allowed to be fortified with vitamin D include cereal flours and related products, milk and products made from milk, and calcium-fortified fruit juices and drinks. Currently, fortified foods provide most of the vitamin D in the American diet (14). The US Department of Agriculture's Nutrient Database (15) lists the vitamin D content of many foods; although it is noteworthy that relatively few foods have been analyzed.

Bioequivalence of Vitamins D₂ and D₃

As previously described, in supplements and fortified foods vitamin D is available in two forms, D₂ (ergocalciferol) and D₃ (cholecalciferol). Vitamin D₂ is manufactured by the UV irradiation of ergosterol in yeast, and vitamin D₃ is manufactured by the irradiation of 7-dehydrocholesterol from lanolin and the chemical conversion of cholesterol. The two forms have traditionally been regarded as bioequivalent based on their ability to cure rickets, but recent evidence suggests that this might not be the case. Following a single large dose, vitamin D₃ raised serum 25-OH D₃ concentrations higher and maintained those levels for a more sustained period of time than did vitamin D₂ (16). However, a subsequent study using lower daily doses found equivalent 25-OH D₃ concentrations with either D₂ or D₃ (17). Because these results conflict, and circulating concentrations of 25-OH D₃ are not a valid functional assessment (in contrast to the earlier bioassay of the healing of rickets), further research is needed to understand the comparative physiologic effects of both forms.

STATUS ASSESSMENT AND DEFICIENCY

Currently, serum concentration of 25-OH D₃ is used to evaluate vitamin D status because it reflects both vitamin D produced cutaneously and that obtained from food and supplements (18) and has a fairly long circulating half-life (15 days) (19). However, serum 25-OH D₃ levels do not reflect the amount of vitamin D stored in other body tissues. Circulating 1,25-OH₂ D₃ is generally not a good indicator of vitamin D status because it has a short half-life (15 hours), and serum concentrations are closely regulated by PTH, calcium, and phosphate (20). Furthermore, levels of 1,25-OH₂ D₃ do not typically decrease until vitamin D deficiency is severe (1). As such, there is considerable discussion of the serum concentrations of 25-OH D₃ that should be used as being indicative of deficiency (e.g., rickets), adequacy for bone health, and optimal overall health. A concentration of <11 ng/mL (or <27.5 nmol/L) is generally considered inadequate; concentrations of >15 ng/mL (>37.5 nmol/L) are recommended. Higher levels (>30 ng/mL or >75 nmol/L) have been proposed by some as desirable for overall health and disease prevention (21), but clinical outcome data are not available to substantiate these higher values. Serum concentrations of 25-OH D₃ consistently above 200 ng/mL (>500 nmol/L) are potentially toxic. Emerging evidence also suggests some increased risk of all-cause mortality and pancreatic cancer associated with 25-OH D₃ levels >75 nmol/L, and evidence-based thresholds of 25-OH D₃ concentrations for deficiency, sufficiency, and adverse outcomes need to be more clearly defined (1).

Standard Reference Material and Quality Control

Because of variability among assays and operators conducting them, a challenge in the field remains the measurement of serum 25-OH D₃ concentrations. Consequently, the National Institute of Standards and Technology recently released a 25-OH D₃ standard reference material that will now permit standardization of values across laboratories (21). Four levels of 25-OH D₃ are available, each with specified values for selected vitamin D metabolites. This material is intended for use as an accuracy control and quality assurances tool and should appreciably improve the accuracy and reliability of future studies focused on reporting vitamin D status.

Deficiency and Prevention

Vitamin D deficiency in humans is usually the result of dietary inadequacy, impaired absorption and use, increased requirements, and/or increased excretion. Vitamin D deficiency can occur when exposure to sunlight is limited. Vitamin D-deficient diets tend to be associated with those consumed in response to milk allergy, lactose intolerance, and veganism.

Rickets and Osteomalacia

Rickets and osteomalacia are the classical vitamin D deficiency diseases, although calcium deficiency can also cause both disorders. In children, vitamin D deficiency causes rickets, a disease characterized by a failure of bone tissue to properly mineralize, resulting in soft bones and skeletal deformities. The fortification of milk with vitamin D has made rickets a rare disease in the United

States. However, it is still reported periodically, particularly among African-American infants and children. For instance, a 2003 report from Memphis described 21 cases of rickets among infants, 20 of whom were African-American (22).

Infancy is a period of substantial risk for vitamin D deficiency, partly because vitamin D requirements cannot always be met by human milk. Indeed, a somewhat recent review of reports of nutritional rickets found that a majority of cases occurred among young, breastfed African-Americans (23). Consequently, the American Academy of Pediatrics (AAP) recommends that exclusively breastfed and partially breastfed infants should begin receiving supplements of 400 IU/day vitamin D shortly after birth and that this should continue until they are weaned and consuming ≥ 1000 mL/day vitamin D-fortified formula or whole milk (24). Similarly, they advise that all nonbreastfed infants ingesting < 1000 mL/day vitamin D-fortified formula or milk should receive a vitamin D supplement of 400 IU/day. The AAP also recommends that older children and adolescents who do not obtain 400 IU/day through vitamin D-fortified milk and foods take a 400 IU vitamin D supplement daily.

Prolonged exclusive breastfeeding without the AAP-recommended vitamin D supplementation is a significant cause of rickets, particularly in dark-skinned infants breastfed by mothers who are not vitamin D replete. Additional causes of rickets include extensive use of sunscreens and placement of children in daycare programs with limited outdoor activity and sun exposure (22). Rickets is also more prevalent among immigrants from Asia, Africa, and the Middle East, possibly because of genetic differences in vitamin D metabolism and/or behavioral differences that lead to less sun exposure (4).

Americans aged 50 years and older are also at increased risk of developing vitamin D insufficiency (2). As people age, skin cannot synthesize vitamin D as efficiently, and the kidneys are less able to convert vitamin D to its active hormone form (18). In fact, it has been reported that as many as half of older adults in the United States with hip fractures could have serum 25-OH D3 levels lower than 12 ng/mL (< 30 nmol/L) (1).

Chronic Disease

As previously mentioned, much recent attention has shifted to the possibility that suboptimal vitamin D status might be related to a variety of chronic diseases. The evidence (or lack thereof) for this is briefly presented here.

Osteoporosis

More than 25 million adults in the United States have or are at risk of developing osteoporosis. Osteoporosis is most often associated with inadequate calcium intake, but insufficient vitamin D also contributes to osteoporosis by reducing calcium absorption. Although rickets and osteomalacia are extreme examples of the effects of vitamin D deficiency, osteoporosis is an example of a long-term effect of calcium and vitamin D insufficiency. Adequate storage levels of vitamin D maintain bone strength and might help prevent osteoporosis in older adults; nonambulatory individuals who have difficulty exercising; postmenopausal women; and individuals chronically taking steroid-containing medications.

Unfortunately, most supplementation trials designed to test rigorously the effects of vitamin D on bone health have also included calcium making it impossible to isolate the independent effects of each nutrient from them. Nonetheless, the authors of a recent evidence-based review concluded that supplements of both vitamin D3 (at 700–800 IU/day) and calcium (500–1200 mg/day) decreased the risk of falls, fractures, and bone loss in elderly individuals (1). The decreased risk of fractures occurred primarily in the oldest women living in nursing homes.

Ironically, although African-Americans have lower circulating levels of 25-OH D3 than Caucasians, they develop fewer osteoporotic fractures. This suggests that factors other than vitamin D provide protection (25). African-Americans have an advantage in bone density from early childhood, a function of their more efficient calcium economy, and have a lower risk of fracture even when they have the same bone density as Caucasians. They also have a higher prevalence of obesity, and the resulting higher estrogen levels in obese women might protect them from bone loss. Likely further reducing their risk of osteoporosis, African-Americans have lower levels of bone-turnover markers, shorter hip-axis length, and superior renal calcium conservation. Despite this advantage in bone density, osteoporosis remains a notable health problem among African-Americans as they age.

Cancer

Laboratory (in vitro) and animal (in vivo) evidence as well as epidemiologic data suggest that vitamin D status could affect cancer risk. Strong biological and mechanistic bases indicate that vitamin D plays a role in the prevention of colon, prostate, and breast cancers. Emerging epidemiologic data suggest that vitamin D has a protective effect against colon cancer, but the data are not as strong for a protective effect against prostate and breast cancer, and are variable for cancers at other sites (26). Studies do not consistently show a protective effect or no effect, however. For instance, researchers studying Finnish smokers found that subjects in the highest quintile of baseline vitamin D status had a threefold *higher* risk of developing pancreatic cancer than those with the lowest vitamin D status (27). Nonetheless, vitamin D emerged as a protective factor for cancer in a prospective, cross-sectional study of 3121 adults who were ≥ 50 years of age (96% men) and undergoing colonoscopy. The investigators found that 10% of the subjects had at least one advanced cancerous lesion and that those with the highest vitamin D intakes had the lowest risk of these lesions (28). However, the Women's Health Initiative found no effect of vitamin D and calcium supplementation on the incidence of colorectal cancers over 7 years (29). More recently, a clinical trial focused on bone health in postmenopausal women found that subjects supplemented daily with calcium (1400–1500 mg) and vitamin D3 (1100 IU) had a significantly lower incidence of cancer over 4 years compared with women taking a placebo (30). The small number of cancers reported ($n = 50$) precludes generalizing about a protective effect from either or both nutrients or for cancers at different sites. This caution is supported by an analysis of 16,618 participants in the National Health and Nutrition Examination Survey (NHANES) III, in which total cancer mortality was found to be unrelated to baseline vitamin D status (31).

However, colorectal cancer mortality was inversely related to serum 25-OH D3 concentrations. Further research, particularly from double-blinded, randomized, clinical trials, is needed to determine both whether greater exposure to vitamin D is generally protective in the population and if some individuals might actually be at *increased* risk of cancer because of greater vitamin D exposure.

Autoimmune disorders

A growing body of research also suggests that vitamin D might play some role in the prevention and treatment of some forms of autoimmune diseases including type 1 diabetes, multiple sclerosis, inflammatory bowel disease, psoriasis, and rheumatoid arthritis. However, most evidence for these roles comes from *in vitro*, animal, and epidemiologic studies, not the randomized clinical trials considered to be more definitive (32).

Infectious disease and all-cause mortality

Poor vitamin D status has also been implicated in increased susceptibility to a variety of infectious diseases. In a meta-analysis using a systematic review of randomized, controlled clinical trials, Yamshchikov and colleagues concluded that there may be a beneficial effect on tuberculosis, influenza, and viral upper respiratory tract illness (33). However, these data are not conclusive and more rigorously designed clinical trials are needed. Similarly, one meta-analysis found that use of vitamin D supplements by middle-aged or elderly individuals was associated with a 7% reduction in all-cause mortality (34), and a systematic evidence-based meta-analysis that only considered the use of oral vitamin D supplements without calcium (400–830 IU/day) by healthy individuals found no relation between vitamin D supplementation and all-cause mortality (32,35). Further, in one of four prospective cohort studies (32) and the NHANES III study (31), lower 25-OH D3 levels were associated with increased risk for all-cause mortality. In summary and as discussed in more detail below, emerging evidence suggests a U-shaped relationship of 25-OH D3 and all-cause mortality with increased risk at both lower and upper extremes.

TOXICITY AND ADVERSE OUTCOMES

Intakes above the Institute of Medicine's Tolerable Upper Intake Level (UL; 2000 IU/day) increase the risk of acute toxicity and adverse health effects (18). Vitamin D toxicity can cause nonspecific symptoms such as nausea, vomiting, poor appetite, constipation, weakness, and weight loss. More seriously, it can raise urinary and blood levels of calcium, the latter of which causes mental status changes such as confusion and heart rhythm abnormalities. Deposition of calcium and phosphate in the kidneys and other soft tissues can also be caused by excessive vitamin D intake. Adverse effects of doses closer to the UL over a longer period time are not well established, although the use of both calcium (1000 mg/day) and vitamin D (400 IU/day) supplements by postmenopausal women was associated with a 17% increase in the risk of kidney stones over 7 years in the Women's Health Initiative (36), and an increased risk of all-cause mortality is suggested in cohort or population studies when 25-OH D3 levels are >75 to

80 nmol/L (32,35). An increased risk of pancreatic cancer was also associated with 25-OH D3 levels >65.5 nmol/L in Finnish male smokers (27). Additional research is needed to determine whether adverse effects occur with higher long-term exposure to vitamin D.

Excessive sun exposure does not result in vitamin D toxicity because the sustained heat on the skin is thought to photodegrade previtamin D3 (precholecalciferol) and vitamin D3 as they are formed. High intakes of dietary vitamin D are very unlikely to result in toxicity unless large amounts of cod liver oil are consumed; toxicity is more likely to occur from excessive supplement use.

RECOMMENDED DIETARY INTAKES

The Institute of Medicine's Food and Nutrition Board has established adequate intake (AI) levels for vitamin D that represent daily intakes thought to be sufficient to maintain bone health and normal calcium metabolism in most healthy people. AIs for vitamin D are listed in both micrograms (μ g) and international units (IU); the biological activity of 1 μ g is equal to 40 IU (Table 2). The AIs for vitamin D are based on the assumption that the entire supply of this vitamin needed by the body is obtained from diet—not synthesized by exposure to sunlight (18). As the Dietary Reference Intake (DRI) values for this nutrient were established in 1997, substantial research has justified a re-evaluation of the values. Specifically, at the time this chapter was written the members of the Board were debating (1) the effects of circulating concentrations of 25-OH D3 on health outcomes, (2) the effects of vitamin D intakes on circulating 25-OH D3 and health outcomes, and (3) levels of intake associated with adverse effects (37). One can find updated information on these discussions and related DRI values online (38).

CONCLUSION

In summary, a growing literature suggests that vitamin D is likely important for many physiologic functions that

Table 2 Dietary Reference Intakes for Vitamin D Through the Lifecycle

Life stage group	μ g/day (IU/day)
Adequate intake (AI) levels	
0–12 mo	5 (200)
1–18 y	5 (200)
19–50 y	5 (200)
51–70 y	10 (400)
70+ y	15 (600)
Pregnancy	5 (200)
Lactation	5 (200)
Tolerable upper intake (UL) levels	
0–12 mo	25 (1000)
1–18 y	50 (2000)
19+ y	50 (2000)
Pregnancy	50 (2000)
Lactation	50 (2000)

Source: Institute of Medicine. Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride. Washington, DC: National Academy Press, 1997.

extend far beyond those directly related to modulation of calcium economy. For example, vitamin D (whether obtained from exogenous or endogenous sources) can regulate gene expression, and this can affect both growth and differentiation of cells. This may be especially important in terms of protecting from and/or predisposing a person to unregulated cell growth that can result in cancer. However, data relating vitamin D status to cancer are conflicting. Furthermore, because of vitamin D's potent immunomodulatory effects, some experts have suggested that perturbations in vitamin D metabolism may be involved in the etiology of a variety of autoimmune disorders, but again the available evidence is limited. Vitamin D is found in very few foods, and recent evidence suggests that several populations, such as breastfed infants and the elderly, might be especially prone to vitamin D deficiency. This has prompted a renewed interest in establishing more rigorous and effective measures of vitamin D deficiency. In addition, scientists are once again re-examining dietary recommendations (including those for supplementation) for this vitamin.

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Vitamin E

Maret G. Traber

INTRODUCTION

Vitamin E was discovered in 1922 by Evans and Bishop (1) and was described as a dietary factor required for reproduction in rodents. Since then, great advances have been made in our understanding of the antioxidant and nonantioxidant roles of vitamin E in human nutrition. Nonetheless, no specific biochemical function, other than that of an antioxidant, has been proven as the mechanism as to why humans require it. Indeed, the nonspecific nature of the vitamin's antioxidant role has led advocates to suggest that amounts far in excess of dietary requirements might be beneficial to promote health, delay aging, and decrease the risk of chronic diseases. This entry will address facts about vitamin E, the gaps in our knowledge, and our expectations for the future.

NAME AND GENERAL DESCRIPTION

Vitamin E [α -tocopherol is called *RRR*- α -tocopherol; or on package labels, d- α -tocopherol; or more formally, 2,5,7,8-tetramethyl-2*R*-(4'*R*,8'*R*,12-trimethyltridecyl)-6-chromanol] is a fat-soluble vitamin (2). Positions 2, 4', and 8' of tocopherols are chiral carbon centers that are in the *R*-conformation in naturally occurring tocopherols (Fig. 1), but theoretically can take on either the *R*- or *S*-conformations. The chemical synthesis of α -tocopherol results in an equal mixture of eight different stereoisomers (*RRR*, *RSR*, *RRS*, *RSS*, *SRR*, *SSR*, *SRS*, and *SSS*). Therefore, synthetic α -tocopherol is called *all rac*- α -tocopherol; or on package labels, dl- α -tocopherol; or more formally, 2,5,7,8-tetramethyl-2*RS*-(4'*RS*,8'*RS*,12-trimethyltridecyl)-6-chromanol.

Dietary components with vitamin E antioxidant activity include α -, β -, γ -, and δ -tocopherols, and α -, β -, γ -, and δ -tocotrienols (2). All these molecules have a chromanol ring and vary in the number of methyl groups on the chromanol ring. Tocopherols have a phytyl tail, while tocotrienols have an unsaturated tail. α -Tocopherol and α -tocotrienol have three methyl groups— β and γ have two, and δ has one.

Importantly, only α -tocopherol meets human vitamin E requirements because only this form has been shown to reverse human vitamin E deficiency symptoms and is recognized preferentially by the hepatic α -tocopherol transfer protein (α -TTP) (2). Defects in the gene for α -TTP result in vitamin E deficiency both in humans and in animal models, as will be discussed later. It is for this reason that vitamin E has been defined for human requirements as α -tocopherol (2).

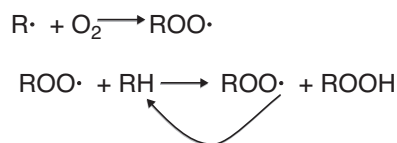
VITAMIN E SUPPLEMENTS

Most vitamin E supplements and food fortificants contain *all rac*- α -tocopherol, and can also have mixtures of tocopherols or tocotrienols. Often, supplements are sold as esters, which protect α -tocopherol from oxidation. These can be acetates, succinates, or nicotinates of α -tocopherol. Either the natural stereoisomer (*RRR*- α -tocopherol) or the synthetic (*all rac*- α -tocopherol) form can be sold as an ester, for example, d- or dl- α -tocopheryl acetate, respectively. However, it is important to note that only half of the vitamin E in synthetic mixtures contains the 2*R*-stereochemistry. Thus, only 50% of *all rac*- α -tocopherol meets human requirements (2).

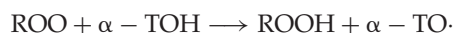
BIOCHEMISTRY AND FUNCTIONS

Antioxidant Activity

Vitamin E is the most potent lipid-soluble antioxidant in human plasma and tissues (3). Hence, it protects polyunsaturated fatty acids within membranes and plasma lipoproteins from oxidation by reactive oxygen species. For example, a peroxy radical (ROO^\bullet) in a membrane is 1000 times more likely to attack a vitamin E molecule than a polyunsaturated fatty acid (RH) (4). In the absence of vitamin E, a chain reaction occurs:



However, if vitamin E (e.g., α -TOH) is present, the hydroxyl group on the chromanol ring reacts with the peroxy radical (ROO^\bullet) to form a tocopheroxyl radical (α -TO $^\bullet$) and a lipid hydroperoxide (ROOH). Thus, vitamin E acts as a chain-breaking antioxidant, thereby preventing further autoxidation of lipids (5).



The tocopheroxyl radical (α -TO $^\bullet$) has a number of possible fates. It can react with another radical to form nonreactive products. Alternatively, it can be further oxidized to tocopheryl quinone, a two-electron oxidation product. Another possibility is "vitamin E recycling," where the tocopheroxyl radical is restored to its unoxidized form by other antioxidants such as vitamin C, ubiquinol, or thiols, such as glutathione (6). This "recycling" process depletes other antioxidants; hence, an adequate intake of other dietary antioxidants is important

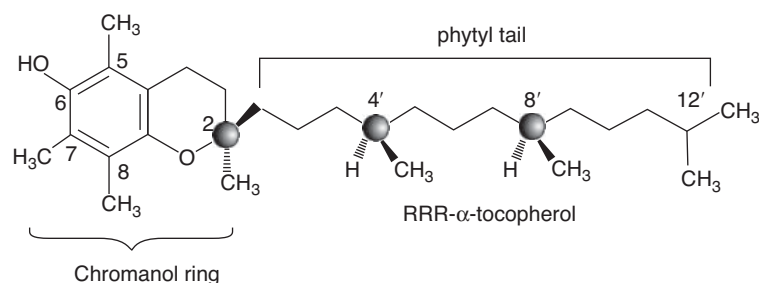


Figure 1 Structure of RRR- α -tocopherol showing three chiral centers with the 2-position important for biologic activity.

to maintain vitamin E concentrations. In addition, the tocopheryoxyl radical, because it is relatively long lived and if there are no other coantioxidants with which it could react, can hypothetically re-initiate lipid peroxidation (7). Upston, Terentis, and Stocker (7) have called this "TMP or tocopherol-mediated peroxidation" and claim it can occur in vivo based on the detection of both oxidized lipids and unoxidized vitamin E in atherosclerotic lesions.

In addition to its antioxidant activity, γ -tocopherol and other non- α -vitamin E forms can also trap reactive nitrogen oxides because they have an unsubstituted position on the chromanol ring (8). Cooney et al. (9) reported that γ -T is more effective in detoxification of NO_2 than α -T. Furthermore, Hoglen et al. (10) demonstrated that 5-nitro- γ -tocopherol (2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-5-nitro-6-chromanol; NGT) is the major reactive product between peroxynitrite and γ -tocopherol. NGT has been reported in the plasma of zymosan-treated rats (11), cigarette smokers (12), patients with coronary artery disease (13), as well as in brains collected postmortem from patients with Alzheimer's disease (14).

All tocopherols and tocotrienols have antioxidant activity, and in some systems many of these have been reported to have higher antioxidant activity than α -tocopherol (15,16). Nonetheless, it must be emphasized that the relationship between biologic activity and antioxidant activity is not clear. α -Tocopherol has the highest biologic activity, suggesting it shows some specific molecular function.

Biologic Activity

Biologic activity is a historic term indicating a disconnection between molecules having vitamin E antioxidant activity and a relative *lack* of in vivo biologic function. Observations in rodent experiments carried out in the 1930s formed the basis for determining the "biologic activity" of this vitamin (17). Although the various molecules with vitamin E activity had somewhat similar structures and antioxidant activities, they differed in their abilities to prevent or reverse specific vitamin E deficiency symptoms (e.g., fetal resorption, muscular dystrophy, and encephalomalacia) (18). α -Tocopherol, with three methyl groups and a free hydroxyl group on the chromanol ring with the phytyl tail meeting the ring in the *R*-orientation (Fig. 1), has the highest biological activity. This specific structural requirement for biological, but not chemical, activity is now known to be dependent upon the hepatic α -TTP (19).

As will be discussed later, α -TTP maintains plasma, and indirectly tissue, α -tocopherol concentrations (20,21).

Molecular Functions

In addition to antioxidant activity, there are claims for specific α -tocopherol-dependent functions that normalize cellular signaling and metabolism in a variety of cells (22). α -Tocopherol has been shown to inhibit the activity of protein kinase C (23), a central player in many signal transduction pathways. Specifically, pathways of platelet aggregation (24,25), endothelial cell nitric oxide production (26,27), monocyte/macrophage superoxide production (28), and smooth muscle cell proliferation (29) were found to be modulated by added α -tocopherol. Regulation of adhesion molecule expression and inflammatory cell cytokine production by α -tocopherol has also been reported (30). The difficulty with these studies is that animals fed vitamin E-deficient diets are genetically lacking α -TTP and have not been reported to have altered expression of any of these pathways.

There have been reports of regulation of the expression of lipoprotein receptors by α -tocopherol. Both the scavenger receptor B1(SR-B1) (31), and its homolog, CD36 (32,33), are decreased by high cellular α -tocopherol and increased by low concentrations.

γ -Tocopherol, as well as its metabolite (γ -CEHC; γ -carboxyethyl hydroxychroman), possesses anti-inflammatory properties, because stimulated macrophages and epithelial cells, treated with γ -tocopherol, have decreased cyclo-oxygenase-2 activity and lower levels of prostaglandin E_2 (PGE_2) synthesis (34). Moreover, in rats fed a high γ -T diet (33 mg/kg chow) and subjected to carrageenan-induced inflammation, PGE_2 and leukotriene B_4 synthesis were decreased by 46% and 70%, respectively (35). Additionally, γ -CEHC has been shown to increase sodium excretion (36).

The in vivo significance of many of these various effects and the role of vitamin E in signaling pathways remain controversial because most of the information in this area has been obtained from in vitro studies. Additionally, microarray technology has been used to show changes in gene expression in response to vitamin E (37,38), but the physiologic relevance has not yet been clearly documented. More studies in humans are needed to relate α -tocopherol intakes and tissue concentrations to optimal tissue responses and gene regulation. It should be recognized, however, that it is very possible that vitamin E itself has no gene regulatory function, as reviewed (39).

PHYSIOLOGY

Absorption and Plasma Transport

Intestinal absorption of vitamin E is dependent upon normal processes of fat absorption. Specifically, both biliary and pancreatic secretions are necessary for solubilization of this vitamin in mixed micelles containing bile acids, fatty acids, and monoglycerides (Fig. 2). α -Tocopheryl acetates (or other esters) from vitamin E supplements are hydrolyzed by pancreatic esterases to α -tocopherol prior to absorption. Low-fat diets limit vitamin E absorption, especially from supplements (40). Following micellar uptake by enterocytes, it is incorporated into chylomicrons and secreted into the lymph. Once in the circulation, chylomicron triglycerides are hydrolyzed by lipoprotein lipase (LPL). During chylomicron catabolism in the circulation, vitamin E is nonspecifically transferred both to tissues and to other circulating lipoproteins (41).

It is not until the vitamin E-containing chylomicrons reach the liver that discrimination between the various dietary vitamin E forms occurs. The hepatic α -TTP preferentially facilitates secretion of α -tocopherol, specifically 2R- α -tocopherols, but not other tocopherols or tocotrienols, from the liver into the plasma in very low density lipoproteins (VLDLs) (42,43). In the circulation, VLDLs are catabolized to low-density lipoproteins (LDLs). During this lipolytic process, all of the circulating lipoproteins become enriched with α -tocopherol.

There is no evidence that vitamin E is transported in the plasma by a specific carrier protein. Instead, the vitamin is nonspecifically transported in all of the lipopro-

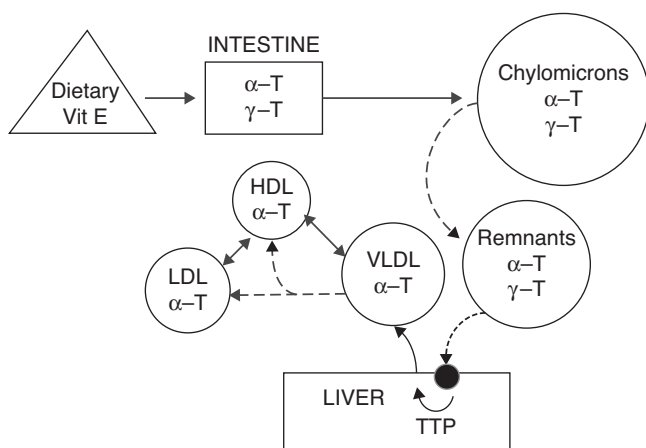


Figure 2 Intestinal vitamin E absorption and plasma lipoprotein transport. Vitamin E absorption requires both biliary and pancreatic secretions for solubilization of vitamin E in mixed micelles. Following micellar uptake by enterocytes, vitamin E (shown as α - and γ -tocopherols, α -T and γ -T) is incorporated into chylomicrons and is secreted into the lymph. During chylomicron catabolism in the circulation, it is nonspecifically transferred both to tissues and to other circulating lipoproteins (not shown). It is not until the vitamin E-containing chylomicrons reach the liver that discrimination between the various dietary vitamin E forms occurs. The hepatic α -TTP preferentially facilitates secretion of α -tocopherol from the liver into the plasma in very low density lipoproteins (VLDLs). In the circulation, VLDLs are catabolized to LDLs. During this lipolytic process, all of the circulating lipoproteins (e.g., LDL and HDL) become enriched with α -tocopherol.

tein fractions (44). An advantage of this transport is that oxidation-susceptible lipids are protected by the simultaneous transport of a lipid-soluble antioxidant. Similarly, delivery of vitamin E to tissues is dependent upon lipid and lipoprotein metabolism. Thus, as peroxidizable lipids are taken up by tissue, the tissues simultaneously acquire a lipid-soluble antioxidant.

Plasma α -tocopherol concentrations in humans range from 11 to 37 $\mu\text{mol/L}$, while γ -tocopherol concentrations are roughly 2 to 5 $\mu\text{mol/L}$ and tocotrienol concentrations are less than 1 $\mu\text{mol/L}$, even in subjects supplemented with tocotrienols (45). When plasma lipids are taken into account, the lower limits of normal level are 1.6 μmol α -tocopherol/ mmol lipid (sum of cholesterol and triglycerides), or 2.5 μmol α -tocopherol/ mmol cholesterol (46).

The apparent half-life of RRR- α -tocopherol in plasma of normal subjects is approximately 48 hours (47), while that of SRR- α -tocopherol is only 15 hours (47), and that of γ -tocopherol is also similar to the SRR- α -tocopherol, about 15 hours (48). This relatively fast turnover of 2 S- α -tocopherol is also accompanied by increased metabolism (49). The comparatively fast disappearance of the 2 S- α -tocopherols indicates that by 48 hours, nearly 90% of the 2 S-forms have been removed from the plasma, while 50% of the 2R-forms remain. It is then no wonder that the plasma disappearance curves of RRR- and all *rac*- α -tocopherols are parallel; they both trace the disappearance of 2R-forms (50–52).

Tissue Delivery

Vitamin E is delivered to tissues by three methods, none of which is specific for vitamin E. But rather its trafficking depends on mechanisms of lipid and lipoprotein metabolism. These include transfer from triglyceride-rich lipoproteins during lipolysis, delivery as a result of receptor-mediated lipoprotein uptake, and exchange between lipoproteins or tissues.

With respect to lipolysis, LPL facilitates the delivery of α -tocopherol from triglyceride-rich lipoproteins to cells, as shown *in vitro* (53). The importance of this pathway was demonstrated *in vivo* when LPL was overexpressed in muscle, resulting in increased vitamin E delivery to muscle (54).

Both low- and high-density lipoproteins (LDL and HDL, respectively) have been shown to deliver vitamin E to tissues. The LDL receptor-mediated uptake of LDL delivers the lipoprotein particle via an endocytic pathway, and vitamin E is released during lipoprotein degradation (55). In contrast, HDL binds to the SR-BI allowing selective delivery of the HDL lipids, including vitamin E, to the cells (56). In SR-BI knockout mice, plasma α -tocopherol concentrations are elevated. Some tissues (e.g., brain (57) and lung (31)) contain decreased α -tocopherol contents, while hepatic tocopherol concentrations are unchanged. But biliary tocopherol excretion is decreased (58). Apparently, SR-BI-mediated hepatic uptake of HDL-associated α -tocopherol is coupled to biliary excretion of vitamin E (58).

Although vitamin E spontaneously exchanges between lipoproteins (59), the phospholipid transfer protein facilitates the exchange of phospholipids between

lipoproteins, as well as the transfer of vitamin E from VLDL to HDL and from lipoproteins into cells (60). Phospholipid transfer protein knockout mice compared with wild types have higher vitamin E in apolipoprotein B-containing lipoproteins (VLDL or LDL) (61). The involvement of the plasma cholesteryl ester transfer protein in this transfer process was ruled out (61).

The regulation of tissue vitamin E is not well understood, but it is seen that α -tocopherol is the predominant form in tissues as a result of its plasma concentrations (20). The ATP-binding cassette transporter (ABCA1) has been shown to participate in the efflux of α -tocopherol from cells to HDL (62). Apparently, excess vitamin E could be removed from cells via ABCA1 facilitating its transfer to apolipoprotein AI, and transport via HDL to the liver where SR-BI could mediate vitamin E transfer into a liver pool destined for excretion in bile.

Metabolism and Excretion

Vitamin E is excreted as intact tocopherols or tocotrienols, oxidized forms, and a metabolic product (41). α - and γ -Tocopherols as well as α - and γ -tocotrienols are metabolized to α - and γ -CEHCs [2,5,7,8-tetramethyl- and 2,7,8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychromans], respectively, by humans (41). CEHCs were first described in rats fed high amounts of δ -tocopherols (63). About 1% of a dose of α -tocopherol or tocotrienol and 5% of a dose of γ -tocopherol or tocotrienol are excreted in the urine as CEHCs (64). On the basis of studies in hepatocytes (65,66), it is likely that the liver synthesizes CEHCs. Studies in renal dialysis patients (67,68) suggest that in addition to urinary excretion (41), bile may be a major route for CEHC excretion. Similarly, CEHCs have been found in both rat urine and bile (69).

Vitamin E metabolism appears to be a key factor in the regulation of vitamin E bioavailability (70). The various forms of vitamin E appear to be metabolized similar to xenobiotics in that they are initially oxidized by P450s, conjugated, and excreted in urine or bile. CEHCs have a shortened phytyl tail, resulting from ω -oxidation, a cytochrome P450 (CYP)-mediated process, followed by β -oxidation (71–73). Hepatic CYP 4F2 is involved in ω -oxidation of α - and γ -tocopherols (73), as is CYP 3A (65,71,72,74). It should be noted that a compound can stimulate CYPs other than those involved in its own metabolic pathway; thus, interactions with a variety of pathways are possible.

CEHCs can be sulfated or glucuronidated (75–77). Both free and conjugated forms have been detected in plasma (76) urine (41), and bile (78). All of the systems involved in vitamin E metabolism could be under PXR regulation (79). However, Cho et al. (80) showed that stimulation of PXR by the mouse PXR activator, pregnenolone 16 α -carbonitrile (PCN), in wild-type compared with PXR-null mice decreased vitamin E metabolism. Thus, it appears that PXR-activation does not increase vitamin E metabolism. They also identified a new CEHC conjugate, a glycoside. Importantly, PCN treatment in wild-type mice, which stimulates PXR, decreased the urinary excretion of α -CEHC glucuronide and γ -CEHC glycoside. Thus, the regulation of vitamin E metabolism by PXR remains unclear.

Dietary vitamin E forms, such as γ -tocopherol (81) or γ -tocotrienol (82), are more actively metabolized to CEHCs than α -tocopherol (49,64,75). In fact, nearly all of the absorbed γ -tocopherol has been estimated to be metabolized to γ -CEHC (75). High α -tocopherol intakes, for example, supplements, lead to both increased α -CEHC (83) and γ -CEHC excretion (64). Thus, vitamin E metabolism may be a key factor in hepatic disposal of excess vitamin E, as well as a key determinant in vitamin E bioavailability.

HUMAN VITAMIN E DEFICIENCY

Vitamin E deficiency was first described in children with fat malabsorption syndromes, principally abetalipoproteinemia, cystic fibrosis, and cholestatic liver disease (84). Subsequently, humans with severe deficit with no known defect in lipid or lipoprotein metabolism were described to have a defect in the α -TTP gene (85).

Erythrocyte fragility, hemolysis, and anemia were described as vitamin E deficiency symptoms in various animals fed diets devoid of this antioxidant (86). However, in humans, the major symptom is a peripheral neuropathy characterized by the degeneration of large caliber axons in the sensory neurons (87).

INDICATIONS AND USAGE

Food Sources

Vitamin E can be readily obtained from food, but relatively few foods have high α -tocopherol concentrations (88). Generally, the richest sources are vegetable oils. Wheat germ oil, safflower oil, and sunflower oil contain predominantly α -tocopherol, while soy and corn oils have mainly γ -tocopherol. All of these oils are polyunsaturated. Good sources of monounsaturated oils, such as olive or canola oils, also have α -tocopherol to a large extent. Whole grains and nuts, especially almonds, are also good α -tocopherol sources. Fruits and vegetables, although rich in water-soluble antioxidants, are *not* good sources of vitamin E. Indeed, desserts are a major source of vitamin E in the American diet (89).

In the past, it was assumed for the purpose of calculating dietary vitamin E intakes in α -tocopherol equivalents (α -TEs) that γ -tocopherol can substitute for α -tocopherol with an efficiency of 10% (90). However, functionally, γ -tocopherol is not equivalent to the latter. Caution should be exercised in applying α -TEs to estimates of α -tocopherol intakes when corn or soybean oils (hydrogenated vegetable oils) represent the major oils present in foods. These oils have high γ -tocopherol contents, and if food tables reporting α -TEs are used to estimate dietary α -tocopherol, intakes of α -tocopherol may be overestimated.

Treatment of Vitamin E Deficiency

Overt vitamin E deficiency occurs only rarely in humans and almost never as a result of inadequate vitamin E intakes. It does occur as a result of genetic abnormalities in α -TTP (87) and various fat malabsorption syndromes (91). Vitamin E supplementation halts the progression of the neurologic abnormalities caused by inadequate

nerve tissue α -tocopherol, and in some cases, has reversed them (92).

Patients with these disorders require daily pharmacologic vitamin E doses for life to overcome and prevent the deficiency symptoms. Generally, subjects with ataxia with vitamin E deficiency are advised to consume 1000 mg *RRR*- α -tocopherol per day in divided doses, those with abetalipoproteinemia 100 mg/kg body weight per day, and for cystic fibrosis 400 mg/day. However, patients with fat malabsorption due to impaired biliary secretion generally do not absorb orally administered vitamin E. They are treated with special forms of vitamin E, such as α -tocopheryl polyethylene glycol succinate, which spontaneously form micelles, obviating the need for bile acids (93).

Chronic Disease Prevention

In individuals at risk for vitamin E deficiency, it is clear that supplements should be recommended to prevent deficiency symptoms. What about vitamin E supplement in normal individuals? Dietary changes such as decreasing fat intakes (94), substituting fat-free foods for fat-containing ones, and increasing reliance on meals away from the home have resulted in decreased consumption of α -tocopherol-containing foods. Therefore, intakes of the vitamin E recommended dietary allowance (RDA)—15 mg α -tocopherol—may be difficult. Estimates of α -tocopherol intakes by Americans suggest that less than 10% consume adequate amounts of the vitamin, and that women have lower intakes than men (95). Increased consumption of nuts and seeds, as well as olive and canola oils, may be useful in increasing dietary α -tocopherol intakes.

The potential role of vitamin E in preventing or ameliorating chronic diseases has prompted many investigators to ask if supplements might be beneficial. When “excess” amounts of many vitamins are consumed, they are excreted and provide no added benefits. Antioxidant nutrients may, however, be different. Heart disease and stroke, cancer, chronic inflammation, impaired immune function, Alzheimer’s disease—a case can be made for the role of oxygen-free radicals and inflammation in the etiology of all of these disorders, and even in aging itself. Do antioxidant nutrients counteract the effects of free radicals and thereby ameliorate these disorders? And if so, do large quantities of antioxidant supplements have beneficial effects beyond “required” amounts? *The 2000 DRI Report on Vitamin C, Vitamin E, Selenium, and Carotenoids* stated that there was insufficient proof to warrant advocating supplementation with antioxidants (2). But it also stated that the hypothesis that antioxidant supplements might have beneficial effects was promising. Despite the lack of positive findings from various intervention studies (96,97), and some more positive findings from others (98–101), the consequences of a long-term increased antioxidant intake in healthy people are not known. Moreover, a study examining the relationship between the genetic background of diabetic women and the benefits of antioxidant supplementation found a marked beneficial effect on coronary artery stenoses in haptoglobin 1 allele homozygotes, but not in those with the haptoglobin 2 allele (102). Moreover, haptoglobin 2–2 diabetic subjects were studied

in a prospective, double-blind, placebo-controlled trial of vitamin E. Vitamin E supplementation reduced cardiovascular events in diabetic individuals and the Hp 2–2 genotype (ClinicalTrials.gov NCT00220831) (103). Thus, it would appear that subjects with high oxidative stress and the appropriate genetic background may benefit from antioxidant supplements, but not in those without these factors.

Dietary Reference Intakes

In 2000, the Food and Nutrition Board of the Institute of Medicine, National Academy of Sciences published the *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium and the Carotenoids* (2). Recommendations for vitamin E intakes are shown in Table 1.

The requirements for vitamin E intakes are based primarily on its long-term (5–7 yr) depletion and repletion studies in humans carried out by Horwitt et al. (104). Serum α -tocopherol concentrations and the corresponding hydrogen peroxide-induced erythrocyte hemolysis were determined at various intervals. Serum concentrations necessary to prevent in vitro erythrocyte hemolysis in response to known levels of vitamin E intake in subjects who had undergone experimentally induced vitamin E deficiency were used to determine estimated average requirements for vitamin E. The RDAs are levels that represent the daily α -tocopherol intakes required to ensure adequate nutrition in 95% to 97.5% of the population and are an overestimation of the level needed for most people in any given group.

Vitamin E Units

The Food and Nutrition Board defined vitamin E for human requirements to include only α -tocopherol and specifically those forms with 2*R*- α -tocopherol stereochemistry (2). According to the *U.S. Pharmacopoeia* (USP), 1 international unit (IU) of vitamin E equals 1 mg *all rac*- α -tocopheryl acetate, 0.67 mg *RRR*- α -tocopherol, or 0.74 mg *RRR*- α -tocopheryl acetate (105). These conversions were estimated on the relative “biologic activities” of the various forms when tested in the rat assay for vitamin E deficiency, the fetal resorption assay. These USP IUs are currently used in labeling vitamin E supplements and food fortificants. It should be noted that the 2000 RDA does not use vitamin E USP units; rather the recommendation is set at 15 mg 2*R*- α -tocopherols. To convert IU to milligram of 2*R*- α -tocopherols, the IU *RRR*- α -tocopherol (or its esters)

Table 1 Estimated Average Requirements (EARs), Recommended Dietary Allowances (RDAs), and Average Intakes (AIs) (mg/day) for α -Tocopherol in Adults and Children

Lifestage	EAR	RDA	AI
0–6 mo			4
7–12 mo			6
1–3 yr	5	6	
4–8 yr	6	7	
9–13 yr	9	11	
14–18 yr	12	15	
Adult (male or female)	12	15	
Pregnancy	12	15	
Lactation	16	19	

Source: From Ref. 2.

is multiplied by 0.65, while the IU *all rac*- α -tocopherol (or its esters) is multiplied by 0.45.

ADVERSE EFFECTS

Upper Tolerable Limits

High vitamin E intakes are associated with an increased tendency to bleed. It is not known if this is a result of decreased platelet aggregation caused by an inhibition of protein kinase C by α -tocopherol (24), some other platelet-related mechanism (106), or decreased clotting due to a vitamin E interaction with vitamin K (107). It has also been suggested that extraordinarily high vitamin E intakes may interfere with activation of vitamin K (108). Individuals who are deficient in vitamin K or who are on anticoagulant therapy are at increased risk of uncontrolled bleeding. Thus, patients on anticoagulant therapy should be monitored when taking vitamin E supplements to ensure adequate vitamin K intakes (109). This "adverse effect" may actually be a benefit for some individuals! Glynn et al. (110) report that vitamin E supplements may decrease the risk of venous thromboembolism, especially in those with a genetic predisposition to clotting.

The 2000 Food and Nutrition Board of the Institute of Medicine, National Academy of Sciences, recommended 1000 mg as an upper limit (UL) of all forms of α -tocopherol in supplements taken by adults 19 years and older, including pregnant and lactating women. The vitamin E UL was set for only supplements because it is impossible to consume enough α -tocopherol-containing foods to achieve a daily 1000 mg intake for prolonged periods of time. The UL was defined for *all* forms of α -tocopherol, not just the 2*R*-forms, because all eight of the stereoisometric forms in *all rac*- α -tocopherol are absorbed and delivered to the liver and therefore potentially have adverse effects. The ULs for supplements containing either *RRR*- or *all rac*- α -tocopherol supplements are 1500 IU *RRR*- α -tocopherol or its esters, or 1100 IU of *all rac*- α -tocopherol or its esters. The UL for *RRR*- α -tocopherol is apparently higher because each capsule of *RRR*- α -tocopherol contains fewer milligram of α -tocopherol than does one containing *all rac*- α -tocopherol.

ULs were set for children and adolescents by adjusting the adult limit on the basis of relative body weight. No UL was set for infants because of lack of adequate data. The 2000 Food and Nutrition Board did recommend that food be the only source of vitamin E for infants. However, a UL of 21 mg/day was suggested for premature infants with birth weight of 1.5 kg, on the basis of the adult UL.

Adverse Interactions of Drugs and Vitamin E

Drugs intended to promote weight loss by impairing fat absorption, such as Orlistat or olestra, can also impair vitamin E and other fat-soluble vitamin absorption. Therefore, multivitamin supplementation is recommended. Supplements should be taken with meals at times other than when these drugs are taken to allow adequate absorption of the fat-soluble vitamins.

The inhibition of cholesterol absorption by Etzetimibe interferes with the function of Niemann-Pick C1-like

1 protein. This drug has also been found to decrease vitamin E absorption in rats (111). It is therefore possible that Etzetimibe also interferes with vitamin E absorption in humans.

Findings from two clinical trials have suggested adverse vitamin E effects. One study was a three-year, double-blind trial of antioxidants (vitamins E and C, β -carotene, and selenium) in 160 subjects on simvastatin-niacin or placebo therapy (112,113). In subjects taking antioxidants, there was less benefit of the drugs in raising HDL cholesterol than was expected (112), while there was an increase in clinical end points [arteriographic evidence of coronary stenosis, or the occurrence of a first cardiovascular event (death, myocardial infarction, stroke, or revascularization)] (113). The other study was the Women's Angiographic Vitamin and Estrogen (WAVE) Trial, a randomized, double-blind trial of 423 postmenopausal women with at least one coronary stenosis at baseline coronary angiography. In postmenopausal women on hormone replacement therapy, all-cause mortality was higher in women assigned to antioxidant vitamins compared with placebo group (HR, 2.8; 95% CI, 1.1–7.2; $P = 0.047$) (114). The reasons for these adverse effects, especially mortality, are unclear because a meta-analysis of more than 80,000 subjects taking part in vitamin E intervention trials did not find increased mortality in those taking vitamin E (115). Other meta-analyses have reported slightly increased risk of mortality, but could not identify any mechanisms of action (116,117) while other researchers have criticized the methodologies used for the meta-analyses and found no evidence of vitamin E adverse effects (118).

CONCLUSIONS

One of the real difficulties in setting requirements or making recommendations for optimal vitamin E intakes is that the function of the antioxidant remains undefined. Certainly, its *in vitro* antioxidant function has been agreed upon for decades, but questions remain as to whether this is the only function of vitamin E, or if indeed antioxidant activity is its *in vivo* function (39,119,120). In addition, if the vitamin functions solely as an antioxidant, then biomarkers of oxidative stress will never be useful for setting requirements because oxidative damage certainly can be modulated by antioxidants in addition to vitamin E. Thus, one of the major thrusts is to establish the function of vitamin E. One important area that is currently under investigation is its role in inflammation (121) and immune function (122). But, here again, the role of oxidative stress confounds the findings because leukocytes release reactive oxygen species and this is attenuated by vitamin E (28). Clearly, defining vitamin E function(s) is the goal of future studies.

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Vitamin K

J. W. Suttie

INTRODUCTION

Vitamin K activity is exhibited by phyloquinone, found in green plants, and by a series of menaquinones, which are synthesized by a limited number of anaerobic bacteria. The metabolic role of this vitamin is as a substrate for an enzyme, the vitamin K-dependent carboxylase, which mediates a post-translational modification of a small number of proteins by converting specific glutamyl residues to γ -carboxyglutamyl (Gla) residues. These include a number of proteins that regulate hemostasis: prothrombin, factor VII, factor IX, factor X, and proteins C, S, and Z. The bone proteins, osteocalcin and matrix Gla protein (MGP), and several other less well-characterized proteins also require vitamin K for their synthesis. The human requirement for vitamin K is low, and the adequate intake for adult men and women is currently set at 120 and 90 $\mu\text{g}/\text{day}$, on the basis of the median intakes of the U.S. population. The classical symptom of a vitamin K deficiency, a hemorrhagic event, is essentially impossible to produce in adults without some underlying factor influencing absorption of the vitamin. However, newborn infants are routinely supplemented with vitamin K to prevent a condition called hemorrhagic disease of the newborn. A small amount of the protein osteocalcin circulates in plasma, and because this protein is not maximally γ -carboxylated at normal levels of intake, there is currently a great deal of interest in a possible role of vitamin K in promoting skeletal health. Supplementation of the diet with 45 mg of menaquinone-4 is a widely used treatment for osteoporosis in Japan and other parts of Asia, but the efficacy of this treatment in North America or Europe has not yet been established. The possibility of an impact of vitamin K status on atherosclerosis outcomes through the action of MGP is also a problem of research interest.

Background, Chemistry, and Dietary Sources

In the early 1930s, Henrik Dam observed that chicks consuming very low lipid diets developed subdural or muscular hemorrhages and that blood taken from these animals clotted slowly. This hemorrhagic disease could not be cured by supplementation with any other known dietary factor, and Dam (1) proposed the existence of a new fat-soluble factor, vitamin K. Subsequent studies by Dam and others (2) established that the antihemorrhagic factor was present both in the lipid extracts of green plants and in preparations of fish meal that had been subjected to bacterial action. The vitamin could be isolated from alfalfa as a yellow oil, and it was characterized as 2-methyl-3-phytyl-1,4-naphthoquinone (3) and synthesized

by Doisy's group at the St. Louis University. The Doisy group also isolated a crystalline form of the vitamin from putrefied fish meal and demonstrated that this compound contained an unsaturated polyprenyl side chain at the 3-position of the naphthoquinone ring.

The term vitamin K is now used as a generic descriptor of 2-methyl-1,4-naphthoquinone (menadione) and in all derivatives of this compound that exhibit an antihemorrhagic activity in animals fed a vitamin K-deficient diet (Fig. 1). The major dietary source of vitamin K, the form found in green plants, is commonly called vitamin K₁, but is preferably called phyloquinone. The compound, 2-methyl-3-farnesylgeranylgeranyl-1,4-naphthoquinone, first isolated from putrefied fish meal, is one of a series of vitamin K compounds with unsaturated side chains called multiprenylmenaquinones, which are produced by a limited number of anaerobic bacteria and are present in large quantities in the lower bowel. This particular menaquinone has 7 isoprenoid units in the

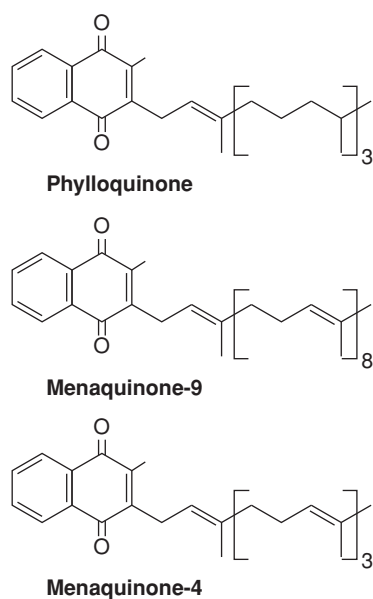


Figure 1 Structures of vitamin K active compounds. Phyloquinone (vitamin K₁) synthesized in plants is the main dietary form of vitamin K. Menaquinone-9 is a prominent member of a series of menaquinones (vitamin K₂) produced by intestinal bacteria and menaquinone-4, while a minor bacterial product can also be synthesized by animal tissues from phyloquinone.

side chain and was once called vitamin K₂. That term is currently used to describe any of the vitamers with an unsaturated side chain, and this compound is more correctly identified as menaquinone-7 (MK-7). The predominant menaquinones found in the gut are MK-7 through MK-9, but smaller amounts of others are also present. Menadione is used as a source of vitamin K activity in poultry and swine rations, and a specific compound, (MK-4), is formed in animal tissues by its alkylation (4). This is the biologically active form of the vitamin present in animal tissues when menadione is used as the dietary form of vitamin K.

Standardized procedures to assay the vitamin K content of foods, and sufficient values (5) to provide reasonable estimates of its daily intake are now available (Table 1). In general, foods with higher phyloquinone content are green leafy vegetables. Those providing substantial amounts of the vitamin to the majority of the population are spinach (380 µg/100g), broccoli (180 µg/100g), and iceberg lettuce (35 µg/100g). Fats and oils are also a major contributor to the vitamin K content of the diet. Soybean oil (190 µg/100g) and canola oil (130 µg/100g) are quite high, while corn oil (3 µg/100g) is a very poor source. The source of fat or oil will influence the vitamin K content of margarine and prepared foods with a high-fat content. The process of hydrogenation to convert plant oils to solid margarines or shortening, converts some of the phyloquinone to 2',3'-dihydrophyloquinone with a completely saturated side chain. The biological activity of this form of the vitamin is not accurately known, but it has been reported that the intake of this form of the vitamin by the American population may be 20% to 25% that of phyloquinone (6).

Vitamin K-dependent Proteins

The first proteins identified as requiring vitamin K for their synthesis were plasma clotting factors, and the classical sign of a vitamin K deficiency has been the development of a hemorrhagic syndrome. Many of the proteins involved in regulating blood coagulation (Fig. 2) are protease zymogens, which are sequentially activated through a series of events, many involving membrane-associated complexes with each other and with accessory proteins (7–9). Prothrombin (clotting factor II) is the circulating zymogen of the procoagulant thrombin, and was the first protein shown to be dependent on vitamin K for its synthesis. Clotting factors VII, IX, and X were all initially identified because their activity was decreased in the plasma of a patient with a hereditary bleeding disorder (10) and were subsequently shown to depend on vitamin K for their synthesis. These four “vitamin K-dependent clotting factors” were the only proteins known to require this vitamin for their synthesis until the mid-1970s. The distinguishing character of vitamin K-dependent proteins is the presence of a post-translational modified glutamic acid (Glu) residue, γ-carboxyglutamic acid (Gla). Proteins C and S were discovered after it had been shown that prothrombin contained Gla residues. They were subsequently shown to have an anticoagulant, rather than a procoagulant, role in hemostasis. The seventh vitamin K-dependent plasma protein, protein Z, is not a protease zymogen and also exhibits an anticoagulant role under some conditions.

Table 1 Phyloquinone Concentration of Common Foods^a

Food item	µg/100g
<i>Vegetables</i>	
Collards	440
Spinach	380
Salad greens	315
Broccoli	180
Brussels sprouts	177
Cabbage	145
Bib lettuce	122
Asparagus	60
Okra	40
Iceberg lettuce	35
Green beans	33
Green peas	24
Cucumbers	20
Cauliflower	20
Carrots	10
Tomatoes	6
Potatoes	1
<i>Protein sources</i>	
Dry soybeans	47
Dry lentils	22
Liver	5
Eggs	2
Fresh meats	<1
Fresh fish	<1
Whole milk	<1
<i>Fats and oils</i>	
Soybean oil	193
Canola oil	127
Cottonseed oil	60
Olive oil	55
Margarine	42
Butter	7
Corn oil	3
<i>Prepared foods</i>	
Salad dressings	100
Coleslaw	80
Mayonnaise	41
Beef chow mein	31
Muffins	25
Doughnuts	10
Potato chips	15
Apple pie	11
French fries	5
Macaroni/cheese	5
Lasagna	5
Pizza	4
Hamburger/bun	4
Hot dog/bun	3
Baked beans	3
Bread	3

^aMedian values.

Source: From Ref. 5.

The amino terminal, “Gla domain,” of the four vitamin K-dependent procoagulants is very homologous, and the 10 to 13 Gla residues in each are in essentially the same position as in prothrombin. These proteins play a critical role in hemostasis, and a large number of genetic variants of these proteins have been identified as risk factors in coagulation disorders (11). The cDNA and genomic organization of each of these proteins is also well documented (11).

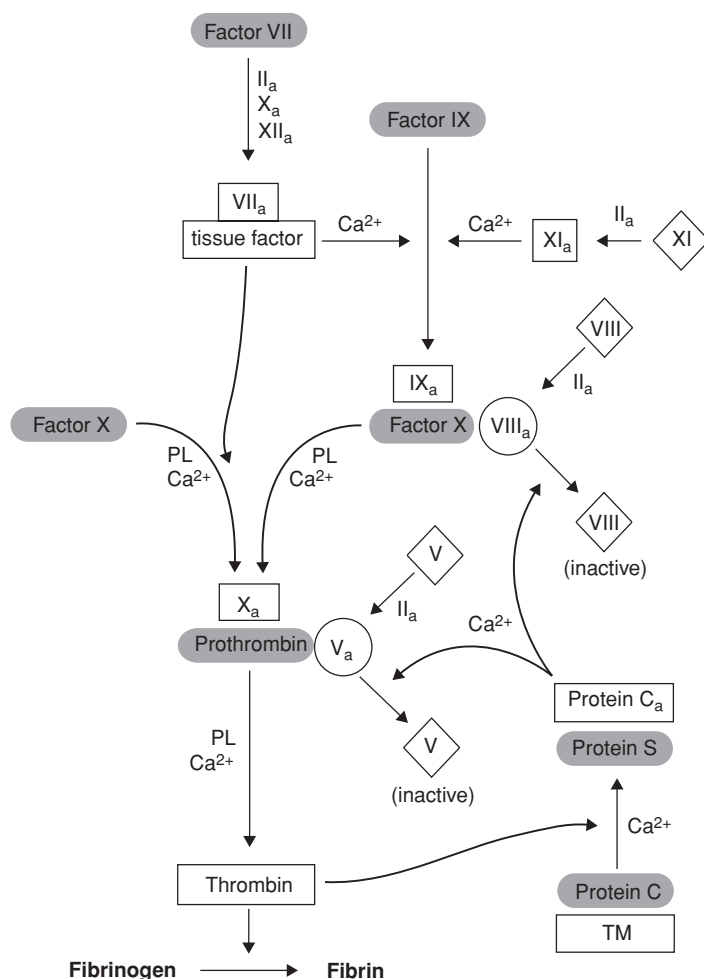


Figure 2 Involvement of vitamin K-dependent clotting factors in blood coagulation. The vitamin K-dependent procoagulants shown as gray ovals (prothrombin, F-VII, F-IX, F-X) circulate as zymogens until converted to their active (subscript a) forms. This process is initiated by an “extrinsic” pathway when vascular injury exposes tissue factor to blood. The product of the activation of one factor can activate a second zymogen, and this cascade effect results in the rapid activation of prothrombin to thrombin and the subsequent conversion of soluble fibrinogen to the insoluble fibrin clot. A number of steps in this series of activations involve an active protease, a second vitamin K-dependent protein substrate, and an additional plasma protein cofactor (circles) to form a Ca²⁺-mediated association with a phospholipid surface. The formation of activated F-X can also take place through an “intrinsic” pathway involving thrombin activation of F-XI and subsequently F-IX. Two vitamin K-dependent proteins, which participate in hemostatic control, function as anticoagulants not procoagulants. Protein C is activated by thrombin (II_a) in the presence of an endothelial cell protein called thrombomodulin (TM). Activated protein C functions in a complex with protein S to inactivate V_a and VIII_a and to limit clot formation.

The first vitamin K-dependent, Gla-containing protein discovered, which was not located in plasma, was the bone protein osteocalcin (12,13). This 49-residue protein contains three Gla residues and has little structural homology to the vitamin K-dependent plasma proteins. Although it is the second most abundant protein in bone, its function is not clearly defined. It does appear to be involved in some manner in the control of tissue mineralization or skeletal turnover, but lack of functional osteocalcin has not been related to poor mineralization, and osteocalcin gene “knockout” mice have been seen to produce more dense bone rather than a defect in bone formation. A second low-molecular-weight (79 residue) protein with five Gla residues was also first isolated from bone (14) and called matrix Gla protein (MGP). This protein is also present in other tissues and is synthesized in cartilage and many other soft tissues. Like osteocalcin, details of its physiological role are unclear. But in studies with MGP “knockout” mice, death ensued from spontaneous calcification of arteries and cartilage.

A limited number of other mammalian proteins have been found to contain Gla residues and are therefore dependent on vitamin K for their synthesis. One is Gas 6, a ligand for the tyrosine kinase Ax1 (15), which

appears to be a growth factor for mesangial and epithelial cells. Four members of a transmembrane Gla protein family (PRGP-1, PRGP-2, TMG-3, and TMG-4) have been cloned (16), but the roles of these cell-surface receptors are not yet known. Two additional Gla-containing bone proteins, periostin (17) and Gla rich protein (18), have been recently identified, but their physiological roles have not yet been clearly defined. Vitamin K-dependent proteins are not confined to vertebrates, and a large number of toxic venom peptides secreted by marine *Conus* snails are rich in Gla residues (19). Gla-containing proteins or peptides have also been identified in some snake venoms, and the carboxylase that is needed to form Gla residues has been cloned from *Drosophila* (20,21). Although no Gla-containing proteins have been identified in *Drosophila*, the presence of this enzyme indicates that Gla is of ancient evolutionary origin and suggests that numerous vitamin K-dependent proteins are yet to be discovered.

Biochemical Role of Vitamin K

The biochemical basis for the early observations that vitamin K was needed to maintain normal prothrombin concentrations was not established until the mid-1970s, and

the observations leading to an understanding of the functional role of vitamin K have been reviewed (22). A circulating inactive form of prothrombin was found in the plasma of patients treated with oral anticoagulants, and it was demonstrated that the prothrombin produced when hypoprothrombinemic rats were given vitamin K and a protein synthesis inhibitor was not radiolabeled if radioactive amino acids were administered at the same time as the vitamin (23). These data suggested the presence of a hepatic precursor protein pool in the hypoprothrombinemic rat, which could be converted to prothrombin by a post-translational modification. The "abnormal prothrombin" isolated from the plasma of cows fed the anticoagulant dicoumarol was shown to lack the specific calcium-binding sites present in normal prothrombin. Acidic peptides obtained by proteolytic enzyme digestion of prothrombin, but not the "abnormal prothrombin," were subsequently seen (24,25) to contain Gla, a previously unrecognized acidic amino acid that was responsible for the calcium-binding properties of prothrombin.

The discovery of Gla residues in prothrombin directly led to the demonstration (26) that crude rat liver microsomal preparations contained an enzymatic activity (the vitamin K-dependent carboxylase) that promoted a vitamin K-dependent incorporation of $\text{H}^{14}\text{CO}_3^-$ into Gla residues of the endogenous precursors of vitamin K-dependent proteins present in these preparations. This

carboxylation reaction does not require adenosine triphosphate, and the energy to drive this process is derived from the oxidation of the reduced, hydronaphthoquinone form of vitamin K (vitamin KH_2) by O_2 to form vitamin K-2,3-epoxide (KO) (Fig. 3).

The primary gene product of the vitamin K-dependent proteins contains a very homologous domain between the amino terminus of the mature protein and the signal sequence that targets the polypeptide for the secretory pathway. This "propeptide" region appears to be both a "docking" or "recognition" site for the enzyme (27), and a modulator of the activity of the enzyme by decreasing the apparent K_m of the Glu site substrate (28). This peptide is cleaved before secretion of the protein, and although the binding affinities of propeptides from different proteins for the carboxylase differ significantly (29), propeptides are required for efficient carboxylation. Glutamate-containing peptides with no homology to vitamin K-dependent proteins have been shown to be good substrates for the carboxylase if a propeptide is attached (30,31). The molecular role of the vitamin in this reaction is to abstract the hydrogen on the γ -carbon of the glutamyl residue to allow attack of CO_2 at this position. The association between epoxide formation, Gla formation, and γ -C-H bond cleavage has been studied, and the reaction efficiency defined as the ratio of Gla residues formed to γ -C-H bonds cleaved has been shown to be independent

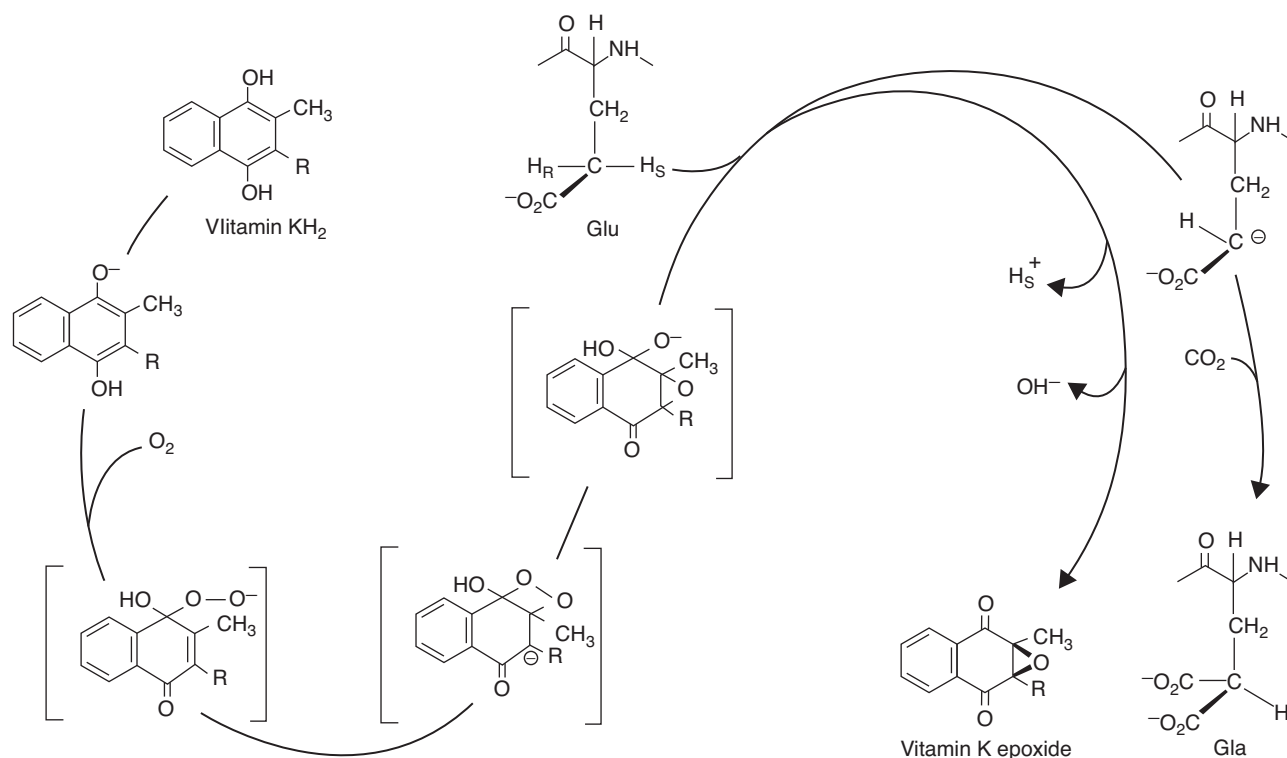


Figure 3 The vitamin K-dependent γ -glutamyl carboxylase. An interaction of O_2 with vitamin KH_2 , the reduced (hydronaphthoquinone) form of vitamin K, generates intermediates leading to an oxygenated metabolite, which is sufficiently basic to abstract the γ -hydrogen of the glutamyl residue. The products of this reaction are vitamin K-2,3-epoxide and a glutamyl carbanion. Attack of CO_2 on the carbanion leads to the formation of a γ -carboxyglutamyl residue (Gla). The bracketed peroxy, dioxetane, and alkoxide intermediates have not been identified in the enzyme-catalyzed reaction but are postulated on the basis of model organic reactions. The available data are consistent with their presence.

of Glu substrate concentrations, and to approach unity at high CO₂ concentrations. These studies have been adequately reviewed (32) and the details of carbanion formation are now available (33).

A key finding essential to a complete understanding of the detailed mechanism of action of this enzyme has been the identification of an intermediate chemical form of vitamin K, which could be sufficiently basic to abstract the γ -hydrogen of the glutamyl residue. It has been proposed (34–36) that the initial attack of O₂ at the naphthoquinone carbonyl carbon adjacent to the methyl group results in the formation of a dioxetane ring, which generates an alkoxide intermediate. The general scheme (37) shown in Figure 3 is consistent with all the available data, but there is no direct chemical evidence for any of these intermediates, and the mechanism remains a hypothesis at this time. Progress in purifying the enzyme was slow (38,39), but the enzyme was eventually purified to near homogeneity (40) and cloned (41). It is a unique 758 amino acid residue protein with a sequence suggestive of an integral membrane protein. The multiple Glu sites on the substrate for this enzyme are carboxylated progressively (42), and they are bound to the enzyme via their propeptide, while the Gla domain undergoes intramolecular movement to reposition each Glu for catalysis. The release of the carboxylated substrate has been reported to be the rate-limiting step in the reaction (43). Further details of the morphology of the enzyme within the membrane and the location and identification of key active site residues are available in recent reviews (29,44–47).

Vitamin K Metabolism

The major route of phyloquinone metabolite excretion is via the feces, and very little unmetabolized vitamin is excreted (48). Details of the metabolic transformation of vitamin K are currently lacking, but it has been shown (49) that the side chains of phyloquinone and MK-4 are shortened by the rat to seven carbon atoms terminating in a carboxylic acid group that is cyclized to form a γ -lactone. This lactone was excreted in the urine as a glucuronic acid conjugate. The cytochrome P450 that metabolizes the vitamin K side chain is likely the same enzyme involved in vitamin E metabolism and may be responsible for the known interference of vitamin K activity by vitamin E (50). Radioactive phyloquinone metabolism has also been studied in humans (51), and it has been shown that about 20% of an injected dose of either 1 mg or 45 μ g of phyloquinone was excreted in the urine in three days, and that 40% to 50% was excreted in the feces via the bile. Two different aglycones of phyloquinone were tentatively identified as the 5- and 7-carbon side-chain carboxylic acid derivatives, and there is evidence that indicates that there are numerous unidentified metabolites. More recent studies (52,53) have shown that a fraction of ingested phyloquinone is completely dealkylated to yield menadione which is secreted in urine and bile as glucuronide or sulfate conjugates.

A major pathway of vitamin K metabolism is that which is involved in the reduction and recycling of the epoxide formed by the carboxylase. Although the epoxide had been demonstrated before the carboxylase was identified (54), the existence and importance of this pathway became clear when it was demonstrated that the hepatic ratio

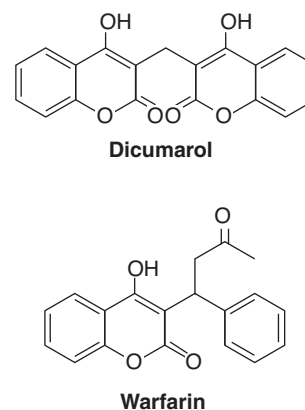


Figure 4 Structure of dicumarol and warfarin. Dicumarol was the compound isolated from sweet clover as a toxic hemorrhagic factor, and warfarin is the most commonly used of a number of 4-hydroxycoumarin anticoagulants.

of the epoxide relative to that of the vitamin was increased in animals administered the 4-hydroxycoumarin anticoagulant warfarin (55). This class of drugs was discovered in the early 1930s, following studies of a hemorrhagic disease of cattle consuming improperly cured sweet clover hay that was prevalent in the American upper Midwest and western Canada. The cause of the prolonged clotting times was found to be a decrease in the prothrombin activity of blood, and the compound was isolated from spoiled sweet clover, characterized (56) as 3-3'-methylbis-4-(hydroxycoumarin), and called dicumarol (Fig. 4). A large number of analogs of dicumarol were synthesized and tested for their anticoagulant activity, and the compound first used as both a rodenticide and as therapy for thrombotic disease was warfarin [3- α -(acetonylbenzyl)-4-hydroxycoumarin]. Several other coumarin derivatives have been developed for clinical use as oral anticoagulants. Although warfarin has a very favorable pharmacologic profile and is essentially the only coumarin derivative prescribed in North America, others are widely used in Europe.

The observations that warfarin increased tissue epoxide levels led to an understanding that its inhibition of vitamin K action was indirect through an inhibition of the KO reductase (57). Blocking of this enzyme prevents the reduction of the epoxide to the quinone form of the vitamin and eventually to the carboxylase substrate, vitamin KH₂. Widespread use of warfarin as an anticoagulant rodenticide led to the appearance of strains of warfarin-resistant rats, and the study of the activity of the epoxide reductase in livers of these animals was a key to an understanding (58,59) of the details of the vitamin K cycle (Fig. 5). Three forms of vitamin K [the quinone, the hydronaphthoquinone (KH₂), and the KO] can feed into this liver vitamin K cycle. In normal liver, the ratio of vitamin K-2,3-epoxide to the less oxidized forms of the vitamin is about 1:10 but can increase to a majority of epoxide in an anticoagulated animal. In addition to the epoxide reductase, the quinone and hydronaphthoquinone forms of the vitamin can also be interconverted by a number of NAD(P)H-linked reductases, including one that appears

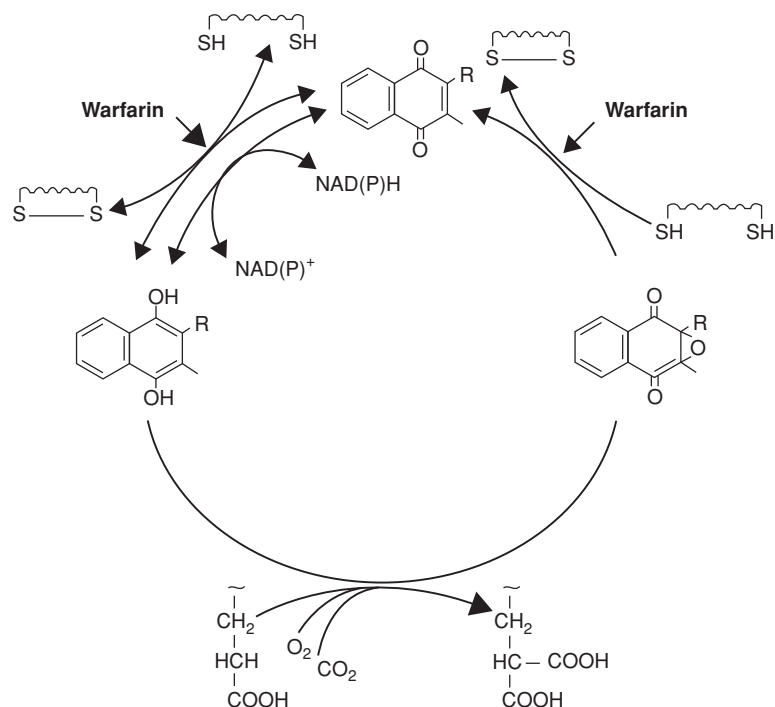


Figure 5 Metabolism of vitamin K tissues in animals. Vitamin K epoxide, which is formed in the carboxylation reaction, is reduced to the quinone form of the vitamin by a warfarin-sensitive pathway, "vitamin K epoxide reductase," which is driven by a reduced dithiol. The naphthoquinone form of the vitamin can be reduced to the hydronaphthoquinone form either by the same warfarin-sensitive dithiol-driven reductase or by one or more of the hepatic NADH or NADPH-linked quinone reductases that are less sensitive to warfarin.

to be a microsomal-bound form of the extensively studied liver DT-diaphorase activity. The epoxide reductase utilizes a sulfhydryl compound as a reductant *in vitro*, but the physiological reductant has not been identified (60). Efforts to purify this enzyme have proceeded slowly, but the identification of the gene for this protein (61,62) has led to a rapid increase in an understanding of this important pathway of vitamin K metabolism, (63,64).

Vitamin K Deficiencies and Requirements

The classic example of a human vitamin K deficiency is that of hemorrhagic disease of the newborn or early "vitamin K deficiency bleeding" (VKDB) occurring during the first week of life in healthy appearing neonates (65). Contributing factors are: low placental transfer of phyloquinone, low clotting factor levels, a sterile gut, and the low vitamin K content of breast milk. The incidence of the disease is low, but the mortality rate from intracranial bleeding is high, and prevention by oral or parenteral administration of vitamin K immediately following birth is the standard cure. Late VKDB is a syndrome occurring between 2 and 12 weeks of age predominantly in exclusively breastfed infants or infants with severe intestinal malabsorption problems. The current recommendations of the American Academy of Pediatrics advise that "vitamin K (phyloquinone) should be given to all newborns as a single, intramuscular dose of 0.5 to 1 mg," and if this advice is followed, the disease will be effectively prevented.

Uncomplicated adult deficiencies of vitamin K are extremely rare, and most diets contain an adequate amount. The historical indication of a vitamin K deficiency depended on a relatively insensitive measure, the "prothrombin time" (PT), to assess adequacy of the vitamin K-dependent clotting factors. Low lipid intake or the

impaired lipid absorption resulting from the lack of bile salts will also adversely affect vitamin K absorption. Depression of the vitamin K-dependent coagulation factors has frequently been reported in malabsorption syndromes and in other gastrointestinal disorders. These reports and numerous cases of the most commonly reported cause of a vitamin K deficiency, a vitamin K-responsive hemorrhagic event in patients receiving antibiotics, have been extensively reviewed (66). These episodes have usually been assumed to be due to decreased utilization of menaquinones produced in the lower bowel by these patients. However, it is possible that some cases may represent low dietary intake alone or an effect of the antibiotics on blood coagulation not related to a vitamin K-induced hypoprothrombinemia. Recently, a number of controlled studies utilizing diets containing approximately 10 µg/day or less of phyloquinone (67–69) have demonstrated alterations using more sensitive markers of vitamin K status, but a clinically significant decrease in PTs was not seen.

The Dietary Reference Intakes project of the Food and Nutrition Board/Institute of Medicine has recently established recommended intakes of vitamin K for the U.S. and Canadian populations (5). There are ample data to establish that essentially all individuals do not consume sufficient vitamin K to maximally γ -carboxylate their circulating osteocalcin and that supplementation with about 1 mg/day of phyloquinone is needed to achieve this response. As the clinical significance of this apparent deficiency has not been established, these indices of adequacy were not used to set a reference value. The only indicator of vitamin K status with known clinical significance is the PT, and alterations in the PT by changes in dietary intake alone are uncommon to nonexistent. As circulating phyloquinone concentration is very dependent on

Table 2 Adequate Intakes of Vitamin K

Population	Vitamin K ($\mu\text{g/day}$)
0–6-mo-old infants	2.0
7–12-mo-old infants	2.5
1–3-yr-old children	30
4–8-yr-old children	55
9–13-yr-old boys and girls	60
14–18-yr-old boys and girls ^a	75
19–70+-yr-old men	120
19–70+-yr-old women ^a	90

^aNo alteration of intake for pregnancy or lactation.

Source: From Ref. 5.

previous day intake, it is also not a satisfactory indicator of an adequate intake. Intakes of vitamin K that are in the range of 10% of those consumed by the general population have been demonstrated to result in decreases in urinary Gla excretion and small increases in under- γ -carboxylated prothrombin. However, no studies have utilized a range of intakes, which would allow the calculation of an estimated average requirement or a recommended dietary allowance. Because insufficient data to determine these values are available, the dietary reference value used was the adequate intake (Table 2). This value is defined as: “the recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate.” Adequate intakes of infants are based on the phyloquinone content of human milk and assume that infants also receive prophylactic vitamin K at birth. Those for children, adolescents, and adults are based on the highest median intake for each age group reported by the Third National Health and Nutrition Examination Survey (NHANES III). On the basis of those data, the intakes of pregnant or lactating women do not differ from those of the general population. At the present time, there are relatively few dietary supplements containing vitamin K, and only a few foods that are fortified with it.

Vitamin K and Chronic Disease

Three of the vitamin K-dependent proteins—osteocalcin, MGP, and protein S—are synthesized in bone, and this has focused attention on the role of vitamin K-dependent proteins in bone. Small amounts of osteocalcin circulate in plasma, and it is clear that a fraction of the protein in individuals within the normal population is not completely γ -carboxylated and can be influenced by vitamin K status (68–72). Depending on assay conditions and the specific epitopes detected by the various assay kits utilized to measure total and under- γ -carboxylated osteocalcin (ucOC), the fraction of ucOC reported in normal populations has ranged from 30 to 40 to less than 10%. Normal dietary intake of vitamin K is not sufficient to maximally γ -carboxylate osteocalcin, and a recent study (73) has established that supplementation with 1 mg of phyloquinone per day ($\sim 10 \times$ the current RDI) is required to achieve maximal γ -carboxylation. Attempts to link this apparent marker of vitamin K insufficiency with bone health have included epidemiologic observations that a low intake is associated with increased hip fracture risk (74,75) and reports that ucOC is correlated with low bone mass (76,77).

These associations do not necessarily imply causation, and they might simply be surrogate markers of general nutrient deficiencies. Patients on oral anticoagulant therapy have very high ucOC levels, and a number of attempts to correlate this treatment with alterations in bone mineral density have not yielded consistent outcomes (78). Available data do not support a link between increased ucOC and decreased mineralization and tend to suggest that functional osteocalcin decreases or controls ectopic mineralization. Rats can be maintained on a protocol where a high intake of warfarin is accompanied by administration of large amounts of phyloquinone maintaining adequate levels of plasma clotting factors, but γ -carboxylation of osteocalcin is effectively blocked. A mineralization disorder characterized by complete fusion of proximal tibia growth plate and cessation of longitudinal growth has been observed utilizing this protocol (79). These data strongly suggest that a skeletal vitamin K-dependent protein regulates the deposition of bone mineral but that the outcome is not decreased mineralization. Studies utilizing transgenic mice lacking the osteocalcin gene (80) have also demonstrated an increase in bone mineralization rather than a decrease.

Although near maximal carboxylation of osteocalcin does not appear to be needed for bone mineralization, vitamin K supplementation is a common therapy for osteoporosis in Japan and other Asian countries. The standard amount supplemented is 45 mg of menaquinone-4 per day, a pharmacological rather than nutritional approach. Positive responses in bone mineral density at specific sites or reduction in fracture rates of postmenopausal osteoporotic women have been reported (81,82), and MK-4 has been reported (83,84) to increase markers of bone formation or bone mineral density in experimental animals or human subjects. It is of interest that menaquinone-4 can be synthesized in animal tissues from phyloquinone, and that many tissues have high concentrations of menaquinone-4 produced by this transformation (85,86). Menaquinone-4 does have effects on cultured bone cells that are not seen with phyloquinone (87). The positive responses in bone health reported to result from the supplementation of 45 mg per day of menaquinone have not been studied with a similar amount of phyloquinone. However, five randomized controlled trials of the impact of phyloquinone supplementation on the skeletal health of elderly subjects have been conducted. The trials utilized 200 μg to 5 mg of phyloquinone and ranged from 12 to 36 months, with the effect on bone mineral density as the major end point (88–92). In only one study at a single anatomical site was a positive effect of supplementation observed. A report (93) of a post-marketing study of over 2000 elderly osteoporotic patients receiving 45 $\mu\text{g/day}$ of MK-4 found that a decrease in new fracture rate was observed in only a small subpopulation of subjects with multiple previous fractures. The results of these vitamin K supplement studies would suggest that although vitamin K may have a role in skeletal metabolism, it is unlikely that increased dietary vitamin K is an effective agent in decreasing osteoporosis.

MGP (94,95) is also found in bone and other tissues with potential for calcification. Studies of the MGP “knockout” mouse indicated that these animals died from massive calcifications of the large arteries within eight weeks of birth (96), and a rapid calcification of the elastic

lamellae of arteries and heart valves has been seen in a rat model where MGP carboxylation was blocked (97). There are reports of an association between low vitamin K intake and aortic calcification (98) and an inverse correlation between menaquinone intake and aortic calcification, myocardial infarction, and sudden cardiovascular death (99). There is a great deal of interest in the role of MGP as a calcification inhibitor (94), and a three-year supplementation of 500 µg/day of phylloquinone has shown a slight slowing of the progression of coronary artery calcification in elderly men and women (100). These data are preliminary, and whether or not individuals with low vitamin K status are at risk for cardiovascular disease is not yet clear. Vitamin K status has also been linked to changes in insulin resistance and to various measures of inflammation (95). A great deal of additional data would be needed to classify low vitamin K intake as a risk factor for cardiovascular disease or other chronic diseases.

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Yohimbe

Joseph M. Betz

INTRODUCTION

Yohimbe is a West African evergreen tree, *Pausinystalia johimbe* (K. Schum.) Pierre ex Beille, a member of the madder family (Rubiaceae). Teas made from the bark have been used in Africa as aphrodisiacs and for other purposes. The bark contains a series of closely related indole alkaloids, with the single-compound yohimbine predominating. In the United States, products that contain yohimbe bark or yohimbe bark extract (alone or in combination with other ingredients) are available as dietary supplements. These products are sold as libido enhancers, for weight loss, and as aids for bodybuilding. There is almost no published scientific research on yohimbe that supports these or any other claims. Instead, there is a very large literature on the single-alkaloid, yohimbine. This literature is of variable quality and encompasses everything from in vitro studies of mechanism of action to animal research to human clinical trials, mostly on the utility of yohimbine in certain types of erectile dysfunction (ED). The modern consensus appears to be that the pure compound yohimbine is effective for treating certain mild types of ED in some men, but does not act as an aphrodisiac. Yohimbine hydrochloride is available as a prescription drug for treatment of certain types of ED in the United States. There are a few older studies that examine yohimbine for weight loss, but results were largely inconclusive. There are concerns about the safety of yohimbine, and especially about the potential of this compound to cause drug/herb interactions.

BACKGROUND

Yohimbe is one of a number of economically and medicinally important plants in the madder family, including coffee (*Coffea arabica* L.), ipecac [*Psychotria ipecacuanha* (Brot.) Stokes], and *Cinchona calisaya* Wedd., the source of quinine.

The raw material is collected from the wild and it enters into commerce in the form of flattened or slightly curled (quilled) pieces 75-cm long and 4- to 8-mm thick. The bark is characterized by an external corky layer of a gray-brown color covered with isolated lichens. When examined in this form, the crude material shows numerous longitudinal and transverse fissures. When the bark is broken, the transverse fracture is of a uniform yellowish-brown to orange-brown color, and presents short, soft fibers like rough velvet (1).

CHEMISTRY AND PREPARATION

According to Tyler (2), yohimbe "enjoys a considerable folkloric reputation as an aphrodisiac (a drug that

enhances sexual performance and desire)," and several authors of herbal use guides intended for lay readers have provided information on traditional use of the bark (3–5).

Tyler (2) provides a recipe that "recommends boiling 6–10 teaspoonfuls of inner bark shavings in a pint of water for a few minutes, straining, sweetening and drinking the beverage." All of these popular authors place yohimbe in the unsafe category due to unpleasant side effects, whereas the German Commission E has published a negative monograph on the plant due to lack of efficacy data and safety concerns (6). Western observations on the use of yohimbe for purposes of sexual enhancement extend back over a century.

In 1900, Oberwarth and Loewy (7) reported that yohimbe exerted a strong aphrodisiac effect in animals and humans. Authentic yohimbe bark has been reported to contain up to 6% total alkaloids. The major alkaloid in the plant and the one most thoroughly studied is yohimbine (17 α -hydroxy-yohimban-16 α -carboxylic acid methylester) (2). Minor alkaloids isolated from *P. johimbe* bark include ajmaline, alloyohimbine, pseudoyohimbine, corynanthine, corynantheine, α -yohimbane, and β -yohimbane (8). In addition to its presence in *Pausinystalia*, yohimbine and its derivatives have been isolated from a number of plant genera in the family Apocynaceae, including *Rauwolfia*, *Amsonia*, *Vallesia*, *Aspidosperma*, and *Vinca*, from *Gelsemium* and *Strychnos* (Loganiaceae), and from *Alchornea* (Euphorbiaceae). Very soon after the compounds responsible for the biological activity of yohimbe were identified and characterized, research shifted away from the effects of the plant to the effects of the isolated compounds. Probably as a result of this trend, no reports of human studies on the effect of crude yohimbe bark or its extracts on sexual performance can be found in the literature. *P. johimbe* bark remains the major source of commercial yohimbine, as synthetic processes are prohibitively expensive.

Unfortunately, the lack of published research on yohimbe, yohimbe extract, and yohimbe products limits the following discussions primarily to the major alkaloid, yohimbine. The contributions of the minor alkaloids and nonalkaloidal constituents to the biological activity of yohimbe cannot be addressed, and the aphrodisiac (vs. ED) activity of the plant is also impossible to evaluate. Any discussion of the use of the bark for sexual enhancement thus begins and ends with folklore. Proponents of botanical medicines will deem this to be sufficient evidence of efficacy and safety, whereas critics (or skeptics) will require modern scientific evidence. Some help is provided by the fairly rich literature on yohimbine, but even this must be tempered by the fact that it represents

only approximately 15% of the total alkaloid of the tree bark and that virtually nothing is known about the non-alkaloidal constituents.

PRECLINICAL STUDIES

Yohimbine (Fig. 1) is a potent α_2 -adrenoreceptor blocker of short duration and a weaker α_1 -adrenergic antagonist with some antidopaminergic properties (9–12). Yohimbine HCl has been available as a prescription drug for the treatment of male impotence for decades, and is still registered for that use in many countries. The drug has been used both in oral dosage forms and as an injection. In addition to this application, the compound has more recently been used as a pharmacological probe to study the involvement of α_2 -adrenoreceptors in the regulation of autonomic function. Yohimbine has also been used to treat female sexual dysfunction, but the few published clinical trials have reported that yohimbine is no better than placebo for this indication.

Animal studies over the years confirm the effects of yohimbine on male sexual behavior. A review by Tam, Worcel, and Wyllie (13) notes that in male rats, the alkaloid decreases the latencies of intromission, mounting, and ejaculation; induces mating behavior and copulatory behavior during sexual exhaustion; and at low doses enhances the ejaculatory response. Similar results have been reported in dogs (14) and golden hamsters (15). The 25th edition of the *United States Dispensatory* (7) noted that yohimbine "has been used by clinicians in neurasthenic impotence, with reports which are generally favorable." The text goes on to state that the compound is of no value when the impotence is caused by organic nerve damage and is harmful when impotence is caused by chronic inflammatory disease of the sexual organs or the prostate gland.

A number of proprietary yohimbine-containing drug products intended for the treatment of impotence were available on the US market prior to the prescription drug review mandated by the 1960 amendment of the Federal Food, Drug, and Cosmetic Act. The most heavily studied of these products was Afrodex[®], which contained a mixture of 5 mg of extract of *Strychnos nux vomica* L. (1–4% strychnine), 5 mg of methyl testosterone, and 5 mg of yohimbine hydrochloride (16). There are numerous published clinical trials of this product, involving thousands of

human subjects. The trials were largely positive, with few reported side effects, but following the prescription drug review, the product disappeared from the US marketplace (although it was available in Africa as late as 1973).

Scientific understanding of yohimbine and its biological activity has evolved within the context of modern investigations into sexual dysfunction. Although there has been a considerable amount of research on the effects of yohimbine on impotence and ED no published studies on the use of this compound for its purported male aphrodisiac ("enhancement of sexual performance and desire") properties could be found. The distinction between therapeutic agents useful for treating a medical disorder (ED) and relatively commonplace ingredients with aphrodisiac properties is significant. Powerful compounds such as sildenafil (Viagra[®]) (or yohimbine HCl), available only with a physician's prescription fall into the former category, whereas "legendary love potions," like Spanish fly, glandular secretions from musk deer and civet cats, oats (*Avena sativa*), ginseng (*Panax spp.*), and oysters fall into the latter (17). Yohimbe-containing dietary supplement products intended to "enhance sexual performance and desire" are widely available in health food stores, supermarkets, and pharmacies, and on the Internet. In addition to the scientific and medical implications of these diverging definitions, there may be regulatory significance as well. A material that affects a disease state like ED is regulated as a drug in the United States, whereas products intended to maintain or enhance libido are marketed as dietary supplements. As noted, there have been a number of ED studies (including meta-analyses) on yohimbine HCl, the drug, but no published human clinical studies on yohimbe bark or any of its crude preparations could be located.

CLINICAL STUDIES: EFFICACY

Yohimbine HCl has been used in the management of select cases of organic impotence. A number of randomized controlled clinical trials have been performed over the past several decades. The quality of the trials and diagnoses of the etiology of the ED have been variable, and so results have been equivocal. A 1994 review notes that yohimbine has "enjoyed a reputation as an aphrodisiac although no effect on sexual drive in humans has been adequately demonstrated" (18). The review also pointed out that yohimbine has been evaluated for effects on erectile disorder in a number of placebo-controlled but otherwise poorly designed clinical trials and been found to have a modest therapeutic benefit over placebo, particularly in psychogenic erectile disorder. More methodologically sound trials performed over the past decade have produced enough data to allow a meta-analysis to be performed.

The absorption and pharmacokinetics of yohimbine are fairly straightforward. The compound is absorbed rapidly from the gastrointestinal tract, and because the free base is highly lipophilic, it quickly crosses the blood-brain barrier into the CNS. Peak plasma levels have been found to be achieved 10 to 45 minutes after ingestion of 10 mg of yohimbine HCl, and it is eliminated rapidly (mean

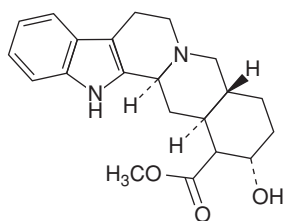


Figure 1 Yohimbine.

half-life 0.58 hours). Oral bioavailability ranges from 7% to 87%, and less than 1% of the orally administered drug is excreted unchanged in the urine. The biological activity of yohimbine lasts longer than the absorption and half-life would suggest, raising the possibility that it is converted into an active metabolite with a longer duration of action than the parent molecule. Major metabolites of yohimbine are 10-hydroxy-yohimbine and 11-hydroxy-yohimbine (18).

The systematic review and meta-analysis of randomized clinical trials published by Ernst and Pittler in 1998 (19) indicates that all authors had reported significant placebo effects, but still concludes that yohimbine is more effective than placebo in treating ED. In their publication, Ernst and Pittler (19) describe the best two trials in some detail. In one trial, 61 subjects who had been treated for secondary ED for at least 6 months were given 5.4 mg of yohimbine HCl or matching placebo three times daily for 8 weeks and then crossed over for 8 more weeks. At 4-week intervals, patients self-reported quality and frequency of erections. After 8 weeks, 36.7% of the drug group and 12.9% of the placebo group ($P < 0.05$) reported good stimulated erections. In the placebo group, 41.9% ($P < 0.02$) of patients reported positive results after crossover to the drug. In the second trial, 82 patients, included regardless of the degree, duration, or etiology of their ED, were divided into groups ranked as having mild, moderate, or severe ED. The initial dose was one 5.4 mg yohimbine HCl tablet or matching placebo four times per day. The dose was raised to two 5.4-mg tablets four times daily on the second day, with dosage reduced by one tablet/day if adverse events occurred. Success was assessed by Derogatis Sexual Functioning Inventory, penile brachial index test, and daytime arousal test. After 4 weeks, 14% of the drug patients had experienced full restoration of erectile function and 20% had partial response. As a caveat to their meta-analysis, Ernst and Pittler did note that publication bias (the tendency to publish positive trials but not negative studies) could not be ruled out.

At least two clinical trials of yohimbine HCl (alone or in combination) in ED have been published since the meta-analysis. Guay et al. (20) administered 5.4 mg of yohimbine HCl three times a day for 4 weeks to 18 men with organic ED. At 4 weeks, the dose was doubled and the trial continued for an additional 4 weeks. There was no placebo group. Fifty percent of the patients in the treatment group responded. Lebreton et al. (21) performed a double-blind, placebo-controlled, three-way crossover study comparing the effects of L-arginine glutamate/yohimbine HCl, yohimbine HCl, and placebo. Forty-eight patients with at least a 3-month history of mild-to-moderate ED were randomized and treated. Doses of 6.0 g of arginine/6.0 mg yohimbine HCl, 6.0 mg yohimbine, or placebo were administered once daily for 2 weeks. The review by Tam, Worcel, and Wyllie (13) reinforces the conclusions found in earlier trials: Yohimbine is an effective therapy to treat certain forms of mild organic ED in some men. In contrast, a 2007 review of dietary supplements and nutraceuticals in the management of andrologic disorders concurred that prescription yohimbine is likely effective in men with certain types of nonsevere ED, but noted that variability of yohimbine content in over-the-counter supplement products recommends against their use (22).

MECHANISM OF ACTION

Several possible mechanisms for the utility of yohimbine in ED have been proposed. Early work focused on the effects of the alkaloid on peripheral blood flow in the penis. Its action on peripheral blood vessels resembles (but is weaker than) that of reserpine, a structurally similar alkaloid found in Indian snakeroot, *Rauwolfia serpentina* (L.) Benth. ex Kurz (Apocynaceae). Penile erection is achieved when the smooth muscle of the corpus cavernosum (spongy erectile tissue) is relaxed and there is an increase in cavernosal blood flow and a decrease in outflow. Penile flaccidity (the usual state) is maintained when the cavernosal smooth muscle is contracted. Smooth muscle contraction is mediated by the α -adrenergic neuroeffector system. As noted previously, yohimbine blocks α_2 -adrenoreceptors. Its peripheral effects on penile hemodynamics could thus be at least partly ascribed to increasing cholinergic and decreasing adrenergic activity, thus increasing inflow and decreasing outflow of blood (i.e., by interfering with the penile detumescence status quo).

Studies in rodents have focused on the central effects of yohimbine on sexual function. Most of the evidence for dominance of CNS-mediated activity is derived from experiments that show that the alkaloid increases sexual motivation even in sexually exhausted rats due to its action on central α_2 -adrenoreceptors found in the locus coeruleus of the brain. Blockage of these brain adrenoreceptors appears to reverse a central negative feedback mechanism that regulates penile erection and maintains detumescence. Some doubts remain about the importance of this proposed mechanism in the overall biological activity of yohimbine because the compound does not increase sexual desire or thoughts in human clinical trials. The combined evidence from human and animal studies indicates that yohimbine is far less potent in stimulating sexual behavior in humans than in rats. One possible explanation for this finding is the existence of powerful and multiple inhibitory controls on sexual behavior in humans that are not present in rats; that is, the cognitive aspects of sex are far more important in humans than the basic instinctive functions observed in animals (23).

Increased understanding of the role of nitric oxide (NO) in vascular smooth muscle function has led to a reinvestigation of the manner in which yohimbine works in ED. Upon sexual stimulation, the terminal axons of the parasympathetic nerves release NO. This compound diffuses into the smooth muscles that line the arteries of the corpus cavernosum and activates guanylate cyclase. This enzyme catalyzes the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP), which causes penile smooth muscle relaxation. The effects of cGMP are eventually reversed (and erection ceases) because cGMP is converted into its inactive form by phosphodiesterase 5 (PDE5), an enzyme found in penile tissue. Sildenafil exerts its biological activity by inhibiting PDE5 and preventing breakdown of cGMP. Filippi et al. (24) found that yohimbine blocks smooth muscle contraction induced by adrenergic agonists and also those induced by nonadrenergic substances such as endothelin-1 (ET-1). They also reported that this effect is unrelated to antagonism at ET-1 receptors. These data

suggest that yohimbine counteracts ET-1 induced contraction by altering NO release from endothelial tissue. This was tested in the in vitro model by mechanically removing endothelial cells (the source of NO) from the experimental preparation. This blocked the relaxant effect of yohimbine on the tissue. Conversely, yohimbine activity was strongly increased by inhibiting cGMP degradation. A study of yohimbine's effect on isolated human corpus cavernosum reported that the compound induces nonadrenergic, non-cholinergic relaxation, probably by activating the nitric-gas-soluble guanylate cyclase pathway and K_{ATP} (25). A sildenafil/yohimbine combination was reported to improve erectile processes (but not sexual motivation) in male rats without additional hypotension, probably because yohimbine exerted its primary effect on the central component of the erectile process. (26).

A study by Ajayi et al. (27) measured the effects of an aqueous extract of the plant bark of *P. johimbe* on renal circulation to test the hypothesis that the extract has effects on NO production and/or ET-1-like actions. Results suggest a strong possibility of postreceptor crosstalk between α_2 -adrenoreceptors and endothelin as well as a direct effect of α_2 -adrenoreceptors on NO production. The study also demonstrates the importance of performing preclinical and clinical trials on plant materials and their extracts rather than the presumed "actives" (in this case, yohimbine). The study used an aqueous extract of yohimbe bark and was therefore relatively devoid of yohimbine and other lipophilic alkaloids, suggesting the presence of as yet unidentified active principles in the bark.

Today, many botanical ingredients (including yohimbe) are being touted in the United States as legal "natural" substances that enhance endogenous androgenic steroid production (28) (and are hence marketed as bodybuilding aids). No published evidence that yohimbe (or yohimbine) has the effect of increasing lean muscle mass in humans could be found in peer-reviewed scientific literature. None of the studies cited previously support the use of yohimbine as an anabolic agent, and Gurguis and Uhde (29) report that its administration has no effect on plasma growth hormone levels. A study of the effects of an aqueous extract of a commercial yohimbe tablet on reproductive health of male swiss albino mice reported that treatment caused an increase in serum testosterone levels. However, treatment also resulted in effects on other hormones, including increases in estradiol and prolactin. The study reported that yohimbe treatment induced chromosomal aberrations, spermatozoal abnormalities, reduction in male fertility, and reduced pre- and postimplantation losses of embryos. The authors postulated that damage to sperm and chromosomes were mediated by increased free radicals in the testes, but also noted that the changes in hormone profiles were likely contributors to the sexual dysfunction reported following yohimbe treatment (30).

There was a flurry of interest in yohimbine and several other compounds (including synephrine) during the late 1980s and early 1990s when it was found that a number of α -adrenergic agents caused increased lipolysis in rodent brown adipocytes in vitro (31–34). A number of small human trials of yohimbine (but not yohimbe) for weight loss have been published, but the results are contradictory and have not been repeated. Subsequent work on human

adipocytes has demonstrated that the basic biochemistry of rodent and human adipocytes is substantially different, and these compounds do not increase lipolysis in human cells in vitro. Because of the small sizes of the trials and their equivocal results, the evidence base for efficacy of yohimbine in weight loss does not currently support this claim (35). No published clinical trials (or studies of any sort) on yohimbe and weight loss could be found in the literature.

ADVERSE SIDE EFFECTS

Clinical trial reports have indicated that the adverse effects of yohimbine in these settings are few and mild (31,36–38). Ernst and Pittler (19) warn that this relatively small number may reflect the strict inclusion criteria used for clinical trials rather than true incidence rates. The known pharmacological actions of yohimbine allow one to predict an increase in adrenergic tone that may cause an increase in blood pressure. In a double-blind, placebo-controlled clinical trial in 10 healthy, normotensive men, Tank et al. (39) found that yohimbine increases blood pressure, heart rate, and attenuates baroreflex-mediated bradycardia. A systematic review of adverse events associated with weight-loss supplements notes that the adverse events reported with the use of yohimbine are well documented and include hypertension, anxiety and agitation (40). A 1-year prospective surveillance study of 275 dietary supplement-related poison control center calls in 2006 found most dietary supplement-related events were minor, but that sympathomimetic toxicity was the most commonly reported toxidrome (41), with almost half of such cases associated with caffeine-containing products and 18% associated with yohimbe-containing products. Yohimbine-containing products have the potential to produce psychiatric symptoms, primarily anxiety and panic, especially in individuals with preexisting panic disorder, and the compound has been used to provoke panic attacks and anxiety in studies of the pathophysiology, psychopharmacology, and treatment of anxiety disorders (42). A clinical trial involving 32 healthy adults tested the effect of yohimbine, hydrocortisone, yohimbine/hydrocortisone, and placebo on panic symptoms, autonomic responses, and attention to threat (42). The investigators found a significant increase in panic symptoms in the yohimbine and yohimbine + hydrocortisone groups, but no increase in the placebo or hydrocortisone groups.

Case reports indicate that yohimbine produces a number of reactions in certain individuals at doses below those required for peripheral α -adrenergic blockade. Manifestations of these effects include antidiuresis and central excitation (elevated blood pressure and heart rate, increased motor activity, nervousness, irritability, and tremors). Dizziness, headache, skin flushing, and orthostatic hypotension have also been reported. The recommended dose of yohimbine HCl for ED is 5.4 mg three times daily. Doses of 20 to 30 mg produce increases in blood pressure and heart rate, piloerection, and rhinorrhea. Paresthesias, incoordination, tremulousness, and dissociative states have been reported in the most severe cases (29,44–29). The apparent monoamine oxidase inhibitory effect of this compound is probably attributable

to weak calcium channel-blocking activity (48). Therefore, yohimbe is contraindicated with tyramine-containing foods, antidepressants, and other mood-modifying drugs (44). In a likely idiosyncratic reaction, yohimbe was reported to have induced a generalized erythrodermic skin eruption, progressive renal failure, and a lupus-like syndrome in a patient being treated for impotence (44). Yohimbe overdose (200 mg) in a 62-year-old man produced tachycardia, hypertension, and anxiety of brief duration (50), consistent with the expectations already outlined. Following treatment with activated charcoal and observation for 19 hours, he was released from hospital with normal blood pressure and heart rate. Ingestion of 5 g of yohimbe by a 37-year-old bodybuilder resulted in severe acute neurotoxicity (51). Symptoms included malaise, vomiting, loss of consciousness, and repeated seizures. At admission to the hospital, his blood pressure was 259/107 and his pulse rate was 140 beats/min. Twelve hours after his admission, his blood pressure was 127/70 and his pulse rate was 94. Over the next 24 hours, all of his vital signs returned to normal. The short duration of the symptoms in both cases can be explained by the pharmacokinetics of yohimbe.

A topic of more recent concern is the likelihood that yohimbe can cause drug/drug interactions. Yohimbe has been reported to raise blood pressure in patients taking tricyclic antidepressants and to produce withdrawal symptoms and anxiety in opioid-dependent individuals (i.e., patients undergoing methadone therapy). As an α_2 -adrenrgic antagonist, yohimbe opposes the effects of clonidine and may potentiate the α_2 -adrenoreceptor-blocking properties of phenothiazines (52). A pharmacological investigation of the biochemical underpinnings of alcohol-seeking and self-administration in male Wistar rats reported that the α_2 receptor antagonist and 5-hydroxytryptamine agonist properties of yohimbe may increase alcohol-seeking behavior in this model (52). The negative German Commission E monograph for the use of yohimbe (as opposed to yohimbe) in ED is based on lack of efficacy studies on the plant material as well as the potential adverse effects outlined (6).

Unfortunately, although the literature on the safety and efficacy of yohimbe is instructive and may provide useful insight into the safety and efficacy of yohimbe, there are questions that cannot be answered without putting the plant material itself or products made from the plant into rigorous preclinical and clinical studies. The effects of the minor alkaloids or of the other chemical constituents (such as the water-soluble compounds) on the safety/efficacy of the plant cannot be known until such research is done. Tyler noted that the bark was used to make a tea (2). The alkaloids of yohimbe are marginally water soluble (if at all), whereas the aqueous extract yohimbe bark used by Ajayi et al. (27) was found to be biologically active in its own right. Very little has been published on the identity or nature of the water-soluble constituents of the plant. If basic questions about the safety and efficacy of the plant itself cannot be answered at this time, the picture for products that contain more than one plant are even more bleak. Yohimbe was an ingredient in a dietary supplement called LipoKinetix that was associated with severe hepatotoxicity in seven individuals. The yohimbe present is unlikely to have been the cause of the toxicity, but its

contribution when combined with the other ingredients is unknown.

Finally, as products have proliferated in the marketplace, various investigators have performed surveys of yohimbe content in yohimbe products (27,55). Yohimbe bark has been reported to contain approximately 0.7% yohimbe and approximately 3.9% total alkaloids. Marketed US dietary supplement products were found to contain 0% to 0.05% yohimbe. Only those in the higher range of yohimbe content were observed to contain other alkaloidal components. Several of the products contained multiple botanical ingredients, but none of these contained much yohimbe. Products purchased via the Internet from various countries were found to contain from 0 to 9.5 mg of yohimbe per unit. In the US products, alkaloid levels in only 2 of 18 tested materials contained amounts of alkaloid that approach clinical relevance (many contained no alkaloid), whereas 4 of the 20 Internet products were found to contain amounts of yohimbe that approach levels that may be a cause of concern.

REGULATORY STATUS

Australia

Yohimbe but not yohimbe is found in the TGA substances that may be used in listed medicines in Australia (56). Yohimbe is permitted as an ingredient in prescription medicines (57).

Canada

Yohimbe and its salts (but not yohimbe) are listed as Schedule F (i.e., prescription only) drug ingredients. Natural products that contain Schedule F substances are not permitted to be marketed as Natural Health Products therefore there is no marketing authorization for yohimbe in Canada (58). No Yohimbe products are listed in the TPD Drug Product Database (59). Health Canada advises consumers not to use unapproved products containing yohimbe or yohimbe bark (60).

China

Yohimbe is not listed in the Pharmacopoeia of the Peoples Republic of China.

European Union

European Medicines Agency: No posted documents were found when the search terms "yohimbe" and "Pausinystalia" were used, indicating that there are no European Union (EU) approved uses or authorized yohimbe products in the EU. No quality standards monograph is published in the European Pharmacopoeia (PhEur), also indicating that there are no authorized products (otherwise there would likely be a quality standards monograph for test and release of the herbal drug yohimbe bark).

Individual EU Member States

Germany

There was a negative monograph decision by the German BfArM's Kommission E, meaning that marketing authorization for yohimbe as an active ingredient is not permitted. (61).

United Kingdom

Yohimbe is not listed in the MHRA "List B consolidated list of substances which are present in authorized medicines for general sale" (62). Yohimbe is listed as a medicinal herb in Schedule I to S.I. 2130 but cannot be sold on the open market without marketing authorization (premarket authorization). There are no yohimbe-containing medicinal products authorized with either a Product License number or with a Traditional Herbal Registration number on the MHRA Web site (63).

Japan

Yohimbe is not listed in the Japanese Pharmacopoeia.

Non-EU-Member European Countries

Switzerland

There are no yohimbe-containing products listed in the Swissmedic zugelassenen Präparate listing (Swissmedic authorized medicinal products) (64).

United States

Yohimbine and its salts are available as prescription drug ingredients in the United States, and there are monographs for Yohimbine Hydrochloride and Yohimbine injection in the United States Pharmacopeia (65). In 1989, US Food and Drug Administration (FDA) published a final regulation that stated that over-the-counter (OTC) drug products that purport to possess aphrodisiac properties are not generally recognized as safe and effective (and thus misbranded). Section 310.528 of the final rule states that any product that bears labeling claims that it will arouse or increase sexual desire, or that it will improve sexual performance, is an aphrodisiac drug product. The rule went on to state that going forward, OTC products that wish to make aphrodisiac claims must go through the drug approval process (66). Yohimbe containing products are available as dietary supplements in the United States, but a 2004 FDA Compliance and Enforcement Guide on dietary supplements stated that FDA officials conducting good manufacturing practice Inspections of dietary supplement manufacturers should inspect labels on site and forward labels or high-quality copies of labels to FDA headquarters if the labels were for yohimbe or *Pausinystalia yohimbe* containing products that claimed "yohimbine HCL" as ingredients (67).

A recent import alert permits FDA inspectors to seize foreign-manufactured products that contain yohimbe bark or yohimbe bark extract at US ports of entry without physical inspection because such products have been found in the past to be or contain unapproved alternatives to anabolic steroids (68).

CONCLUSIONS

There is little documentation of efficacy of yohimbe dietary supplement products for any use in the peer-reviewed scientific literature, whereas products that purport to contain yohimbe are associated with a large number of adverse event reports. Quality control remains an is-

sue, with some products containing undetectable amounts of the principle alkaloid. As long as case reports for adverse reactions as well as efficacy (many at the level of anecdote) rely on inconsistent products in an ever-changing marketplace, the utility and safety of yohimbe for any use will remain in dispute.

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Zinc

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INTRODUCTION

Zinc is the most abundant intracellular trace element. It is present in every living cell in the body and has many diverse biological functions. Because zinc is a component of many enzymes, it is involved in the synthesis and degradation of carbohydrates, lipids, proteins, nucleic acids, and gene expression, as well as in the metabolism of other nutrients (1). It is also an essential cofactor required for the structure and function of many proteins. Thus, it has a multitude of physiologic and biochemical functions, notably in embryogenesis, immunity, and growth. Zinc is associated with proteins in the food supply; good food sources of protein tend to be good sources of zinc. However, zinc's absorption depends on the presence or absence of substances in foods, such as phytate, which can bind zinc and make it unavailable for absorption. In 1961, human zinc deficiency was demonstrated in rural Iranian boys who consumed food that contained zinc, but which was not readily absorbed (2). Evidence of zinc deficiency has been reported in populations in developing countries subsisting on cereal-based diets. Treatment with supplements (zinc sulfate, zinc gluconate, etc.) appears effective in the short term, but in longer term, more sustainable solutions are required.

BIOCHEMISTRY AND PHYSIOLOGIC FUNCTIONS

Zinc has a 2+ charge (Zn^{2+}). It has an atomic number of 30 and an atomic weight of 65.37 (isotopic mean). In the pure form, it is a bluish white metal. Zinc is a strong Lewis acid, meaning it is an electron acceptor. It has a particular affinity for thiol and hydroxy groups and for amine electron donors (3). The metal readily forms complexes with amino acids, peptides, proteins, and nucleotides. It does not exhibit redox chemistry.

Catalytic, Structural, and Regulatory Functions

Zinc functions as a catalytic, structural, and regulatory component of nearly 300 enzymes, in which it maintains structural integrity and plays a role in regulating gene expression (4). In humans, the element is critical for the proper function of about 60 enzymes. Enzymes that require zinc for their catalytic function are found in all six enzyme classes (ligases, isomerases, lyases, hydrolases, transferases, and oxidoreductases) (5). An enzyme is considered to be a zinc metalloenzyme if removal of zinc reduces enzyme activity without affecting the protein structure per se, and if reconstitution with zinc restores activity (6). The metal may serve a catalytic function for these

enzymes by serving as an electron acceptor. Zinc is essential for catalytic activities of RNA polymerase, alkaline phosphatase, and carbonic anhydrase.

In its structural role, zinc helps proteins fold into their three-dimensional configurations; this folding is required for these proteins to have biological activity. "Zinc fingers," or loops formed by binding zinc to the amino acids, cysteine, and histidine, enable the folding to take place (7). Examples of proteins requiring zinc for normal structure include transcription factors (e.g., metal transcription factor 1), nuclear receptors (e.g., retinoic acid receptors), and enzymes (e.g., copper-zinc superoxide dismutase). Zinc finger proteins are widely distributed in the cell and also bind to RNA molecules and proteins. This interaction with other molecules allows for transcriptional and translational control and signal transduction. This ability to regulate gene expression may explain the tight homeostatic control of cellular zinc levels. The broad impact of zinc fingers on metabolism probably contributes to the nonspecific symptoms that characterize zinc deficiency (8).

Zinc and sulfur join to form thiolate clusters in many proteins such as metallothionein. These clusters allow metallothionein to exchange electrons and react with oxidants (nitric oxide, glutathione/glutathione reductase) in the body. When oxidized, thiolate clusters release zinc and the amount of free zinc increases in the cells. Too much free zinc can be toxic to the cells. Shifts in the activity of nitric oxide synthase (9) with changes in free zinc account for the increase in oxidative stress (10).

As a regulatory agent, zinc controls the expression of various genes (e.g., the metallothionein gene) (11) programmed cell death or apoptosis (12) and synaptic signaling (13). Zinc binds to zinc finger domains on a protein called metal response element transcription factor (MTF1). The zinc-MTF1 complex then binds to metal response elements (MREs) on the promoter area of the gene (14) and thereby regulates gene expression (15). The variable human responses to zinc depletion may be linked to shifts in the regulation of the *MTF1* gene (16). Zinc is also involved in regulating genes associated with apoptosis and immune function (17).

Zinc regulates immune function in part by receptor-mediated signal transduction. Zinc bound to a T-cell receptor joins with the protein tyrosine kinase enzyme (18) to activate the T cell. The transfer of information between and within cells that activates or inhibits communication with other cells, or that initiates signal transduction, allows an ion channel or a second messenger system to initiate immune cell function, differentiation, and proliferation (19). Zinc also functions as a signal molecule when

released in the synaptic vesicles of “zinc-containing” nerve cells found almost exclusively in the brain (20). Zinc-containing neurons bind zinc and glutamate suggesting that zinc may regulate the function of glutamatergic synapses (20).

Protein and Nucleic Acid Metabolism

Zinc plays a fundamental role in protein and nucleic acid metabolism, and cell division and differentiation. Zinc deficiency has a greater effect on cell division, a function of nucleoprotein synthesis, than on cell growth, which is a function of protein synthesis. Zinc inadequacy adversely affects gene expression and DNA or RNA metabolism, which in turn impair cell division and cause growth failure (21). Severe zinc deficiency will limit protein synthesis and increase amino acid oxidation. Proteins with short half-lives will be affected by zinc deficiency more readily than those with longer half-lives. For example, zinc deficiency is associated with a reduction in serum retinol, a protein whose half-life is about 1.5 hours (22).

Lipid and Carbohydrate Metabolism

Zinc-dependent enzymes are involved in the metabolism of carbohydrate and lipid, and release of energy. They also are involved in the synthesis of long-chain fatty acids from their dietary precursors and the formation of various prostaglandins (23). Zinc helps stabilize membranes by binding directly to protein and lipid components. Impaired glucose tolerance is associated with zinc deficiency, possibly because zinc affects insulin structure, secretion, and/or function (24).

METABOLISM

Zinc Distribution in Humans

Zinc is present in all tissues, organs, fluids, and secretions of the body. The total body content is approximately 1.5 g in females and 2.5 g in males. Zinc is located in the lean body mass with an average overall concentration of 30 $\mu\text{g}/\text{kg}$. Tissues with the highest concentrations of zinc include the bone, liver, muscle, prostate, and kidneys; the brain, skin, heart, and plasma have lower concentrations (25). Bone and skeletal muscle contain 86% of total body zinc. Approximately, 95% of the total body zinc is intracellular (26).

Zinc Absorption

Advances in knowledge regarding the molecular understanding of zinc absorption by the gastrointestinal tract contribute to and support previous studies of zinc metabolism, absorption kinetics, and homeostatic regulation. Zinc transporters play a key role in regulating cellular zinc metabolism. Changes in dietary zinc, hormones, or cytokines may upregulate or downregulate transporter gene expression. Tissue specificity and the location of the transporters within cells permit considerable flexibility in managing zinc homeostasis (27).

Two families of zinc transporter proteins exist. The ZnT (ZnT1–ZnT9) and ZIP family (at least 15 mammalian ZIP transporters) each have numerous members (28,29). ZnTs facilitate zinc efflux across the plasma membrane or into intracellular vesicles. In contrast, ZIPs enable zinc

influx into cellular cytoplasm from circulation or from cellular vesicles. The expression of ZnT and ZIP genes vary with physiologic conditions and body zinc status (30,31). Some of the transporters bind multiple divalent ions. This may explain some metal–metal interactions (e.g., zinc and iron) (32).

Zinc is absorbed along the entire intestinal tract (33), but the jejunum has the highest rate of absorption (34). However, a considerable amount of zinc is absorbed in the duodenum because the duodenal lumen has the highest initial zinc concentration after a meal (35). Zinc is resecreted into the digestive tract from sloughed-off intestinal and pancreatic cells or endogenous secretions. The estimated average requirement (EAR) and recommended dietary allowance (RDA) for zinc are based on the amount needed in the diet to replace endogenous zinc losses with intestinal losses being the major contributor. In establishing the RDA, it is assumed that the apparent zinc absorption averages 33% (36). However, apparent zinc absorption varies with the sources of dietary zinc (1). Factors that impair the regulation of endogenous intestinal zinc secretion, such as pancreatic insufficiency and inflammatory bowel disease, will reduce the ability to maintain zinc homeostasis.

Homeostatic Regulation

Total body zinc is primarily controlled by intestinal absorption. When dietary zinc intake decreases, fractional zinc absorption increases although the total amount of zinc absorbed declines (37,38). At the same time, endogenous losses decrease to achieve zinc balance (36). With severe dietary zinc restriction, endogenous fecal and urinary losses can be reduced to less than 1 mg/day (39). Zinc absorption is increased with marked increases in demand such as infant growth or lactation (37,40).

Zinc Turnover and Transport

Plasma zinc comprises only 0.1% of total body zinc and is maintained within a narrow range (10–15 $\mu\text{mol}/\text{L}$). A severe reduction in zinc intake that prevents zinc balance will cause a decline in plasma zinc concentrations (41,42). Plasma zinc levels also fall with infection due to tissue zinc uptake for the synthesis of immune proteins (43). Trauma or severe starvation leading to the catabolism of lean tissue may increase plasma zinc concentrations and zinc losses in the urine and feces (44). Food intake causes a decline in plasma zinc over the next 2 to 3 hours. This may reflect tissue uptake of circulating zinc to facilitate metabolism (45). Plasma zinc is bound primarily to the proteins, albumin (6), and α_2 -macroglobulin. Approximately, 70% of it in plasma is bound to albumin and exchanges easily ($K_d = 7.5 \text{ M}$) with it. α_2 -Macroglobulin, a protease inhibitor and carrier of growth factors, binds the metal tightly and represents most of the remaining protein-bound zinc in plasma (6,46). A very small amount of plasma zinc is bound to free amino acids. Zinc in the plasma turns over completely approximately 130 times/day reflecting the important role zinc plays in numerous metabolic processes (47).

The concentration of zinc in blood cells is higher in leukocytes (6 mg of Zn/ 10^6 cells) than erythrocytes (1 mg of Zn/ 10^6 cells) (48). However, because there are many more erythrocytes than leukocytes, approximately 75% to

88% of the total zinc in human blood is in erythrocytes; only 3% is in leukocytes and the remainder (12–22%) is in the plasma. Leukocytes actively make proteins, and cDNA array analyses have shown that leukocyte genes are very sensitive to zinc (17). Kinetic data, from radioactive and stable isotopic tracer studies, provide a model of the distribution of zinc among the main body pools and their respective turnover rates. Basically, the total body zinc can be divided into two pools, a more rapid pool that turns over in ≈ 12.5 days and a slow pool that turns over in 300 days (49,50). Zinc in the liver, pancreas, kidney, and spleen turns over rapidly, whereas a slower turnover is found in muscle, erythrocytes, bone, and the nervous system. An exchangeable zinc pool (EZP) has been defined in human subjects as that amount of whole body zinc, which exchanges with the isotopic tracer within 2 days (51). The size of the EZP declines with severe zinc depletion, but it may remain unchanged with marginal depletion (52,53). The EZP has been proposed as a marker of zinc status, but the degree of change in EZP during zinc depletion is usually smaller than the change in plasma zinc concentrations and the EZP is much more challenging to measure than is plasma zinc (52).

Zinc Reserves

Zinc has no specific storage site. However, it appears that all cells have a small zinc reserve stored in membrane-bound vesicles or “zincosomes” (28). Robust homeostatic mechanisms, such as a reduction in endogenous fecal excretion or urinary losses, help maintain tissue zinc levels when zinc intakes are low. Supplementation studies show that zinc reserves can be used to support growth during subsequent periods of low zinc intake (54). Red blood cells contain between 20 and 40 $\mu\text{g Zn/g hemoglobin}$ (48,55) and the average circulating hemoglobin content is 750 g in adults. This represents a red blood cell zinc pool of 15 to 30 mg. The average lifespan of a red blood cell is 120 days. Therefore, turnover for this zinc pool is between 0.12 and 0.25 mg/day. This suggests that a meaningful amount of zinc needs to be supplied for erythropoiesis. The zinc demand for maintaining leukocytes is probably much greater than that for erythrocytes as the turnover rate for leukocytes is much more rapid.

Excretion

The major route of zinc excretion is secretion into the gastrointestinal tract. This is the combined contribution from pancreatic secretions (enterohepatic circulation), sloughing of mucosal cells into the intestinal lumen, and transepithelial flux of intestinal zinc from the serosa to the mucosa (6). The metal lost via pancreatic secretions is undefined but certainly includes zinc metalloenzymes involved in digestion. It is not surprising, therefore, that pancreatic zinc secretion is stimulated by meals. Gastrointestinal excretion varies with zinc intake. The amount lost is <1 mg/day in a severe dietary zinc restriction state (0.3 mg/day) (39). At typical intakes of 7 to 15 mg/day, endogenous fecal losses are approximately 3.0 to 4.6 mg/day (36,56), and it increases proportionately at higher intakes. Urinary zinc output is low (<1 mg/day) and is refractory to change over a wide intake range (4–25 mg/day) (39,57). Starvation/trauma and other conditions that

increase muscle protein catabolism increase urinary zinc as the load of amino acids filtered by the kidney increases. Some supplements with tightly bind zinc (e.g., zinc picolinate) may promote urinary zinc losses (58). Glucagon has been shown to regulate zinc reabsorption by the renal tubular system (59). A number of zinc transporters are expressed in kidney. It has been proposed that ZnT1, an efflux transporter, is oriented such that it contributes to zinc reabsorption (60). Changes in the expression of zinc transporters are involved in adjusting intestinal endogenous losses and the renal reabsorption of zinc with changes in zinc intake.

Other sources of zinc loss include integumental losses from sweat and sloughed skin cells (~ 1 mg/day), semen (~ 1 mg/ejaculate), and menstruation (0.1–0.5 total) (25).

ZINC INADEQUACY

Identification of Inadequacy

Diagnosis of zinc deficiency is difficult due to the lack of a specific, sensitive biomarker. Plasma or serum zinc concentrations are used most widely to assess zinc status. However, plasma concentrations do not respond readily to modest changes in dietary zinc, and they are affected by metabolic conditions unrelated to zinc status (e.g., stress, infection, food intake, and hormonal state). However, until more definitive markers are available, the status can be estimated from the dietary zinc intake and plasma zinc concentration. An individual with a low nonfasting plasma zinc level, that is, less than 65 $\mu\text{g/dL}$, and a usual zinc intake that is less than the EAR are at risk of poor zinc status. Zinc deficiency is more common in developing countries (61,62) in conjunction with the intake of cereal- and grain-based diets that are high in phytate. Groups vulnerable to zinc deficiency include individuals with increased needs such as growing infants and children, pregnant and lactating women. The elderly may also be at increased risk due to lower zinc intakes, reduced absorption, or altered utilization (61).

Symptoms of Inadequacy

Symptoms of severe zinc deficiency include dermatitis, alopecia (loss of hair), delayed wound healing, and apathy (25). More moderate deficiencies cause poor growth in children, loss of appetite, impaired immune function, and cognitive changes (63). Factors predisposing an individual to zinc depletion include increased requirement (demands for growth, pregnancy and lactation, and infection), inadequate dietary supply (poor food choices, lack of animal food sources, alcoholism, and low socioeconomic status), decreased absorption (diet high in zinc chelating agents such as phytate, gastrointestinal dysfunction or disease), or increased losses (diarrheal fluid loss, postinfection or trauma, burns, surgery, and uncontrolled diabetes).

INDICATIONS AND USAGE

Zinc Sources

Major dietary sources of zinc include shellfish, red meats, liver, poultry and dairy products, whole grains, legumes,

and fortified cereals. Because zinc functions as a cofactor or component of enzymes and proteins, it is not surprising that it is found primarily in animal food sources. In the United States, approximately 25% of the dietary zinc intake comes from beef (64,65). Cereal grains also contain zinc in the outer layers of the kernel and the germ. Thus, refined cereals have less zinc than do whole grains. Infant formulas, infant cereals, and ready-to-eat breakfast cereals are often fortified with zinc in the United States. The average zinc intake of Americans from food is 13 mg zinc/day for men and 9 mg zinc/day for women (36).

Bioavailability

The proportion of dietary zinc that is available for absorption in the small intestine varies depending on the other components of the diet. Phytate, a storage molecule for phosphorus in grains and seeds, strongly binds zinc in the gut, making it unavailable for absorption (66). The presence of calcium in the diet may enhance the binding between phytate and zinc, and further reduce zinc absorption (67,68). On the other hand, protein and amino acids also bind zinc in the gut and increase the amount absorbed (69). Because phytate is the primary dietary factor influencing zinc absorption, the availability of zinc for absorption can be predicted from the phytate/zinc molar ratio of the diet, which is calculated as follows (70):

$$\frac{\text{mg phytate}/660}{\text{mg zinc}/65.4}$$

Diets with a phytate/zinc molar ratio below 15 are considered to have relatively high zinc availability, whereas those with a molar ratio greater than 25 have low availability (71). Because animal food sources do not contain phytate, meat-based diets have phytate:zinc ratios below 15. Cereal-based diets lacking animal food sources tend to have ratios above 25.

Supplement Use

Approximately 15% of Americans augment their dietary intake of zinc with zinc supplements (72,73). The amount of zinc in multivitamin/multimineral supplements ranges from 10 to 25 mg/dose. Oral supplements may also be purchased that provide as much as 100 mg of zinc per tablet. When the average zinc intake from food and supplements is compared with the average intake from food alone, it is increased from 10 to 11 mg zinc/day. However, the top 5% of intake from food and supplements in the United States ranges from 33 to 45 mg/day due to the high amounts of zinc in some supplements.

Recommended Intake Levels

In 2002, the U.S. Food and Nutrition Board of the National Academy of Sciences reviewed its recommendations for zinc intake (36). A factorial approach was used to set the RDA meaning that the recommended intake is based on the amount of zinc required to replace zinc losses from the body each day. The RDA represents a daily intake that ensures adequate nutrition for 95% to 97.5% of the population. The recommendations are summarized in Table 1.

Table 1 Recommended Dietary Allowance (RDA)^a

Age	RDA (mg/day)	
	Male	Female
0–6 mo ^b		
7–12 mo	3	3
1–3 yr	3	3
4–8 yr	5	5
9–13 yr	8	8
14–18 yr	11	9
19–50 yr	11	8
≥51 yr	11	8
<i>Pregnancy</i>		
≤18 yr		13
19–50 yr		11
<i>Lactation</i>		
≤18 yr		14
19–50 yr		12

^aU.S. Food and Nutrition Board of the Institute of Medicine, 2002.

^bRecommended intake is equivalent to average human milk zinc content.

Treatment of Zinc Depletion

Supplementation with a modest amount of zinc readily corrects zinc depletion (74,75). The amount used should not exceed the zinc upper levels (ULs) (Table 2).

Prevention of Zinc Depletion Among Children Worldwide

From the results of a series of randomized trials performed around the world, it is evident that modest zinc supplementation frequently improves linear growth (74), and reduces the incidence of diarrheal disease (76,77), pneumonia (78), and malaria (75,79) among children living in developing countries. In populations with evidence of dietary zinc deficiency and with high rates of stunting and/or low plasma zinc concentrations, supplementation should be implemented to promote children's growth, particularly for children under 24 months of age. Criteria for identifying populations at risk for zinc deficiency are summarized in Table 3. If >20% of the population studied meet the criterion for stunting, adequacy of zinc intakes,

Table 2 Tolerable Upper Intake Levels (UL) for Zinc

Age group	UL (mg/day)
0–6 mo	4
7–12 mo	5
1–3 yr	7
4–8 yr	12
9–13 yr	23
14–18 yr	34
19–50 yr	40
>50 yr	40
<i>Pregnancy</i>	
≤18 yr	34
>18 yr	40
<i>Lactation</i>	
≤18 yr	34
>18 yr	40

Table 3 Criteria for Identifying Populations at Risk for Zinc Deficiency

Indicator	Criterion	Recommended cut-off
Rates of stunting among children under 5 years	Length-for-age or height-for-age Z-score < -2	≥20% of the population
Adequacy of dietary zinc intakes	Proportion of population with intakes below two-thirds of the RDA	≥20% of the population
Plasma or serum zinc	Proportion of population below nonfasting concentration of 65 µg/dL (70 µg/dL fasting)	≥20% of the population

From Ref. 1.

or plasma zinc, then that population should be considered at risk for zinc deficiency. The suggested dosage of supplemental zinc is 5 mg/day for children under 6 years of age and 10 mg/day for those over 6 and adults.¹

The World Health Organization (WHO) advocates providing 20 mg/day of zinc supplementation for 10 to 14 days (10 mg/day for infants under 6 months) for treating acute diarrhea (80). No recommendations have been made to use supplemental zinc to prevent diarrhea in at-risk populations.

Disease Treatment

Common Cold

The use of zinc lozenges within 24 hours of the onset of cold symptoms and continued every 2 to 3 hours while awake has been advocated for reducing the duration of the common cold (81). Ten randomized controlled trials have been conducted to evaluate the use of zinc lozenges on the duration of colds; five showed a positive effect and five reported no effect. A meta-analysis of the effectiveness of the lozenges in reducing the duration of the common cold failed to find a significant effect (82). Taking these lozenges every 2 to 3 hours while awake results in a total zinc intake that exceeds the recommended upper zinc level (UL) of 40 mg/day (Table 2). Some individuals have reported gastrointestinal disturbances and mouth irritation while using them (83).

Alzheimer's Disease

Brains of patients with Alzheimer's disease are filled with numerous deposits of a substance called amyloid. The primary component of amyloid is a protein called A-beta, which is normally found in many body fluids such as plasma and cerebrospinal fluid. A-beta binds to zinc very strongly at levels that are slightly higher than those normally found in the human brain. Possibly, abnormal brain zinc metabolism in Alzheimer's patients causes A-beta to deposit as amyloid (84,85). In a study of 12 nuns with Alzheimer's disease, plasma zinc levels were measured approximately 1 year before their deaths. Those nuns with low blood zinc levels had more brain plaques at autopsy. This does not indicate that poor zinc nutrition is a cause of Alzheimer's disease. Instead, it may indicate that people with this disorder metabolize zinc differently.

HIV/AIDS

Zinc is required for the synthesis of immune proteins and for maintaining normal immune function. In the later

stages of acquired immunodeficiency syndrome (AIDS), excessive losses of zinc from diarrhea increase the requirement for zinc (25). Furthermore, plasma zinc concentrations may be low as a result of cytokine-directed redistribution of zinc to the liver, bone marrow, and other tissues. In a randomized controlled trial of zinc supplementation (45 mg zinc/day for 1 month), opportunistic infections were reduced in AIDS patients in comparison to a placebo group. However, as the human immunodeficiency virus (HIV) requires zinc, there is some concern that zinc supplementation may enhance disease progression. In one observational study, supplemental zinc was associated with poorer survival among AIDS patients. Until further information is available on optimal zinc intakes for HIV-infected individuals, the amount of supplemental zinc taken by these patients should not exceed the UL (Table 2).

Other Diseases

Prepubertal children with sickle cell disease (SCD) may have zinc deficiency and may benefit from zinc supplementation to improve linear growth and weight gain. SCD children supplemented with 10 mg zinc/day had significantly greater increases in height (0.66 ± 0.29 cm/yr), sitting height (0.97 ± 0.40 cm/yr), knee height (3.8 ± 1.2 mm/yr), and arm circumference (0.27 ± 0.12 cm/yr) (86).

Wilson's disease is an autosomal recessive inherited disorder in which excessive copper is stored in the liver and later in the brain and other organs leading to hepatic and/or neuropsychiatric disease. Treatment requires life-long administration of copper chelators (*d*-penicillamine and trientine). Oral zinc supplements are frequently used as an alternative treatment. However, prospective randomized controlled studies have not been conducted to confirm that supplemental zinc improves the outcome of chelation therapy in Wilson's disease patients (87,88).

Contraindications

At present, the incidence of zinc toxicity is far less frequent than that of moderate-or-mild zinc deficiency. However, with increased use of zinc supplements and zinc-fortified foods, the tolerance for high intakes may be exceeded, and toxicity may occur. In 1998, approximately one-half of the toddlers in the United States reported zinc intakes that were greater than the UL recommended by the Institute of Medicine (89).

Isolated outbreaks of acute toxicity have occurred as a result of consumption of food or beverages contaminated with zinc from galvanized containers or from accidental exposure to zinc oxide fumes. The overt toxicity symptoms include nausea, vomiting, epigastric pain, lethargy, and fatigue (25,36).

Chronic overdosage of supplemental zinc (i.e., 100–300 mg zinc/day) is of greater concern than acute toxicity because it is more common and is less readily detected. A chronic excessive zinc intake has a negative effect on copper absorption and status. Intakes as low as 60 mg zinc/day have been shown to reduce erythrocyte copper–zinc superoxide dismutase (ESOD) activity, a sensitive marker of copper status (90). Doses between 50 and 100 mg zinc/day have lowered high-density lipoprotein cholesterol in some (91,92), but not all, studies (93). The zinc ULs based on the effect of supplemental zinc on the

activity of a copper-dependent enzyme, ESOD, or some other marker of copper status, such as serum copper or ceruloplasmin (36).

ADVERSE INTERACTIONS

Zinc deficiency may also be related to a competitive interaction with iron. The interactions vary, however, possibly due to the form of iron, whether the additional iron is taken with a meal or not, and the amounts of iron and zinc fed. In general, large quantities of supplemental iron (i.e., greater than 50 mg) appear to reduce zinc absorption (94), and the effect is greater when iron and zinc are given together under fasting conditions than when consumed with food or as part of a meal (95,96). Because supplemental iron, but not supplemental zinc, is often prescribed for pregnant women, infants, and children, there is a concern that their zinc status could be impaired, but this has not been confirmed in studies of zinc absorption in the presence of supplemental iron (97). It is unlikely, however, that high iron intakes from food interferes with zinc absorption.

Alcohol and Drugs

Alcohol and other drugs may have an adverse effect on zinc status by reducing zinc absorption or by increasing zinc excretion. When alcohol is used on a regular basis, it appears to increase zinc losses and lower tissue zinc levels. This does not occur with occasional alcohol use. The detrimental effect of alcohol on zinc occurs with chronic, excessive use of alcohol.

A number of drugs can affect zinc status. Some anticancer drugs and aspirin chelate zinc and make it less available for absorption. Other drugs, such as penicillamine, chelate zinc within the tissues and increase zinc losses. Prophylactic coadministration of 25 mg of zinc may be indicated when these drugs are used.

CONCLUSIONS

Groups at risk of zinc deficiency include those with high zinc needs such as growing infants, children, and adolescents, and pregnant and lactating women. Dietary zinc intakes in the United States are adequate for most groups, with the exception of vegans, who do not consume fortified cereals, and, possibly, the elderly. Supplemental zinc has been used to treat a variety of diseases with inconsistent results. Individuals experiencing trauma and tissue catabolism due to burns, wounds, or surgery lose zinc; supplemental zinc may improve their recovery. Supplements may also be indicated for those taking drugs that chelate zinc. The WHO advocates providing 20 mg/day of zinc supplementation for 10 to 14 days (10 mg/day for infants under 6 months) for treating acute diarrhea (80).

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Second Edition

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