Chapter 1
Vitamin C: An Introduction
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Structure and Chemistry of Vitamin C (L-Ascorbic Acid)

The chemical name for L-ascorbic acid is 2,3-didehydro-L-threo-hexano-1,4-lactone. Carbon 5 of ascorbic acid (Fig. 1.1) is asymmetric, making two enantiomeric forms possible; L-ascorbic acid is the naturally occurring and biologically active form. L-Ascorbic acid is a water-soluble 6-carbon α-ketolactone with two enolic hydrogen atoms (pK\textsubscript{a1} at carbon 3 = 4.17 and pK\textsubscript{a2} at carbon 2 = 11.57; Fig. 1.1) (1,2). At physiologic pH, >99% of L-ascorbic acid is ionized to L-ascorbate, which can donate a hydrogen atom (H\textsuperscript{+} + e\textsuperscript{−}) to produce the resonance-stabilized ascorbyl radical (Fig. 1.1). The ascorbyl radical can donate a second electron to form the 2-electron oxidation product of ascorbate, dehydroascorbic acid (DHA), or dismutate to form ascorbate and DHA (Fig. 1.1). Alternatively, the ascorbyl radical may be enzymatically reduced back to ascorbate by NADH-dependent semidehydroascorbate reductase or the NADPH-dependent selenoenzyme, thioredoxin reductase. DHA can be reduced back to ascorbate by the glutathione-dependent enzyme, glutaredoxin, or thioredoxin reductase (3). If not recycled to ascorbate, DHA is irreversibly hydrolyzed to 2,3-diketo-L-gulonic acid (DKG), which does not function as an antioxidant. Further degradation of DKG results in the formation of oxalic acid and L-threonic acid. Other catabolites include, among many others, L-xylonic acid, L-lyxonic acid, and L-xylose (4). The term vitamin C is generally used to describe all compounds that qualitatively exhibit the biological activity of ascorbate, including ascorbate and DHA (2).

Fig. 1.1. Oxidation of ascorbate (AscH\textsuperscript{−}) by two successive one-electron oxidation steps to give the ascorbyl radical (Asc\textsuperscript{*−}) and dehydroascorbic acid (DHA), respectively.
Biological Activities of Vitamin C

Electron Donor for Enzymes

The physiologic functions of vitamin C are related to its efficacy as a reducing agent or electron donor. Vitamin C is known to be a specific electron donor for eight human enzymes (1). Three of those enzymes (proline hydroxylase, lysine hydroxylase, and procollagen-proline 2-oxoglutarate 3-dioxygenase) participate in the post-translational hydroxylation of collagen, which is essential for the formation of stable collagen helices (5). Many of the manifestations of the vitamin C–deficiency disease, scurvy, are related to defective collagen synthesis, including blood vessel fragility (petechiae, ecchymoses, and inflamed bleeding gums), tooth loss, bone and connective tissue disorders, and impaired wound healing. Vitamin C is also necessary for the maximal activity of two dioxygenase enzymes ($\gamma$-butyrobetaine 2-oxoglutarate 4-dioxygenase and trimethyllysine 2-oxoglutarate dioxygenase) required for L-carnitine biosynthesis. L-Carnitine is required for the transport of activated fatty acids across the inner mitochondrial membrane and plays critical roles in modulating energy metabolism (6). Fatigue and lethargy, which are early signs of scurvy, likely are related to carnitine deficiency. Vitamin C is also a cosubstrate for dopamine-$\beta$-monooxygenase, the enzyme that catalyzes the conversion of the neurotransmitter dopamine to norepinephrine. Neuropsychiatric changes associated with scurvy, including depression, mood swings, and hypochondria, could be related to deficient dopamine hydroxylation (3). Two other enzymes are known to require vitamin C as a cosubstrate, although their connection to the pathology of scurvy has not been established. Peptidyl glycine $\alpha$-amidating monooxygenase is required for peptide amidation and 4-hydroxyl-phenylpyruvate dioxygenase is required for tyrosine metabolism (5).

Vitamin C may also play a role in the metabolism of cholesterol to bile acids and in steroid metabolism as a cosubstrate of the enzyme 7$\alpha$-monooxygenase (7). The hydroxylation of xenobiotics and carcinogens by the cytochrome P450 family of enzymes is also enhanced by reducing agents, such as vitamin C (8). Vitamin C has been found to enhance the activity of endothelial nitric oxide synthase (eNOS) by maintaining its cofactor tetrahydrobiopterin in the reduced, and thus active, form (9). Synthesis of nitric oxide (NO) by eNOS plays a critical role in maintaining normal endothelial function (10). The finding that vitamin C enhances eNOS activity through its activity as a reducing agent supports the idea that sufficient vitamin C may contribute to the prevention of cardiovascular diseases (see below).

Recent research suggests that a family of oxygen-dependent prolyl hydroxylase enzymes plays an important role in the ability of cells to recognize and respond to hypoxia (11). Under normoxic conditions, hydroxylation of a conserved proline residue on the hypoxia-inducible transcription factor $\alpha$-subunit (HIF-1$\alpha$) results in rapid proteasome-mediated degradation. Hypoxia inhibits prolyl hydroxylation, allowing HIF-1$\alpha$ to accumulate and migrate to the nucleus where it activates hypoxia-responsive genes (12). Although ascorbate is known to increase the activity of these
oxygen-dependent prolyl hydroxylase enzymes \textit{in vitro} (11,13), its significance to this “oxygen sensing” pathway \textit{in vivo} remains to be determined.

\textbf{Antioxidant Activity}

An antioxidant has been defined as “a substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (14). Several properties make vitamin C an ideal antioxidant in biological systems. First, the low one-electron reduction potentials of ascorbate and the ascorbyl radical enable these compounds to react with and reduce virtually all physiologically relevant reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide, hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone, nitrogen dioxide, nitroxide radicals, and hypochlorous acid (15). Hydroxyl radicals also react rapidly, although not preferentially, with vitamin C; hydroxyl radicals are so reactive that they combine indiscriminately with any substrate at a diffusion-limited rate. Vitamin C also acts as a co-antioxidant by regenerating $\alpha$-tocopherol from the $\alpha$-tocopheroxyl radical. This may be an important function because \textit{in vitro} experiments have found that $\alpha$-tocopherol can act as a prooxidant in the absence of co-antioxidants such as vitamin C (16). Another property that makes vitamin C an ideal antioxidant is the low reactivity of the ascorbyl radical formed when ascorbate scavenges ROS or RNS. The ascorbyl radical is neither strongly oxidizing nor strongly reducing and it reacts poorly with oxygen. Thus, when a reactive radical interacts with ascorbate, a much less reactive radical is formed. The ascorbyl radical scavenge another radical or rapidly dismutates to form ascorbate and DHA. Alternatively, the ascorbyl radical and DHA can be reduced enzymatically or recycled back to ascorbate (see above).

\textbf{Dietary Iron Absorption}

The ability of ascorbate to maintain metals ions in a reduced state is critical to the function of the mono- and dioxygenases discussed above (7,8). Concomitant consumption of vitamin C from food or supplements enhances nonheme iron absorption from a single meal in a dose-dependent manner (17), probably because the reduction of iron by ascorbate makes it less likely to form insoluble complexes with phytate and other ligands (1). Iron deficiency is the most common nutrient deficiency in the world and is associated with a number of adverse health effects (18). Consequently, the potential for increased vitamin C intake to improve iron nutritional status by increasing the bioavailability of dietary nonheme iron has received considerable attention. Despite consistent findings of enhanced iron absorption from a single meal in the presence of vitamin C, several intervention studies were not able to demonstrate that increasing vitamin C intake improved iron nutritional status (19,20). More recent research indicates that the enhancing effect of vitamin C on iron absorption from a complete diet, rather than a single meal, may be offset partially by dietary inhibitors of
iron absorption (21). Further research is required to determine whether increasing vitamin C intake is an effective strategy for improving iron nutritional status.

Uptake, Distribution, and Metabolism of Vitamin C

Intracellular Transport

Ascorbate is actively transported into cells via Sodium-dependent Vitamin C Transporters known as SVCT1 and SVCT2 (22). SVCT1 is expressed mainly on the epithelial surfaces of the intestine and kidney, and in the liver, whereas SVCT2 expression has been found in a number of tissues, including neurons, endocrine tissue, and bone. This distribution suggests that SVCT1 is involved with bulk transport of ascorbate, whereas SVCT2 is involved in tissue-specific ascorbate uptake (23). DHA can be transported into cells by facilitated diffusion via the glucose transporters, GLUT1, GLUT3 and to some extent, GLUT4. Intracellularly, DHA is immediately reduced to ascorbate through chemical reduction by glutathione or enzymatic reduction (4).

Intestinal Absorption

Intestinal absorption of ascorbate appears to occur mainly through active transport via sodium-dependent ascorbate transporters on the apical side of enterocytes, whereas the transport mechanism responsible for basolateral efflux of ascorbate from enterocytes is not yet known (23). Intestinal absorption of DHA has not been well characterized in humans (4,5). Although DHA has antiscorbutic effects when administered to humans, at least one study suggests that the absorption of DHA is less than that of ascorbate (24). Glucose has been found to inhibit ascorbate and DHA uptake by human small intestinal brush border membrane vesicles (25) and by human neutrophils (26).

Bioavailability is defined in pharmacokinetic terms as the difference between the increase in plasma levels of a substance after a dose given intravenously and that after the same dose given orally. The only study examining the true bioavailability of ascorbate calculated that the bioavailability of a liquid solution of ascorbate given to fasting men at steady state was >80% for doses ≤100 mg, 78% for a 200-mg dose, 75% for a 500-mg dose, and 62% for a 1250-mg dose (27).

Plasma Concentrations

Plasma ascorbate concentrations in people without scurvy generally range from 11 to 90 µmol/L, whereas DHA is not generally detectable in plasma (<2% of total ascorbate). Plasma ascorbate concentrations <11 µmol/L indicate vitamin C deficiency, and concentrations between 11 and 28 µmol/L represent marginal vitamin C status (2). Data on plasma ascorbate levels collected from 1988 to 1994 during the third National Health and Nutrition Examination Survey (NHANES III) indicated that the prevalence of vitamin C deficiency in the United States ranges from 9% in women to 13%...
in men and the prevalence of marginal vitamin C status ranges from 17% in women to 24% in men (28).

In two pharmacokinetic studies conducted in healthy young men and women, steady-state plasma ascorbate concentrations increased rapidly at vitamin C doses between 30 and 100 mg/d, suggesting that varying vitamin C intake within that range may result in significant differences in the availability of ascorbate to tissues (29,30). At doses of 200 mg/d, the rate of increase in steady-state plasma ascorbate concentrations decreased, and plasma ascorbate levels increased very little at doses >400 mg/d.

**Tissue concentrations**

Tissue ascorbate concentrations vary greatly depending on the tissue type, with the highest concentrations found in adrenal and pituitary glands and slightly lower concentrations found in liver, spleen, lens, pancreas, kidney, and brain (4). Intracellular ascorbate concentrations in lymphocytes, neutrophils, and monocytes obtained from healthy young men and women were saturated at vitamin C doses of 100 mg/d and reached concentrations of 1–4 mmol/L, i.e., at least one order of magnitude higher than plasma concentrations (29,30).

**Excretion**

Urine is the primary route of excretion for ascorbate and its metabolites in humans. In the kidney, ascorbate is filtered by the glomerulus and actively reabsorbed by sodium-dependent ascorbate transporters in the proximal tubules (4,23). Active reabsorption of ascorbate is saturable, and human plasma ascorbate concentrations appear limited by the capacity for renal reabsorption. In healthy men and women at steady-state conditions, the threshold for urinary ascorbate excretion was observed at vitamin C intakes between 60 and 100 mg/d, and most of the ascorbate from intakes ≥500 mg/d was excreted in the urine within 24 h (29,30). Because DHA cannot be detected in plasma, no information is available regarding renal excretion or reabsorption of DHA (4). Limited research in healthy men indicates that high doses of supplemental vitamin C ranging from 1000 to 10,000 mg/d increases urinary oxalate excretion, but not above normal reference ranges of 20–60 mg/d (28).

**Sources and Intake Recommendations**

**Recommended Intake of Vitamin C**

Unlike most mammals, humans and other primates obtain vitamin C exclusively from their diets because they lost the ability to synthesize vitamin C from glucose during the course of evolution. This defect is due to mutations in the gene encoding the final enzyme of the vitamin C biosynthetic pathway, L-gulonolactone oxidase (1). To prevent scurvy, an adult must consume ~10 mg/d of vitamin C, an amount easily obtained from as little as one serving/d of most fruits and vegetables. Although the amount of vitamin C required for the prevention of scurvy in humans has been well
established, the optimal intake of vitamin C is likely to be considerably higher and to vary with life stage, gender, and disease state.

The current recommended dietary allowance (RDA) for vitamin C is 90 mg/d for men and 75 mg/d for women (31). The recommended intake for smokers is 35 mg/d higher than for nonsmokers, because ascorbate turnover is ~35 mg/d greater in smokers, presumably due to increased oxidative stress and other metabolic differences. The previous RDA of 60 mg/d for men and women was based in part on the prevention of scurvy with a 4-wk margin of safety (1). The current RDA is based on the vitamin C intake required for 80% neutrophil saturation with little urinary loss in healthy men. At the time the recommendation was made, similar data were not available for women, and the RDA was extrapolated on the basis of body weight. Recently, the results of a similar study in women were published along with a recommendation that the RDA for vitamin C be raised to 90 mg/d for women as well (30).

Although the current RDA is no longer based solely on the prevention of scurvy, it continues to be based primarily on the prevention of deficiency, rather than the prevention of chronic disease and the promotion of optimal health. Pharmacokinetic studies in healthy young men and women found that leukocytes generally became saturated at vitamin C intakes between 100 and 200 mg/d, and these intake levels have been associated epidemiologically with decreased risk of chronic disease, particularly cancer, heart disease, and stroke. Thus, for healthy individuals, a vitamin C intake of at least 200 mg/d should be considered on the basis of preventing chronic disease and promoting optimal health. The amounts of vitamin C required to maintain optimal body levels in special populations, such as children, pregnant women, and older adults, have not been established. Similarly, the amounts of vitamin C required to derive therapeutic benefits in individuals affected by chronic diseases are not known and most probably are higher than current recommendations for healthy individuals.

**Dietary Sources of Vitamin C**

A daily vitamin C intake of at least 200 mg is easily obtained by consuming 5 servings of fruits and vegetables. Increased fruit and vegetable intakes have been consistently associated with decreased risk of chronic diseases (32–34), and indeed most of the epidemiologic evidence associating increased vitamin C intake with decreased chronic disease risk is based on vitamin C consumption from fruits and vegetables. Therefore, fruits and vegetables should be considered the preferred vehicle for increasing one’s vitamin C intake. Fresh vegetables, fruits, and juices are the richest sources of vitamin C. The vitamin C content in foods may be decreased by prolonged storage and some cooking practices. Boiling vegetables has been found to result in vitamin C losses from 50 to 80%. Steaming vegetables in minimal amounts of water or cooking them in a microwave oven substantially decreases the loss of vitamin C from cooking (35,36).
Supplements

Vitamin C supplements are a significant source of vitamin C intake in the United States. Data from NHANES III indicate that ~40% of the U.S. population surveyed had taken at least one nutritional supplement in the past month (37). The most common ingredient in those supplements was vitamin C, which was present in 45% of the supplements. In general, studies comparing the bioavailability of vitamin C from foods with that from supplements have found little difference (38). Studies comparing the bioavailability of vitamin C from different types of vitamin C supplements (ascorbic acid, mineral ascorbates, ascorbate plus vitamin C metabolites, and ascorbate with flavonoids) have not generally found differences (39). One exception is a study that found plasma ascorbate levels and 24-h urinary ascorbate excretion to be increased by 35% when ascorbate was taken with a citrus extract containing bioflavonoids (40). The significance of these results is unclear given recent findings that flavonoids may inhibit cellular uptake of ascorbate (41,42).

Milestones in Vitamin C Research

Recognition of the Biological Importance of Vitamin C in the Prevention of Scurvy and Associated Symptoms

Diseases likely to be scurvy have been reported throughout written history. Known as the “calamity of sailors,” scurvy has also been recorded during famines, sieges, imprisonment, and long expeditions over land. James Lind reported the benefits of citrus fruits in treating scurvy in his “Treatise on Scurvy” in 1753, but it was not until 1795 that the British admiralty mandated a daily dose of citrus juice for British seamen, the origin of the term, “limey” (43). Although it was acknowledged that citrus fruits could prevent and cure scurvy, the concept that the disease was caused by the lack of an essential nutrient in the diet was not generally accepted at the beginning of the 20th century. Reports by Axel Holst and Theodor Fröhlich in 1907 that scurvy could be produced experimentally in guinea pigs by feeding a diet lacking fresh fruits or vegetables, and the proposal by Casimir Funk in 1912 that scurvy, pellagra, rickets, and beriberi were due to dietary deficiencies of factors he called “vitamines” led to 20 years of intensive efforts toward isolating the antiscorbutic factor (44). The complementary findings of two research groups, that of Charles King at the University of Pittsburgh in the United States and that of Albert Szent-Györgyi at the University of Szeged in Hungary, led to the discovery of vitamin C as the antiscorbutic factor in 1932 (45,46). In 1933, E.L. Hirst and W.N. Hayworth elucidated the structure of vitamin C, using material isolated by Szent-Györgyi, and vitamin C was first synthesized the same year (47). Szent-Györgyi was awarded the 1937 Nobel Prize for Physiology or Medicine, in part, for his work in isolating vitamin C as the antiscorbutic factor, and Hayworth was awarded the Nobel Prize for Chemistry the same year. The isolation, identification, and synthesis of ascorbic acid laid the foundation for research into the role of vitamin C in health and disease that continues today.
Recognition of the Role of Vitamin C as an Important Biological Antioxidant

In the 1950s, the free radical theory of aging hypothesized that free radicals arising from enzymatic and nonenzymatic reactions inside and outside cells contributed to the aging process (48). However, the presence of free radicals in biological systems was not generally considered likely until the discovery of superoxide dismutase in 1969 (49). Currently, there is a great deal of evidence that ROS and RNS play significant roles in aging and a number of chronic diseases. Ascorbate and the ascorbyl radical readily scavenge virtually all physiologically relevant ROS and RNS (see above). Ascorbate has also been found to be the most effective endogenous aqueous-phase antioxidant in human plasma under many different oxidizing conditions (50,51). Although other endogenous antioxidants are able to decrease the rate of detectable lipid peroxidation, only ascorbate is reactive enough to intercept oxidants before they can cause detectable oxidative damage to lipids. These experimental data are in agreement with a thermodynamic hierarchy or “pecking order,” indicating that vitamin C is the terminal small molecule antioxidant (15).

Stimulation of Interest in the Use of Vitamin C to Prevent and Treat Diseases Other than Scurvy

During the late 1960s, Linus Pauling, the only person ever to receive two unshared Nobel Prizes (Chemistry in 1954 and Peace in 1962), became fascinated with the role of vitamin C in maintaining health. In 1970, he wrote the book, *Vitamin C and the Common Cold*, which generated a great deal of public interest as well as scientific controversy over the potential health benefits of supplemental vitamin C. Later, Dr. Pauling became increasingly interested in potential roles of vitamin C and other micronutrients to prevent and treat certain chronic and hereditary—as opposed to deficiency—diseases, a scientific discipline he termed “orthomolecular medicine.” To that end, he published several more books and established the Linus Pauling Institute of Science and Medicine (52). After Linus Pauling’s death in 1994, the Linus Pauling Institute moved to the campus of Oregon State University, his undergraduate alma mater, where scientists continue to conduct research into the roles of vitamins, micronutrients, and phytochemicals in disease prevention and treatment.

On the basis of cross-species comparisons, evolutionary arguments, and the amount of vitamin C likely consumed in a raw plant food diet, Dr. Pauling reasoned in 1970 that the optimum daily vitamin C intake would be at least 2300 mg/d for an adult with an energy requirement of 2500 kcal/d (53). Scientific evidence accumulated since then from epidemiologic, biochemical and clinical studies, many of which are reviewed in this book, has established that much more moderate vitamin C intakes of between 100 and 200 mg/d are associated with tissue saturation in healthy adults and reduced risk from chronic disease (1–3). Although several of Dr. Pauling’s views on vitamin C and health have been proved incorrect, his pioneering efforts in stimulating scientific, medical, and popular interest in the roles of micronutrients, and vitamin C
in particular, in promoting optimal health and preventing chronic disease have had a lasting effect, for better or for worse, and cannot be ignored.

**Beneficial Effects of Dietary Vitamin C in Chronic Disease Prevention**

**Cardiovascular diseases.** Numerous large epidemiologic studies have found a significant inverse relationship between vitamin C intake and cardiovascular or cerebrovascular disease risk (3). Those studies that did not find a relationship between vitamin C intake and cardiovascular disease risk often compared individuals who were already consuming close to 100 mg/d of vitamin C with those consuming higher amounts. Because human leukocytes in healthy young men and women are saturated at vitamin C doses of ~100 mg/d (29,30), it is possible that once tissue saturation has been achieved, additional protective effects of vitamin C against cardiovascular diseases are small and, therefore, difficult to detect. Consistent with this notion, numerous prospective studies have found low plasma ascorbate levels at baseline to be associated with a subsequent increase in the risk of heart disease or stroke (3,54,55).

Endothelium-derived NO is a critical molecule for maintaining healthy endothelial function (56). In addition to inducing vasodilation by stimulating vascular smooth muscle relaxation, NO inhibits other potentially atherogenic processes, such as smooth muscle proliferation, platelet aggregation, and leukocyte-endothelial cell interactions. Endothelium-dependent vasodilation of the brachial artery can be measured noninvasively in humans. Treatment with vitamin C has consistently resulted in improved endothelium-dependent vasodilation in individuals with coronary artery disease, angina pectoris, hypercholesterolemia, hypertension, or diabetes (3). One study found endothelium-dependent vasodilation in patients with coronary artery disease to improve by 40% after oral supplementation with 500 mg/d of vitamin C for 4 wk (57). As previously noted, ascorbate has been found to increase NO synthesis in endothelial cells by maintaining the NOS cofactor, tetrahydrobiopterin, in the reduced form, thereby enhancing eNOS activity (9).

Hypertension significantly increases the risk of cardiovascular and cerebrovascular diseases. Plasma ascorbate levels were inversely correlated with systolic and diastolic blood pressure in a cross-sectional study of >500 men and women in the U.K. (58). In healthy people who were fed a vitamin C–deficient diet for 30 d and a vitamin C–sufficient diet for another 30 d, plasma ascorbate levels were also inversely correlated with diastolic blood pressure (59). At least two intervention studies in patients with mild-to-moderate hypertension have found that 4 wk of oral supplementation with 500 mg/d of vitamin C resulted in significant decreases in systolic or diastolic blood pressure (60,61).

**Diabetes mellitus.** A number of observational studies have found that people with diabetes mellitus have plasma or serum ascorbate levels at least 30% lower than those without the disease (62). Plasma ascorbate levels were also significantly and inversely correlated with glycosylated hemoglobin (HbA1c) levels in a cross-sectional study of >6000 British men and women (63). However, it is not yet clear whether lower plasma
Ascorbate concentrations observed in diabetics are related to an increased requirement for vitamin C or other factors, such as diet and lifestyle. A large population-based study found that differences in serum ascorbate concentrations among >200 individuals with newly diagnosed diabetes and 1800 individuals without diabetes disappeared after adjustment for vitamin C consumption and cigarette smoking (64). Diabetes represents a condition of increased oxidative stress (65,66), which may be related to the fact that diabetic individuals are at more than twice the risk of death from cardiovascular and cerebrovascular diseases (67). Vitamin C supplementation has also been found to improve endothelium-dependent vasodilation (see above), which is commonly impaired in diabetic individuals. Thus, ensuring sufficient vitamin C intake may be beneficial in preventing some of the complications of diabetes mellitus.

Cancer. A number of well-designed epidemiologic studies have suggested a protective role for dietary vitamin C, especially with respect to cancers of the lung and digestive tract. For the most part, dietary vitamin C intake, mainly from fruits and vegetables, rather than supplements, appeared to be the source of the protective effect (3,34). Such studies are the basis for dietary guidelines endorsed by the U.S. Department of Agriculture and the National Cancer Institute, which recommend at least five servings of fruits and vegetables per day. In general, prospective studies in which the lowest intake group consumed >86 mg/d of vitamin C did not observe significant differences in cancer risk, whereas those studies that observed significant cancer risk reductions found them in people consuming at least 80–110 mg/d of vitamin C (3). One prospective study that followed 870 men for 25 y found that those who consumed >83 mg/d of vitamin C had a striking 64% reduction in lung cancer compared with those who consumed <63 mg/d (68).

A number of observational studies have found increased dietary vitamin C intake to be associated with decreased risk of stomach cancer, and laboratory experiments have shown that vitamin C inhibits the formation of carcinogenic N-nitroso compounds in the stomach (69). Infection with the bacteria, *Helicobacter pylori*, is known to increase the risk of stomach cancer and also appears to lower the vitamin C content of stomach secretions (70). Although two intervention studies did not find a decrease in the occurrence of stomach cancer with vitamin C supplementation (31), more recent research suggests that vitamin C supplementation may be a useful addition to standard *H. pylori* eradication therapy in reducing the risk of gastric cancer (71).

Cataracts. Cataracts are a leading cause of blindness in the United States, occurring more frequently and becoming more severe with age. Some, but not all, studies have observed increased dietary vitamin C intake (72,73) and increased blood levels of vitamin C (74) to be associated with a decreased risk of cataracts. Two intervention trials examined the effect of vitamin C supplementation in combination with other nutrients on cataract risk. Supplementation of >2000 men and women in Linxian, China with 120 mg/d of vitamin C and 30 µg/d of molybdenum for 5 y resulted in a nonsignificant 23% reduction in cataract risk, whereas in a separate but similar trial, multivita-
min and mineral supplementation resulted in a significant 36% risk reduction (75). More recently, a 7-y intervention trial of a daily antioxidant supplement containing 500 mg of vitamin C, 400 IU of vitamin E and 15 mg of β-carotene in >4000 men and women found no difference between the antioxidant combination and a placebo on the development and progression of age-related cataracts (76). Consequently, the relationship between vitamin C intake and cataract risk requires further clarification before specific recommendations can be made.

Recognition of a Vitamin C Deficiency Disease Other than Scurvy

The creation of mice deficient in the ortholog (Slc23a1) of the sodium-dependent ascorbic acid transporter, SVCT2, has recently led to the observation of a vitamin C deficiency syndrome other than scurvy. Despite maternal vitamin C supplementation, ascorbate was undetectable or markedly reduced in the tissues of newborn Slc23a1+/− mice, indicating that the Slc23a1 is required for ascorbate transport across the placenta and into many fetal tissues (77). Slc23a1 deficient mice died within minutes of birth due to respiratory failure and intraparenchymal brain hemorrhage. Newborn Slc23a1+/− mice did not exhibit the generalized vascular fragility that is characteristic of scurvy, and normal skin 4-hydroxyproline levels indicated that the post-translational processing of collagen was not affected, also suggesting they were not suffering from scurvy. The lethal effects of this ascorbate transporter deficiency in newborn mice provide evidence of previously unrecognized roles for vitamin C in perinatal development.

Conclusion

The consequences of insufficient vitamin C intake have been recorded throughout history, yet ascorbic acid was not isolated and recognized as the antiscorbutic factor until 1932. Although the need for small amounts of vitamin C to prevent scurvy is now widely recognized, optimal intakes of vitamin C for health promotion and chronic disease prevention and treatment remain controversial. Determining the optimal vitamin C intakes and understanding underlying biochemical mechanisms will require a plethora of scientific approaches, including biochemical, pharmacokinetic, toxicologic, epidemiologic, and intervention studies, some of which are detailed in the chapters that follow this introduction.

References


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Chapter 2

Vitamin C Pharmacokinetics in Healthy Men and Women

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Introduction

Recommended Dietary Allowances (RDA) for vitamin C were increased in 2000 by the Food and Nutrition Board of the U.S. National Academy of Sciences as part of new Dietary Reference Intake guidelines (1,2). Independent of these guidelines, recommended vitamin C intake was also increased in many countries (3–5). These increases were based in part on new pharmacokinetics data in healthy subjects. Here we describe why these pharmacokinetics studies were undertaken and what they showed.

Before 2000, the RDA for vitamin C were based on prevention of deficiency with a margin of safety (6,7). Recommendations were not based on biochemical function. We recognized that preventing deficiency might not be equivalent to ideal nutrient intake (8). Therefore, we proposed in 1986 and 1987 that the RDA for vitamin C and other water-soluble vitamins should be based on vitamin function in relation to concentration (8,9). Several data sets must be obtained to achieve this goal (10,11). It is necessary to know vitamin C concentrations in humans in relation to dose across a wide dose range. Once relevant concentrations in humans have been measured, several functional outcomes must be characterized in relation to these concentrations. Functional outcomes must be determined in cells and tissues rather than simply for isolated vitamin C–dependent reactions in vitro. Findings must then be extended to humans, with targeted biochemical and clinical outcomes measures. Such data represent specific concentration-function relationships, the foundation of recommendations for ideal nutrient intake.

A key part of this proposal is the relationship between ingested vitamin and the resulting concentrations achieved in plasma and tissues. Information was available about vitamin C concentrations in humans (12–22). However, it was difficult to interpret the overall data because of flaws in study design, execution, or analyses. The flaws included the following: use of vitamin C assays that were not specific or sensitive at low concentrations; narrow dose range of administered vitamin C; use of a diet deficient in other vitamins and minerals; use of radiolabeled vitamin C without verification of radiolabel metabolism in vivo; lack of verification of steady state for vitamin
C dose; and outpatient or uncertain dietary control of vitamin C ingestion. To obtain information about a variety of doses, it became necessary to combine data from different studies in which there was variability in analytical techniques and dietary control, making comparisons unwieldy. Only limited and incomplete data were available from studies in which there was dietary control of vitamin C ingestion in inpatients (14). The problems in data interpretation using such comparisons are evident in a recent meta-analysis (22). Thus, the goal of consistent dose-concentration data for vitamin C in humans was elusive.

Therefore, in 1991, we enrolled men in an inpatient study to determine vitamin C plasma and tissue concentrations as a function of doses over a wide dose range. Vitamin C was measured using a sensitive high-performance liquid chromatography (HPLC) coulometric electrochemical detection assay that was validated using human blood samples under clinical sampling conditions (23,24). Healthy men \((n = 7)\) completed this study (25), with hospitalization of each subject for ~5 mo. In 1995, the study was extended to women. Women \((n = 15)\) completed the study (26), with hospitalization of each subject for ~6 mo. The results of these studies are described here.

### Subjects and Methods

The study was approved by the Institutional Review Board, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. Written informed consent was obtained from all enrolled subjects. Men \((n = 7)\) ages 20–26 y and women \((n = 15)\) ages 19–27 y enrolled and completed the study. Complete study details are described elsewhere (25–28).

Subjects were initially screened by written questionnaire and telephone interview by physicians. Potentially acceptable subjects were then screened in person. Subjects underwent complete history, full physical examination, laboratory testing, psychological testing, and interviews by several staff members. Selected subjects were in good health, were nonsmokers, used no medications or supplements, and did not use alcohol or illicit drugs.

Subjects were hospitalized on an endocrinology-metabolism ward to control nutrient intake. Men were hospitalized 146 ± 23 d and women were hospitalized 186 ± 28 d. For the entire hospitalization, subjects consumed a vitamin C–deficient diet that contained <5 mg of vitamin C daily and utilized a computerized 14-d cycle selective menu design with >300 menu choices. The diet was sufficient in energy, protein, fat, and saturated fat. Other vitamin and mineral deficiencies were prevented by supplement administration so that only vitamin C intake was restricted (27). Pure vitamin C, when administered, was given twice daily in water (pH adjusted to 6.5) to subjects who had fasted for at least 2 h before breakfast and dinner.

Upon hospital admission, subjects began the depletion phase of the study. Consumption of the study diet caused vitamin C depletion. When plasma concentrations declined to 7–8 \(\mu\)mol/L, subjects were depleted without clinical scurvy. Neutrophils were isolated to measure vitamin C content, 36-h bioavailability sampling
for vitamin C (15 mg) was performed, and 24-h urine samples were collected to measure excreted vitamin C, creatinine, and other metabolites.

Subjects then entered the repletion phase of the study. Vitamin C (15 mg) twice daily was administered until subjects achieved steady state for this dose (30 mg daily). Bioavailability sampling (36-h) for the daily dose was performed, and subjects underwent apheresis (cell separation) to obtain monocytes, lymphocytes, and platelets for analyses of vitamin C content. After 24-h urine samples were collected and neutrophils were isolated, the vitamin C dose was increased and the sequence repeated at the new dose. Using this study design, subjects received in succession, daily vitamin C doses of 30, 60, 100, 200, 400, 1000, and 2500 mg, with bioavailability sampling for vitamin C doses of 15, 30, 50, 100, 200, 500, and 1250 mg. All measurements for vitamin C were analyzed by HPLC with coulometric electrochemical detection. All vitamin C samples were analyzed in triplicate with SD < 5% of the mean. No vitamin C degradation occurred under processing and storage conditions. Dehydroascorbic acid was <2% of plasma vitamin C and could not be distinguished from 0. Plasma data are predose values from morning samples from subjects who were fasting.

All experimental results are displayed as mean ±SD. When not displayed, the SD was less than symbol size. Data were not available for all subjects at all doses for the following reasons: inability of some subjects to remain hospitalized for the entire study; intravenous access limitations; and inadvertent nursing errors or sample loss. Numbers of subjects for whom data were available are indicated in figure legends.

Results

Fasting plasma vitamin C concentrations as a function of vitamin C dose and study day are shown in Figure 2.1A and B for men and women, respectively. Plasma vitamin C concentrations are shown at all doses. Vitamin C plasma concentrations were determined at least 2–3 times/wk in all subjects. Subjects required different amounts of time to achieve steady state at doses <100 mg/d. Because some subjects remained at doses longer than others, gaps are displayed between doses.

Steady state was attained when plasma vitamin C concentrations reached equilibrium for a given dose. Steady-state plasma concentration was defined as the mean of at least 5 plasma samples over at least 7 d with <10% SD, and the first sample included in the steady-state calculation was ≥90% of the mean. The steady-state value for the highest dose in one male subject was based on four samples. For men, 86% of steady-state values were based on ≥6 samples. For women, all steady-state values were based on ≥6 samples, with 85% of steady-state values based on ≥7 samples. Each subject achieved steady state for a dose before the next dose was given. Steady state was evident from visual inspection of data and was always confirmed using the calculations described. An example of steady state at the 60-mg dose is shown in Figure 2.2.

Steady-state plasma values were calculated for every subject at every dose and displayed as a function of dose (Fig. 2.3A and B for men and women, respectively). There was a sigmoid relationship between dose and steady-state plasma concentra-
Fig. 2.1. Vitamin C plasma concentration as a function of days at dose. Doses are indicated at the top of each panel. Each symbol represents a different subject. There is a 1-d gap between all doses for bioavailability sampling. See text for details. (A) Plasma concentration as a function of days at dose in men. Doses through 400 mg/d were received by 7 subjects, through 1000 mg/d by 6 subjects, and through 2500 mg/d by 3 subjects. 

Source: Ref. 25. (B) Plasma concentration as a function of days at dose in women. Doses through 200 mg/d were received by 15 subjects, through 1000 mg/d by 13 subjects, and through 2500 mg/d by 10 subjects. Source: Ref. 26.
tion at doses <400 mg/d for both men and women. However, the curve for women was shifted to the left compared with that for men (Fig. 2.3C). By repeated-measures analysis of variance, plasma concentrations for women at doses of 30–100 mg daily were higher than for men ($P^2 = 0.01$), but differences disappeared at doses >100 mg/d. The first dose beyond the steep (linear) portion of the sigmoid curve for both sexes was 200 mg/d. This dose produced a plasma concentration of ~70 µmol/L, similar to the concentration at which the sodium vitamin C transporter (SVCT)2 approaches $V_{\text{max}}$ (29,30). Several factors could be responsible for the sigmoid shape of the relationship between dose and plasma concentration, i.e., vitamin C uptake, absorption, and excretion. These were investigated in turn as described below.

Tissue uptake of vitamin C was determined by measuring vitamin C concentrations in cells that could be obtained without harming subjects. Circulating cells and platelets found in blood were ideal indicators of vitamin C distribution in tissues. Vitamin C concentrations were determined at steady state over the dose range in neutrophils, monocytes, and lymphocytes for both sexes, and additionally in platelets of women (Fig. 2.4A, B). Most cells saturated at doses of 100–200 mg/d.

Bioavailability, or the fraction of the dose absorbed, was determined from oral and intravenous vitamin C administration when steady state for a dose was reached. Data have been calculated for men, and analysis is ongoing for data from women.

Fig. 2.2. Determination of steady-state plasma vitamin C concentration at 60 mg/d for one female subject. Source: Ref. 26. See Ref. 25 for male subject data.
Fig. 2.3. Steady-state plasma vitamin C concentrations as a function of dose for all doses for all men (A) and for all women (B), and comparison of values for men (A) and women (B) at doses of 0–200 mg/d (C). See Figure 2.1 and text for details. Sources: Ref. 25,26.
Fig. 2.4. Intracellular vitamin C concentrations (mmol/L) in circulating cells as a function of dose. Cells were isolated when steady state was achieved for each dose. (A) Cells from male subjects. Numbers in parentheses at each dose indicate the number of men from whom neutrophils were obtained. Numbers in brackets at each dose indicate the number of men from whom lymphocytes and monocytes were obtained. See Ref. 25 for details. (B) Cells and platelets from female subjects. For neutrophils, samples were available from 13 women at doses of 0–200 mg/d; from 11 women at doses of 400 and 100 mg/d; and from 10 women at 2500 mg/d. For lymphocytes, monocytes, and platelets, samples were available from 13 women at 30 mg/d; from 12 women at 60 mg/d; from 6 women at 100 mg/d; from 2 women at 400 and 1000 mg/d; from 9 women at 2500 mg/d. See Ref. 26 for details.
Examples of data obtained for bioavailability determinations are shown in Figure 2.5. As a first approach, area under curve (AUC) calculations (linear trapezoidal analyses) were used to calculate bioavailability at doses of 200, 500, and 1250 mg (Table 2.1). At each of these three doses, bioavailability was calculated as the ratio of the area under the oral dose divided by the area under the intravenous dose. By simple visual inspection, it can be seen that the AUC was similar at the 200-mg dose orally and intravenously, but was much less for the oral dose compared with the intravenous dose.

![Graph showing Vitamin C bioavailability in plasma](image)

**Fig. 2.5.** Vitamin C bioavailability in plasma. Upper panel: bioavailability in one male subject for 200 mg. Lower panel: bioavailability in one male subject for 1250 mg. For each dose, vitamin C was administered at 8:00 AM orally; sample values (○) are shown for the times indicated. Baseline is indicated by the dashed line with larger spaces. After 24 h, the same dose was given intravenously and samples were taken for the times indicated (●). Baseline is indicated by the dashed line with smaller spaces. For oral doses, samples before zero time and between 13 and 24 h are not shown for clarity. Bioavailability was calculated using the linear trapezoidal method. Bioavailability was the ratio of the area of the oral dose divided by the area of the intravenous dose. The area after the curve returned to baseline was assumed to equal zero. See Ref. 25 for details.
dose at 1250 mg (Fig. 2.5). AUC calculations are based on pharmacokinetics assumptions that volume of distribution and clearance are constant. However, these assumptions are not valid at vitamin C doses <200 mg. A more sophisticated pharmacokinetics model was developed and is the first to include the three parameters of nonlinear absorption, nonlinear elimination, and nonlinear tissue distribution (28). This model was used to calculate bioavailability based on plasma values when vitamin C was administered orally and intravenously for all doses at steady state (Table 2.2). Data analyzed using the model indicated that as vitamin C dose increased, bioavailability (absorption) decreased.

Plasma was nearly saturated with vitamin C at an oral dose of 200 mg/d (Fig. 2.3). However, bioavailability did not decrease proportionally at higher doses (Table 2.1). One potential explanation was urinary excretion of absorbed vitamin C. During bioavailability sampling when vitamin C was administered orally and intravenously, urine was collected from men and women and vitamin C excretion was measured (Fig. 2.6A, B, and insets). The threshold dose for urinary excretion of vitamin C was between 60 and 100 mg for both sexes. With intravenous administration of 500 and 1250 mg, nearly the entire dose was excreted in urine for both sexes. With oral administration of these doses, urine excretion was less than that of intravenous administration, likely because bioavailability was less at higher doses compared with lower ones (Table 2.1) (25). Considered together, the data show that at doses ≥500 mg/d, when plasma is saturated, most of absorbed vitamin C is excreted in urine.

### TABLE 2.1
Vitamin C Bioavailability in Men at Doses of 200, 500, and 1250 mg Determined by the Linear Trapezoidal Method (Area Under the Curve)\(^a\)

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Bioavailability (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>112 ± 25</td>
</tr>
<tr>
<td>500</td>
<td>73 ± 27</td>
</tr>
<tr>
<td>1250</td>
<td>49 ± 25</td>
</tr>
</tbody>
</table>

\(^a\)Source: Ref. 25.

### TABLE 2.2
Vitamin C Bioavailability in Men at All Doses Calculated Using a Pharmacokinetics Model with Nonlinear Absorption, Elimination, and Tissue Distribution\(^a\)

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Bioavailability (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>85 ± 20</td>
</tr>
<tr>
<td>30</td>
<td>85 ± 20</td>
</tr>
<tr>
<td>50</td>
<td>84 ± 20</td>
</tr>
<tr>
<td>100</td>
<td>82 ± 20</td>
</tr>
<tr>
<td>200</td>
<td>78 ± 22</td>
</tr>
<tr>
<td>500</td>
<td>75 ± 24</td>
</tr>
<tr>
<td>1250</td>
<td>62 ± 34</td>
</tr>
</tbody>
</table>

\(^a\)Source: Ref. 28.
Fig. 2.6. Urinary vitamin C excretion as function of single vitamin C doses at steady state in men (A) and women (B). Urine was collected during determination of vitamin C bioavailability for each dose. Vitamin C excretion was determined after administration of vitamin C given either orally (○) or intravenously (●). (A) Urine was collected from oral sampling for 24 h, and then from intravenous sampling for 9–10 h. Data from oral and intravenous administration were available from 7 subjects at doses of 15, 30, 50, and 100 mg; from 6 subjects at 200 mg; from 6 subjects at 500 mg; from 3 subjects at 1250 mg. See Ref. 25 for details. (B) Urine was collected from oral and intravenous sampling for 24 h. Data from oral and intravenous administration were available from 11 subjects at doses of 15, 30, 50, and 200 mg; from 10 subjects at 100 mg; from 8 subjects at 500 mg; and from 9 subjects at 1250 mg. See Ref. 26 for details. For both panels: Inset A shows vitamin C excretion for single oral (○) or intravenous (●) doses of 15–100 mg. The x-axis indicates dose and the y-axis indicates amount (mg) excreted in urine. Inset B shows the fractional excretion (the fraction of the dose excreted) after intravenous administration of single doses of vitamin C. The x-axis indicates dose and the y-axis indicates fractional excretion (vitamin C excreted in urine in milligrams divided by the vitamin C dose in milligrams). The minimum amount of ascorbate excreted was <0.4 mg.
Discussion

The data presented here describe vitamin C pharmacokinetics in healthy young men and women, and define the range of physiologic vitamin C concentrations at which functional outcomes are possible. In both sexes, there was a steep sigmoid relationship between dose and steady-state plasma concentration at doses between 30 and 100 mg/d. By 400 mg/d, plasma at steady state was saturated, with little effect of higher doses. Cells saturated at lower doses. Plasma saturation was due to several factors, including cell and tissue saturation at daily doses >100–200 mg, decreased bioavailability at doses >200 mg, and increased renal excretion at doses >100–200 mg (28). Some of these findings have already provided a new basis for recommended dietary allowances of vitamin C (2–5).

Several aspects of the data were surprising. One striking finding was the observed tight control of plasma and tissue concentrations as a function of dose. Healthy humans apparently strive to reach plasma concentrations of 70–80 µmol/L; once this concentration range is achieved, it is not exceeded despite large increases in oral ingestion. Ascorbate doses at ≥400 mg/d had virtually no effect on increasing steady-state plasma and tissue concentrations, and 200 mg/d of vitamin C resulted in nearly complete saturation of plasma and tissues.

Why is there such tight control of vitamin C concentrations, particularly in plasma? There are two general explanations, i.e., either tight control is necessary to avoid harm or it is beneficial directly or indirectly. For the first possibility, tight control of plasma concentrations may be to avoid harm arising perhaps via potential prooxidant toxicity. Theoretically, ascorbic acid could act as a prooxidant under certain conditions especially at higher concentrations. For the second possibility, tight control of extracellular concentrations may allow higher local concentrations to occur under certain conditions, for example, by release of ascorbate from cells into tissue. These concentrations might act in a paracrine or autocrine fashion, perhaps participating in signal transduction. Such local ascorbate release has been described in cell systems and animals with respect to the adrenal gland (31,32) and brain (33). If there were strong advantages to tight control for these or other reasons, it would be predicted that vitamin C transporters would have less genetic variation than normally predicted, for example, as determined by single nucleotide polymorphisms in the known vitamin C transporters SVCT1 and SVCT2. Such genomic analyses are underway.

Another surprising finding was that tight control of vitamin C concentrations could be bypassed for several hours when vitamin C was given intravenously. Obviously, intravenous administration is not relevant physiologically, but may have unexpected implications pharmacologically (34,35). Vitamin C given intravenously results in vasodilatation, although differences between oral physiologic and intravenous pharmacologic administration have sometimes been misunderstood (36,37). Nevertheless, independent of recommendations for physiological benefit, vitamin C given pharmacologically achieves far higher concentrations, and these perhaps might have therapeutic importance. By analogy, oral penicillin may be ineffective in treat-
ment of life-threatening meningitis, whereas intravenous penicillin is effective because far higher concentrations are achieved. There are theoretical possibilities for therapeutic use of vitamin C intravenously, and these possibilities should be explored rigorously.

An additional surprising finding was that vitamin C dose-concentration relationships in plasma were shifted to the left for women compared with men. In hindsight, these differences might be due to differences in muscle mass. Muscle mass is greater in men than women. Although mRNA for vitamin C transporters have not been reported in muscle, muscle contains higher ascorbic acid concentrations than plasma. Although ascorbate concentrations in muscle are lower than in many other tissues, muscle mass contributes significantly to total body weight, and therefore muscle is predicted to contain a substantial total amount of ascorbate. Because women have less muscle mass than men, women should achieve ascorbate saturation in muscles at lower doses than men. This in turn would permit ascorbate plasma concentrations in women to rise higher compared with men at low doses, which was what was observed. In future studies, lean body mass will be measured to verify these possibilities.

There are limitations to these pharmacokinetics data. Prolonged hospitalization was necessary; thus, large numbers of subjects could not be studied. The subjects were healthy adult nonsmokers <28 y old. Pharmacokinetics are not known in older subjects, smokers, diabetics, patients with cardiovascular disease, patients with end-stage kidney disease on dialysis, and patients with other conditions. Prolonged hospitalization is not practical or possible for many of these relevant populations. It is possible that under some conditions or in some patients, vitamin C pharmacokinetics curves are shifted to the right, or that saturation values differ from healthy subjects. Methods should be developed to study at least some of the relevant patient populations, for example by decreasing hospitalization time.

Another limitation of the pharmacokinetics data is that pure ascorbic acid was used to determine steady-state plasma concentrations and bioavailability. However, recommended dietary allowances provide guidelines for ingesting vitamin C in the diet from foods. It is possible that other substances in foods rich in vitamin C could decrease absorption. For example, vitamin C is found in high amounts in many fruits and vegetables. These foods also contain many other compounds, with flavonoids as one example. Recent evidence shows that flavonoids inhibit the intestinal vitamin C transporter SVCT1 (38). Flavonoid inhibition occurred when this transporter was expressed in expression systems, when cells were transfected to overexpress the transporter, and when bioavailability was determined in animals given vitamin C and flavonoids. It is unknown whether flavonoids or other compounds in foods inhibit vitamin C absorption in humans. If such inhibition of absorption occurred, vitamin C dose concentration curves would be shifted to the right.

There is substantial evidence that fruits and vegetables are beneficial to human health. Five or more servings of fruits and vegetables daily may be protective in heart disease, stroke, cancer, cataract development, and longevity (39,40). Five or more
daily servings of fruits and vegetables will provide 210–300 mg of vitamin C. This amount of vitamin C should produce near-saturation of cells and plasma, even if flavonoids decreased absorption, without causing harm. In certain circumstances, fruits and vegetables may be ill-advised or contraindicated, for example, in patients with end-stage renal disease on dialysis or patients with inflammatory bowel disease. Otherwise, for most adults, the best advice is to eat five varied servings of fresh fruits and vegetables daily to maximize health and to obtain vitamin C from these foods.

References


Chapter 3

Biochemical and Physiological Interactions of Vitamin C and Iron: Pro- or Antioxidant?

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Vitamin C

Vitamin C is an essential micronutrient for humans, who have lost the ability to synthesize ascorbic acid due to a mutation in the gene coding for L-gulono-γ-lactone oxidase (1). A lack of vitamin C in the human diet causes the deficiency disease scurvy. The current Recommended Dietary Allowance (RDA) for vitamin C is 75 mg/d for women and 90 mg/d for men (2). The RDA for men is based on near-maximal neutrophil concentration and minimal urinary excretion of vitamin C at daily doses of between 60 and 100 mg (2,3); that for women is by extrapolation from the RDA for men on the basis of relative body mass (2).

The molecular mechanisms underlying the antiscorbutic effects of vitamin C are largely understood. For example, vitamin C is a co-substrate for enzymes involved in procollagen, carnitine, and catecholamine synthesis (1,4). Prolyl and lysyl hydroxylases, two enzymes involved in procollagen synthesis, require vitamin C for maximal activity (1). A deficiency of vitamin C results in a weakening of the collagenous triple helix structure with tooth loss, joint pains, and poor wound healing, all characteristic signs of scurvy (4). Two dioxygenases in the biosynthesis of carnitine also require vitamin C for maximal activity (1,4). Carnitine is essential for the transport of long-chain fatty acids into mitochondria for β-oxidation; not surprisingly, fatigue and lethargy are early symptoms of scurvy (3,4). Vitamin C also acts as a co-substrate for dopamine β-hydroxylase, which converts dopamine to norepinephrine (1,4).

The role of vitamin C as a co-substrate in the above enzyme reactions is to maintain the active center metal ions in the reduced, enzymatically active form (1). This same electron-donating activity of ascorbate also makes it a powerful antioxidant, i.e., vitamin C readily scavenges reactive oxygen species (ROS) (5–8) such as superoxide (O$_2^•$•−), hydroperoxy radicals, aqueous peroxyl radicals, singlet oxygen, hypochlorous acid and ozone, reactive nitrogen species (RNS) (9–11) such as nitrogen dioxide and dinitrogen tetroxide, and antioxidant-derived radicals (5,12) such as thiyl and urate radicals (Table 3.1), thereby protecting biological macromolecules such as proteins, lipids, and DNA from oxidative damage (13–16). Vitamin C also acts as a co-antioxidant by regenerating α-tocopherol (vitamin E) from the α-tocopheroxyl radical in lipoproteins and membranes (15–17). This is a potentially important function because
<table>
<thead>
<tr>
<th>Chemical species scavenged by ascorbate</th>
<th>Rate constant(^a) (mol/L(^{-1}).s(^{-1}))</th>
<th>Ref.</th>
<th>Redox couple</th>
<th>(E^o)(^b) (mV)</th>
<th>(\Delta E^o)(^c) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reactive oxygen species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxyl radical ((^\bullet)OH)</td>
<td>(1.1 \times 10^{10})</td>
<td>(5)</td>
<td>(^\bullet)OH, H(^+)/H(_2)O</td>
<td>2310</td>
<td>2028</td>
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<tr>
<td>Aliphatic alkoxy radical (RO(^\bullet))</td>
<td>(1.6 \times 10^{9})</td>
<td>(5)</td>
<td>RO(^\bullet), H(^+)/ROH</td>
<td>1600</td>
<td>1318</td>
</tr>
<tr>
<td>Alkylperoxyl radical (ROO(^\bullet))</td>
<td>(1–2 \times 10^{6})</td>
<td>(5)</td>
<td>ROO(^\bullet), H(^+)/ROOH</td>
<td>1000</td>
<td>718</td>
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<tr>
<td>Superoxide anion/Hydroperoxyl radical (O(_2)(^\bullet)(^-)/HO(_2)(^\bullet))</td>
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<td>O(_2)(^\bullet)(^-), 2H(^+)/H(_2)O(_2)</td>
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<td>658</td>
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<td>(6 \times 10^{6})</td>
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<td>Ozone (O(_3))</td>
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<td>Singlet oxygen ((1)O(_2))</td>
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<td><strong>Reactive nitrogen species</strong></td>
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<tr>
<td>Nitrogen dioxide ((^\bullet)NO(_2))</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinitrogen trioxide/Dinitrogen tetroxide (N(_2)O(_3)/N(_2)O(_4))</td>
<td>(1.2 \times 10^{9})</td>
<td>(10)</td>
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<td></td>
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<tr>
<td>Peroxynitrite/Peroxynitrurous acid (ONOO(^-)/ONOOH)</td>
<td>(235)</td>
<td>(11)</td>
<td></td>
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<tr>
<td><strong>Antioxidant-derived radicals(^d)</strong></td>
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<tr>
<td>Thiol/Sulphenyl radical ((RS)(^\bullet)/(RSO)(^\bullet))</td>
<td>(6 \times 10^{8})</td>
<td>(5)</td>
<td>RS(^\bullet), H(^+)/RSH (cysteine)</td>
<td>920</td>
<td>638</td>
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<tr>
<td>Urate radical ((UH)(^\bullet))</td>
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<td>UH(^\bullet), H(^+)/UH(_2)(^-)</td>
<td>590</td>
<td>308</td>
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<tr>
<td>(\alpha)-Tocopheroxyl radical ((\alpha)-TO(^\bullet))</td>
<td>(2 \times 10^{5})</td>
<td>(5)</td>
<td>(\alpha)-TO(^\bullet), H(^+)/(\alpha)-TOH</td>
<td>500</td>
<td>218</td>
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<tr>
<td>(\beta)-Carotene radical cation ((\beta)-C(^{\bullet\bullet}))</td>
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<td></td>
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</tbody>
</table>

\(^a\)The approximate rate constant for the reaction with ascorbate at pH 7.4 is given, if known.

\(^b\)The one-electron reduction potential at pH 7.0 is given, if known (from Ref. 15).

\(^c\)The difference in one-electron reduction potentials for the reaction with ascorbate is shown (the one-electron reduction potential for the ascorbyl radical/ascorbate monoanion couple is 282 mV); if \(E^o\) is positive, the reaction is “thermodynamically feasible,” assuming equimolar concentrations of the reactants.

\(^d\)A number of other small molecule antioxidants can be regenerated from their respective radical species by ascorbate.
in vitro experiments have shown that α-tocopherol in low-density lipoprotein (LDL) can act as a prooxidant in the absence of vitamin C (17–19), and evidence for a prooxidant effect of α-tocopherol in vivo is mounting (19).

There are several properties that make ascorbate such a strong physiologic antioxidant, including the low one-electron reduction (or redox) potentials of ascorbate (282 mV) and its one-electron oxidation product, the ascorbyl radical (−174 mV) (15). These low reduction potentials enable ascorbate and its radical to spontaneously react with and reduce most physiologically relevant radicals and oxidants, i.e., these reactions are energetically or thermodynamically feasible (Table 3.1). Indeed, it has been stated that vitamin C acts “as the terminal water-soluble small molecule antioxidant” in biological systems (15). Second, the second-order rate constants for the reactions of ascorbate and most physiologically relevant ROS and RNS are greater than $10^5$ (mol/L)$^{-1}$·s$^{-1}$ (Table 3.1), which makes these reactions highly competitive with those of the same ROS and RNS with biological macromolecules. Third, the ascorbyl radical formed from ascorbate upon one-electron oxidation is quite stable and unreactive, due to resonance stabilization of the unpaired electron (15). Importantly, the ascorbyl radical does not react with molecular oxygen (O$_2$) to form a more reactive peroxyl radical. Instead, the ascorbyl radical readily dismutates to ascorbate and dehydroascorbic acid (DHA). DHA is reduced back to ascorbate in biological systems, e.g., by glutathione, glutaredoxin, or thioredoxin reductase, or is rapidly and irreversibly hydrolyzed (1,20) (Scheme 3.1). Thus, a fourth reason that ascorbate is an effective physiologic antioxidant is that it can be regenerated from its oxidized forms, either by spontaneous chemical reactions or enzymatically.

Iron

Iron is the most abundant transition metal in biological systems. The total amount of iron in a normal adult human has been estimated to be ~4.5 g (21), most of it in hemoglobin. Iron is also one of the most abundant elements in the earth’s crust; however, the large amount of iron in all living cells cannot be explained by this fact alone. The preference of iron as a biologically relevant metal is related to its unique physicochemical properties, allowing it to vary its oxidation state, redox potential, and elec-

![Scheme 3.1](image-url)
tronic spin configuration in response to different coordinating environments. These properties enable iron, in contrast to many other metals, to play an essential role in a plethora of biological reactions. Iron exerts its functions either in the form of heme proteins or nonheme-containing proteins, such as iron-sulfur clusters. Iron-containing proteins catalyze many key reactions in energy metabolism, including respiration, O₂ delivery to tissues, DNA synthesis, and regulation of the citric acid cycle. Several other biological reactions, such as those with O₂ (monooxygenases and dioxygenases, see above) and peroxides (peroxidases, catalase and ferrioxidase), are also catalyzed by iron-containing enzymes.

In biological systems, iron is commonly found in three oxidation states, i.e., Fe(II), Fe(III), and in some cases Fe(IV). At physiologic pH, Fe(II) is water soluble, whereas Fe(III) precipitates as oxyhydroxide polymers. On the other hand, Fe(II) is unstable in aqueous solutions and tends to react with O₂ to form Fe(III). Thus, to use iron effectively as a cofactor in enzymatic systems, coordinating ligands such as O, N, S, or other metalloid atoms are used to shield iron from O₂ and the surrounding media (22,23). When O₂ is bound to iron in the active site of an enzyme, reactions of the bound O₂ and reduced oxygen intermediates become less energetically favorable. In addition, steric factors are well controlled in proteins, thus preventing the occurrence of undesirable side reactions between bound reactive intermediates and nonsubstrate molecules. The redox potential of iron is highly dependent on its coordination, and many enzymes “use” this property of iron as a fundamental means of controlling oxidation and reduction reactions of biomolecules.

Just as for O₂, which can be converted to ROS, the widespread use of iron in living organisms also gave rise to a paradox. On the one hand, by serving its multifunctional roles in biological systems, iron represents a great advantage for the complex chemical reactions of life; on the other hand, if not appropriately shielded, iron can readily participate in one-electron transfer reactions that can produce toxic free radicals. To overcome these problems, single-cell organisms first evolved molecules known as siderophores, which are secreted into the extracellular medium where they complex Fe(III) and are then assimilated by the cells via a receptor-mediated mechanism. In turn, multicellular organisms developed iron-binding proteins known as transferrins, which complex iron, transport it in the circulation (serum transferrin) or in other media (ovotransferrin, lactoferrin), and are taken up by peripheral cells, usually by a receptor-mediated mechanism.

Once transferrin is taken up and iron is released inside the cell, numerous mechanisms are in place whereby iron can be utilized in a form that is soluble under physiological conditions, bioavailable and nontoxic. “Nontoxic” means that iron is not able to elicit the well-known Fenton reaction, which gives rise to hydroxyl radicals (•OH) from hydrogen peroxide (H₂O₂) and Fe(II) (see below, Reaction 3.1b). Thus, the complexation of iron within a cell necessitates that the iron storage protein, ferritin, must guard its iron in a form that will be assimilated into the many other proteins and molecules whose function depends on iron, but cannot be released indiscriminately to elicit oxidative damage and, thus, potentially pathological consequences.
Many studies have shown that iron can be released from iron-containing proteins under specific conditions. The release of iron from ferritin, the most concentrated source of iron [up to 4500 Fe(III) atoms per molecule], has been studied extensively (24). Any compound capable of reducing Fe(III) within the iron core of ferritin in the presence of a suitable chelator is capable of releasing iron from ferritin, particularly O$_{2}^{•−}$, nitric oxide (•NO), and the “redox-cycling” class of xenobiotics. Ascorbate-mediated release of iron from ferritin seems to be due mainly to O$_{2}^{•−}$ generated during oxidation of ascorbate catalyzed by iron bound to ferritin (25,26). In addition to this reductive release from ferritin, iron can also be released in an oxidative manner from heme-proteins or heme (27). It is thus conceivable that “free” iron is immediately conveyed into an intermediate, labile iron pool, and that this pool represents a steady-state exchangeable and readily chelatable iron compartment.

**Studies of Biochemical Interactions Between Vitamin C and Iron**

*Autoxidation of Ascorbate and Other Biomolecules*

It is widely believed that many biomolecules undergo autoxidation. Even though the reaction of O$_{2}$ with a biomolecule may be thermodynamically favorable, it does not occur at an appreciable rate because of a kinetic spin restriction of O$_{2}$ (23). For O$_{2}$ to react with a biomolecule, it must be activated either enzymatically or photochemically, or by sequential one-electron reductions to partially reduced oxygen species (i.e., ROS), including O$_{2}^{•−}$, H$_{2}$O$_{2}$, and •OH. Transition metals, such as the reduced form of iron, are able to activate O$_{2}$. The sequential reduction of O$_{2}$ by iron is best exemplified by the iron-mediated Haber-Weiss reaction (28).

Coordination of iron to biomolecules almost always involves the $d$ orbitals of the metal. Because O$_{2}$ can also bind to iron through the $d$ orbitals of the metal (23), iron may simultaneously bind to biomolecules and O$_{2}$, effectively serving as a “bridge” between the two. It has been postulated that ascorbate, iron, and O$_{2}$ can form such a ternary complex (23). Ascorbate is both an iron chelator and an iron reductant; therefore, it can bind Fe(III) and subsequently reduce it to Fe(II). The Fe(II) can then reduce O$_{2}$ to O$_{2}^{•−}$, with regeneration of Fe(III). Thus, it appears as if ascorbate autoxidizes, when in fact the reaction is catalyzed by iron. Accordingly, the “autoxidation” of ascorbate and numerous biogenic amines, such as epinephrine, is completely inhibited by strong metal chelating agents (29). Therefore, it has been suggested that biomolecules do not “autoxidize” but that the oxidation of biomolecules is mediated by trace amounts of transition metals, such as iron. In fact, it has been demonstrated (30) that the rate of ascorbate “autoxidation” in a given solution is proportional to the concentration of (contaminating) metal ions. To distinguish between Fe and Cu contamination, EDTA can be added to the assay solution, because Fe-EDTA is an excellent catalyst of ascorbate oxidation, whereas Cu-EDTA is a very poor catalyst. Measuring ascorbate oxidation spectrophotometrically at 265 nm, the iron levels in phosphate
buffer can be estimated to a lower limit of ~100 nmol/L (30). Using electron spin resonance (ESR) spectroscopy, the detection limit can be further lowered to ~5 nmol/L (31). For this method, EDTA is added to the solution, which converts iron into a catalytic form, followed by the addition of ascorbate and measurement of the steady-state concentration of the ascorbyl radical by ESR. This method is useful not only because of its high sensitivity, but also if the solution to be assayed is colored or turbid and thus not suitable for standard colorimetric analysis of iron, or if only “loosely bound” iron is to be estimated (31).

The Ascorbate-Driven, Iron-Catalyzed Oxidation of Biological Macromolecules

In light of its important role as an antioxidant, it seems paradoxical that under certain conditions ascorbate can promote the generation of the very same ROS it is known to scavenge. This represents just another “paradox” of redox chemistry, which applies to any good reductant or antioxidant. Recognition of this property of ascorbate was an outgrowth of the pioneering studies of Udenfriend et al. (32) on the conversion of tyramine to hydroxytyramine by adrenal medullary homogenates. This work eventually led to the discovery that a nonenzymatic system comprised of ascorbate, O₂, ferrous iron, and EDTA catalyzes the hydroxylation of a number of aromatic and heterocyclic compounds with the formation of products that are similar to those generated in animals in vivo.

It is now generally agreed that the prooxidant activity of ascorbate is due to its capacity to reduce transition metal ions, such as iron or copper (Reaction 3.1a) (1,14,15,33). The reduced metal ions may react with H₂O₂ to form highly damaging •OH, a process known as Fenton chemistry (3.1b). Lipid hydroperoxides may also react with reduced metal ions to form lipid alkoxyl radicals (3.1c), which can initiate and propagate the chain reactions of lipid peroxidation (14,15).

The “classical” prooxidant mixture (also called “Udenfriend system”) of ascorbate, redox-active iron, and H₂O₂ (or lipid hydroperoxides, e.g., preexisting in membrane preparations) has been, and continues to be used to induce oxidative stress in vitro (1,14,15,33). The same or similar systems are also employed to induce oxidation in cell culture systems, whereby the redox-active metal ions often are supplied by the culture media. For example, Ham’s F-10 medium contains trace amounts of transition metal ions, which are required for cell-mediated LDL oxidation by vascular cells (34). The ascorbate-driven, metal-catalyzed oxidation (MCO) system, because of its simplicity and because it mimics in every important aspect the properties of enzyme mixed function oxidation systems (35), has been used extensively to study oxidation of biological macromolecules, such as lipids, nucleic acids, and proteins.
Lipid peroxidation. The study of lipid oxidation, generally referred to as lipid peroxidation (because lipid peroxides are intermediates in the process), has been a topic of much research. Many investigators have proposed that iron is involved in the initiation of lipid peroxidation, although considerable controversy exists concerning its role in biological systems. One commonly proposed mechanism is that iron is responsible for catalyzing the generation of \( \cdot \text{OH} \) via Fenton chemistry and the Haber-Weiss reaction.

The key step in the initiation of lipid peroxidation is the abstraction of a hydrogen atom from the bis-allylic site of a polyunsaturated fatty acid. The \( \cdot \text{OH} \) is possibly the most powerful oxidant that can be formed in biological systems. As such, it can easily abstract a hydrogen atom from polyunsaturated fatty acids. It is, therefore, not surprising that \( \cdot \text{OH} \) has received so much consideration as the initiating species, although it is known that many other ROS and RNS can also initiate lipid peroxidation, e.g., hydroperoxyl radicals, peroxynitrite (\( \text{ONOO}^- \)), and nitrogen dioxide. From a theoretical point of view, the ability of \( \cdot \text{OH} \) to initiate lipid peroxidation is unquestionable. However, it is imperative to realize that the indiscriminate, diffusion-limited reactivity of \( \cdot \text{OH} \) toward sugars, nucleotides, proteins, and any other biomolecules is a limitation to the idea that lipid peroxidation is initiated by \( \cdot \text{OH} \). Some investigators have addressed this problem by proposing that \( \cdot \text{OH} \) is formed directly at the site of attack (36).

Another possible mechanism by which iron could be involved in initiating lipid peroxidation involves the formation of an Fe(III):Fe(II) complex. This mechanism has been proposed by Aust’s group (37), and recently other researchers have arrived at similar conclusions (38,39). Interest in the initiation of lipid peroxidation by an iron complex started with the observation (37) that ADP:Fe(II) promoted the peroxidation of phospholipid liposomes only after a lag phase. Catalase, superoxide dismutase, and “\( \cdot \text{OH} \) scavengers” did not extend the lag phase or inhibit the subsequent rate of lipid peroxidation, indicating that the reaction was not initiated by ROS. Interestingly, the lag phase was eliminated by the addition of ADP:Fe(III), which led to the proposal that the necessary species being generated during the lag phase was Fe(III). Furthermore, another study (40) showed that maximal rates of lipid peroxidation occurred when ~50% of the Fe(II) was oxidized, i.e., the Fe(II):Fe(III) ratio was ~1:1.

Consistent with the requirement for both Fe(II) and Fe(III), ascorbate, by reducing Fe(III) to Fe(II), stimulated iron-catalyzed lipid peroxidation; however, when the ascorbate concentration was high enough to reduce all of the Fe(III) to Fe(II), ascorbate inhibited lipid peroxidation (33). Exogenously added \( \text{H}_2\text{O}_2 \) also either stimulated or inhibited ascorbate-dependent, iron-catalyzed lipid peroxidation, apparently by altering the ratio of Fe(II):Fe(III). Thus, it appears that the prooxidant effect of ascorbate is related to its ability to promote the formation of the proposed Fe(II):Fe(III) complex and not due to ROS production, whereas the antioxidant effect of ascorbate may be due to complete reduction of Fe(III) to Fe(II) (33).

DNA oxidation. It has been suggested that the genotoxicity of many chemicals is enhanced by their ability to decompartmentalize cellular iron (41). Iron has been
implicated as a causative agent in numerous cancers (28). One mechanism by which iron could be involved in the initiation or promotion of cancer is through the oxidation of DNA, causing mutations. DNA can be modified by free radicals resulting in single- and double-strand breaks, depurination, and depyrimidation, or chemical modification of the bases or phosphate-sugar backbone (42). Several ROS have been shown to oxidatively modify DNA, including \( \cdot \text{OH} \), singlet oxygen, and \( \text{ONOO}^\cdot \) (43). In contrast, \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \) are not capable of oxidizing DNA in the absence of adventitious metals (42), suggesting that the role of \( \text{O}_2^\cdot \) in DNA oxidation is simply as a constituent of the metal-driven Haber-Weiss reaction to produce \( \cdot \text{OH} \). In addition, it has been demonstrated that the addition of any chemical that will act as an alternate reactant for \( \cdot \text{OH} \), such as organic-based buffers or \( \cdot \text{OH} \) traps,” inhibits the oxidation of DNA (38). Conversely, the presence of chemicals that increase the iron-mediated production of \( \cdot \text{OH} \) will promote DNA oxidation (38).

Some researchers have postulated that the iron-mediated oxidation of DNA is a site-specific process (41,42). They propose that iron or an iron chelate binds to the DNA, either at phosphate on the backbone or to the purine or pyrimidine bases, where the iron can serve as a center for recurring formation of \( \cdot \text{OH} \), resulting in modification of the DNA (42,44,45). Experiments using purified DNA or isolated nuclei (46–48) confirm that in the presence of added metal ions, ascorbate acts as a prooxidant in vitro. In the absence of added metal ions, however, vitamin C inhibits oxidative DNA damage in purified DNA and cells (47,49–53), although there are a few exceptions (54–56). The latter are likely explained by “contaminating” metal ions in the cell culture media used.

The bleomycin-iron complex was the first well-studied system to damage DNA site specifically in a metal-dependent manner (44,45). Bleomycin-iron cleaves DNA to release \( N \)-propenal–substituted derivatives of thymine, cytosine, adenine, and guanine, which are believed to be responsible for some of the cytotoxic effects of bleomycin (45). The bleomycin-mediated cleavage of DNA is proposed to occur via a ternary bleomycin-DNA-iron complex. It is not known whether bleomycin binds to DNA first and then Fe(II) binds to the bleomycin-DNA complex, or whether a bleomycin-Fe(II) complex forms and then binds to DNA. Either way, oxidation of the complexed Fe(II) results in site-specific oxidation of DNA, presumably via \( \cdot \text{OH} \) production (57). In the presence of reducing agents such as ascorbate, Fe(III) is reduced back to Fe(II), thus continuing the oxidation of the DNA.

**Protein oxidation.** Studies of the metal-mediated denaturation of proteins were an outgrowth of investigations into the regulation of protein turnover in bacteria (35). These studies led to the discovery that degradation occurs when the protein has been oxidized (35). Similar to DNA, iron-mediated oxidation of protein may be a site-specific process. This notion is supported by the findings that the oxidation of proteins by MCO systems involves modification of only a few amino acid residues, in particular proline, histidine, arginine, lysine, and cysteine, whereas reactions of proteins with ROS generated by ionizing radiation are more or less random events, leading to the modification of many or all amino acid residues. Furthermore, metal ion-catalyzed
oxidation reactions in proteins are not sensitive to inhibition by ROS scavengers (35). The site-specific nature of metal ion-catalyzed reactions is consistent with the fact that enzymes that require divalent metals for activity, and therefore must possess metal-binding sites, are particularly sensitive to inactivation by MCO systems. The concept of site specificity is also supported by the studies of Stadtman (35) showing that the ascorbate-mediated modification of glutamine synthetase involves the conversion of a single histidyl residue to an asparaginyl residue, and an arginyl residue to a glutamylsemialdehyde residue; furthermore, both of these amino acid residues are situated at the metal-binding site of the enzyme.

Oxidation of the amino acid side chain can lead to the conversion of some amino acids to carbonyl derivatives, loss of catalytic activity, and increased susceptibility of the protein to proteolytic degradation (35). Although it is very probable, it remains uncertain whether •OH is the species responsible for the oxidation of proteins by MCO. Because the metal ion-catalyzed reactions lead to the conversion of some amino acid side chains (viz., prolyl, arginyl, and lysyl) to carbonyl derivatives, the concentration of protein carbonyl groups can be used as a measure of the extent of oxidative damage. Interestingly, protein carbonyl content increases with age and is associated with a number of pathological states (58).

**The Crossover Effect for Ascorbate as an Anti- or Prooxidant**

In general, low concentrations of ascorbate are required for prooxidant activity, whereas high concentrations are required for antioxidant activity. Thus, there is a “crossover effect,” i.e., at a certain concentration, ascorbate “switches” from pro- to antioxidant activity. In the literature, a wide range of concentrations has been reported at which this switch occurs (5). The crossover effect can be rationalized as follows: In the presence of ascorbate, catalytic metals will initiate radical chain reactions. However, due to the hydrogen-donating activity of ascorbate, the chain length of these radical processes will be short as long as the ascorbate concentration is relatively high, resulting in little oxidative damage. As the ascorbate concentration decreases, the initiation processes are slowed somewhat, but more importantly, the antioxidant reactions of ascorbate are slowed as well. Thus, the chain length of these radical processes becomes longer and more oxidative damage will occur. It has been proposed (5) that the variability observed in the literature for the crossover effect is a result of the variability in the concentration and form of the catalytic metals present. Thus, at very low levels of catalytic metals, ascorbate will nearly always serve as antioxidant. However, if the levels of available metals should increase, then ascorbate may exert deleterious effects.

**Studies on the Interaction of Vitamin C and Iron Under Physiologically Relevant Conditions**

Vitamin C is known to increase the gastrointestinal absorption of nonheme iron by reducing it to a form that is more readily absorbed (59). It appears, however, that even
at high intakes of vitamin C, iron uptake is tightly regulated in healthy people (59). Nevertheless, low dietary levels of vitamin C may be advantageous in cases of iron overload, such as homozygous hemochromatosis and β-thalassemia, because the excess iron can cause tissue damage (60,61). Individuals with iron overload generally have low plasma levels of vitamin C, possibly due to interaction with the elevated levels of “catalytic” iron in these individuals (60,61). Therefore, vitamin C administration has been claimed to be harmful in these patients (14,62). Iron overload has also been implicated in the sequelae of atherosclerosis, although the data are conflicting and inconsistent, and individuals with iron overload generally do not suffer from premature atherosclerosis (63,64). In addition, several vitamin C and iron cosupplementation studies, in both animals and humans, have shown that vitamin C inhibits, rather than promotes iron-dependent oxidative damage (summarized in Table 3.2).

**Studies Using Plasma and Cultured Cells**

*In vitro* experiments have shown that human serum and interstitial fluid strongly inhibit metal ion–dependent lipoprotein oxidation (65). These findings were attributed

<table>
<thead>
<tr>
<th>TABLE 3.2</th>
<th>Role of Vitamin C in Iron-Mediated Oxidative Damage*</th>
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<tbody>
<tr>
<td><strong>Study system</strong></td>
<td><strong>Challenge</strong></td>
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<tr>
<td><strong>In vitro</strong></td>
<td></td>
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<tr>
<td>Human plasma</td>
<td>Iron</td>
</tr>
<tr>
<td>Human plasma, lymph, synovial fluid</td>
<td>None</td>
</tr>
<tr>
<td>Human plasma</td>
<td>Iron/EDTA/H₂O₂</td>
</tr>
<tr>
<td>Human plasma</td>
<td>Iron-EDTA</td>
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<tr>
<td>Human plasma</td>
<td>Iron/H₂O₂</td>
</tr>
<tr>
<td>3T3 fibroblasts</td>
<td>Iron</td>
</tr>
<tr>
<td><strong>In vivo (animals)</strong></td>
<td></td>
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<tr>
<td>Guinea pig liver</td>
<td>Iron +</td>
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<tr>
<td>Guinea pig plasma, liver</td>
<td>Iron</td>
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<tr>
<td>Guinea pig serum</td>
<td>Iron</td>
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<tr>
<td><strong>Ex vivo autooxidation</strong></td>
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<tr>
<td><strong>In vivo (humans)</strong></td>
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<tr>
<td>Leukocytes</td>
<td>Iron (12 wk)</td>
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<td></td>
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<tr>
<td>White blood cells</td>
<td>Iron (6 wk)</td>
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<td></td>
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<tr>
<td>Preterm infant plasma</td>
<td>(BDI), none</td>
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</tbody>
</table>

*aAbbreviations: ↑, increased damage; ↓, decreased damage; ↔, no change; BDI, bleomycin-detectable iron; LOOH, lipid hydroperoxides; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances.

to the presence of metal binding proteins in these fluids, rather than vitamin C, because enzymatic removal of endogenous vitamin C did not alter the results. However, when sufficient exogenous iron (as ferrous ammonium sulfate) is added to plasma to saturate transferrin, resulting in the appearance of non-protein bound, bleomycin-detectable iron, endogenous and exogenous vitamin C strongly inhibits, rather than promotes lipid peroxidation (66) (Table 3.2). This finding is supported by an earlier study in which vitamin C acted as an antioxidant in serum to which excess copper had been added (67). Two other studies carried out with plasma, lymph, and synovial fluid showed that vitamin C can catalyze the formation of •OH, but only when the catalytically active form of iron, iron-EDTA, was added (68,69), not ferrous ammonium sulfate (69). Recently, we found that even when H₂O₂ is added to plasma, in addition to ascorbate and excess metal ions to constitute the complete Udenfriend system, ascorbate protects against iron- or copper-induced lipid peroxidation (J. Suh, B.-Z. Zhu, and B. Frei, unpublished observations). Ascorbate also did not enhance metal-dependent oxidation of plasma proteins under these conditions, as measured by protein carbonyl formation. These results demonstrate that even in the presence of high concentrations of transition metal ions and H₂O₂, ascorbate acts as an antioxidant that inhibits, and does not promote lipid and protein oxidation in biological fluids such as plasma.

Reports about the effects of ascorbate in cultured cells are conflicting, with some showing inhibition of cell death or apoptosis by ascorbate, and others suggesting that ascorbate is cytotoxic and induces apoptosis (70). Using three different cell types and two different culture media (Dulbecco’s modified Eagle’s medium and RPMI 1640), Clement et al. (71) found that the toxicity of ascorbate is due to ascorbate-mediated production of H₂O₂, to an extent that varies with the cell culture medium used. For example, in Dulbecco’s modified Eagle’s medium, 1 mmol/L ascorbate (a highly unphysiological, extracellular concentration) generated 161 ± 39 μmol/L H₂O₂ and induced apoptosis in 50% of HL60 cells, whereas in RPMI 1640 medium, only 83 ± 17 μmol/L H₂O₂ was produced and no apoptosis was observed. Apoptosis was prevented by catalase, and direct addition of H₂O₂ at the above concentrations to the cells mimicked the effects of ascorbate. These studies show that ascorbate itself is not toxic to cultured cells, and caution that the effects of ascorbate observed in cultured cells in vitro are of little or no relevance in vivo. The ability of ascorbate to interact with different cell culture media components, most probably contaminating metal ions, to produce H₂O₂ at different rates could account for most, if not all of the conflicting results reported (71,72).

**Animal Supplementation Studies**

Two animal studies have reported an antioxidant role for vitamin C in guinea pigs cosupplemented with vitamin C and iron (Table 3.2): (i) ex vivo autoxidation of liver microsomes obtained from iron-supplemented guinea pigs resulted in increased accumulation of malondialdehyde compared with microsomes obtained from control animals or animals cosupplemented with iron and vitamin C (73); and (ii) plasma and
liver F$_{2\alpha}$-isoprostanes, markers of *in vivo* lipid peroxidation, were increased in vitamin C–deficient guinea pigs loaded with iron, but reduced by vitamin C supplementation (74). In the latter study, hepatic vitamin C levels, in contrast to iron levels, were inversely associated with hepatic F$_{2\alpha}$-isoprostane levels (74). Another recent study using rats showed an antioxidant effect of vitamin C when given before a paraquat challenge, but a prooxidant effect when given after the challenge, as determined by expiratory ethane (75). The prooxidant effect was attributed to the paraquat-mediated release of metal ions from damaged cells. That study (75), therefore, suggests that vitamin C may have different effects depending on when it is added to the system under study, as has been observed previously with copper-dependent lipid peroxidation in LDL (76,77). Another recent study (78) found that large doses of intravenous ascorbate increased the levels of loosely bound iron and *in vitro* oxidation of serum obtained from iron-loaded guinea pigs, but not control animals (Table 3.2). Susceptibility of LDL to *ex vivo* oxidation increased after vitamin C injection in the control group, but there was no further increase in the iron-loaded group.

**Human Supplementation Studies**

A study carried out in humans to assess the effects of simultaneous iron and vitamin C supplementation yielded mixed results with respect to formation of various types of oxidized DNA bases in leukocytes (Table 3.2). Reanalysis of the data from that study (79) shows an inverse association between the plasma concentration of vitamin C and total DNA base damage. In addition, there was a positive correlation between the concentration of plasma vitamin C and the percentage of transferrin saturation, possibly due to a vitamin C–dependent increase in iron bioavailability (59), but no correlation was observed between the percentage of transferrin saturation and total base damage. These correlations are analogous to those observed in the above study using guinea pigs and suggest that vitamin C acts as an antioxidant, rather than a prooxidant, *in vivo* in the presence of iron.

Decreased levels of serum vitamin C and increased levels of lipid and protein oxidation products have been detected in hemochromatosis and β-thalassemia patients (60,61), which was attributed to the iron overload condition. However, these conclusions are not supported by a study in preterm infants, who often have excess iron in their plasma (66). In that study, plasma levels of F$_{2\alpha}$-isoprostanes and protein carbonyls were not correlated with levels of bleomycin-detectable iron, even in the presence of high concentrations of vitamin C (Table 3.2). It was found recently (80) that supplementation of either vitamin C or vitamin C plus iron did not cause a rise in total oxidative DNA damage measured by gas chromatography-mass spectrometry. However, a significant decrease was observed in the levels of the purine base oxidation product, 8-oxoguanine, after ascorbate supplementation. 5-Hydroxymethyl uracil levels were also decreased by either ascorbate or ascorbate plus iron supplementation, relative to the presupplemental levels, but not relative to the placebo group. In addition, levels of 5-hydroxymethyl hydantoin and 5-hydroxy cytosine increased significantly by ascorbate plus iron supplementation relative to the presup-
plementation period (Table 3.2). However, no consistent or compelling evidence for a prooxidant effect of vitamin C supplementation, with or without iron cosupplementation, on DNA base damage was observed (80). In another study by the same group (81), iron supplementation failed to affect any of the iron status variables measured, including serum ferritin, transferrin-bound iron, and the percentage of saturation of transferrin, and there were no detrimental effects on oxidative damage to DNA in healthy individuals with high plasma ascorbate levels. Finally, a recent study investigated the effect of a daily combined iron (100 mg/d as fumarate) and vitamin C (500 mg/d as ascorbate) supplement on plasma lipid peroxidation in pregnant women during the third trimester (82). In the supplemented group, plasma iron levels were higher than in the control group and plasma levels of thiobarbituric-acid reactive substances (TBARS) were significantly enhanced, suggesting that pharmacologic doses of iron, associated with high vitamin C intakes, may result in oxidative damage in vivo. However, TBARS are a poor measure of lipid peroxidation in biological fluids because there are many interfering substances and the basal levels reported in human plasma vary widely (83).

Conclusions

It is evident that ascorbate can exhibit both antioxidant and prooxidant activities. Although its role as an antioxidant is well documented, there is little, if any evidence that it serves as a prooxidant under physiological conditions. A majority of the studies that specifically addressed the interaction of vitamin C with iron in physiological fluids or in vivo (Table 3.2) found either no effect of vitamin C or decreased oxidative damage. Vitamin C played a prooxidant role in biological fluids only if iron-EDTA was added in vitro (68,69).

It should be noted that the levels of ascorbate vary substantially from tissue to tissue, ranging from 30 to 120 µmol/L in the plasma of normal individuals to millimolar intracellular concentrations in eye lens, brain, lung, and adrenals. These concentrations are certainly sufficient to provoke radical generation. The limiting factor is most likely the availability of metal ions, which are absolutely required for the prooxidant activity of ascorbate to occur. Although the total iron concentration of most tissues is quite high, iron exists almost entirely tightly sequestered in protein complexes (viz., transferrin, lactoferrin, hemoglobin, and ferritin) and is, therefore, not readily available for ROS generation (85,86). Conditions that facilitate the release of iron from these complexes most likely also promote ascorbate-mediated radical damage. Thus, the release of iron, which is often associated with tissue damage, would be expected to (secondarily) provoke ROS generation via the ascorbate-iron system.

On the basis of the studies on the biochemical interactions between ascorbate and iron, it is likely that the ratio of the concentration of iron and ascorbate will determine the ability of ascorbate to express prooxidant or antioxidant activity. At very low levels of catalytic iron under normal physiological conditions, ascorbate will act mainly as an antioxidant; however, if the levels of available iron increase significantly under certain pathologic conditions, ascorbate may exert prooxidant activity.
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References


Chapter 4

Vitamin C and Cancer

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Introduction

The role of vitamin C in treating and preventing cancer remains very controversial in spite of a number of studies that have been conducted over the last 25 years. In 1974, Cameron and Pauling (1) suggested that vitamin C might play a role in the supportive care of cancer patients. A study conducted by Cameron and Campbell (2) on 50 terminal cancer patients appeared to support this concept. They showed that large doses of vitamin C given orally, intravenously, or by a combination of both routes could significantly improve survival in advanced cancer patients. In a subsequent report by these authors, 10 of the original patients with unusual cancers were replaced with 10 new ones randomly selected from the records of vitamin C–treated patients from the same area. Furthermore, almost 50% of the 1000 control subjects were replaced because data on some of the initial control population were considered to be unreliable (3). The second study appeared to show an even greater benefit of vitamin C treatment. Because of the potential for bias in these nonrandomized studies, a group of researchers at the Mayo Clinic performed a randomized study to evaluate the efficacy of vitamin C in patients with advanced cancer (4). A second community-based study was reported at the American Society of Clinical Oncologists in 1983 (5). Finally, a third placebo-controlled study was conducted in cancer patients at the Mayo Clinic and reported in 1986 (6). Similar results were obtained in all three studies, i.e., there was no significant effect of vitamin C therapy on patient comfort or survival.

Vitamin C is an efficient water-soluble one-electron reducing agent that would be predicted to have efficacy in preventing oxidative DNA damage. A number of studies have in fact shown a reduction in oxidative damage to DNA, although many of these studies used methodology that was flawed (7). In contrast, cancer chemoprevention studies have consistently shown that there is no benefit from vitamin C supplementation in terms of cancer outcome (8,9). This contrasts with studies that consistently show a reduction in disease risk with diets that are rich in antioxidants such as vitamin C (10).

It is thought that lipid peroxidation of polyunsaturated fatty acids (PUFA) plays an important role in the degenerative diseases of aging such as cancer (11,12). The formation of lipid hydroperoxides from PUFA by free radical processes is a complex
process, which leads to a number of different regioisomers and stereoisomers (13). It would be predicted that vitamin C would inhibit this pathway. However, lipoxygenase (LOX)-mediated oxidation of PUFA results in the formation of lipid hydroperoxides (14), and vitamin C would not be predicted to inhibit this pathway. Lipid hydroperoxides undergo homolytic decomposition to bifunctional electrophiles (15) that can react with DNA (12). Therefore, we were interested in determining whether vitamin C could mediate lipid hydroperoxide–mediated DNA damage.

In previous studies, we examined the homolytic decomposition of 13-[S-(Z,E)]-9,11-hydroperoxyoctadecadienoic acid (13-HPODE; a prototypic n-6 PUFA lipid hydroperoxide) in the presence of the DNA bases 2′-deoxyadenosine (dAdo) and 2′-deoxyguanosine (dGuo). From structures of the resulting DNA-adducts, we proposed that the major covalent modifications arose through generation of 4-oxo-2-nonenal from 13-HPODE. The same adducts were formed when DNA bases were treated with synthetic 4-oxo-2-nonenal (16,17). Subsequently, 4-oxo-2-nonenal was confirmed as a major breakdown product of homolytic lipid hydroperoxide decomposition (18), a finding that was recently confirmed by Spiteller et al. (19). Surprisingly, 4-hydroperoxy-2-nonenal was also characterized as a product of 13-HPODE decomposition by our laboratory and by Schneider et al. (18,20). 4-Hydroperoxy-2-nonenal was subsequently shown to be a precursor in the formation of 4-oxo-2-nonenal and 4-hydroxy-2-nonenal (21). The other major bifunctional electrophile identified in homolytic 13-HPODE decomposition was 4,5-epoxy-2(E)-decanal (21), a recently identified product from the autoxidation of arachidonic acid (22).

An environmental contaminant in water and food [see Ref. (23) for discussion], trans,trans-2,4-decadienal is also a product of lipid peroxidation through α-cleavage of the alkoxy radicals derived from 9-hydroperoxy-(E,E)-10,12-octadecadienoic acid or 11-hydroperoxy-(Z,Z,E,E)-eicosa-5,8,12,14-tetraenoic acid (19,22). Recent studies have shown that the reaction of peroxide-treated trans,trans-2,4-decadienal with dAdo or dGuo results in the formation of 1,N6-etheno-dAdo (23) and 1,N2-etheno-dGuo (24), respectively. We reasoned that 4,5-epoxy-2(E)-decanal could have been formed when trans,trans-2,4-decadienal was treated with peroxides (22,25) and that this bifunctional electrophile was in fact the precursor to the formation of etheno-adducts from lipid hydroperoxides.

**Formation of Lipid Hydroperoxides**

Lipid hydroperoxides are formed nonenzymatically by reactive oxygen species (ROS) such as superoxide (O₂•−), peroxide (O₂²−), and hydroxyl radical (HO•). The endogenous pathways for ROS generation include normal mitochondrial aerobic respiration, phagocytosis of bacteria or virus-containing cells, peroxisomal-mediated degradation of fatty acids, and cytochrome P₄₅₀-mediated metabolism of xenobiotics DNA (26). Mixtures of vitamin C and transition metal ions (27), and the conversion of catechols to quinones (28) can also cause ROS formation from molecular oxygen. Antioxidant defense systems in vivo that can detoxify ROS include the following: superoxide dis-
(mutase, catalase, and reduced glutathione (GSH)-dependent peroxidases (26). Also, endogenous processes such as the sequestration of hydrogen peroxide–generating enzymes or the chelation of free transition metal ions by transferrin, ferritin, and ceruloplasmin can protect against ROS-mediated damage. However, it is always possible that cellular macromolecules and lipids can be damaged by the ROS that escape from these defense systems. It has been suggested that ROS generation is a major contributor to the degenerative diseases of aging, including cardiovascular disease, cancer, immune-system decline, and brain dysfunction (26). ROS-mediated formation of lipid hydroperoxides is a complex process, which involves initiation by the abstraction of a \( \text{bis-allylic} \) methylene hydrogen atom of PUFA followed by addition of molecular oxygen (13). This results in the formation of 9- and 13-hydroperoxyoctadecadienoic acid (HPODE) isomers from linoleic acid, the major n-6 PUFA present in plasma lipids. Lipid hydroperoxides can also be formed enzymatically from LOX (14) and cyclooxygenases (COX) (29) with much greater stereoselectivity than is observed in the free radical mechanism (Fig. 4.1). Human 15-LOX convert linoleic acid mainly to 13-HPODE (30). COX-1 and COX-2 produce mainly 9-[R-(E,Z)]-10,12-hydroperoxyoctadecadienoic acid (9-HPODE) and 13-HPODE (31). The other C\(_{18}\) PUFA including linolenic acid (n-3) and dihomo-\( \gamma \)-linolenic acid (n-6) and all C\(_{20}\) PUFA undergo 15-LOX–mediated conversion to hydroperoxides. The products that arise from 5-LOX and COX-derived metabolism of C\(_{20}\) PUFA are prostaglandins, thromboxanes, and leukotrienes rather than lipid hydroperoxides.

**Transition Metal Ion-Mediated Decomposition of Lipid Hydroperoxides**

Lipid hydroperoxides undergo transition metal ion–induced decomposition to the \( \alpha,\beta \)-unsaturated aldehyde genotoxins that can react with DNA (12). We determined previously that 4-oxo-2-nonenal was a major product from homolytic decomposition of 13-HPODE (18), which was confirmed by Spiteller et al. (19). 4-Hydroperoxy-2-nonenal was also characterized as a product of 13-HPODE decomposition (18,20). We recently developed a liquid chromatography (LC)/atmospheric pressure chemical ionization (APCI)/mass spectrometry (MS) methodology to identify the \( \alpha,\beta \)-unsaturated aldehydes by gas chromatography–mass spectrometry (GC/MS).

![Fig. 4.1. Enzymatic formation of lipid hydroperoxide. Abbreviations: 13(S)-Z,E-HPODE, 13-[S-(Z,E)]-9,11-hydroperoxyoctadecadienoic acid; LOX, lipoxygenase; COX, cyclooxygenase.](image)

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Dic bifunctional electrophiles that could potentially be formed during homolytic lipid hydroperoxide decomposition (21). Using this methodology, the aldehydes resulting from FeII-mediated (50 or 500 µmol/L) decomposition of 13-HPODE (400 µmol/L) were analyzed. Four major products were observed in the ultraviolet (UV; 226 nm) chromatogram at 10.6, 11.0, 12.3, and 17.1 min, respectively (Fig. 4.2A-e). The LC/MS characteristics of the earliest eluting aldehyde with a protonated molecular ion (MH+) at m/z 169 were identical to authentic trans-4,5-epoxy-2(E)-decenal. cis-4,5-Epoxy-2(E)-decenal was also observed in the ion chromatogram for m/z 169 with a retention time of 11.7 min (Fig. 4.2A-a). The aldehyde with a retention time of 11.0 min and MH+ at m/z 155 was identified as 4-oxo-2-nonenal (Fig. 4.2A-b). The LC/MS characteristics of the most abundant aldehyde with [MH–OH]+ at m/z 156 were identical to authentic 4-hydroperoxy-2-nonenal (Fig. 4.2A-c). The last eluting product with MH+ at m/z 157 was identified as 4-hydroxy-2-nonenal (Fig. 4.2A-d). At a higher concentration of FeII (500 µmol/L), the major products were trans-4,5-epoxy-2(E)-decenal, 4-oxo-2-nonenal, and 4-hydroxy-2-nonenal (Fig. 4.2B). Under these conditions, the 4-hydroperoxy-2-nonenal was undetectable by MS or UV (Fig. 4.2B-c,e).

The initial formation of 4-hydroperoxy-2-nonenal at lower FeII concentrations and subsequent decline at higher concentrations suggested that it was a precursor to the formation of 4-oxo-2-nonenal and 4-hydroxy-2-nonenal. To test this possibility, authentic 4-hydroperoxy-2-nonenal was treated with increasing concentrations of FeII. As the concentration of FeII increased, there was a gradual decline in the amount of

![Fig. 4.2. Decomposition of 13-[S-(Z,E)]-9,11-hydroperoxyoctadecadienoic acid (13-HPODE) with transition metal ions. Abbreviations: 4,5-EDE, 4,5-epoxy-2(E)-decenal; 4-ONE, 4-oxo-2-nonenal; 4-HPNE, 4-hydroperoxy-2-nonenal; 4-HNE, 4-hydroxy-2-nonenal; UV, ultraviolet.](image-url)
4-hydroperoxy-2-nonenal and a concomitant increase in the formation of 4-oxo-2-nonenal and 4-hydroxy-2-nonenal. Stoichiometric amounts of 4-oxo-2-nonenal and 4-hydroxy-2-nonenal were formed in a ratio that was identical to that from the reaction of 13-HPODE with FeII. These findings confirmed that one of major products in homolytic 13-HPODE decomposition was 4,5-epoxy-2(E)-decenal, which was recently identified as a product from the autoxidation of arachidonic acid (22). We have also shown that 4-hydroperoxy-2-nonenal was an important precursor to the formation of 4-oxo-2-nonenal and 4-hydroxy-2-nonenal.

**Transition Metal Ions, Vitamin C, and ROS**

Transition metal ion–mediated Haber Weiss reactions are known to produce ROS, which then cause oxidative damage to the DNA. Vitamin C is used as an antioxidant because it can prevent such damage (32). When vitamin C reacts with hydrogen peroxide, the vitamin C radical anion and a hydroxyl radical are produced. The reactive hydroxyl radical is then detoxified by reaction with vitamin C radical anion or vitamin C itself to give a water molecule. If the vitamin C radical anion reacts with the hydroxyl radical, dehydro-vitamin C is also formed. However, biological buffers contain substantial amounts of transition metal ions and it is paradoxical that ROS formation is enhanced in the presence of vitamin C (27). We have assessed the transition metal ion contamination in typical aqueous buffers by monitoring the decline in absorbance of vitamin C solutions at 265 nm as suggested by Buettner and Jurkiewicz (27). As can be seen in Figure 4.3A, normal 3-(N-morpholino)propanesulfonic acid (MOPS) buffer solutions cause a substantial decline in absorbance at 265 nm over 2 h resulting from oxidation of vitamin C to its dehydro form. Vitamin C proved to be quite stable in Chelex-treated MOPS buffer solutions (Fig. 4.3A). However, when vitamin C was dissolved in Chelex-treated MOPS buffer and 500 nmol/L CuII was added, the same decline in absorbance was observed as in non-Chelex–treated buffer. This indicated that normal buffers contained the equivalent of 500 nmol/L CuII in transition metal ion contamination. Chelex-treated MOPS buffer containing vitamin C showed no decline in absorbance at 265 nm unless it contained ≥50 nmol/L of added CuII (Fig. 4.3B). This suggested that the maximum amount of CuII in Chelex-treated MOPS buffer was <50 nmol/L. Concentrations of Cu and Fe in the buffer were determined subsequently by graphite furnace atomic absorption spectrophotometry and inductively coupled plasma/MS, respectively. Chelex treatment of the MOPS buffer reduced the Cu from 234 to 16 nmol/L and Fe from 250 to <36 nmol/L. No other transition metal ions were detected.

**Vitamin C–Induced Decomposition of Lipid Hydroperoxides**

When vitamin C reacts with a lipid hydroperoxide, an alkoxy radical is formed by a mechanism analogous to the formation of hydroxyl radicals from hydrogen peroxide (Fig. 4.4). The alkoxy radical could then be detoxified by the vitamin C radical anion...
or vitamin C itself to give a nontoxic hydroxy acid. This is similar to the formation of water from hydroxyl radicals. Alternatively, it could rearrange in a manner analogous to alkoxy radicals formed from transition metal ions. Therefore, the same bifunctional electrophiles produced by transition metal ion–mediated decomposition of lipid hydroperoxides could potentially be formed by vitamin C. To test this possibility, 13-HPODE was treated with vitamin C in the transition metal ion–free MOPS buffer at 37°C. The same bifunctional electrophiles as those formed by transition metal ion–mediated decomposition of lipid hydroperoxides were produced by vitamin C (21). Although the MOPS buffer contained <50 nmol/L of transition metal ions, we determined whether Cu or Fe had an additive effect on the vitamin C–mediated decomposition of 13-HPODE. When 1 µmol/L of either CuII or FeII was added to the reaction mixture, there was no increased formation of bifunctional electrophiles from 13-HPODE. This confirmed that trace amounts of these transition metal ions did not substantially affect the reaction between vitamin C and 13-HPODE. In separate experiments, we established that synergistic effects between vitamin C and transition metal ions occurred only at concentrations that were two orders of magnitude greater than the two present in the Chelex-treated MOPS buffer (10–20 µmol/L).
The lipid hydroperoxide–derived \( \alpha,\beta \)-unsaturated aldehydes were formed by two quite different pathways (Fig. 4.5). The first pathway is based on that described previously by Pryor and Porter (33) and is initiated by alkoxy radical formation. Complex rearrangements of the alkoxy radical, together with the addition of molecular oxygen results in the formation of 4-oxo-2-nonenal, 4-hydroxy-2-nonenal, and 4,5-epoxy-2(\(E\))-decenal. The formation of 4-hydroperoxy-2-nonenal cannot be rationalized by any previously proposed mechanism. However, we have now established that 4-hydroperoxy-2-nonenal undergoes both transition metal ion– and vitamin C–mediated breakdown to 4-oxo-2-nonenal and 4-hydroxy-2-nonenal, which provides an addition- al route to these genotoxins.

DNA-Adducts from Lipid Hydroperoxides

The compound \textit{trans,trans}-2,4-decadienal is an \( \alpha,\beta \)-unsaturated aldehydic decomposition product from 9-hydroperoxy-(\(E,E\))-10,12-octadecadienoic acid or 11-hydroperoxy-(\(Z,Z,E,E\))-eicosa-5,8,12,14-tetraenoic acid (19,22). Recent studies have shown that the reaction of peroxide-treated \textit{trans,trans}-2,4-decadienal with dAdo or dGuo results in the formation of 1,\(N^6\)-etheno-dAdo (23) and 1,\(N^2\)-etheno-dGuo (24), respectively. We reasoned that \textit{trans}-4,5-epoxy-2(\(E\))-decenal could have been formed when \textit{trans,trans}-2,4-decadienal was treated with peroxides (22,25) and that this bifunctional electrophile was in fact the precursor to the formation of etheno-adducts from lipid.

Fig. 4.4. Potential formation of DNA-reactive bifunctional electrophiles by vitamin C-mediated decomposition of 13-[\(S-(Z,E)\)]-9,11-hydroperoxyoctadecadienoic acid (13-HPODE).

DNA-reactive bifunctional electrophiles
hydroperoxides. The reaction of dAdo and dGuo with trans-4,5-epoxy-2(E)-decalen formed unsubstituted etheno-adducts (34). The structure of 1,N⁶-etheno-dAdo was confirmed by LC/MS analysis (Fig. 4.6A) and by multiple tandem mass spectrometry (MS^n) analyses (Fig. 4.6B). The structure of 1,N²-etheno-dGuo was established by hydrolysis to the corresponding guanine (Gua)-adduct and comparison with authentic etheno-Gua isomers. N²,3-etheno-Gua and 1,N²-etheno-Gua eluted with retention times of 8.0 min and 8.7 min, respectively (Fig. 4.7A). The APCI/MS spectra of 1,N²-etheno-Gua and N²,3-etheno-Gua were identical (data not shown). MS² analysis of 1,N²-etheno-Gua (m/z 176) gave rise to product ions at m/z 148 [MH⁺−CO] and m/z 121 [MH⁺−CO−CNH]. For N²,3-etheno-Gua, no product ions were observed when

![Diagram](image_url)

**Fig. 4.5.** Formation of 4-oxo-2-nonenal (4-ONE), 4-hydroperoxy-2-nonenal (4-HPNE), 4-hydroxy-2-nonenal (4-HNE), and 4,5-epoxy-2(E)-decalen (4,5-EDE) from 13-[5-(Z,E)]-9,11-hydroperoxyoctadecadienoic acid (13-HPODE).

**Fig. 4.6.** Analysis of 1,N⁶-etheno-dAdo from the reaction of trans-4,5-epoxy-2(E)-decalen with dAdo. (A) Liquid chromatography/mass spectrometry chromatograms showing the total ion current chromatogram (total ion current; upper), the ion chromatogram for MH⁺ (m/z 276; middle), and selected reaction monitoring chromatogram for MH⁺ (m/z 276) → BH₂⁺ (m/z 160; lower). (B) Product ion spectrum of MH⁺ (m/z 276).

Abbreviation: TIC, total ion current.
MS2 analysis of m/z 176 was conducted. The dGuo adduct from the reaction with trans-4,5-epoxy-2(E)-decenal was isolated by preparative high-performance liquid chromatography and subjected to acid hydrolysis (1N HCl, 100°C, 1 h). After depuration, the retention time of the adduct was 8.7 min (Fig. 4.7B) and the MS spectrum exhibited an intense MH+ ion at m/z 176 (Fig. 4.7C). The MS2 spectrum showed two major product ions at m/z 148 [MH+–CO] and m/z 121 [MH+–CO–CHN] (Fig. 4.7D). This confirmed that the adduct was 1, N2-etheno-dGuo rather than the isomeric N2,3-etheno-dGuo.

Transition metal ion-free buffers were used in the reactions of dAdo and dGuo with trans-4,5-epoxy-2(E)-decenal and the pH was maintained at 7.4. Under these conditions, trans-4,5-epoxy-2(E)-decenal was quite stable; thus, the unsubstituted etheno-adducts could not have been formed from further breakdown products of the epoxide. 2,3-Epoxyoctanal, used in the synthesis of trans-4,5-epoxy-2(E)-decenal, is much more efficient at converting both dAdo and dGuo to unsubstituted etheno-adducts (data not shown). Therefore, we considered the possibility that the trans-4,5-
epoxy-2(E)-decenal was contaminated with 2,3-epoxyoctanal. A normal phase LC/MS assay was developed that would detect trace amounts of 2,3-epoxyoctanal. LC/APCI/selected ion monitoring (SIM)/MS analysis of the two epoxides employed MH\(^+\) for 2,3-epoxyoctanal and trans-4,5-epoxy-2(E)-decenal at \(m/z\) 143 and \(m/z\) 169, respectively. Under these conditions, there was a clear separation of the two epoxides with no interfering signals at the retention times of either analyte. A calibration curve was obtained by analyzing standard solutions containing known amounts of 2,3-epoxyoctanal and trans-4,5-epoxy-2(E)-decenal (20 \(\mu\)g). A typical regression line was 
\[
y = 6.0 \times 10^6 + 194385, \quad r^2 = 0.9995 \text{ (Fig. 4.8)}.
\]
The amount of 2,3-epoxyoctanal in the authentic trans-4,5-epoxy-2(E)-decenal (20.0 \(\mu\)g, 2 \(\mu\)g on column) was then determined. The 2,3-epoxyoctanal was below the detection limit of the assay (<2 ng on column, <0.1%). At this level of contamination, there would have been no significant contribution from 2,3-epoxyoctanal to the formation of unsubstituted etheno-adducts. Furthermore, trans-4,5-epoxy-2(E)-decenal was incubated in Chelex-treated MOPS buffer (pH 7.4) for 24, 48, or 72 h at 37°C. We confirmed that the LC/MS response for trans-4,5-epoxy-2(E)-decenal at each time point was identical to that observed before the sample was placed in the incubator, and 2,3-epoxyoctanal was not formed during prolonged incubations of trans-4,5-epoxy-2(E)-decenal.

In a recent series of experiments, we explored the potential for unsubstituted etheno-dAdo and etheno-dGuo formation to occur in intact DNA. Calf thymus DNA (1.5 mg, 5.03 \(\mu\)mol) in 100 mmol/L MOPS containing 150 mmol/L NaCl (pH 7.4, 500 \(\mu\)L) was treated with trans-4,5-epoxy-2(E)-decenal (10 mg, 60.0 \(\mu\)mol). The reaction mixture was incubated at 37°C for 24 h after sonication for 15 min. Samples were placed in ice for 30 min, the DNA was precipitated by adding ice-cold ethanol, and the DNA pellet was removed. It was then hydrolyzed enzymatically under very mild conditions using methodology that we developed previously (17). The hydrolysate was applied directly to a solid-phase extraction cartridge and the etheno-adducts were
eluted with methanol/water. The eluates were evaporated to dryness under nitrogen and dissolved in water. Using both LC/MS and LC/selected reaction monitoring (SRM)/MS, it was possible to detect 1,N²-etheno-dGuo (Fig. 4.9A) and 1,N⁶-etheno-dAdo (Fig. 4.9B) in the DNA hydrolysate. The MS² spectrum of etheno-adducts from trans-4,5-epoxy-2(E)-decalen–modified calf thymus DNA showed exclusive product ion at m/z 176 (retention time of 15.2 min, BH₂⁺ for 1,N²-etheno-dGuo, Fig. 4.9C) and at m/z 160 (retention time of 18.0 min, BH₂⁺ for 1,N⁶-etheno-dAdo, Fig. 4.9D). These were identical to the LC/MS characteristics of authentic 1,N²-etheno-dGuo and 1,N⁶-etheno-dAdo. Based on the signal of authentic standards, the signal for 1,N²-etheno-dGuo corresponded to 1.3 adducts/10⁵ normal bases and the signal for 1,N⁶-etheno-dAdo corresponded to 3.7 adducts/10⁵ normal bases.

**Summary**

1,N⁶-Etheno-dAdo has been detected in human tissues (35) as well as in the liver of vinyl chloride–treated rats (36). 1,N⁶-Etheno-dAdo is highly mutagenic in mammalian cells and much more mutagenic than lesions that arise from oxidative damage such as 7,8-dihydro-8-oxo-dGuo (37). This most likely stems from the ability of atypical

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**Fig. 4.9.** Liquid chromatography/mass spectrometry (LC/MS) and LC/selected reaction monitoring (SRM)/MS analysis of the DNA hydrolysate after the treatment of calf thymus DNA with trans-4,5-epoxy-2(E)-decalen for 24 h at 37°C. (A) The ion chromatogram for MH⁺ of 1,N²-etheno-dGuo (m/z 292; upper), and SRM chromatogram for MH⁺ (m/z 292) → BH⁺ (m/z 176; lower). (B) The ion chromatogram for MH⁺ of 1,N⁶-etheno-dAdo (m/z 276; upper), and SRM chromatogram for MH⁺ (m/z 276) → BH⁺ (m/z 160; lower). (C) Product ion spectrum for MH⁺ (m/z 292) of 1,N²-etheno-dGuo. (D) Product ion spectrum for MH⁺ (m/z 276) of 1,N⁶-etheno-dAdo.
mammalian DNA-polymerases to perform translesional synthesis, which results in A to T transversions (38). \(N^2,3\)-Etheno-Gua was also found in the liver DNA of rats treated with vinyl chloride (39). \(1,N^2\)-Etheno-Gua was identified in chloroacetaldehyde-treated DNA (40) and both \(N^2,3\)-etheno-Gua and \(1,N^2\)-etheno-Gua were isolated from 2-halooxirane-treated DNA (41). \(1,N^2\)-Etheno-dGuo was shown to be mutagenic in mammalian cells (42), although the mutation profile was much more complex than for \(1,N^6\)-etheno-dAdo (37). In vitro studies with DNA bases have demonstrated that peroxide-treatment of 4-hydroxy-2-nonenal and \(\text{trans,trans-2,4}\)-decadienal results in the formation of etheno DNA-adducts (23,24,43). However, it is not clear that such reactions could occur in vivo because of the competition between detoxication by glutathione-S-transferases and aldo-keto reductases (44) and activation by peroxidation. We have now shown that the reaction of dAdo and dGuo with \(\text{trans-4,5-epoxy-2(E)}\)-decenal results in the formation of unsubstituted etheno-adducts (Fig. 4.10), which provides an important link between a primary product of lipid peroxidation and a mutagenic DNA-lesion (37,42) that was detected in human tissue DNA (35).

We have shown that lipid hydroperoxide-derived 4-oxo-2-nonenal reacts with dGuo and dAdo to give substituted etheno-adducts (45,46). The mechanism for the formation of the etheno-dGuo adducts involves highly regioselective nucleophilic addition of \(N^2\) of the dGuo to the C-1 aldehyde of 4-oxo-2-nonenal followed by reaction of \(N^1\) at C2 of the resulting \(\alpha,\beta\)-unsaturated ketone. The intermediate etheno-

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**Fig. 4.10.** Formation of etheno-adducts from lipid hydroperoxide–derived 4-oxo-2-nonenal and \(\text{trans-4,5-epoxy-2(E)}\)-decenal. Abbreviations: PUFA, polyunsaturated fatty acids; 13-HPODE, 13-[\(S-(Z,E)\)]-9,11-hydroperoxyoctadecadienoic acid; 15-HPETE, 15\(S\)-hydroperoxy-5,8,11,13(\(Z,Z,Z,E\))-eicosatetraenoic acid.
adducts then dehydrate to a single substituted etheno-adduct (Fig. 4.10) (16). Similarly, initial nucleophilic addition of $N^6$ of dAdo to the C-1 aldehyde of 4-oxo-2-nonenal is followed by reaction of N1 at C2 of the resulting $\alpha,\beta$-unsaturated ketone to generate a mixture of two ethano-adducts that interconvert. The ethano-adducts subsequently dehydrate to give a single heptanone etheno-dAdo adduct (Fig. 4.10) (17,47). The reaction of 4-oxo-2-nonenal with calf thymus DNA was also shown to result in the formation of heptanone-etheno-dGuo and dAdo adducts (17). There was almost three times as much of the dGuo adduct as the dAdo adduct at all time points up to 12 h after the addition of the 4-oxo-2-nonenal.

Reactions between dGuo and the $\alpha,\beta$-unsaturated aldehydes, 4-hydroxy-2-nonenal and malondialdehyde (MDA, another breakdown product of lipid peroxidation) result in the formation of exocyclic propano adducts (48). In contrast to 4-oxo-2-nonenal, Michael addition occurs initially at the $\beta$-carbon, which is followed by nucleophilic addition of dGuo at the aldehyde carbon. When the $\alpha,\beta$-unsaturated aldehyde has a substituent at the $\beta$-carbon such as in 4-hydroxy-2-nonenal, the steric hindrance inhibits nucleophilic attack from N1. Kinetic control of the reaction favors the regiosomer in which $N^2$ is attached to the $\beta$-carbon atom and N1 is attached to the aldehyde carbon. This results in the formation of two pairs of diastereomeric hexanol-1, $N^2$-propano-dGuo adducts from 4-hydroxy-2-nonenal and pyrimido[1,2-$\alpha$]purin-10(3H)-one from MDA. When MDA reacts with dAdo, an acyclic $N^6$-oxopropenyl adduct is generated (49,50). This adduct is thought to be a consequence of initial 1,4-addition to the $\beta$-hydroxyacrolein form of MDA followed by dehydration. 4-Hydroxy-2-nonenal does not appear to react very efficiently with dAdo. However, the epoxide derivative, 2,3-epoxy-4-hydroxynonanal, has been shown to form substituted and unsubstituted etheno-adducts with dAdo through the same mechanism by which heptanone-etheno-dAdo adducts are formed from 4-oxo-2-nonenal (43,51,52). 1, $N^2$-Etheno-dGuo, a substituted 1, $N^2$-etheno-adduct, and a tetracyclic adduct containing two 5-membered fused rings at N1 and $N^2$ atoms of guanine are also formed from the reaction between 2,3-epoxy-4-hydroxynonalal and dGuo (51,53). DNA-adducts have not yet been identified from the reaction of DNA bases with the potential genotoxic bifunctional electrophile 4-hydroperoxy-2-nonenal.

This study provides a mechanism for vitamin C–mediated decomposition of lipid hydroperoxides to genotoxic bifunctional electrophiles without the need for free transition metal ions. Future studies will focus on determining whether vitamin C can induce such genotoxin formation in vivo.

Acknowledgments

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References


37. Levine, R.L., Yang, I.-Y., Hussain, M., Pandya, G., Grollman, A.P., and Moriya, M.


Ascorbic Acid and Endothelial NO Synthesis

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Introduction

Endothelium-derived nitric oxide (NO) was originally discovered as a vasodilator product and is now known as a central regulator of vascular homeostasis and a principal factor involved in the antiatherosclerotic properties of endothelial cells (1–4). Experimental studies have shown that NO interferes with key events of atherosclerosis such as monocyte and leukocyte adhesion to the endothelium, platelet-endothelium interactions, smooth muscle cell proliferation, and increased endothelial permeability. In agreement with these findings, a dysfunction of the endothelium with a decreased generation of NO caused accelerated atherosclerosis in experimental models (5). Moreover, a reduced NO-dependent vasodilation was detectable in patients with atherosclerosis or with a cardiovascular risk profile even when the coronary vessels were angiographically still normal (6,7). All major risk factors for atherosclerosis including hypercholesterolemia, hypertension, hyperhomocysteinemia, and cigarette smoking have been associated with impaired vascular NO synthesis (8–11). Because these conditions are also correlated with increased oxidative stress (12), particularly increased production of superoxide radicals and elevated levels of oxidized lipoproteins, which can directly inactivate NO (13,14), antioxidants have been thought to improve endothelial dysfunction. Accordingly, epidemiologic studies demonstrated that a diet high in antioxidant vitamins is associated with lower cardiovascular morbidity and mortality (15).

With regard to ascorbic acid, it has been shown that plasma or leukocyte ascorbate levels are reduced in patients with an unstable coronary syndrome or angiographically documented coronary artery disease, respectively (16,17). Moreover, a number of clinical studies have demonstrated that ascorbic acid can reverse NO-dependent endothelial dysfunction present in coronary or peripheral arteries of patients with atherosclerosis and several conditions that predispose patients to atherosclerosis (18–37). Protective ascorbic acid effects have been seen with different stimuli of NO-dependent vasodilation such as acetylcholine or metacholine (18,20,22–27,33,35–37), flow (19,21,22,28–30,32) or arginine infusion (31), and with both ascorbate infusion (18,20–28,31–37) and oral ascorbate supplements (19,26,29,30). Concerning the underlying mechanisms, several possibilities have been discussed (38–40). Ascorbic acid could interfere with the oxidation of low density lipoprotein (LDL) (41,42) or protect endothelial NO synthesis from the effects of oxidized LDL (14,43–47).
Ascorbate could also enhance endothelial-dependent vasodilation by sparing intracellular thiols (48), which in turn may stabilize NO through the formation of biologically active $S$-nitrosothiols (49). The latter likely serve as a reservoir of NO in plasma (50,51) and, interestingly, ascorbate has been shown to release NO from $S$-nitrosothiols and to improve the delivery of NO to the vasculature (52–54). Given the importance of the superoxide anion as a mechanism of endothelial dysfunction (40), several investigators have also assumed that ascorbic acid exerts its beneficial effect by scavenging superoxide anion and protecting NO from inactivation. A recent report, however, showed that ascorbate concentrations ≥10 mmol/L would be required to compete efficiently with the reaction of NO and superoxide (55). These concentrations are potentially achievable in plasma by ascorbate infusion and may account for the beneficial effects of ascorbic acid seen in the respective studies (18,20–28,31–37). Plasma ascorbate levels after oral supplementation are in the range of 100 µmol/L (26,30), however, and are unlikely to prevent NO inactivation by superoxide. Thus, if superoxide scavenging is involved in the beneficial effect of physiologic doses of ascorbic acid, it should possibly occur in the intracellular milieu where ascorbate concentrations are likely in the low millimolar range (42,56).

Little is known about the effects of antioxidants and especially of ascorbic acid on the synthesis of NO in endothelial cells. NO is generated from the conversion of L-arginine to L-citrulline by the enzymatic action of an NADPH-dependent NO synthase (NOS) that requires Ca$^{2+}$/calmodulin, FAD, FMN, and tetrahydrobiopterin as cofactors (57–60). The endothelial NOS isoform (eNOS) is constitutively expressed and activated upon cell stimulation with calcium-mobilizing agonists and fluid shear stress (60). Optimal NO formation has been shown to be dependent on the availability of intracellular cofactors (61–63) and the membrane localization of the enzyme (64). eNOS activity is regulated at the transcriptional level and by a variety of modifications such as acylation, which enables membrane targeting, and phosphorylation, which is involved in shear stress–dependent enzyme activation. Moreover, protein-protein interactions support either activation or inactivation of eNOS (65). Within plasmalemmal caveolae, eNOS is quantitatively associated with caveolin, the structural coat component of these microdomains (64). This complex formation has been shown to inhibit enzyme activity, and the inhibitory effect was reversed upon binding of Ca$^{2+}$/calmodulin. The activation of eNOS is also facilitated by interactions of the enzyme with heat shock protein 90 and with dynamin. On the other hand, eNOS binding to the bradykinin B2 receptor participates in its inactivation (65).

Generally, changes in the intracellular redox state could affect NO generation at different levels. Oxidized LDL, for example, which is likely to induce oxidative stress in the cells, has been shown to inhibit the NO-dependent vasorelaxation (66), and the underlying mechanisms are thought to involve a decrease in eNOS expression (43), an uncoupling of Gi protein-dependent signal transduction (44), a limited availability of L-arginine (45), and changes in the subcellular eNOS localization (46,47). An alteration of the intracellular redox state might also affect the availability of reduced cofactors for eNOS. In particular, tetrahydrobiopterin seems to be a cofactor that can limit
NO synthesis (67–70). Tetrahydrobiopterin acts as a redox-active cofactor (71–74) and additionally has profound effects on the structure of eNOS, including the ability to shift the heme iron to its high spin state, the promotion of arginine binding and the stabilization of the active dimeric form of the enzyme (58,59). A number of experimental (75–78) and clinical studies (79–83) have shown that low tetrahydrobiopterin levels were associated with decreased NO formation and impaired endothelium-dependent relaxation and, conversely, that tetrahydrobiopterin supplementation was capable of restoring NO production and endothelium-dependent vasodilation.

The present study was designed to examine whether ascorbic acid affects NO synthesis in human endothelial cells and to investigate possible underlying mechanisms. We show that saturated ascorbic acid levels in endothelial cells are necessary to protect the eNOS cofactor tetrahydrobiopterin from inactivation and to provide optimal conditions for cellular NO synthesis. A detailed description of the methods and results presented here was published earlier (84,85).

**Materials and Methods**

**Materials.** Medium 199 (M199), human serum (HS), fetal calf serum (FCS), and collagenase were from BioWhittaker Europe (Verviers, Belgium). L-[2,3,4,5-3H]Arginine monohydrochloride (61 Ci/mmol), L-[U-14C]arginine monohydrochloride (303 mCi/mmol), L-[carboxyl-14C]ascorbic acid (16 mCi/mmol), and [3H]cGMP Biotrak radioimmunoassay systems were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were from Pharma Biotechnology (Hannover, Germany). NADPH, tetrahydrobiopterin, sepiapterin, and L-nitroarginine methylester (L-NAME) were obtained from Alexis Corporation (Läufelfingen, Switzerland). All other biochemical reagents were purchased from Sigma Chemical (Deisenhofen, Germany). Endotoxin contamination of ascorbic acid solutions was measured with the coagulation Limulus amebocyte lysate assay and was proved to be below the detection limit of the kit (0.05 u/mL).

**Cell cultures.** Human umbilical cord vein endothelial cells (HUVEC) were prepared with 0.05% collagenase and cultured in M199 containing 15% FCS, 5% HS, and 7.5 µg/mL endothelial cell growth supplement. Experiments were carried out with monolayers of the first to second passage. Preincubation of cells with L-ascorbic acid, L-gulonolactone, dehydroascorbic acid, sepiapterin, 2,4-diamino-6-hydroxyprimidine (DAHP), or the combination of TNF-α, IFN-γ, and lipopolysaccharide (LPS) was performed in culture medium. Cell stimulation with ionomycin (2 µmol/L, 15 min) or thrombin (1 U/mL, 15 min) was carried out in Hapes buffer (10 mmol/L Hapes, 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO₄, 10 mmol/L glucose, 1.5 mmol/L CaCl₂, pH 7.4) in the absence of ascorbic acid, sepiapterin, DAHP, or cytokines.

**Measurement of citrulline and cGMP formation.** Endothelial cells were stimulated with ionomycin or thrombin in Hapes buffer (pH 7.4) containing 10 µmol/L
L-[\textsuperscript{3}H]arginine (0.33 Ci/mmol) for the measurement of citrulline formation or 0.5 mmol/L isobutylmethylxanthine for cGMP determinations. The [\textsuperscript{3}H]citrulline generated was separated from [\textsuperscript{3}H]arginine by cation exchange chromatography [Dowex AG50WX-8 (Na\textsuperscript{+} form)] and quantified by liquid scintillation counting (84,85). The cGMP accumulated was measured in cellular extracts by radioimmunoassay following the instructions of the manufacturer.

\textsuperscript{14}C]Ascorbic acid uptake in endothelial cells. Endothelial cells were incubated in culture medium containing 100 µmol/L [\textsuperscript{14}C]ascorbic acid (16 mCi/mmol). After various times, cells were washed with cold Hepes buffer (pH 7.4) containing 100 µmol/L phloretin and lysed with 100 mmol/L NaOH, 2% Na\textsubscript{2}CO\textsubscript{3}, and 1% sodium dodecylsulfate. The radioactivity of cell lysates was measured by liquid scintillation counting.

\textit{Determination of eNOS activity.} Experiments were performed with tetrahydrobiopterin-free eNOS that was expressed in yeast \textit{Pichia pastoris} and purified as described (86). The assay solution (100 µL) contained 50 mmol/L Tris-HCl buffer (pH 7.4), 0.3 µg eNOS, 100 µmol/L L-[\textsuperscript{3}H]arginine (100,000 cpm), 0.5 mmol/L CaCl\textsubscript{2}, 0.2 mmol/L NADPH, 5 µmol/L FAD, 5 µmol/L FMN, 10 µg/mL calmodulin, 10 nmol/L–100 µmol/L tetrahydrobiopterin, and 0.2 mmol/L CHAPS. The [\textsuperscript{3}H]citrulline generated was separated from [\textsuperscript{3}H]arginine by ion exchange columns and quantified as described above.

\textit{Measurement of biopterin derivatives.} Culture medium was collected and endothelial monolayers were detached with trypsin/EDTA (0.05%/0.02%, vol/vol). Aliquots of 5 × 10\textsuperscript{6} cells and 1-mL aliquots of medium were oxidized with 0.02 mol/L KI/I\textsubscript{2} in 0.1 mol/L HCl or in 0.1 mol/L NaOH for 1 h in the dark. The precipitates were removed by centrifugation and excess iodine was destroyed by the addition of 0.02 mol/L ascorbic acid. Quantification of biopterin in supernatants was performed by high-performance liquid chromatography (HPLC) as described (87). The amount of 5,6,7,8-tetrahydrobiopterin was calculated from the difference in biopterin concentrations measured after oxidation in acid (total biopterins) and base (7,8-dihydrobiopterin + biopterin). Additionally, nonoxidized supernatants were used to determine biopterin.

\textit{Determination of GTP cyclohydrolase I (GTP-CH I) expression and activity.} Extraction of total RNA from endothelial cells, electrophoresis on 1% agarose/6% formaldehyde gels, Northern blotting, and hybridization of the blots with 10\textsuperscript{6} cpm/mL [\textsuperscript{32}P]dCTP-labeled probe for human GTP cyclohydrolase I, obtained by polymerase chain reaction using consensus primers to GTP cyclohydrolase I from \textit{Escherichia coli}, mouse and human, were performed according to standard protocols. GTP-CH I activity in cytosolic fractions from endothelial cells was measured as described (87).
Statistical analysis. All data are given as means ± SEM, n = 3–5 independent experiments. To determine the statistical significance of the described results, analysis of variance with Bonferroni’s correction for multiple comparisons or Student’s t-test for paired data was performed. A P value of < 0.05 was accepted as significant.

Results and Discussion

Ascorbic Acid Potentiates NO Synthesis in Endothelial Cells

NO synthesis in our study was measured as the formation of citrulline, which is produced stoichiometrically with NO, and as an accumulation of intracellular cGMP, which is generated when NO activates the soluble guanylate cyclase of the cells. Both parameters are increased after cell stimulation with calcium-mobilizing agonists or shear stress, and this increase can be prevented by eNOS inhibition. To investigate whether ascorbic acid in concentrations corresponding to physiologically achievable plasma levels (26,30,88) affects agonist-induced NO synthesis, we preincubated endothelial cells for 24 h with 0.1–100 µmol/L ascorbate before cell stimulation with ionomycin or thrombin. Figure 5.1 shows that both agonist-induced citrulline and cGMP formation were increased in a dose-dependent fashion by pretreatment of cells with the compound, thus indicating a potentiation of endothelial NO synthesis by ascorbic acid. These results were obtained not only in HUVEC but also in coronary artery endothelial cells (data not shown, see Ref. 84). Our data are also in good agreement with findings reported by Huang et al. (89) who measured a potentiation of A23187-induced cGMP accumulation in ascorbate-pretreated porcine aortic endothelial cells, whereas ascorbic acid did not affect the eNOS-independent cGMP formation.

Because cell stimulation was performed in the absence of extracellular ascorbate, the effect on endothelial NO synthesis was most likely due to an increase of intracellular ascorbic acid concentrations. Indeed, under normal culture conditions, cells are unlikely to be saturated with ascorbic acid because its concentration in culture media is generally low. Using 100 µmol/L [14C]-labeled ascorbate, we found an uptake of the compound into endothelial cells that was time dependent and saturated between 12 and 24 h (Fig. 5.2A). Assuming that ascorbate in nonsupplemented cells was negligible, the maximal intracellular ascorbate concentration was 21.5 ± 3.7 nmol/mg protein as calculated from the specific radioactivity of the added compound. Thus, these data confirm that endothelial cells can accumulate ascorbic acid in the low millimolar range at a medium concentration related to the normal plasma concentration of the antioxidant (42,56). A saturation of intracellular ascorbate levels at an extracellular concentration of 100 µmol/L might also explain the lack of further NO synthesis potentiation with higher ascorbic acid supplements in our study. No differences in ionomycin-induced citrulline production were seen between a 24-h pretreatment of endothelial cells with 100 µmol/L or 1 mmol/L ascorbate (561 ± 40 or 574 ± 40 fmol [3H]citrulline/mg cell protein, respectively, n = 3). The ascorbic acid uptake is most probably mediated by sodi-
um-dependent transporters, which have recently been cloned from rat and human cDNA libraries (90,91). Although these transporters have not yet been characterized in endothelial cells, the involvement of an active transport mechanism for ascorbic acid in these cells has already been demonstrated (56). Interestingly, the time dependence of the ascorbate effect on endothelial NO synthesis followed a kinetics similar to the ascorbate uptake (Fig. 5.2B), thus emphasizing the importance of intracellular ascorbate accumulation for the observed effects of ascorbic acid on NO synthesis. Moreover, our data suggest that the reductive capacity of the compound may be essential for the potentiation of NO formation by ascorbate. The molecular structure of L-ascorbic acid consists of an unsaturated \(\gamma\)-lactone ring with an enediol configuration conjugated with a carbonyl group (Fig. 5.3). L-Gulonolactone, an ascorbic acid precursor molecule, is lacking the redox-active enediol configuration and cannot be transformed into ascorbic acid in human cells.

Fig. 5.1. Influence of ascorbic acid on ionomycin- or thrombin-induced citrulline (left panel) and cGMP (right panel) formation. Endothelial cells from human umbilical veins were preincubated for 24 h with 0.1–100 \(\mu\)mol/L ascorbic acid in culture medium. Subsequently, cells were stimulated with ionomycin (2 \(\mu\)mol/L, 15 min) or thrombin (1 U/mL, 15 min) in Hepes buffer (pH 7.4) containing 10 \(\mu\)mol/L L-[\(^{3}\)H]arginine (0.33 Ci/mmol) for the measurement of citrulline formation or 0.5 mmol/L isobutylmethylxanthine for cGMP determinations. The \(^{3}\)H]citrulline generated was separated from \(^{3}\)H]arginine by cation exchange chromatography and quantified by liquid scintillation counting. The accumulated cGMP was measured in cellular extracts by radioimmunoassay. Data are shown as agonist-induced increases in \(^{3}\)H]citrulline formation or cGMP production calculated from the differences between stimulated and unstimulated cells (means ± SEM, \(n = 4\)); cells with and without ascorbate pretreatment were compared, *\(P \leq 0.05\).
due to the absence of the enzyme gulonolactone oxidase. Accordingly, it did not affect ionomycin-induced citrulline or cGMP synthesis when incubated with endothelial cells. On the other hand, dehydroascorbic acid, which is partially converted back to ascorbate by glutathione-dependent reactions (92), exerted a partial stimulatory effect (Fig. 5.3).

**Fig. 5.2.** Time-dependence of the uptake of $[^{14}C]$ascorbic acid and the effect of ascorbate on ionomycin-induced citrulline and cGMP formation. Endothelial cells were preincubated with 100 µmol/L $[^{14}C]$ascorbic acid (16 mCi/mmol) (A) or 100 µmol/L unlabeled ascorbate (B) for the indicated times. Then, cells were washed, solubilized, and analyzed for the cell-associated radioactivity (A). Alternatively, cells were stimulated with ionomycin (2 µmol/L, 15 min) and either citrulline or cGMP formation was measured (B). Data are shown as cpm incorporated $[^{14}C]$ascorbic acid/mg cell protein (means ± SEM, n = 3) (A) or as ionomycin-induced increases in $[^{3}H]$citrulline or cGMP production calculated from the differences between stimulated and unstimulated cells (means ± SEM, n = 4) (B). *P < 0.05 vs. untreated control cells. Reprinted with permission from Ref. 84.
Ascorbic Acid Enhances the Availability of the NOS Cofactor Tetrahydrobiopterin

The data reported above demonstrated that ascorbic acid potentiates agonist-induced NO formation in cultured endothelial cells in a dose- and time-dependent fashion. The effect was saturated within physiologically relevant concentrations, related to an intracellular ascorbate accumulation and dependent on the redox-active enediol group of ascorbic acid. We next performed experiments to investigate mechanisms responsible for the observed effects. In agreement with other studies (89,93), preincubation of endothelial cells with ascorbate neither induced the expression of eNOS nor affected its subcellular distribution between membrane and cytosolic fractions (data not shown, details in Ref. 84). Similarly, an increased availability of the eNOS substrate L-arginine did not account for the potentiation of NO synthesis because ascorbic acid did not improve the cellular uptake of this amino acid (data not shown, details in Ref. 84). However, we found that the effect of ascorbate on agonist-stimulated citrulline and cGMP production was mimicked by pretreatment of the cells with increasing concentrations of sepiapterin (0.001–10 µmol/L, 24 h) (Fig. 5.4). This compound is readily taken up by cells and converted into tetrahydrobiopterin via a salvage pathway (94). Its potentiating
Effect on endothelial NO formation indicates that eNOS is not saturated with its cofactor tetrahydrobiopterin thus confirming previous studies from cultured endothelial cells (68, 70). Interestingly, sepiapterin abolished the potentiating effect of ascorbic acid on NO production in a concentration-dependent manner (Fig. 5.4), suggesting that ascorbate exerts its effect on NO synthesis only under suboptimal intracellular tetrahydrobiopterin concentrations. Accordingly, we hypothesized that ascorbic acid may either enhance the availability of tetrahydrobiopterin in endothelial cells or increase its affinity for eNOS.

To test the latter possibility, we performed experiments with tetrahydrobiopterin-free eNOS expressed in and purified from Pichia pastoris as described recently (86). The enzyme was inactive in the absence of exogenous tetrahydrobiopterin. The addition of the pteridine (1 nmol/L–100 µmol/L) stimulated the formation of citrulline in a concentration-dependent manner with a 50% effective concentration (EC₅₀) of 0.31 ± 0.036 µmol/L and a maximal effect at ~100 µmol/L (Fig. 5.5). The presence of 100 µmol/L ascorbic acid in the assay solution resulted only in a slight decrease of the EC₅₀ to 0.16 ± 0.014 µmol/L without significant increase in maximal enzyme activity. From these data, we concluded that ascorbate

Fig. 5.4. Influence of sepiapterin on ascorbic acid–induced potentiation of citrulline and cGMP formation. Endothelial cells were preincubated for 24 h with 0.001–10 µmol/L sepiapterin in culture medium in the absence or presence of 100 µmol/L ascorbic acid. Subsequently, cells were stimulated in Hepes buffer (pH 7.4) for 15 min with 2 µmol/L ionomycin and either citrulline or cGMP formation was measured. Data are shown as agonist-induced [³H]citrulline formation or cGMP production calculated from the differences between stimulated and unstimulated cells (means ± SEM, n = 4); cells with and without ascorbate pretreatment were compared, *P < 0.05. Reprinted with permission from Ref. 85.
does not substantially modify the pterin affinity of the enzyme. Interestingly, ascorbate did not activate eNOS in the absence of exogenous tetrahydrobiopterin, indicating that it does not act as a cofactor for eNOS itself.

We next investigated the influence of ascorbic acid on intracellular tetrahydrobiopterin concentrations. We found that the preincubation of endothelial cells with ascorbic acid (100 µmol/L, 24 h) increased intracellular tetrahydrobiopterin levels from 0.38 ± 0.04 to 1.14 ± 0.09 pmol/mg protein (n = 20). The effect of ascorbic acid on endothelial tetrahydrobiopterin levels was concentration dependent and saturable at 100 µmol/L (Fig. 5.6). Thus, there is a close relationship between intracellular ascorbic acid accumulation, the potentiation of agonist-induced citrulline and cGMP synthesis, and the increase of tetrahydrobiopterin levels induced by ascorbate, suggesting that intracellular tetrahydrobiopterin concentration and, consequently, NO formation are critically dependent on the tissue levels of ascorbate. From the data presented in our study, we can speculate that intracellular ascorbate levels of ~2 mmol/L and tetrahydrobiopterin levels in the range of 200 nmol/L provide optimal reaction conditions for NO formation in endothelial cells.
Our results are in agreement with two recent reports showing that an enhanced eNOS activity after ascorbic acid pretreatment was associated with an increase of intracellular tetrahydrobiopterin levels in porcine aortic endothelial cells and HUVEC (89,93). Moreover, conditions that are thought to be associated with tetrahydrobiopterin deficiency (i.e., coronary artery disease or smoking) have been characterized by low ascorbic acid levels in plasma or leukocytes (16,17,95,96) and are known to be associated with an impaired NO-dependent vasodilation. Taken together, these data led us to the suggestion that increased availability of tetrahydrobiopterin could be a common mechanism underlying the improvement of endothelial dysfunction in patients with chronic oral ascorbic acid administration.

**Ascorbic Acid Protects Tetrahydrobiopterin from Oxidation**

The findings presented so far have related the potentiating effect of ascorbic acid on endothelial NO synthesis to an increase in intracellular tetrahydrobiopterin levels. We next attempted to understand whether the improved availability of tetrahydrobiopterin was due to an enhanced synthesis or to a decreased degradation of the compound. Tetrahydrobiopterin is synthesized *de novo* from GTP by the sequential action of three enzymes, GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase, and sepi-
apterin reductase (Fig. 5.7). GTP cyclohydrolase I has been shown to be the rate-limiting enzyme of the *de novo* pathway and to be regulated by cytokines such as TNF-α, IFN-γ, and interleukin-1β in a number of cell types including endothelial cells (68,70,97,98). We first investigated whether ascorbic acid affects the expression and activity of GTP cyclohydrolase I. Because the expression of this enzyme in endothelial cells is generally low, experiments were carried out with cells pretreated without and with cytokines (250 U/mL TNF-α, 250 U/ml IFN-γ and 1 µg/mL LPS, 24 h) to induce enzyme expression. Figure 5.8A shows that the mRNA expression of GTP cyclohydrolase I was upregulated by cytokines but no differences were seen between ascorbate-treated cells and their respective controls. Similarly, ascorbic acid did not affect GTP cyclohydrolase I activity when added to cytosolic fractions of cytokine-treated cells, suggesting that the compound does not act as a direct cofactor of the enzyme (Fig. 5.8B). These results suggest that the effect of ascorbic acid on intracellular tetrahydrobiopterin level is not due to an increased synthesis of the compound. Accordingly, inhibition of tetrahydrobiopterin formation by DAHP, an inhibitor of GTP cyclohydrolase I (99), did not prevent the ascorbate-mediated increase of the pteridine although it substantially decreased tetrahydrobiopterin levels in both control and ascorbic acid-treated endothelial cells (Fig. 5.9). In parallel, ionomycin-stimulated formation of citrulline and cGMP was decreased upon pretreatment of the cells with

**Fig. 5.7.** Biosynthetic pathways and oxidative degradation of 5,6,7,8-tetrahydrobiopterin.

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DAHP in controls and in the presence of ascorbic acid, but the potentiating effect of ascorbate was maintained (Fig. 5.9).

Because ascorbic acid did not affect tetrahydrobiopterin synthesis, we speculated that it might act by preventing the degradation of the compound. 5,6,7,8-Tetrahydrobiopterin is oxidized intracellularly to the quinonoid 6,7-[8H]-dihydrobiopterin, which spontaneously rearranges to 7,8-dihydrobiopterin (100). The latter is further degraded to biopterin (Fig. 5.7). To investigate whether ascorbic acid prevents degradation of tetrahydrobiopterin, the levels of tetrahydrobiopterin, 7,8-dihydrobiopterin, and biopterin in cells and cell supernatants were measured and balanced on the basis of pmol pteridines/dish. The experiments were performed in cells coincubated with ascorbate (1–100 µmol/L ascorbic acid, 24 h) and cytokines (250 U/mL TNF-α, 250 U/mL IFN-γ and 1 µg/mL LPS, 24 h) to increase pteridine production. Fig. 5.10 shows that the sum of all biopterin derivatives in cells and media was not influenced by ascorbate, confirming the lack of an effect on pterin biosynthesis. However, the ascorbic acid–induced increase of intracellular tetrahydrobiopterin was paralleled by a decrease of 7,8-dihydrobiopterin + biopterin in cells and cell supernatants. Interestingly, ~90% of the dihydrobiopterin + biopterin formed in endothelial cells

Fig. 5.8. Effect of ascorbic acid on GTP cyclohydrolase mRNA expression and activity. (A) Total RNA was extracted from endothelial cells preincubated for 24 h with 100 µmol/L ascorbic acid in the absence or presence of a mixture of cytokines (250 U/mL tumor necrosis factor-α, 250 U/mL interferon-γ) and lipopolysaccharide (1 µg/mL). After electrophoresis on 1% agarose/6% formaldehyde gels (20 µg/lane) the RNA was blotted on nylon membranes and hybridized overnight with [32P]dCTP-labeled probes for human GTP cyclohydrolase I (GTP-CH I) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). One typical experiment out of three is shown. (B) GTP-CH I activity was measured in cytosolic fractions from cytokine-treated endothelial cells in the absence or presence of 100 µmol/L ascorbate. Data are expressed as pmol neopterin/(mg cytosolic protein·min) (means ± SEM, n = 3). Panel (A) was reprinted with permission from Ref. 85.

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was released into the medium in control cells, whereas tetrahydrobiopterin was not detectable in supernatants of both control and ascorbate-treated cells. Our data suggest that a chemical stabilization of the fully reduced pterin by ascorbate is the underlying mechanism for the increased intracellular tetrahydrobiopterin concentration. The stabilizing function of ascorbate is most likely due to a chemical reduction of the quinonoid 6,7-[8H]-dihydrobiopterin to tetrahydrobiopterin, which had already been demonstrated for other reducing compounds such as dithioerythritol and NADPH. Moreover, Toth and co-workers (101) confirmed in a recent study that ascorbate mediated the reductive reversal of the autoxidation process of tetrahydrobiopterin. Another possible mechanism is the regeneration of tetrahydrobiopterin from its trihydrobiopterin radical by ascorbate, which has been shown in in vitro experiments (102). This action could possibly also support eNOS activation because the trihydrobiopterin radical is formed in the catalytic mechanism of NOS. However, the protective effect of ascorbate in our study was prominent in intact cells and only minimal with the purified eNOS, although this was possibly due to the presence of reducing

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**Fig. 5.9.** Influence of 2,4-diamino-6-hydroxypyrimidine (DAHP) on ascorbic acid–mediated increase of tetrahydrobiopterin levels and potentiation of citrulline and cGMP formation. Endothelial cells were preincubated for 24 h with 0.5–1.0 mmol/L DAHP in culture medium in the absence or presence of 100 µmol/L ascorbic acid. Subsequently, cells were stimulated for 15 min in Hepes buffer (pH 7.4) with 2 µmol/L ionomycin and processed for either citrulline or cGMP measurement. Additionally, biopterin levels were measured by reversed-phase high-performance liquid chromatography after oxidation of cells with 0.02 mol/L KI/I\(_2\) in 0.1 mol/L HCl or 0.1 mol/L NaOH; tetrahydrobiopterin was calculated from the difference. Data are shown as means ± SEM (n = 4). Controls and DAHP-treated cells (*) and cells with and without ascorbate pretreatment (+) were compared, **P < 0.05. Reprinted with permission from Ref. 85.
agents in the enzyme assay. On the other hand, a stimulating ascorbate effect has been seen in lysates from HUVEC and in bovine eNOS preparations with higher ascorbate concentrations and under different assay conditions (89,93). It is not yet known which agents cause an increased tetrahydrobiopterin oxidation in vivo. In vitro studies have shown that tetrahydrobiopterin is a primary target for peroxynitrite-catalyzed oxidation but does not significantly react with hydrogen peroxide (103). Interestingly, a cell membrane-permeable superoxide dismutase mimetic was not able to increase tetrahydrobiopterin levels in endothelial cells, although a significant effect of ascorbic acid was observed in that study (93). Thus, superoxide anion might not be involved in tetrahydrobiopterin oxidation in endothelial cells and moreover, the stabilizing effect

**Fig. 5.10.** Effect of ascorbic acid on tetrahydrobiopterin stability in intact cells. Endothelial cells were preincubated for 24 h with 250 U/mL tumor necrosis factor-α, 250 U/mL interferon-γ and 1 µg/mL lipopolysaccharide in the absence or presence of 1–100 µmol/L ascorbic acid. Aliquots of 2 × 10⁶ cells and 1-mL aliquots of cell supernatants were oxidized with 0.02 mol/L KI/I₂ in 0.1 mol/L HCl or 0.1 mol/L NaOH and the resulting biopterin was quantified by reversed-phase high-performance liquid chromatography. Biopterin levels after oxidation in base indicate the amount of dihydrobiopterin + biopterin, whereas tetrahydrobiopterin was calculated from the difference in biopterin concentration after oxidation in acid and base. To balance biopterin derivatives in cells and medium, pteridine levels were calculated in pmol/dish. Data are shown as means ± SEM from 3 experiments. *P < 0.05 vs. levels of biopterin derivatives in untreated control cells. Reprinted with permission from Ref. 85.

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of ascorbate on tetrahydrobiopterin may not involve superoxide scavenging. Finally, in this context, it is worth noting that the increased availability of tetrahydrobiopterin in endothelial cells saturated with ascorbic acid will not only potentiate NO formation but also decrease superoxide formation by eNOS by preventing uncoupling of oxygen reduction and arginine oxidation (86,104,105), thereby further decreasing oxidative stress in endothelial cells.

Summary

There is strong indication that a decreased bioavailability of endothelium-derived NO predisposes to atherosclerosis and related disease states. Among other factors, oxidative stress might be responsible for the development of endothelial dysfunction, implying that atherogenesis and progression of atherosclerosis can be inhibited by antioxidants. This concept is supported by the consistent finding that ascorbic acid can facilitate endothelium-dependent vasodilation. The data presented and discussed in this paper provide evidence that one of the possible mechanisms by which ascorbate might prevent or ameliorate endothelial dysfunction is its potentiating effect on endothelial NO synthesis. This is due to the ability of ascorbic acid to protect tetrahydrobiopterin, an essential cofactor of eNOS, from oxidation and requires saturated intracellular ascorbate levels as well as the reductive capacity of the compound. The role of ascorbic acid in increasing eNOS activity and possibly preventing endothelial dysfunction could provide a rationale for optimizing its dietary intake or for oral ascorbate supplementation and must be confirmed in clinical long-term studies. In this context, it seems to be important that not only plasma levels but also tissue concentrations (in leukocytes for example) of ascorbic acid will be measured to evaluate the body status of ascorbate and its relationship to NO synthesis and bioavailability.

Acknowledgments

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References


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Primarily as an S-Nitroso Adduct of Serum Albumin, Proc. Natl. Acad. Sci. USA 89,
7674–7677.
Loscalzo, J. (1994) In Vivo Transfer of Nitric Oxide Between a Plasma Protein-Bound
the Metabolism of S-Nitroso-glutathione, FEBS Lett. 389, 149–152.
the Biological Activity of S-Nitrosoglutathione, Hypertension 36, 291–295.
Interaction of Superoxide and Nitric Oxide Only at Very High Physiological
Human Umbilical Vein Endothelial Cells and Its Effect on Oxidant Insult, Biochem.
Pharmacol. 50, 1339–1346.
57. Mayer, B., and Hemmens, B. (1997) Biosynthesis and Action of Nitric Oxide in
Nitric Oxide Synthase, Biochemistry 63, 734–743.
Structure, Function and Inhibition, Biochem. J. 357, 593–615.
Oxide Synthase Activity, Cardiovasc. Res. 43, 274–278.
Strategy in the Treatment of Endothelial Dysfunction? Am. J. Physiol. 280,
H2448–H2488.
64. Feron, O. (1999) Intracellular Localization and Activation of Endothelial Nitric Oxide
65. Kone, B.C. (2000) Protein-Protein Interactions Controlling Nitric Oxide Synthases,
Impairment of Endothelial-Dependent Arterial Relaxation by Lysolecithin in Modified
Tetrahydrobiopterin-Dependent Formation of Endothelium-Derived Relaxing Factor
(Nitric Oxide) in Aortic Endothelial Cells, Biochem. J. 281, 297–300.
68. Werner-Felmayer, G., Werner, E.R., Fuchs, D., Hausen, A., Reibnegger, G., Schmidt,


Chapter 6

Serum Ascorbic Acid and Disease Prevalence in U.S. Adults: The Third National Health and Nutrition Examination Survey (NHANES III)

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Introduction

Ascorbic acid is an essential nutrient required for multiple biologic functions. During the course of evolution, humans along with other primates lost the ability for the hepatic biosynthesis of ascorbic acid due to a mutation in L-gulonolactone oxidase, the enzyme that controls the conversion of glucose to ascorbic acid (1,2). This inborn error of carbohydrate metabolism for our species has resulted in the dependence of humans on dietary consumption to achieve blood and tissue levels sufficient for the maintenance of health (1). The clinical features of scurvy, resulting from severe ascorbic acid deficiency, are well described (3). In addition to preventing scurvy, ascorbic acid functions as a water-soluble antioxidant (4) and has other important biologic actions that may be unrelated to its antioxidant properties.

To examine the relation of ascorbic acid to a number of health conditions, we undertook an examination of data collected in the Third National Health and Nutrition Examination Survey (NHANES III), a cross-sectional survey based on a probability sample of the U.S. population conducted between 1988 and 1994 by the National Center for Health Statistics (5). Although NHANES III collected dietary intake information using a 24-h recall and a food-frequency questionnaire, it importantly also measured serum ascorbic acid levels using modern high-performance liquid chromatography (HPLC) (6), an improvement over the previous colorimetric assay employed in NHANES II (7). Having serum levels of ascorbic acid, which reflect dietary and supplement intake, permitted a more precise and accurate estimation of the relation between ascorbic acid and health conditions than might typically be available using dietary intake estimations only. This chapter will review and summarize the findings of our research efforts using the NHANES III data set and will present some new findings relating to the association between ascorbic acid and nontraditional cardiovascular disease (CVD) risk factors.
Serum Ascorbic Acid and Cardiovascular Disease

Ascorbic acid may reduce the risk of CVD through several mechanisms. First, ascorbic acid is a highly effective water-soluble antioxidant (4) capable of inhibiting lipid peroxidation (8–12), which has been hypothesized to be an important factor in atherogenesis (13,14). Some studies have reported that ascorbic acid may increase concentrations of high density lipoprotein cholesterol and decrease concentrations of total cholesterol, at least under certain conditions (15–18). Ascorbic acid also promotes endothelial prostacyclin (19–21) [a prostaglandin that decreases vascular tone and inhibits platelet aggregation (22)] and nitric oxide production, thereby resulting in vasodilatation (23, 24).

An association between ascorbic acid status and CVD, however, has been reported inconsistently (15,25). James Lind’s (3) descriptions of sudden cardiac death among sailors with scurvy in 1757 provide the earliest intriguing evidence that ascorbic acid status may influence coronary heart disease (CHD) events. Recently published longitudinal data from the NHANES II Mortality Study, in fact, suggest that low to marginally low serum levels of ascorbic acid may indeed be a risk factor for CHD mortality (25).

In our examination of data from the earlier NHANES II, conducted between 1976 and 1980, we reported that serum ascorbic acid levels were independently associated with a decreased prevalence of self-reported CHD (defined as angina and myocardial infarction) and stroke; a 0.5 mg/dL increase in serum ascorbic acid level was associated with an 11% reduction in CHD and stroke prevalence (26). In NHANES III, we detected an interaction between serum ascorbic acid concentration and alcohol intake; thus, we performed analyses stratified by drinking status (27). Among participants who reported no alcohol consumption, serum ascorbic acid concentrations were not associated with CVD prevalence. However, among participants who consumed alcohol, serum ascorbic acid concentrations consistent with tissue saturation (1.0–3.0 mg/dL) were associated with a decreased prevalence of angina [multivariate odds ratio (OR) = 0.48; 95% confidence interval (CI) 0.23–1.03; \( P \) for trend = 0.06], but were not significantly associated with myocardial infarction or stroke prevalence. Because the metabolism of alcohol may be linked to ascorbic acid status, a biologic interaction is indeed plausible (28). Blood ascorbic acid concentrations have been reported to be strongly correlated with the activity of hepatic alcohol dehydrogenase, the principal enzyme in alcohol metabolism (29), and both alcohol and ascorbic acid are known to have vasodilatory properties (19,30). It is conceivable, therefore, that alcohol and ascorbic acid may interact synergistically to affect angina, as we found in NHANES III.

Serum Ascorbic Acid and Gallbladder Disease

Gallbladder disease is highly prevalent among adult Americans, and as many as 20 million Americans are estimated to have gallstones (31). Most gallstones are composed either partially or entirely of cholesterol (31–33) and form when bile that is supersaturated with cholesterol becomes destabilized (34). In the guinea pig, an animal that like humans lacks the ability to biosynthesize ascorbic acid (35), ascorbic acid affects the activity of cholesterol 7α-hydroxylase, the enzyme regulating the rate-
limiting step in the catabolism of cholesterol to bile acids (36–39). Ascorbic acid supplementation increases cholesterol 7α-hydroxylase activity by as much as 15-fold compared with ascorbic acid–deficient guinea pigs (39), which typically develop cholesterol gallstones (40–43). Because of the animal evidence and the observation that gallbladder disease risk factors in humans are frequently associated with ascorbic acid status, I hypothesized in 1993 that ascorbic acid status might also be a risk factor for human gallbladder disease (44).

To ascertain whether ascorbic acid status is associated with gallbladder disease in humans, and particularly with the presence of asymptomatic gallstones, we analyzed data collected in NHANES III that included serum ascorbic acid levels and information on gallbladder disease among >13,000 American adults. A total of 11% of women and 4% of men had a history of clinical gallbladder disease (i.e., either symptomatic gallstones or a cholecystectomy). Of the NHANES III participants without a history of clinical gallbladder disease (or abdominal pain consistent with gallbladder disease), 8% of women and 6% of men had asymptomatic gallstones. Among women, serum ascorbic acid level was inversely related to the prevalence of clinical and asymptomatic gallbladder disease independent of other gallbladder disease risk factors, such as age, race, diet, and body mass index. Each 0.5 mg/dL increase in serum ascorbic acid levels was associated with an ~13% lower prevalence of both clinical gallbladder disease ($P = 0.006$) and asymptomatic gallstones ($P < 0.05$) (45). We found no significant relation between serum ascorbic acid level and gallbladder disease among men although the OR estimates were consistent with a small protective effect (0.97 for clinical gallbladder disease and 0.91 for asymptomatic gallstones). The findings among men may reflect the lower prevalence of gallbladder disease among men and, as a consequence, the associated reduced statistical power to detect such an association.

The data on asymptomatic gallstones are particularly important. From the NHANES III cross-sectional data, we cannot exclude the possibility that participants changed their diets after being told they had gallbladder disease. Although it seems unlikely that women would consume less ascorbic acid–containing foods and supplements as a consequence of learning of gallbladder disease (thereby producing our findings), this possibility cannot be excluded. However, because NHANES III also collected abdominal ultrasound data on its participants, our findings relating to the presence of asymptomatic gallstones strengthen the hypothesis that ascorbic acid status may indeed be an important risk factor for gallstone formation, at least among women. Furthermore, the magnitude of the association between serum ascorbic acid levels and gallbladder disease prevalence was the same for both clinical and asymptomatic gallbladder disease among women, providing additional circumstantial evidence that the observed association reflects a true biological relationship. These NHANES III findings are the fourth study to report an association between ascorbic acid intake or blood levels and prevalence of gallbladder disease in women (45–48). It is the only one to have data on the presence of asymptomatic gallstones.
Serum Ascorbic Acid, Bone Mineral Density, and Fractures

Osteoporosis, affecting both women and men, is highly prevalent among older Americans, and is an important risk factor for clinical fractures (49). Ascorbic acid deficiency has been associated with decreased collagen synthesis and bone mineral density (BMD) in a few experimental animal studies (50,51). In animals, such deficiency affects vitamin D metabolism and binding and, in turn, the risk of osteoporosis (52). Ascorbic acid has also been reported to affect markers of osteoblast activity (53). In humans, some observational studies (54–61), but not all (62) have reported an association between ascorbic acid intake or blood levels and BMD. Although marked abnormalities in bone metabolism and growth have been described among children with scurvy (63), the relation of ascorbic acid to BMD over a wide range of intakes and blood levels has not been reported previously among a representative sample of the U.S. population. On the basis of the observation that ascorbic acid is a nutrient essential for collagen formation and normal bone development (63), we hypothesized that ascorbic acid status would be associated with BMD.

We analyzed data collected in NHANES III to examine whether dietary ascorbic acid intake and serum ascorbic acid levels were associated with BMD (measured at the proximal femur) and the prevalence of self-reported fractures of the hip, wrist, and spine (64). Because other investigators have described possible interactions between ascorbic acid and calcium intake (60), smoking (61), and postmenopausal estrogen therapy (59) on BMD or fracture, we also were interested in exploring whether these factors modified the association between ascorbic acid, BMD, and self-reported fractures. We identified three-way interactions between smoking, history of estrogen use, and dietary and serum ascorbic acid among postmenopausal women and therefore, analyzed the data for postmenopausal women stratified by smoking and estrogen use. We found that dietary ascorbic acid intake was independently associated with greater BMD among premenopausal women \( (P = 0.002) \). Among men, serum ascorbic acid was associated in a nonlinear fashion with BMD \( (P < 0.05) \), and dietary ascorbic acid intake was associated in a nonlinear fashion with self-reported fracture \( (P = 0.05) \) (see Fig. 6.1). Among postmenopausal women without a history of smoking or estrogen use, serum ascorbic acid was unexpectedly associated with lower BMD \( (P = 0.01) \). However, among postmenopausal women with a history of both smoking and estrogen use, serum ascorbic acid was associated with a 49% decrease in fracture prevalence \( (P = 0.001) \).

Ascorbic acid deficiency has been associated with osteoporosis in Black South African men in several older studies (54–56). More recent studies have examined dietary and supplement intake of ascorbic acid as a correlate of BMD (58–60, 65–68) or hip fracture (61). With the exception of the Honolulu Heart Study, which included men of Japanese ancestry (65), these other studies included only women (58–61,66–68) and none examined the relation of blood ascorbic acid levels to BMD or fracture. Our findings suggest that the relation between ascorbic acid, BMD, and prevalence of fractures differs between men and women and may be modified among postmenopausal women by use of tobacco and postmenopausal hormones.
Fig. 6.1. Serum ascorbic acid and hip bone mineral density (A) and dietary ascorbic acid and self-reported fracture (B) among men. The relation of predicted hip bone mineral density (BMD) (gm/cm²) among men enrolled in the Third National Health and Nutrition Examination Survey (NHANES III), 1988–1994 is expressed as a function of serum ascorbic acid (mg/dL) (A) and that of self-reported fracture as a function of dietary ascorbic acid intake (B). The figures are based on a multivariate model that adjusted for age, race, level of education, physical activity, body mass index, use of thiazide diuretics, dietary intake of calories, fat, protein, calcium, caffeine, and alcohol; history of smoking (never/past/current), history of diabetes, and serum levels of thyroid stimulating hormone, vitamin D, and vitamin E. Multivariate analyses revealed significant linear and quadratic terms for serum ascorbic acid and dietary ascorbic acid intake (both \( P < 0.05 \)).
Serum Ascorbic Acid and Lead Poisoning

Lead pollution is an important public health problem (69). Because millions of American children are believed to have elevated blood lead levels, screening programs for childhood lead exposure have been established by the Centers for Disease Control (69). In 1984, as many as 3–4 million children were estimated to have blood lead levels >15 µg/dL (70) and even lower levels of lead exposure among children have been associated with adverse neuropsychological development (71). Work-related lead exposure has also been targeted as an area of concern by the Occupational Safety and Health Administration (69).

Calcium EDTA and other chelators are standard treatments for lead poisoning. Several animal studies have examined the effect of ascorbic acid on lead toxicity. In rats fed a lead-containing diet, combined dietary supplementation with iron and ascorbic acid prevented growth depression and anemia, and lowered detectable lead levels in liver, kidney, and bone (72). These beneficial effects, however, were transient (73). Comparing the chelating effects of oral ascorbic acid and parenterally administered EDTA in lead-poisoned rats, Goyer and Cherian (74) reported that ascorbic acid and EDTA had equivalent chelating properties. The few published older studies among humans have yielded inconsistent results. Two case series reported significant clinical improvement among 337 workers with occupational lead exposure after daily administration of 100 mg of ascorbic acid (75,76). In an uncontrolled trial, the combined administration of zinc and ascorbic acid was reported to have reduced blood lead levels among 1000 psychiatric outpatients (77). In another study of 85 subjects who volunteered to consume a lead-containing drink, ascorbic acid supplementation produced small reductions in lead retention (78). Two other small clinical trials, however, concluded that ascorbic supplementation did not lower blood lead levels (79,80). In one of these studies, 52 subjects were assigned to receive either ascorbic acid supplementation or placebo (79). Although reported to have no effect, 8 wk of ascorbic acid supplementation resulted in (nonsignificant) improvement. Compared with the placebo group, subjects treated with ascorbic acid were more likely to have a decrease of >5 µg/dL in blood lead level (relative risk = 0.64; 95% CI, 0.36–1.11; \(P = 0.10\)). In the second study of 45 men (80), 3 mo of ascorbic acid treatment resulted in (nonsignificant) 11–23% lower blood lead levels compared with levels in the placebo-treated group. It is possible that these two studies might have yielded significant results had larger numbers of participants been enrolled. Most importantly, a recent small randomized trial by Dawson and colleagues that studied 75 male smokers revealed that participants randomized to receive 1000 mg of ascorbic acid daily had an 81% decrease in their blood lead levels after 4 wk of treatment (\(P < 0.001\)) (81). Because there was no difference in urinary lead levels, the authors concluded that ascorbic acid supplementation likely decreased the absorption of lead from the gastrointestinal tract.

To ascertain whether ascorbic acid status was associated with blood lead levels and particularly with prevalence of elevated blood lead levels, we analyzed data collected in NHANES III that included serum ascorbic acid levels and blood lead levels
for >19,000 Americans. To our knowledge, there have been no previous reports examining the relation between ascorbic acid and lead toxicity among a population-based sample of American adults and youth.

We found that a total of 57 adults (0.4%) and 22 youth (0.5%) (6–17 y old) had elevated lead levels (≥20 µg/dL for adults and ≥15 µg/dL for youth). Blood lead levels ranged from 0.5 to 56 µg/dL among adults and from 0.5 to 48.9 µg/dL among youth (82). Among youth, unadjusted, age-adjusted, and multivariate models revealed that serum ascorbic acid levels were inversely associated with prevalence of elevated blood lead levels. Youth in the highest serum ascorbic acid tertile were 89% less likely (95% CI, 65–96) to have elevated blood lead levels compared with youth in the lowest serum ascorbic acid tertile (multivariate \( P \) for trend < 0.002). Among adults, serum ascorbic acid level was also associated with prevalence of elevated blood lead levels; all models revealed that serum ascorbic acid levels were inversely associated with the prevalence of elevated blood lead levels. Compared with adults in the lowest serum ascorbic acid tertile, adults in the upper two tertiles were ~65–68% less likely to have elevated blood lead levels (multivariate \( P \) for trend = 0.03); ~4% of youth and 2% of adults with the lowest serum ascorbic acid levels had elevated blood lead levels (see Fig. 6.2).

In the context of the recent report by Dawson and colleagues (81) and the previous, albeit inconsistent, older reports regarding the effect of ascorbic acid on blood lead levels, we believe that the relation that we observed in NHANES III is likely real and not the result of residual confounding or bias. The increased consumption of ascorbic acid–containing foods and supplements represents a low-risk and potentially highly efficacious public health intervention with the potential to lower blood lead levels of the American population, including those segments of the population most at risk.

**Serum Ascorbic Acid and Nontraditional CVD Risk Factors**

The antioxidant theory of atherosclerosis hypothesizes, in part, that the oxidative modification of low density lipoprotein cholesterol over time results in vascular damage and ultimately, atherosclerosis (13,83). If this theory is correct, low blood levels of antioxidants, such as ascorbic acid, may be important modifiable risk factors for vascular disease (84). Traditional CVD risk factors, such as high blood cholesterol, hypertension, diabetes, and smoking, only partially account for CVD incidence, and additional risk factors continue to be identified. Some of these nontraditional CVD risk factors include markers of inflammation, such as C-reactive protein, plasma fibrinogen, and leukocyte count (85). The mechanisms linking some other factors, such as serum uric acid, homocysteine, creatinine, and albumin, to CVD have yet to be fully explained.

Because antioxidant status may be correlated with inflammation (86–88), and additionally, because several studies have indicated that ascorbic acid may have a uricosuric effect (89–91), we were interested in examining the relation of ascorbic acid to several nontraditional CVD risk factors. We postulated that if significant associations were identified, then controlling for differences in ascorbic acid status might be
Fig. 6.2. The relation between serum ascorbic acid concentration in mg/dL and prevalence of elevated blood lead levels among (A) 4213 youth ages 6–16 y and (B) 15,365 adults age ≥ 17 y enrolled in the Third National Health and Nutrition Examination Survey (NHANES III), 1988–1994 based on a multivariate model.
important when considering whether these nontraditional risk factors were independent predictors of CVD. To ascertain whether serum ascorbic acid levels are associated with nontraditional CVD risk factors, we again analyzed data collected from NHANES III.

A total of 7345 women and 6390 men, aged 20–90 y enrolled in NHANES III with complete data were available for these analyses. Analyses of the relation of serum ascorbic acid levels to nontraditional CVD risk factors are presented in Table 6.1 for women and Table 6.2 for men. Among both women and men, serum ascorbic acid levels were inversely associated with serum levels of creatinine ($P$ for trend $< 0.001$) and homocysteine (both $P$ for trend $\leq 0.02$) after adjustment for potential confounders. Among women, serum ascorbic acid levels were also inversely associated with plasma levels of fibrinogen ($P$ for trend $< 0.01$), whereas among men, serum ascorbic acid levels were directly associated with higher levels of serum albumin ($P$ for trend $= 0.02$). We found no association, however, between serum ascorbic acid levels and levels of serum uric acid or white blood cell count.

Most participants in NHANES III had nondetectable C-reactive protein levels. Using multivariable logistic regression models, we examined whether serum ascorbic acid levels were associated with detectable levels of C-reactive protein. Compared with women in the lowest serum ascorbic acid quartile, women in the highest quartile had a (nonsignificant) decreased odds of having detectable levels of C-reactive protein ($OR = 0.81; 95\% CI, 0.64–1.02; P = 0.08$). Using multivariable linear regression models, serum ascorbic acid was inversely associated with C-reactive protein levels

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**TABLE 6.1**

Relation of Serum Ascorbic Acid to Nontraditional Cardiovascular Disease Risk Factors Among 7345 Women Enrolled in the Third National Health and Nutrition Survey (NHANES III) 1988(1994)

<table>
<thead>
<tr>
<th>Quartile of serum ascorbic acid</th>
<th>(n = 1894)</th>
<th>(n = 1835)</th>
<th>(n = 1791)</th>
<th>(n = 1825)</th>
<th>$P$ for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (mg/dL)</td>
<td>0.24</td>
<td>0.68</td>
<td>0.97</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>(Range)</td>
<td>(0.0–0.47)</td>
<td>(0.48–0.83)</td>
<td>(0.84–1.11)</td>
<td>(1.12–2.95)</td>
<td></td>
</tr>
<tr>
<td>Adjusted mean levels$^a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log serum albumin (g/dL)</td>
<td>1.48</td>
<td>1.48</td>
<td>1.49</td>
<td>1.49</td>
<td>0.16</td>
</tr>
<tr>
<td>Log serum creatinine (mg/dL)$^b$</td>
<td>-0.028</td>
<td>-0.034</td>
<td>-0.054</td>
<td>-0.060</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Plasma fibrinogen (mg/dL)$^b$</td>
<td>302.7</td>
<td>298.2</td>
<td>293.5</td>
<td>286.5</td>
<td>$&lt;0.01$</td>
</tr>
<tr>
<td>Log serum homocysteine (µmol/L)$^b$</td>
<td>2.12</td>
<td>2.06</td>
<td>2.01</td>
<td>1.98</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Log serum uric acid (mg/dL)</td>
<td>1.73</td>
<td>1.74</td>
<td>1.74</td>
<td>1.71</td>
<td>0.11</td>
</tr>
<tr>
<td>Log leukocyte count ($\times 10^9$/L)</td>
<td>1.82</td>
<td>1.82</td>
<td>1.81</td>
<td>1.81</td>
<td>0.30</td>
</tr>
</tbody>
</table>

$^a$Adjusted for age, race, body mass index, level of physical activity, current tobacco use, level of education, history of hypertension, history of diabetes, alcohol consumption, and aspirin use in the prior month. Uric acid analyses are additionally adjusted for diuretic use; homocysteine analyses are additionally adjusted for red blood cell folate, dietary intake of vitamin B$_{6}$, and serum vitamin B$_{12}$ levels.

$^b$The number of women participants with complete data available for these analyses was 7329 for serum creatinine, 4085 for plasma fibrinogen, and 3605 for serum homocysteine.
TABLE 6.2  
Relation of Serum Ascorbic Acid to Nontraditional Cardiovascular Disease Risk Factors  

<table>
<thead>
<tr>
<th>Quartile of serum ascorbic acid</th>
<th>(n = 1638)</th>
<th>(n = 1587)</th>
<th>(n = 1614)</th>
<th>(n = 1551)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (mg/dL)</td>
<td>0.16</td>
<td>0.52</td>
<td>0.81</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>(Range)</td>
<td>(0.0–0.32)</td>
<td>(0.33–0.67)</td>
<td>(0.68–0.95)</td>
<td>(0.96–2.87)</td>
<td></td>
</tr>
<tr>
<td><strong>Adjusted mean levels</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log serum albumin (g/dL)</td>
<td>1.52</td>
<td>1.53</td>
<td>1.53</td>
<td>1.54</td>
<td>0.02</td>
</tr>
<tr>
<td>Log serum creatinine (mg/dL)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.231</td>
<td>0.229</td>
<td>0.214</td>
<td>0.198</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma fibrinogen (mg/dL)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>287.9</td>
<td>286.5</td>
<td>287.4</td>
<td>286.8</td>
<td>0.90</td>
</tr>
<tr>
<td>Log serum homocysteine (µmol/L)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.38</td>
<td>2.37</td>
<td>2.31</td>
<td>2.30</td>
<td>0.02</td>
</tr>
<tr>
<td>Log serum uric acid (mg/dL)</td>
<td>1.95</td>
<td>1.98</td>
<td>1.97</td>
<td>1.94</td>
<td>0.29</td>
</tr>
<tr>
<td>Log leukocyte count (×10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>1.82</td>
<td>1.79</td>
<td>1.80</td>
<td>1.79</td>
<td>0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Adjusted for age, race, body mass index, level of physical activity, current tobacco use, level of education, history of hypertension, history of diabetes, alcohol consumption, and aspirin use in the prior month. Uric acid analyses are additionally adjusted for diuretic use; homocysteine analyses are additionally adjusted for red blood cell folate, dietary intake of vitamin B<sub>6</sub>, and serum vitamin B<sub>12</sub> levels.  

<sup>b</sup>The number of men participants with complete data available for these analyses was 6371 for serum creatinine, 3738 for plasma fibrinogen, and 2721 for serum homocysteine.

Among women with measurable levels (P < 0.001). Men in the highest serum ascorbic acid quartile had a significant decrease in the odds of having detectable levels of C-reactive protein compared with men in the lowest serum ascorbic acid quartile (OR = 0.67; 95% CI, 0.49–0.91; P = 0.01). Similar to the findings among women, serum ascorbic acid levels were independently and inversely associated with C-reactive protein levels among men with measurable levels (P < 0.01).

**Serum Ascorbic and Helicobacter pylori**

Chronic infection with *Helicobacter pylori* is an important risk factor for peptic ulcer disease (92) and possibly gastric cancer (93,94). Some epidemiologic studies have also linked lower dietary ascorbic acid consumption with an increased risk for gastric cancer (95,96). In one animal study, increased consumption of ascorbic acid inhibited the growth of *Helicobacter pylori* (97). To ascertain whether serum ascorbic acid is associated with serologic evidence of infection with *Helicobacter pylori* and particularly with strains expressing the cagA virulence factor, we recently analyzed data collected from NHANES III that included serum ascorbic acid levels and *Helicobacter pylori* serology for >6000 American adults (unpublished results).

**Conclusions**

Although ascorbic acid functions as an important water-soluble antioxidant, it has effects separate and apart from its antioxidant properties. We used the NHANES III
data set to examine the relation of ascorbic acid to CVD and nontraditional CVD risk factors, gallbladder disease, lead toxicity, bone mineral density, and serologic evidence of infection with Helicobacter pylori.

These analyses have a number of strengths and limitations. First, NHANES III data are available for analysis to the general public for analysis, with the caveat that special statistical software and programming expertise are required. Because NHANES III surveyed a large probability sample of Americans using standardized questionnaire and laboratory protocols, findings derived from NHANES III should be generalizable to the U.S. population. Quantitative dietary information was limited by a single 24-h dietary recall. However, NHANES III measured serum ascorbic acid levels using modern HPLC on a very large sample of the U.S. population, thereby permitting a more reliable assessment of ascorbic acid status as a correlate of disease. However, as with other observational epidemiologic studies, we cannot exclude the possibility of residual confounding. Finally, the cross-sectional nature of survey data mandates that inferences regarding causality be made cautiously.

Data generated from analyses of observational epidemiologic studies, such as those from NHANES III, have to be integrated with evidence from bench research and animal studies. In the final analysis, hypotheses generated from such analyses will still require (when ethical and feasible) to be confirmed in humans using modern clinical trial methodology.

References


Chapter 7

Vitamin C Status and Cardiovascular Disease: A Review of Prospective Studies

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Introduction

Vitamin C is an essential nutrient for humans and its deficiency has long been known to cause scurvy (1). Less well understood is the role vitamin C plays in preventing cardiovascular disease (CVD), the leading cause of mortality in the United States for over 50 years (2). As an antioxidant, vitamin C may protect lipids, particularly low density lipoproteins (LDL), from oxidation. Oxidized LDL may contribute to the development of atherosclerotic lesions through several mechanisms (3,4). The recruitment of monocytes to the vascular intima is increased by oxidized LDL and monocytes can develop into macrophages. Oxidized LDL are more readily taken up by macrophages than nonoxidized LDL, a process in which macrophages can be converted to foam cells with later progression to fatty streaks and plaques. Oxidized LDL inhibit macrophage motility, preventing macrophages from leaving the intima and facilitating continued uptake of oxidized LDL. Oxidized LDL are also cytotoxic, leading to cell death, endothelial loss and denudation of the artery. Some antioxidants can neutralize oxygen-derived free radicals through hydrogen donation, preventing the chain reaction in which LDL are oxidized (4,5). Vitamin C is the only antioxidant that has been shown to prevent the initiation of the oxidation chain reaction in lipids (6), trapping free radicals in the aqueous phase before they can diffuse into lipids such as LDL.

Vitamin C can also regenerate oxidized vitamin E by reducing it back to its active form (7); vitamin E has been shown to slow the rate of LDL oxidation (4,6). Additionally, vitamin C may protect against both the initiation and progression of coronary heart disease (CHD) through other potential mechanisms. Because vitamin C is required for collagen synthesis (1), it may be important in maintaining vascular integrity (8). Vitamin C may also have antithrombotic and antiplatelet effects through its involvement in the synthesis of prostacyclin by the vascular wall endothelium (8). Vitamin C may affect serum cholesterol concentrations, possibly through its role in the production of enzymes involved in the biosynthesis or hydroxylation of cholesterol (9).

However, findings from epidemiologic studies examining the relationship between vitamin C and CVD have been inconsistent; consequently, the role that vita-
min C plays in the etiology of CVD remains controversial. Previous prospective cohort studies have varied in methods for measuring vitamin C status, definitions used to classify morbidity and mortality, range of intake and serum levels within the study population, length of follow-up, cohort size and composition (age, gender, exclusions), and ability to control for potential confounders. Such variability may account for the inconsistent findings regarding vitamin C status and cardiovascular disease. The purpose of this chapter is to review the evidence from prospective cohort studies examining the relationship between vitamin C status and CVD mortality, taking into account the variability in study design.

Materials and Methods

We limited our review to prospective cohort studies that measured vitamin C status before ascertainment of morbidity and mortality and that used appropriate statistical techniques to estimate relative risk (RR). We also limited studies to those that excluded prevalent cases at study entry to avoid potential biases resulting from their inclusion; prevalent cases may be more likely to change their dietary habits as a result of their disease or their disease may affect blood ascorbate levels. Restriction of the cohort to those free of disease at baseline also allows examination of the benefits of vitamin C in primary prevention of CVD. We included studies assessing vitamin C status measured by both blood assay or dietary intake (including or excluding supplement use). We reviewed studies examining various end points, and studies are reviewed in descending order of inclusiveness of CVD end points. We also address whether adjustment was made for potentially confounding variables, particularly smoking, and how these may affect interpretation of findings.

Studies Using Blood Ascorbate Concentrations

Three studies have examined the relation of blood ascorbate levels to CVD, with two finding significant associations (Table 7.1). Using the broadest definition of CVD [9th revision of the International Classification of Disease (ICD-9), codes 390–459], Simon et al. (10) found that 30- to 75-y-old participants in the second National Health and Nutrition Examination Survey (NHANES II) with saturated (≥1.1 mg/dL) or normal (0.5–1.0 mg/dL) compared with marginal (<0.4 mg/dL) serum ascorbate levels had a 34 and 33% reduced risk, respectively, of dying from CVD over 12–16 y. In a cohort of roughly the same age but more than twice as large, Khaw et al. (11) found a significant association between plasma ascorbate quintile and CVD mortality (excluding rheumatic heart disease and diseases of arterioles and veins from previous definition) after adjustment for age during 4 y of follow-up. Levels identified as most protective were consistent with saturation but multivariate-adjusted RR for quintiles were not presented. However, the multivariate-adjusted RR was 0.64 (0.51–0.78) in men and 0.81 (0.62–1.06) in women per increase of 0.35 mg/dL serum ascorbate. In the same NHANES II cohort used in the study by Simon et al. (10), Loria et al. (12) did not find an association between serum ascorbate quartile and a narrower definition of
CVD, including end points likely to be affected by antioxidant mechanisms (e.g., ischemic heart disease, cerebrovascular disease, sudden death, and diseases of arteries). Adjustment for potential confounders in multivariate analyses from all three studies were generally comparable; however, the first two studies used smoking status (i.e., current, past, never) whereas the last study used average number of cigarettes smoked per day.

Two of four studies found a significant relation between blood ascorbate and CHD mortality defined using ICD-9 codes 410–414 (Table 7.1). Plasma ascorbate quintiles were associated with CHD mortality during 4 y of follow-up; however, these data were adjusted only for age; multivariate-adjusted RR in men was 0.63 (0.42–0.94) and women 0.56 (0.36–0.87) per increase of 0.35 mg/dL serum ascorbate (11). In a smaller study of free-living men and women ≥60 y old, Sahyoun et al. (13) found that participants with plasma ascorbate 1.0–1.5 mg/dL vs. 0.9 mg/dL had a decreased risk of heart disease mortality; participants with levels ≥1.6 mg/dL also had an increased risk although it was not significant. Although this study adjusted for a number of confounders, adjustment was not made for smoking. Two other studies examining CHD mortality in middle-aged men (14) and elderly men and women (15) found no significant association with plasma ascorbate. Another study examining the relation of plasma ascorbate with nonfatal and fatal CHD in middle-aged men failed to find a significant association (16). Only one study examined the relation between plasma ascorbate and stroke; elderly men and women in the highest (>0.49 mg/dL) compared with the lowest (≤0.21 mg/dL) tertile had a 30% decreased risk of dying from stroke although the RR risk was borderline significant and was adjusted only for age and sex (15).

Studies Using Vitamin C Intake

Two studies examined the relation between vitamin C intake and broad definitions of CVD in representative samples of U.S. adults (Table 7.2). The first study, using data from NHANES I with 13–16 y of follow-up, found that vitamin C intakes >50 mg/d in combination with vitamin C supplement use had a standardized mortality ratio (SMR) of 0.7 (17). However, SMR, unlike RR estimates, are estimated using an external comparison group and do not permit adjustment for potential confounding factors. In NHANES II, vitamin C intakes of 32–73 mg/d among men were associated with an increased (60%) risk of CVD mortality after adjustment for CVD risk factors, supplement use, and total energy intake, but there was no increased risk in the middle two dietary intake quartiles (Loria, unpublished data). There was no association between vitamin C intake and CVD mortality among women in NHANES II. This study also used a narrower definition of CVD than the NHANES I study, excluding rheumatic, hypertensive and pulmonary heart disease, and diseases of veins.

Four studies examined the relation between vitamin C intake and fatal and nonfatal CHD; however, none yielded significant associations. In a study of U.S. male health professionals who were 40–75 y of age and followed for 4 y, total vitamin C intakes (diet and supplements) were not associated with the risk of CHD after adjust-
### Table 7.1
Prospective Cohort Studies Examining Serum or Plasma Ascorbate Concentrations and Cardiovascular Disease (CVD)\(^a\)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cohort</th>
<th>Gender</th>
<th>Age range (y)</th>
<th>Cohort size</th>
<th>Follow-up (y)</th>
<th>Endpoints (ICD-9)</th>
<th>Exposure (mg/dL)</th>
<th>RR</th>
<th>95% CI</th>
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<td>1.1–2.7</td>
<td>0.66</td>
<td>0.4–0.99</td>
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<tr>
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<td>2–6</td>
<td>400–438</td>
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<td>0.7</td>
<td>0.6–1.5</td>
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<td>RR (95% CI)</td>
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*Abbreviations: ICD-9, International Classification of Diseases, 9th Revision; RR, relative risk; CI, confidence interval; CHD, coronary heart disease; NS, nonsignificant; MI, myocardial infarction.*
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<tr>
<th>Reference</th>
<th>Cohort</th>
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<th>Age range (y)</th>
<th>Cohort size</th>
<th>Follow-up (y)</th>
<th>Endpoints (ICD-9)</th>
<th>Exposure (mg/d)</th>
<th>RR</th>
<th>95% CI</th>
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<td></td>
<td>50 + nosup</td>
<td>1.00</td>
<td>0.9–1.1</td>
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*Abbreviations: ICD-9, International Classification of Diseases, 9th revision; RR, relative risk; CI, confidence interval; sup, supplement use; CHD, coronary heart disease; MI, myocardial infarction; CABG, coronary artery bypass grafting; Q, quintile; NS, nonsignificant.*
ment for CHD risk factors and intakes of vitamin E and carotene (18). Among 30- to 55-y-old female nurses in the United States followed for 8 y, the risk of CHD was lower for women in the highest compared with the lowest quintile of total intake although this association was not significant (19). A study of middle-aged men living in Wales failed to find significant associations (20). Another study examining morbidity and mortality from acute MI did not yield significant associations in Dutch adults followed for 4 y (21).

Five studies examined the relation between vitamin C intake and fatal CHD; only one found a significant association although another tended to be significant. Dietary vitamin C intake was significantly associated with CHD mortality (ICD-8, codes 410–414) among Finnish women but not Finnish men (22). Women in the middle (<61 mg/d) and highest (61–85 mg/d) tertile had a 50% lower risk of CHD mortality than women in the lowest tertile (<60-61 mg/d) (22). Middle-aged men in Chicago followed for 24 y who had intakes between 113 and 393 mg/d compared with 21–82 mg/d had a decreased risk of dying from acute MI (ICD-9, codes 410–412) although it only was borderline significant (23). The RR was significant, however, in nonsmokers when analyses were stratified (23). Another study examining CHD mortality failed to find a significant association in elderly adults (15). In a smaller study of free-living men and women (60 y old, Sahyoun et al. (13) did not find a significant relation between total vitamin C intake and heart disease mortality, presumably CHD. Another study examining mortality from acute MI failed to find a significant association in Iowa women followed for 6 y (24).

One of three studies that examined the relation between vitamin C intake and stroke found a significant association, whereas another reported only a borderline significant finding. Adults ≥65 y old followed for 20 y who consumed >45 mg vitamin C/d had a 60% reduced risk of dying from stroke compared with those consuming <28 mg/d (15). However, RR estimates were adjusted for age and sex only and did not account for other potential confounders. Middle-aged men in Chicago followed for 30 y who had intakes between 123 and 393 mg/d compared with 22–74 mg/d had a decreased risk of stroke although it only was borderline significant (25). In a study of Iowa women 55–69 y of age, there was no increased risk of dying from stroke after 11 y (26).

Discussion

The evidence regarding vitamin C status and CVD from prospective cohort studies was reviewed, taking into account the variability in study design. Findings were more likely to be positive when serum ascorbate concentration vs. intake was used to assess vitamin C status. This observation is not surprising given that these indicators may measure quite different aspects of vitamin C status, i.e., estimated body ascorbic acid pool vs. vitamin C intake from foods and supplements. The relationship between vitamin C intakes and serum ascorbate level is complex because serum levels are saturable (1,27), absorption is dose dependent (1), and may be modified by other factors,
such as cigarette smoking, disease state, and medications (1,4,28–30). On the basis of this review of prospective studies, available stores may be the more important aspect of vitamin C status with respect to CVD than intake level, although the potential for misclassification associated with dietary assessment methods may also account for greater inconsistency in studies examining intake levels.

Findings also tended to be positive when a wider definition of CVD was used; this pattern was observed in studies using both serum concentrations and intake data. Studies that examined CHD, either fatal or nonfatal, as an end point yielded inconsistent findings, although those using serum ascorbate concentrations tended to be positive compared with those using vitamin C intake. The evidence for a relationship between stroke and vitamin C status was also weak. No studies examined the association of vitamin C on morbidity alone; it is possible that vitamin C may affect morbidity differently than mortality. A tendency to positive associations with wider definitions including end points both likely and less likely to be affected by antioxidant mechanisms suggests that vitamin C may be protective of CVD through multiple mechanisms, including but not limited to oxidation.

Most of the studies reviewed controlled for important potential confounders; however, some analyses were adjusted only minimally for age and sex and these results should be interpreted with caution, especially those that did not adjust for cigarette smoking. In one of the two most recent studies examining serum ascorbate in a large cohort, Khaw et al. (11) adjusted only for age and sex in analyses by ascorbate quintile but presented multivariate-adjusted models for continuous ascorbate level. In two different analyses of the other recently published large cohort, NHANES II, one study controlled for smoking status (i.e., current, past, never) and found a significant association (10), whereas the other adjusted for number of cigarettes smoked and failed to find a significant association (12). This discrepancy raises the question of possible residual confounding by smoking although these studies also used differing end points, vitamin C status classification, inclusion criteria, and classification of other adjustment variables, all of which may have contributed to the discrepant findings. More research is required on appropriate adjustment for cigarette smoking in statistical models, given the important association of smoking with serum ascorbate levels. Cigarette smoking decreases serum ascorbate levels (28,31) and increases CVD risk possibly by generating oxygen-derived free radicals (1,32). Further consideration of whether smoking should be treated as a potential confounder, effect modifier, part of the causal mechanism, or some combination of these is warranted.

Summary

Findings from prospective cohort studies regarding vitamin C status and CVD were slightly more consistent when stratifying by vitamin C assessment method and CVD end points. On the other hand, data from such studies remain inconclusive possibly because of limitations of cohort studies, including limited ability to adjust adequately for potential confounders or effect modifiers.
References

Potential Adverse Effects of Vitamins C and E

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Introduction

Vitamins C and E are effective free radical scavengers. Water-soluble vitamin C is an excellent antioxidant in plasma, whereas lipid-soluble vitamin E protects cell membranes from peroxidation. One third of U.S. adults ingest a vitamin and/or mineral supplement daily. A majority of supplement users (65%) consume broad-spectrum multivitamin/mineral products. However, 4 and 8% of U.S. adults specifically ingest vitamin E and vitamin C supplements, respectively, on a daily basis (1). These two vitamins are the most commonly supplemented micronutrients in the United States (2). Of all supplement users, the average vitamin C intake in the form of supplements is ~135 mg; but 10% of supplement users (3% of U.S. adults) consume >1000 mg of vitamin C daily (1,3). This level of intake is 10-fold greater than the recommended dietary allowance (RDA) for vitamin C, 75–90 mg/d. The average intake of vitamin E among supplement users is 10 mg daily1 and 10% of supplement users consume ~140 mg vitamin E daily (3), nearly 1000% of the RDA, 15 mg/d. The safety of such doses has been questioned, yet there is little evidence directly indicating that these levels of intake are harmful for healthy individuals in the general population. Rather, high dietary intakes of vitamin C have been associated with reduced risk of degenerative diseases, and high-dose vitamin E decreases platelet adhesion, inhibits oxidation of low density lipoproteins (LDL), and is promoted for the prevention and treatment of coronary artery disease.

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1Vitamin E is defined herein as the natural α-tocopherol as presented by the Food and Nutrition Board of the Institute of Medicine in the year 2000 to establish the Dietary Reference Intakes for vitamin E. Thus, vitamin E derived from supplements was assumed to be all rac-α-tocopherol (RRR-, RSR-, RSS- and RSS-α-tocopherol) and a factor of 0.50 was applied to convert mg of this synthetic form of vitamin E to mg natural α-tocopherol. To convert IU to mg natural α-tocopherol, a factor of 0.45 was applied to the 2R-stereoisomers of tocopherol and a factor of 0.67 was applied to the natural RRR-α-tocopherol.
Supplementation and Mortality

Vitamin C Supplement Use

All-cause mortality in a sample of the U.S. population [National Health and Nutrition Examination Survey (NHANES) I participants, n = 10,550; 749 deaths] was inversely related to vitamin C intake [standardized mortality ratio as a function of the dietary vitamin C index, 0.58; 95% confidence interval (CI), 0.36–0.80] (4). The vitamin C index ranged from persons consuming <50 mg vitamin C daily to those consuming ≥50 mg vitamin C daily from diet alone and those consuming ≥50 mg vitamin C daily from diet plus an average of ~800 mg daily from supplementation. After adjustment for age, sex, and 10 potentially confounding variables (race, history of serious disease, education, cigarette smoking, recreational exercise, alcohol consumption, energy consumed, fat, serum cholesterol, and dietary vitamin A), the inverse relationship of vitamin C intake to mortality remained strong (standardized mortality ratio, 0.62; 95% CI, 0.36–0.88). This inverse relation of total mortality to vitamin C intake was stronger and more consistent in this population than the relation of total mortality to serum cholesterol and dietary fat intake (4). In a separate population, there were no differences in mortality between individuals consuming above and below 250 mg vitamin C/d; however, those consuming >750 mg vitamin C/d experienced only 40% of the total cohort death rate during the 10-y study period (3).

In the NHANES II Mortality Study (n = 8453; 1327 total deaths), all-cause mortality was reduced 29% (P < 0.001) in individuals with high serum ascorbate (serum values ≥1.1 mg/dL) after multivariate adjustment for potential confounding variables, but vitamin C supplement use was not significantly associated with mortality (P = 0.19) (5). The risk of cardiovascular disease death was reduced 25% (P = 0.09) in individuals with high serum ascorbate, but this risk reduction was not related to the use of vitamin C supplements (5). However, the risk of cancer death in men specifically using vitamin C supplements was reduced 65% (P = 0.046) compared with a 31% reduction in men with high serum ascorbate. Fatal cancer risk was not associated with vitamin C supplement use in women. In an American Cancer Society cohort (n = 711,891; 4404 deaths from colorectal cancer), long-term vitamin C supplementation (≥10 y) lowered risk of rectal cancer mortality (−60%) but did not affect risk of colon cancer mortality (6).

Losconzy et al. (7) reported no significant effect of vitamin C supplementation on all-cause mortality, coronary disease mortality, or cancer mortality, yet vitamin supplement use may have been underreported because subjects completed questionnaires for nonprescription medications only, not vitamin supplementation specifically. Two other large, prospective population studies did not specifically address vitamin C supplementation use, yet men in the highest stratum for vitamin C status, also representing the greatest number of vitamin C supplement users, had significantly reduced risk of all-cause mortality, cardiovascular disease mortality, and cancer mortality (8,9). Women in the highest stratum for vitamin C status had
reduced risk of all-cause mortality and cardiovascular disease in one of these studies (8) but not the other (9). These data indicate the general safety of vitamin C in that mortality end points for the major causes of death in the United States are either unaltered or perhaps favorably affected by high-dose vitamin C ingestion.

**Vitamin E Supplement Use**

In an elderly cohort (n = 11,178), vitamin E supplementation alone reduced all-cause mortality risk 20% independently of other factors (7), and risk for coronary disease mortality and cancer mortality were reduced 36 and 28%, respectively, in vitamin E users (7). In the American Cancer Society cohort, short- or long-term vitamin E supplement use was not associated with reduced risk of colorectal cancer; however, long-term vitamin E use was associated with decreased risk of colorectal cancer mortality in participants who were current cigarette smokers (−40%) (6). Two large, prospective populations studies showed a reduced risk for all-cause mortality in men ingesting ≥113 mg vitamin E daily (−28%, P = 0.06) (10) and trends toward a reduction in the risk of all-cause mortality (−13%) and cardiovascular mortality (−42%) in women ingesting ≥45 mg vitamin E/d (11).

The prospective, randomized, placebo-controlled Alpha-Tocopherol, Beta Carotene Cancer Prevention Study (n = 29,133; 876 new lung cancer diagnosis and 1415 other cancer diagnoses) showed no effect of vitamin E supplementation (25 mg/d) on the incidence of lung cancer in male smokers (12). However, in this trial, there was a beneficial effect of vitamin E use against prostate cancer (34% lower incidence) and colorectal cancer (17% lower incidence). Importantly, a potential adverse effect of vitamin E use was observed in these male smokers, i.e., a 5% increased incidence of hemorrhagic stroke (12). In this same cohort, there was a nonsignificant increased risk of fatal coronary heart disease in smokers ingesting vitamin E supplements (+33%) (13). In the Heart Outcomes Prevention Evaluation Study (n = 9541; 1511 primary cardiovascular events), a high dosage of vitamin E (180 mg/d for 5 y) had no effect on risk of cardiovascular mortality or all-cause mortality in high-risk patients (14). There was a nonsignificant increased risk of stroke mortality among the vitamin E users (+17%, P = 0.13), but there was no increase in hemorrhagic stroke among the vitamin E users.

In 196 hemodialysis patients with preexisting cardiovascular disease who were randomized to receive 360 mg vitamin E daily or placebo, risk of cardiovascular disease mortality was not significantly reduced, but the difference in the survival curves for cardiovascular disease mortality by treatment tended to be significant (P = 0.06) (15). Despite a slight reduction in cardiovascular disease mortality risk, risk for all-cause mortality did not differ by treatment due to a nonsignificant increase in noncardiac mortality in patients ingesting vitamin E, including two cases of death associated with hemorrhage (15). Ascherio et al. (16) specifically examined the effect of regular vitamin E supplement ingestion (113 mg) on risk of ischemic and hemorrhagic strokes in mostly nonsmoking men (n = 43,738) participating in the Health Professionals Follow-up Study. After multivariate adjust-
ments, there was a slight but nonsignificant risk of hemorrhagic stroke (+3%) and ischemic stroke (+16%) in users of vitamin E.

Hence, intervention trials to date do not support a regimen of high-dose vitamin E ingestion for reducing risk of cardiovascular and/or cancer death in high-risk populations. Furthermore, there is some evidence indicating a potential adverse effect on risk for hemorrhagic stroke in users of vitamin E supplements. Hemorrhagic stroke is classified as subarachnoid hemorrhage or intracerebral hemorrhage, and the risk profiles of these two subtypes are distinct. Subarachnoid hemorrhage is typically caused by rupture of an arterial aneurysm and has been related to a smoking-induced elastase/α-antitrypsin imbalance (17), whereas intracerebral hemorrhage may be related more to hypertension and necrosis of small arterioles (18). The number of cigarettes smoked increased the risk of subarachnoid hemorrhage but not intracerebral hemorrhage (18). In smokers, vitamin E supplementation raised the risk of fatal subarachnoid hemorrhage (+181%, \( P = 0.005 \)) but did not alter risk of fatal intracerebral hemorrhage (19); furthermore, a high serum vitamin E concentration was related to a 53% lower incidence of intracerebral hemorrhage in smokers (18). Thus, the antiplatelet and anticoagulant actions of vitamin E may, to some degree, exacerbate an arterial rupture, particularly in smokers, but these actions, as well as the antioxidant action of vitamin E, might lesson the development of fibrinoid necrosis (18). The antiplatelet and anticoagulant actions of vitamin E are discussed in a later section.

**Adverse Reactions to Supplemental Vitamin C**

In the general population, the data overwhelmingly indicate that regular ingestion of vitamin C supplements is unlikely to have major adverse effects. The low toxicity of supplemental vitamin C can probably be attributed to decreased bioavailability and increased urinary excretion as the dose is increased. In one study, ~70% of a 500-mg dose was absorbed and >50% of the absorbed dose was excreted in urine unmetabolized. At the 1250-mg dosage level, only 50% of the dose was absorbed and nearly all of the absorbed dose was excreted (20). Furthermore, the oxidized form of vitamin C, dehydroascorbic acid, is not highly reactive and is effectively cleared from fluids and rapidly reduced to ascorbic acid (21,22). Nonetheless, concerns have been raised regarding adverse effects of high doses of vitamin C.

**Systemic Conditioning (Rebound Scurvy)**

There is some evidence that systemic conditioning (the accelerated metabolism or disposal of ascorbic acid) may occur after prolonged supplementation of high doses of vitamin C. The physiologic relevance of accelerated vitamin C metabolism in otherwise healthy individuals, however, has not been addressed adequately. In human subjects consuming a vitamin C–deficient diet (5 mg/d) in a live-in metabolic unit, abrupt withdraw of vitamin C supplementation (600 mg/d for 3 wk) was associated with a significant reduction in the mean leukocyte vitamin C concentration (16.8 ± 4.37 and 9.4 ± 5.33 \( \mu g \) vitamin C/10^8 cells, presupplementation and at 4 wk after withdrawal of...
supplementation, respectively) (23). Both of these leukocyte vitamin C concentrations were indicative of vitamin C depletion. Yet simply removing vitamin C–rich fruits and vegetables from the diets of healthy free-living adults can lower mean plasma vitamin C concentrations into the depleted or deficient range within 1–3 wk (24). Thus, even in adults not supplementing their diets with vitamin C, removal of vitamin C from the diet (in the form of supplements or foods), results in rapid vitamin C depletion, reflecting how poorly vitamin C status is maintained in the absence of a dietary source.

Well-nourished subjects receiving vitamin C supplements may also display accelerated vitamin C metabolism; however, because indices of vitamin C nutrition remain within normal ranges, the term “rebound scurvy” is misleading. In guinea pigs consuming a standard guinea pig diet containing 0.1% vitamin C (w/w), chronic vitamin C administration [1 g/(kg body.d) intraperitoneally for 4 wk] appeared to be associated with an increased rate of vitamin C turnover. Mean plasma vitamin C concentrations fell significantly below control values at wk 2 and 5 after the abrupt withdrawal of the administered vitamin C (25); however, all mean plasma vitamin C concentrations measured postwithdrawal were well within normal ranges. In human subjects consuming normal diets, the mean plasma vitamin C concentration did not fall significantly below the presupplement value at 7 or 23 d after the abrupt withdrawal of vitamin C supplements (2 g/d for 9 d) (26).

**Kidney Stone Formation**

Almost 75% of kidney stones are calcium oxalate, and a lesser number are uric acid (5%). High doses of vitamin C may moderately increase urinary oxalic acid and urinary uric acid levels; yet, an association between high intakes of vitamin C and the actual formation of kidney stones has never been demonstrated.

In human subjects with hyperuricemia, an acute dose of vitamin C, 0.5 or 2.0 g, did not significantly alter urinary clearance of uric acid. However, a single 4.0-g dose of vitamin C significantly increased mean uric acid clearance by 71% in these subjects (27); it was unclear, however, whether this rise in uric acid clearance was associated with urinary uric acid concentrations above the normal range (1.5–4.4 mmol/d). In three subjects ingesting 8 g of vitamin C daily for up to 7 d, urinary uric acid was elevated over the normal range in one subject (27). In subjects with and without gout, vitamin C infusion (2.5-10 mg/min to achieve plasma levels from 3 to 12 mg/100 mL) raised urinary urate clearance only moderately (28), and there were no differences in response between subjects with and without gout. In a carefully controlled inpatient trial, mean urinary uric acid was not elevated in seven young men after chronic daily ingestion of 200 or 400 mg vitamin C (20). Chronic daily ingestion of 1000 mg of vitamin C did significantly raise mean urinary uric acid to a concentration outside the normal range (~5.5 mmol/d).

Vitamin C intake has been related to urinary oxalate \( (r = 0.33, P < 0.001) \) (29). Mean urinary oxalate was not significantly altered in seven men consuming 200 or 400 mg vitamin C daily for 4–5 wk (20); although chronic daily ingestion of 1000 mg
vitamin C raised urinary oxalate nearly 50% \((P < 0.05)\), urinary oxalate values remained within the normal range \((228–684 \mu \text{mol/d})\). In six healthy subjects, vitamin C supplementation, 10 g/d, did not alter urinary oxalate in five of the subjects, and the elevated level noted in the remaining subject was within the normal range (30).

These data indicate that high doses of vitamin C alter urinary excretion of uric and oxalate acid; however, diets high in purines, organ meats and fish, or chronic low-dose aspirin, can cause hyperuricosuria, and foods rich in oxalates (e.g., tea, coffee, nuts, beans, chocolates) can increase oxalate concentrations in urine to levels above the normal range (31). Hence vitamin C is one of many diet constituents associated with alterations in urinary constituents of renal stones. Furthermore, diets high in animal protein or low in fluids, bacterial products, and heavy physical exertion have also been implicated in renal stone disease. Because uric acid can precipitate in the absence of hyperuricosuria, and urinary saturation with calcium oxalate is common in the general population, factors other than urinary levels of uric acid and oxalic acid are crucial for stone formation, including renal epithelial cell responses, acid urine, and proteins of renal tubular origin (31).

In the Harvard Prospective Health Professional Follow-Up Study (45,251 men; 751 incident cases of kidney stones after 6 y), the relative risk of developing kidney stones in users of vitamin C supplements \((\geq 1500 \text{ mg vitamin C daily vs. } <250 \text{ mg daily})\) was 0.89 after multivariate adjustment (32). Using data from NHANES II, Simon and Hudes (33) found no associations between serum vitamin C and prevalence of kidney stones in women or men. In the Nurses’ Health Study cohort (85,557 women; 1078 incident cases after 14 y), supplemental vitamin C was not associated with risk of kidney stones \((\text{multivariate relative risk, } 1.06)\) (34). Nonetheless, high doses of vitamin C could invalidate urinary measures of uric acid and oxalic acid, and probably should not be taken by individuals predisposed to renal calculi or with renal failure (35).

**Prooxidant Activity**

Ascorbic acid nonenzymatically detoxifies a variety of reactive radicals and oxygen species, including hydroxyl, superoxide, peroxyl and nitrogen dioxide radicals, and in the interstitial fluids, plasma, or the cytosol, ascorbic acid acts as a primary antioxidant to scavenge free radicals that are generated by biological interactions or by cellular metabolism. In the scavenging process, ascorbic acid loses a single electron to form the ascorbate free radical, which is only weakly reactive and which may be quickly reduced back to ascorbic acid, or, with the loss of a second electron, is converted to dehydroascorbic acid. In vivo, dehydroascorbic acid may be recycled back to ascorbic acid in erythrocytes or numerous other cell types, or may incur irreversible ring opening to form 2,3-diketo-1-gulonic acid, a nonreactive metabolite. Given these metabolic pathways, vitamin C is an efficient protective antioxidant and unlikely to demonstrate prooxidant activity in vivo (36).

In vitro, however, vitamin C reduces unbound transition metals, which then participate in free radical generation, most notably the reduction of ferric iron to the fer-
rous form, which is active in the Fenton reaction and the decomposition of hydrogen peroxide to form hydroxyl radicals (37). The extremely reactive hydroxyl radical quickly reacts with most molecules including DNA, and is implicated in mutagenesis and the initiation of cancer. In vivo, most transition metals are bound to protein, thus preventing metal-dependent free radical reactions. Experiments in iron-overloaded plasma demonstrated that iron, even in excess of the amount bound by transferrin, was unable to participate in a Fenton reaction in the presence of ascorbic acid (38). The complexity of plasma, including the presence of catalase or ferroxidase activity, may provide an environment inhibiting a Fenton reaction when transition metals are unbound (38). In a separate study, when excess iron was added to adult plasma, endogenous and exogenous ascorbic acid delayed the onset of iron-induced lipid peroxidation in a dose-dependent manner (39). Furthermore, LDL incubated in buffer were protected from metal ion–dependent oxidative modification by dehydroascorbic acid and from metal ion–independent oxidative modification by ascorbic acid (40). Thus, in biological systems, ascorbic acid acts as an antioxidant, even in the presence of iron overload.

In iron-loaded guinea pigs, significantly less oxidative damage, as indicated by reduced levels of liver or plasma F₂-isoprostanes, was recorded in animals fed high-dose vitamin C vs. animals fed low-dose vitamin C (41). In humans, vitamin C supplementation (260 mg/d for 6 wk) was associated with significant reductions in several DNA adducts that are markers of oxidized DNA base damage, i.e., 8-oxo-guanine and 5-OH methyl uracil (42). Total base damage (compiled data for 12 different DNA adducts), however, was unaffected by vitamin C supplementation. Vitamin C supplementation (260 mg/d) with iron (14 mg/d) was associated with a significant reduction in the 5-OH methyl uracil DNA adduct but with significant increases in 5-OH methyl hydantoin and 5-OH cytosine. However, total base damage was unaltered by the vitamin C plus iron supplementation regimen.

In a controversial report, prooxidant effects of supplemental vitamin C were reported in healthy volunteers consuming 500 mg of vitamin C/d for 6 wk (43). Lymphocytes extracted from these individuals displayed an increase in oxidative damage to DNA as indicated by a significant rise in mean levels of 8-oxo-adenine during the supplementation period. Yet, the same report clearly depicted a significant, 50% decrease in levels of 8-oxo-guanine over the same time period. This is noteworthy because the oxidation of guanine has been highly correlated with mortality from heart disease ($r = 0.95$), colorectal cancer ($r = 0.91$), pancreatic cancer ($r = 0.82$), and lung cancer ($r = 0.32$) (44). Furthermore, hydroxyl radicals and singlet oxygen directly target guanine residues, and the resulting $G\rightarrow T$ transversion mutations have been observed in ras protooncogenes and p53 tumor-suppressor genes, cancer-related genes that are implicated in 40–50% of human cancers (45,46). There are few or no data in the literature implicating 8-oxo-adenine in mutations of cancer-related genes. Other reports have also demonstrated that vitamin C supplementation (250 or 500 mg/d for 28 d) significantly reduced oxidative damage to DNA in vivo as indicated by reduced tissue levels of 8-oxo-guanine (47,48).
Recently, Lee et al. (49) demonstrated that ascorbic acid in the absence of transitional metals reduced lipid peroxides in vitro, producing the genotoxin 4,5-epoxy-2(E)-decenal which reacts with DNA bases to form etheno-DNA adducts. In vivo, etheno-adducts in white blood cell DNA were inversely correlated with vegetable or vitamin E consumption (50) and positively associated with linoleic acid intake (51). Furthermore, citrus fruits, berries, or vitamin C intake were reportedly inversely related to hprt mutation frequency in peripheral blood lymphocytes, a marker of genotoxic effects due to lipid peroxidation (52).

**Iron Overload and Iron Toxicity**

Vitamin C promotes the absorption of dietary nonheme iron and contributes to the prevention of iron deficiency anemia (53,54). Mealtime vitamin C in the range of 50–100 mg has the most marked effect on iron absorption, and supplemental vitamin C beyond these levels had minimal additional effects on iron status in healthy individuals (55). Whether vitamin C supplementation might enhance the uptake and storage of nonheme iron in individuals with iron-overload pathologies (e.g., hereditary hemochromatosis and thalassemia major) is unclear. Moreover, these patients have measurable nontransferrin-bound iron in serum, which is associated with oxidative stress and products of lipid peroxidation (56,57). Theoretically, vitamin C could act as a prooxidant in the presence of unbound iron and fuel the production of lipid peroxides.

A recent case report of a 36-y-old woman with hereditary hemochromatosis resulting in end-stage cardiomyopathy, suggests that high-dose vitamin C supplement use (>1000 mg/d) may have predisposed her to a more fulminant disease course (58). In fact, vitamin C deficiency may have a protective role in some patients with severe iron overload (59). Vitamin E supplementation (300–600 mg/d), however, improved measures of oxidative stress in β-thalassemia patients, significantly reducing levels of LDL-conjugated dienes and red blood cell malondialdehyde, and increasing the resistance of red blood cells to osmotic lysis (60,61). In a rat model of hemochromatosis, vitamin E supplementation reduced lipid peroxidation in iron-loaded livers by 50% (62). This therapeutic dichotomy between vitamin C and vitamin E supplementation is noteworthy.

**Gastrointestinal Effects**

Gastrointestinal symptoms such as nausea, abdominal cramps, and diarrhea may occur in ~10% of subjects when vitamin C doses exceed 2 or 3 g/d. These symptoms have no long-term consequences, and buffered ascorbate salts consumed with a meal would alleviate these effects. However, osmotic diarrhea and gastrointestinal disturbances were the only selected critical end points for the establishment of a Tolerable Upper Intake Level (UL) for vitamin C, 2000 mg/d (63). That is, the only adverse effect of high-dose vitamin C considered by the Food and Nutrition Board in the formulation of the UL for vitamin C was osmotic diarrhea.
Other Adverse Effects

Several other commonly cited adverse effects of high-dose vitamin C, hemolysis related to glucose-6-phosphate dehydrogenase deficiency (64) and destruction of vitamin B₁₂ (65), have never been demonstrated under controlled, experimental conditions. Conversely, serum vitamin B₁₂ and serum vitamin C were directly related (33,65); each 57 pmol/L rise in vitamin C was associated with a 27 pmol/L increase in vitamin B₁₂ ($P < 0.001$) (33).

Adverse Reactions to Supplemental Vitamin E

Meydani et al. (66) demonstrated the safety of vitamin E supplementation (27–360 mg/d for 4 mo) using numerous toxicity indices including nutrient status, liver enzyme function, serum autoantibodies, plasma lipoprotein concentrations, bleeding time, and neutrophil cytotoxicity. Other large intervention trials reported no significant adverse events related to vitamin E supplementation (180–360 mg/d) compared with placebo, providing reassurance for the conduct of large, long-term trials to address the efficacy of vitamin E supplementation (14,15). It is clear, however, that there are a few populations in which caution should be employed regarding vitamin E supplementation, mainly populations at risk of clinically important bleeding (67). Regular cigarette smoking has been associated with aneurysm formation and hemorrhage (17,68), possibly due to reduced levels of serum α₁-antitrypsin with increased arterial collagen catabolism, a situation exacerbated by reduced platelet activity (68,69). Because there was a slight increased risk of fatal subarachnoid hemorrhage in smokers supplementing with vitamin E (see above), vitamin E supplementation should be carefully considered for these individuals.

Antiplatelet Effects

Vitamin E supplementation in adults (150 mg/d for 8 wk) increased platelet vitamin E content 70% and decreased platelet aggregation induced by ADP or arachidonic acid (70). Using increasing dosages of vitamin E (50–268 mg/d), Mabile et al. (71) reported that the inhibition of platelet aggregation occurred at the 50 mg dosage and was not further affected by the higher dosages. Thus low-dose vitamin E is sufficient to reduce platelet activity. Vitamin E also reduced platelet adhesion to human endothelial cells in vitro (72). The antiplatelet effects of vitamin E may be associated with decreased cyclooxygenase activation due to the antioxidant properties of vitamin E, or vitamin E may function to inhibit protein kinase C independent of antioxidant properties (73,74). Although this inhibition of platelet activation may potentially benefit patients at risk of developing thrombotic complications (75), supplementary vitamin E is not currently recommended for either primary or secondary prevention of myocardial infarction (76). Anecdotal reports indicate that the likelihood of postoperative hemorrhage may increase after the preoperative use of vitamin E (67,77). Moreover, patients receiving antithrombotic
therapy (e.g., warfarin or aspirin) should be carefully monitored if using vitamin E supplements.

In a study of male smokers, vitamin E use (25 mg/d), alone or in combination with aspirin use (>100 mg/d), increased the risk of clinically important bleeding (78). The extent of bleeding was greater in smokers supplementing with vitamin E than in those who took neither vitamin E nor aspirin (27.1 and 25.8%, respectively, \( P = 0.05 \)). Bleeding tendency was similar in users of aspirin and nonusers of vitamin E or aspirin. The extent of bleeding was greatest in the smokers using both vitamin E and aspirin (33.4%). Bleeding times, however, were not prolonged by vitamin E supplementation (360 mg/d for 1–4 mo) in healthy adults (66,79).

**Prooxidant Activity**

When protecting against the propagation of fatty acid peroxidation, vitamin E is oxidized to the \( \alpha \)-tocopherol radical. *In vivo*, vitamin C and ubiquinol (coenzyme Q) reduce the \( \alpha \)-tocopherol radical and regenerate vitamin E (80,81). Dihydrolipoic acid also plays a prominent role in vitamin E recycling; however, its role is indirect in that dihydrolipoic acid reduces dehydroascorbic acid, oxidized vitamin C, and maintains high levels of ascorbic acid to provide for effective recycling of vitamin E (80).

Vitamin C levels in plasma are variable and dependent on dietary intake, and dietary vitamin C depletion has been associated with reduced vitamin E concentrations *in vivo* (82,83). About 30% of Americans have marginal vitamin C status, reflecting a diet low in fruits and vegetables or conditions of high oxidative stress, such as smoking or diabetes (84,85). Vitamin E supplementation under these conditions may lead to a redox imbalance by further depleting vitamin C causing prooxidative effects (85,86).

Brown *et al.* (85) examined the effect of a wide range of supplemental vitamin E (0, 70, 140, 560, or 1050 mg/d for 20 wk) on lipid peroxidation in male smokers and in men who had never smoked. Red blood cell vitamin E concentrations were significantly elevated in all groups ingesting vitamin E. The susceptibility of red blood cells to hydrogen peroxide–induced lipid peroxidation *in vitro* was reduced substantially (−63 to −73%, \( P < 0.05 \)) in smokers ingesting 70–1050 mg vitamin E. In the nonsmokers, lipid peroxidation was decreased in those ingesting 70, 140, or 560 mg vitamin E; however, lipid peroxidation was significantly elevated (+36%) in nonsmokers consuming the highest dosage of vitamin E, 1050 mg/d. Moreover, the erythrocyte vitamin E:plasma vitamin C ratio was highest in this group, indicating the highest degree of antioxidant imbalance of all groups. Thus, the increased susceptibility to red blood cell peroxidation in this group may reflect a decrease in vitamin C regeneration of tocopherol and, hence, a prooxidative effect of vitamin E (85). A redox imbalance was also cited as contributing to a 44% increase in platelet-leukocyte coaggregates *ex vivo* in type 2 diabetics ingesting 536 mg vitamin E daily for 6 mo (86).

It seems likely that individuals consuming high-fat diets and who have higher blood lipid concentrations are more likely to respond to vitamin E supplementation because vitamin E absorption and transport would be optimized under these circumstances (87). Thus, a prooxidant effect of vitamin E (or perhaps more appropriately
termed, a redox imbalance) might occur only when certain conditions overlap, i.e., inadequate vitamin C nutrition, high blood lipids, and high dietary fat (85).

Necrotizing Enterocolitis

Newborn infants, especially premature infants, often have low plasma vitamin E concentrations and were once considered to suffer from a vitamin E deficiency manifesting as hemolytic anemia, the retinopathy of prematurity, and/or bronchopulmonary dysplasia. Prophylactic use of high-dose vitamin E in these populations, however, did not prevent anemia (88) or retinopathy (89). Furthermore, vitamin E use by neonates has been associated with an increased incidence of necrotizing enterocolitis as well as hemorrhagic complications of prematurity (89,90). The adequate level of vitamin E in infant formulas is set at 0.5 mg/100 kcal and an upper limit has been proposed at 10 mg/100 kcal (63,91).

Supplementation and Drug Interactions

The anticoagulants aspirin and warfarin (Coumadin) are commonly prescribed to patients at risk of heart attacks or stroke. High-dose vitamin E can magnify the anticoagulant effects of these drugs and increase the possibility of clinically significant bleeding (78). Bleeding with anticoagulant drug use occurs more frequently in older patients and in patients at risk of vitamin K deficiency due to antibiotic therapy or fat malabsorption syndromes (92). Thus, high-dose vitamin E therapy might be contraindicated in these patient populations.

β-Hydroxyl-β-methyl glutarate-CoA reductase inhibitors (statins) in combination with niacin are often the initial therapy prescribed for dyslipidemia to reduce LDL cholesterol and raise high density lipoprotein (HDL) cholesterol. A recent clinical trial examining the efficacy of different lipid-altering and/or antioxidant strategies on coronary artery disease indices demonstrated that when statin/niacin therapy was combined with antioxidant use (β-carotene, 12.5 mg; vitamin C, 500 mg; vitamin E, 268 mg) the beneficial response of HDL to the statin/niacin therapy was markedly attenuated ($P = 0.057$) (93). Although these results must be verified, they demonstrate the importance of investigating interactive effects of popular drug therapies and high-dose vitamin supplementation.

Concern has been expressed regarding high-dose vitamin C use by cancer patients because high intracellular concentrations of vitamin C may enhance the tumor’s ability to resist the oxidative stress associated with radiation therapy and/or chemotherapy (94). However, clinical trials have not addressed this theoretical concern. In fact, vitamin C has been shown to contribute to the antitumor effects of mitomycin C in cultured leukemia cells (HL 60) by acting as an electron donor to mitomycin C (95). Finally, it has been documented that high urinary vitamin C can interfere with routine urinalysis for hemoglobin or glucose if dipstick tests rely on redox indicator systems (96) or with the measurement of oxalate in urine and plasma (97). Protocols to avoid vitamin C interference in these measurements should be followed.
References


Chapter 9

Vitamin E: An Introduction

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Introduction

Eighty years ago vitamin E was discovered to be an essential micronutrient by Evans and Bishop (1). With the later recognition that this vitamin is not only necessary for successful reproduction but also the major lipophilic antioxidant in the human body, and subsequently, the causative role of oxidative stress in the pathogenesis of age-related and common chronic diseases, intense research has been conducted to evaluate the possible protective and curative potential of vitamin E. In the meantime, several other specific biological functions of vitamin E have been revealed apart from its antioxidant function including modulation of cell signaling [e.g., protein kinase C (PKC) inhibition], gene expression, and cell proliferation. Nonetheless, there are still many gaps in understanding the physiologic role of vitamin E in humans. Except in the case of nutrient deficiency, not much is clearly proven about the functional importance of vitamin E for human health and disease prevention. The following introduction provides a brief overview of the known biological functions of vitamin E, focusing on α-tocopherol as the major form of vitamin E in the human body, and highlights some milestones in the past eight decades of vitamin E research.

Molecular Structure of Vitamin E

Vitamin E is the generic name for a group of eight plant-derived, lipid-soluble substances (“tocols”) including four tocopherols and four tocotrienols (Fig. 9.1). The molecular structure of vitamin E is comprised of a chromanol ring with a side chain located at the C2 position. Tocopherols have a saturated phytol side chain and tocotrienols have an unsaturated isoprenoid side chain. The number and position of methyl groups located around the chromanol ring vary among the different tocopherols and tocotrienols, and account for the designation as α-, β-, γ-, or δ-forms. The α-forms of tocopherol and tocotrienol contain three methyl groups, whereas the β- and γ-forms have two, and the δ-forms have one methyl group. Natural and natural-source tocopherols occur in RRR-configuration only (formerly designated as \(d\)-α-tocopherol). In contrast, synthetic α-tocopherol consists of an equal racemic mixture of eight stereoisomers (RRR, RSR, RRS, RSS, SRR, SSR, SRS, SSS) arising from the
three chiral centers of the molecule at positions C2, C4′ and C8′ and designated as all-
\textit{rac}-\alpha\textit{-}
tocopherol (or \textit{dl}-\alpha\textit{-}
tocopherol) (2).

**Biological Activity of Vitamin E**

The various forms of vitamin E differ in their biological activities. \(\alpha\)-Tocopherol is
the most common form of vitamin E occurring in human blood and tissues, and it has
the highest biological activity among all tocopherols and tocotrienols. Moreover, the
human body preferentially accumulates the \textit{RRR}- or 2R-forms of vitamin E (3–5).
Thus natural \textit{RRR}\textit{-}\alpha\textit{-}
tocopherol has a higher bioavailability and “biological activity”
than synthetic all-
\textit{rac}\textit{-}\alpha\textit{-}
tocopherol. This was considered in the new guidelines on vit-
amin E intake as published in 2000 by the Food and Nutrition Board (FNB), U.S.
National Academy of Sciences (6). According to the FNB, only \textit{RRR}\textit{-}\alpha\textit{-}
tocopherol itself, or the \textit{RRR}- and 2R-stereoisomers of all-
\textit{rac}\textit{-}\alpha\textit{-}
tocopherol meet the vitamin E
requirements in humans, resulting in a twofold higher potency of natural vs. synthetic
\alpha\textit{-}
tocopherol sources. This value has been challenged by Hoppe and Krennrich (7).
The biological activity of vitamin E in dietary supplements is usually expressed as international units (IU). As published in the United States Pharmacopeia (USP), 1 IU is defined as the biological activity of 1 mg all-rac-α-tocopheryl acetate. Other biological activities of vitamin E are 1 mg all-rac-α-tocopherol = 1.1 IU; 1 mg RRR-α-tocopherol = 1.49 IU; 1 mg γ-tocopherol = 0.15 IU (8). Formerly, the biological activities of different vitamin E forms were often reported as α-tocopherol equivalents (α-TE). One α-TE was defined, for example, as 1 mg α-tocopherol, or 2 mg β-tocopherol, or 10 mg γ-tocopherol, or 3.3 mg α-tocotrienol (9).

**Dietary Sources, Supplements, and Recommended Intake of Vitamin E**

Vegetable oils and lipid-rich plant products (e.g. nuts, seeds, grains) are the main dietary sources of vitamin E (10,11). In Western diets, vitamin E intake derives mainly from fats and oils contained in margarine, mayonnaise, salad dressing, and desserts, and increasingly also from fortified food (e.g., breakfast cereals, milk, fruit juices) (12–14). It is noteworthy that the U.S. diet contains large amounts of γ-tocopherol compared with populations in other Western countries, which is a result of the high consumption of soybean and corn oils containing more γ- than α-tocopherol (15). Vitamin E used for food fortification or dietary supplements consists mainly of α-tocopherol, derived either from natural sources (i.e., methylated γ-tocopherol from vegetable oil) or from synthetic production; it is usually esterified to increase stability.

The most recent data available on vitamin E intake in the United States were reported in 1999 from the Third National Health and Nutrition Examination Survey (NHANES III, 1988–1994) among >16,000 U.S. adults showing a mean vitamin E intake of 10.4 α-TE in men and 8.0 α-TE in women (16). With the new definition of vitamin E activity from the FNB (6) distinguishing among α-tocopherol stereoisomers, these intake values of vitamin E are likely not as high. New studies are required to update these data on vitamin E intake because dietary patterns have changed during recent years and the consumption of fortified food and vitamin E supplements has increased.

The FNB indicates a recommended daily allowance (RDA) for adults of 15 mg vitamin E including food, fortified food, and supplements (6). This RDA refers to α-tocopherol because it is the only form of vitamin E that has been shown to reverse deficiency symptoms in humans. The recommendations are based largely on in vitro studies of M. Horwitt dating back to 1960; these studies are still considered to offer the most adequate data for defining the physiologic status and health benefits of vitamin E in humans. In these studies, prevention of H₂O₂-induced erythrocyte hemolysis was used as test system (17). However, except when vitamin E is clearly deficient the erythrocyte hemolysis test is not a useful indicator of vitamin E functional status. Thus, the guidelines of the FNB were discussed critically in the literature and it was strongly agreed that other assays and new biomarkers were required in the future to define the physiologic role and beneficial potential of vitamin E in humans (18,19).

The recommended daily intake of 15 mg vitamin E is considered rather unlikely to be achieved by North Americans and other Western countries through diet alone.
Although universal dietary supplementation has not been recommended, a dose of 200 mg/d vitamin E (RRR-α-tocopherol) has been suggested to saturate plasma levels and elevate tissue levels; this may have possible health effects in the long term (20). In the future, subpopulations at risk for vitamin E deficiency in well-nourished populations such as the United States and other Western countries have to be defined. These specific groups, e.g., the elderly or individuals suffering from chronic diseases, may benefit in particular from regular intake of vitamin E supplements.

As an upper limit for supplemental vitamin E intake, the FNB published a dose for adults of 1 g/d α-tocopherol (i.e., 1500 IU RRR- or 1100 IU all-rac-α-tocopherol); this dose is considered safe, showing no apparent side effects (6). In human intervention studies, various doses of vitamin E up to 3600 IU/d have been used (21). Nevertheless, conclusive evidence from long-term studies regarding biological effects and safety of chronic intake of pharmacologic doses of vitamin E are lacking. In the Alpha-Tocopherol, Beta Carotene (ATBC) Cancer Prevention Study (22) with Finnish smokers consuming 50 IU/d vitamin E for 6 y, an increase in mortality from hemorrhagic stroke was observed; however, other intervention studies did not report such an adverse effect (23,24). It was suggested that pharmacologic doses of vitamin E are contraindicated in persons with blood coagulation disorders because vitamin E might exacerbate defects in the blood coagulation system due to its inhibitory effects on platelet aggregation (19,25).

**Uptake, Distribution and Metabolism of Vitamin E**

Together with dietary fat, α-tocopherol and all other forms of vitamin E are absorbed in the digestive tract, incorporated into chylomicrons and transported in the lymphatic system. Part of the absorbed stereoisomers is taken up into extrahepatic tissues by the action of lipoprotein lipase, and the remainder is delivered in chylomicron remnants to the liver (26). The distribution of vitamin E into the circulation is regulated by a cytosolic α-tocopherol transfer protein (α-TTP) in the liver, which is selective for α-tocopherol in its RRR- or 2R-forms (27). Other tocopherols and tocotrienols exert much less affinity for α-TTP as assessed by ligand specificity, e.g., 38% for β-tocopherol, 9% for γ-tocopherol, 2% for δ-tocopherol, and 12% for α-tocotrienol (28). α-TTP plays an important role in maintaining plasma levels of vitamin E (29). The function of α-TTP and the mechanism of α-tocopherol regulation were reviewed recently (30,31).

In the liver, vitamin E is incorporated into very low density lipoproteins (VLDL) and released to the systemic blood circulation. Excess amounts of α-tocopherol along with the other absorbed forms of tocopherols and tocotrienols are metabolized or eliminated by the biliary tract. VLDL are converted into low density lipoproteins (LDL), and excess surface components including α-tocopherol are transferred to high density lipoproteins (HDL). Delivery of α-tocopherol to peripheral tissues takes place via binding of LDL to LDL receptors and subsequent cellular uptake. Vitamin E tends to accumulate in adipose tissues. The metabolism and kinetics of vitamin E as well as the function of vitamin E regulatory proteins have been reviewed (32,33). Cytosolic toco-
pherol-associated proteins (TAP) have been reported, showing α-tocopherol–specific binding characteristics and also nuclear translocation and transcriptional activation in various mammalian cell types and organs (34,35). TAP seems to play an important role in vitamin E–induced gene expression (36); however, its biological functions are not widely known. A further cytosolic vitamin E regulatory protein, i.e., tocopherol-binding protein (TBP) has been reported with yet unknown functions (37,38).

Data on serum levels of vitamin E from population-based studies are scarce. Analysis of the data obtained from the NHANES III study shows mean serum levels of α-tocopherol of 26.8 (0.65–232.18) µmol/L and a mean α-tocopherol:cholesterol ratio of 5.1 (16). Although no differences in gender have been reported, significant differences were found among races, with the highest serum levels in Caucasians and the lowest in African-Americans. Approximately one third of the NHANES III study population had low vitamin E concentrations (<20 µmol/L). A recent study in five European countries among 350 healthy adults showed mean α-tocopherol serum levels of 26.4 (13.23–46.40) µmol/L and a mean α-tocopherol:cholesterol ratio of 6.5 µmol/mmol (39). The lowest acceptable vitamin E level in plasma has been determined to be 11.6 µmol/L (0.5 mg/dL) and a ratio of vitamin E:cholesterol of 2.25 µmol/mmol (40).

It should be mentioned that serum levels of vitamin E correlate with cholesterol levels, and hence do not necessarily correlate with vitamin E intake (16). However, except for non- or poorly responding subjects, serum levels of vitamin E can usually be increased up to threefold by intake of dietary supplements reaching a saturation level (20).

**Milestones in Vitamin E Research**

Four milestones of vitamin E research have been recognized, namely, (i) its biological importance in reproduction and essentiality as a micronutrient; (ii) its unique role as lipophilic antioxidant in lipoproteins and cell membranes; (iii) its effects on cell signaling and gene expression; and (iv) the identification of vitamin E deficiency diseases and the recognition of beneficial effects of vitamin E in human health and disease prevention.

*Milestone I: Biological importance of vitamin E in reproduction and essentiality.* Initially, vitamin E was recognized as a nutritional factor required to ensure normal reproduction in rats (1) and was named according to a consecutive alphabetical order preceded by the discovery of vitamins A–D (41). Later, vitamin E was called α-tocopherol from the Greek term “tokos” (childbirth), “phero” (to bear), and –ol, indicating an alcohol (42).

Rats fed a diet low in vitamin E had reduced fertility and a high rate of fetal resorption. However, when animals were fed a lipophilic fraction from lettuce or, as later shown, wheat germ oil, their fertility was retained and a successful implantation of the embryo was observed (42,43).

The biological activity of vitamin E is based on this rat resorption-gestation assay. The family of natural vitamin E molecules as well as the stereoisomers of all-
rac-α-tocopherol all exhibit vitamin E activity to varying degrees in this bioassay. Unfortunately, this assay of reproductive activity in pregnant rats may have limited relevance to human health.

Milestone II: Unique role of vitamin E as a lipophilic antioxidant in lipoproteins and cell membranes. In the 1950s, it was recognized under the leadership of A.L. Tappel’s group that vitamin E is the body’s major lipid-soluble antioxidant protecting the lipoproteins and membranes in which it resides against free radical–mediated lipid peroxidation which, if not prevented or interrupted by vitamin E, causes widespread oxidative molecular damage and pathology (44,45). All natural isoforms and synthetic stereoisomers of vitamin E exhibit to varying degrees the ability to inhibit lipid peroxidation as a “chain-breaking” antioxidant (46–48).

Vitamin E destroys primarily peroxyl radicals and thus protects polyunsaturated fatty acids (PUFA) from oxidation (49). Additionally, vitamin E scavenges a variety of oxygen-derived free radicals including alkoxyl radicals, superoxide, and other reactive oxygen species (ROS) such as singlet oxygen and ozone, and it reacts with nitrogen species (50). The antioxidant action of vitamin E has been documented in numerous human and animal studies, including protection of lipids (51–55) and DNA (56–58). Only one study investigated in an animal model of diabetic pregnancy the effect of vitamin E on the formation of oxidatively modified proteins showing reduction of protein carbonyls by combined treatment with vitamins E and C (59).

Vitamin E participates in an antioxidant network (a concept advanced by L. Packer’s group); thus, vitamin E radicals can be recycled or regenerated back to their native form, e.g., by vitamin C (60–62). When vitamin C radicals form as a result, they may be regenerated by thiol or polyphenol antioxidants in the body. These interactions between redox antioxidant substances and enzymes form the basis for the body’s underlying antioxidant defense system (Fig. 9.2). All naturally occurring analogs and synthetic stereoisomers of vitamin E exhibit redox cycling activity to varying degrees in the system of antioxidant defense against oxidative stress (63–66).

When vitamin E interacts with lipid peroxyl and other lipid radicals in cell membranes or lipoproteins, a tocopheryl or tocotrienyl radical is formed (67,68). However, like other natural antioxidants such as vitamin C or polyphenols, vitamin E radicals are not as dangerous a species as the ones they have destroyed because the free electron is delocalized around their chemical ring structure. This is the unique feature of such biological antioxidants.

“Oxidative stress” is the term particularly designated by H. Sies to call attention to the imbalance that arises when exposure to oxidants changes the normal redox status of tissue antioxidants (69,70). Oxidative stress occurs from an increased formation of ROS and/or from a diminished ability to inactivate these species. So-called “free radicals” or reactive oxygen and nitrogen species are continuously generated under physiologic conditions from metabolism or arise from strenuous and traumatic exercise, and from exposure to environmental stress (e.g., ultraviolet light, cigarette smoke, pollution, and chemicals), infectious microorganisms, viruses, parasites, and during the aging process. These highly reactive species continuously interact in bio-
logical systems, and there is evidence that both oxidants and antioxidant defense must be kept in balance to minimize molecular, cellular, and tissue damage (71,72).

Direct cellular defenses against oxidative stress are comprised of antioxidant enzymes such as superoxide dismutase, catalase, glutathione (GSH), and thioredoxin reductase/peroxidase on the one hand, and of antioxidants on the other hand. Secondary defenses include proteolytic and repair processes that degrade and eliminate damaged molecules and thus reduce damage. If antioxidant defenses are overwhelmed due to a huge amount of oxidative stress, or if defense systems are impaired due to direct damage, age-dependent degradation processes, or lack of micronutrient supply, accumulation of oxidative damage occurs. All of the major aqueous low-molecular-weight antioxidants such as GSH (or its oxidation product glutathione disulfide, GSSG), vitamin C (ascorbate or its 1- or 2-electron oxidized form, the ascorbate radical or dehydroascorbate), protein thiols such as thioredoxin and glutaredoxin, and the lipophilic antioxidants ubiquinol (and its 1-electron oxidation product, the ubiquinone semiquinone radical, or 2-electron oxidation product, ubiquinone), and vitamin E as tocopherols or tocotrienols (and their 1-electron oxidation product, tocopheroxyl, or tocotrienoxyl radicals or 2-electron oxidation product, tocopherylquinones) are in a redox balance equilibrium, which can be shifted to also reflect “oxidative stress.” Recovery of the oxidative imbalance seen in oxidative stress

![Diagram of antioxidant network concept](https://example.com/diagram.png)

**Fig. 9.2.** The antioxidant network concept.
requires NADPH- and NADH-dependent reactions of reducing metabolism. Indeed, all of the redox-based antioxidants appear to interact with one another through an antioxidant network of nonenzymatic and enzymatic reactions.

Mild oxidative stress affects cell signaling pathways and gene expression and is involved in the regulation of cellular functions (73). However, if an excess of oxidative stress occurs and the rate of oxidation exceeds the rate of repair, the strength of the entire antioxidant system will be weakened and concentrations of antioxidants will decrease (74). It is hypothesized that people with low amounts of antioxidants may be more prone to oxidative tissue damage, and thus to disease. The concept of oxidative stress has been implicated in many diseases including infectious diseases, age-related degenerative diseases, and chronic diseases (75–78).

Oxidative stress is recognized as an important factor in aging and age-related diseases. The free radical theory of aging, first postulated by Harman in 1956 (79), suggests that degeneration processes occur due to the accumulation of oxidative damage over a life span. This damage results physiologically from oxidant by-products of normal metabolism and may be increased by environmental factors resulting in accelerated aging processes and disease. Molecular markers of tissue damage to lipids (e.g., isoprostanes, age pigment, or lipofuscin), protein (carbonyl and nitrotyrosine derivatives of protein) and DNA (products of DNA fragmentation and oxidized bases, e.g., 8-hydroxy-2-deoxyguanosine) accumulate during aging, and tissues of aged individuals are more susceptible to oxidative damage (75). This can result from an impaired antioxidant defense system because antioxidant enzymes are less active in aging persons and dietary antioxidants are often insufficient.

**Milestone III: Effects of vitamin E on cell signaling and gene expression.** In the early 1990s, inhibition of PKC activity by vitamin E was suggested by Azzi’s group as the crucial factor for inhibition of cell proliferation in smooth muscle cells (80–83). PKC plays a major role in cell signaling and modulates gene expression during cell growth, proliferation, and differentiation. Thus, PKC activity is an important factor contributing to disorders such as vascular disease, cancer, diabetes, and other age-related degenerative diseases (84–87).

Vitamin E was found to inhibit PKC activity in many cell types including smooth muscle cells, monocytes, macrophages, neutrophils, fibroblasts, and mesangial cells, and the effects were confirmed repeatedly in animal studies (88–90). Inhibition of PKC activity by vitamin E occurs indirectly via activation of a phosphatase that cleaves the active, phosphorylated form of PKC, or by modulating diacylglycerol kinase activity (91,92). What is novel is that inhibition of PKC is apparently independent of the antioxidant activity of vitamin E (93). Hence, the biological role of vitamin E goes beyond its antioxidant function.

Several other mechanisms have been proposed suggesting that vitamin E modulates cell signaling. Recent advances in molecular biology and the availability of microarray techniques for studying effects of vitamin E on gene expression have revealed novel vitamin E–sensitive genes and signal transduction pathways as recently reviewed by Rimbach and co-workers (94).
Vitamin E regulates at the transcriptional level the expression of several genes including collagen \( \alpha_1 \) and \( \alpha \)-TTP in liver (95,96), collagenase in skin (97), adhesion molecules and chemokines such as vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemoattractant protein 1 (MCP-1) in endothelial cells (98), different integrins in erythroleukemia cells (99), \( \alpha \)-tropomyosin in smooth muscle cells (100), and scavenger receptors class A (SR-A) and CD 36 in macrophages and smooth muscle cells (101,102). At the post-translational level, vitamin E regulates the expression of cyclooxygenase in monocytes leading to a decrease in prostaglandin E\(_2\) levels (103). Because this latter effect was also observed using other vitamin E homologues compatible with antioxidant activity, this function of vitamin E may involve redox-signaling.

These newly discovered actions of vitamin E may help to explain the observed beneficial effects of vitamin E in the chronic and degenerative diseases of aging. Future experimental studies are required to achieve the following: (i) to clarify the role of PKC inhibition in the described activities of vitamin E, and the effects of TAP on gene expression and biological activity; (ii) to determine to what extent antioxidant function, i.e., proton and/or electron transfer, plays a role in the effects of vitamin E on cell signaling and gene expression; and (iii) to investigate potentially different effects on cell signaling and biological activities among isoforms of vitamin E as well as stereoisomers of synthetic \( \alpha \)-tocopherol.

**Milestone IV: Recognition of vitamin E deficiency diseases and the beneficial effects of vitamin E supplements in human health and disease prevention.** Clinically manifest vitamin E deficiency is rare. Clear indications of vitamin E deficiency have been found in premature infants and in children suffering from an abnormal ability to absorb vitamin E as in abetalipoproteinemia, chronic cholestatic liver disease, cystic fibrosis, and short-bowel syndrome (104,105).

A mutation in the \( \alpha \)-TTP gene as found in “familial isolated vitamin E deficiency” results in a failure to deliver \( \alpha \)-tocopherol to the systemic circulation (106,107). In these cases, clinical vitamin E deficiency is recognized in infants showing symptoms of retrolental fiblasia and intraventricular hemorrhage, and in children suffering from muscular dystrophy, ataxia, and other disorders (108,109). High-dose vitamin E supplementation can reduce or eliminate clinical symptoms in these patients (110); however, to achieve amelioration of neurological symptoms, early diagnosis and early start of treatment are crucial.

More frequently, a chronic suboptimal supply of vitamin E (i.e., theoretically an intake below RDA levels) occurs population wide and in all age groups (16,111). This may cause impaired defense against oxidative stress and increased susceptibility to oxidative injury and adverse health effects. In most diseases that have been examined with any degree of scrutiny, evidence for “oxidative stress” and oxidative damage has been observed in some stage of disease initiation or progression. Therefore it seems obvious that vitamin E, like other antioxidants, may prevent or delay disease progression. However, can vitamin E at dietary levels or as supplements given during a short period of our life span effectively prevent chronic diseases and delay age-related degenerative disorders? Indeed, much evidence from observational, clinical, and
<table>
<thead>
<tr>
<th>Study</th>
<th>Institution</th>
<th>End Points</th>
<th>Participants</th>
<th>Phase</th>
<th>Type and design</th>
<th>Treatment</th>
<th>Follow-up</th>
<th>Expected completion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E to treat uveitis-associated macular edema</td>
<td>National Eye Institute (NEI), Bethesda, MD</td>
<td>Cystoid macular edema; uveitis</td>
<td>80</td>
<td>I</td>
<td>Treatment, randomized, double-blind</td>
<td>1600 IU/d vitamin E, or placebo, for 4 mo</td>
<td>mo 4 and 5</td>
<td>N/A</td>
</tr>
<tr>
<td>Donepezil and vitamin E to prevent CD after cranial radiation therapy</td>
<td>Multicenter</td>
<td>Small cell lung cancer; depression; delirium</td>
<td>104</td>
<td>III</td>
<td>Supportive care randomized, double-blind, two-arm</td>
<td>Vitamin E twice daily; and donepezil once daily, or placebo, for at least 1 mo</td>
<td>Every 6 mo</td>
<td>2004</td>
</tr>
<tr>
<td>Memory impairment study</td>
<td>Multicenter</td>
<td>MCI</td>
<td>720 ≥ 55 y old</td>
<td>III</td>
<td>Prevention, treatment, randomized, double-blind, three-arm</td>
<td>2000 IU/d vitamin E or donepezil, or placebo every 6 mo</td>
<td>mo 3 and 6, then every 6 mo</td>
<td>2002</td>
</tr>
<tr>
<td>SELECT 4</td>
<td>Multicenter</td>
<td>Prostate cancer</td>
<td>32,400 men, ≥ 55 y old</td>
<td>III</td>
<td>Prevention, randomized, double-blind, four-arm</td>
<td>400 IU/d vitamin E and/or 200 µg/d selenium, or placebo</td>
<td>Every 6 mo for 7 y</td>
<td>2012</td>
</tr>
<tr>
<td>Lymphedema study</td>
<td>Royal Marsden Hospital, London, UK</td>
<td>Lymphedema</td>
<td>100 women</td>
<td>II</td>
<td>Supportive care; randomized, double-blind, two-arm</td>
<td>Vitamin E and pentoxifylline, or placebo twice a day for 6 mo</td>
<td>At 6 mo and 1 y</td>
<td>N/A</td>
</tr>
<tr>
<td>Lung cancer study</td>
<td>Multicenter</td>
<td>Lung cancer</td>
<td>60 former smokers</td>
<td>II</td>
<td>Supportive care; prevention, randomized, double-blind, three-arm</td>
<td>Vitamin E and/or isotretinoin</td>
<td>Annually for 2 y</td>
<td>N/A</td>
</tr>
<tr>
<td>Vitamin E as add-on therapy for children with epilepsy</td>
<td>The Children’s Hospital, Denver, CO</td>
<td>Epilepsy</td>
<td>50 1–18 y old</td>
<td>IV</td>
<td>Treatment, randomized, double-blind, two-arm</td>
<td>Vitamin E or placebo</td>
<td>6 mo</td>
<td>N/A</td>
</tr>
<tr>
<td>Carotid atherosclerosis trial</td>
<td>University of Texas, Dallas, TX</td>
<td>Cardiovascular</td>
<td>120</td>
<td>II</td>
<td>Treatment, randomized, double-blind</td>
<td>1200 IU/d vitamin E, or placebo, for 2 y</td>
<td>Every 6 mo for 2 y</td>
<td>N/A</td>
</tr>
<tr>
<td>Study</td>
<td>Institution</td>
<td>Disease</td>
<td>Treatment</td>
<td>Prevention, Treatment, Randomized, Double-blind, Two-arm</td>
<td>Interim and Final Data Collection</td>
<td>Final Data Collection</td>
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<tr>
<td><strong>Diet and PSA levels Memorial Sloan-Kettering Cancer Center, New York, NY</strong></td>
<td>Prostate cancer</td>
<td>154</td>
<td>Prevention, treatment, randomized, double-blind, two-arm</td>
<td>“Intensive nutritional intervention or general nutritional instruction” for 18 mo</td>
<td>mo 1 and 3, then every 3 mo</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Laser and medical treatment of diabetic macular edema NEI, Bethesda, MD</strong></td>
<td>Diabetes mellitus; macular edema</td>
<td>60</td>
<td>Treatment, randomized</td>
<td>1600 IU vitamin E, or placebo, pre- and postphotocoagulation</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AREDS Multicenter Cataracts; age-related macular degeneration</strong></td>
<td>4757</td>
<td>Treatment, randomized, double-blind, four-arm</td>
<td>Antioxidants (500 mg vitamin C, 400 IU vitamin E, 15 mg β-carotene) and/or 80 mg zinc daily, or placebo</td>
<td>Every 6 mo for at least 5 y</td>
<td>2005</td>
<td></td>
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<tr>
<td><strong>WHS Multicenter Cardiovascular; cancer</strong></td>
<td>39,876 women, ≥ 45 y old</td>
<td>Prevention, randomized, double-blind, 2 x 2 factorial</td>
<td>600 IU vitamin E and/or aspirin on alternate days, or placebo</td>
<td>At least 5 y</td>
<td>2004</td>
<td></td>
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<tr>
<td><strong>PHS II Multicenter Cardiovascular; cancer; eye disease</strong></td>
<td>15,000 male physicians, ≥ 55 y old</td>
<td>Prevention, randomized, double-blind, 2 x 2 x 2 factorial</td>
<td>400 IU vitamin E and/or 50 mg β-carotene on alternate days, and/or 500 mg/d vitamin C and/or multivitamin daily, or placebo</td>
<td>Annually for at least 5 y</td>
<td>2002 (extended until 2007)</td>
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<tr>
<td><strong>WACS Multicenter Cardiovascular</strong></td>
<td>8000 women, ≥ 40 y old</td>
<td>Prevention, randomized, double-blind, 2 x 2 x 2 factorial</td>
<td>600 IU vitamin E and/or 50 mg β-carotene on alternate days, and/or 500 mg/d vitamin C, or placebo</td>
<td>Annually for at least 5 y</td>
<td>2002 (extended until 2006)</td>
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<tr>
<td><strong>SUVMAX Multicenter Cardiovascular</strong></td>
<td>13,000 women ≥ 35 y old, men ≥ 45 y old</td>
<td>Prevention, randomized, double-blind</td>
<td>30 mg/d vitamin E, 120 mg/d vitamin C, 6 mg β-carotene, 100 µg/d selenium and 20 mg/d zinc, or placebo</td>
<td>Annually for 8 y</td>
<td>2003</td>
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</tbody>
</table>

*(continued)*
### TABLE 9.1 (continued)
Ongoing Clinical Trials Involving Vitamin E (July 2002)\(^a\)

<table>
<thead>
<tr>
<th>Study(^b)</th>
<th>Institution</th>
<th>End Points</th>
<th>Participants</th>
<th>Phase</th>
<th>Type and design</th>
<th>Treatment</th>
<th>Follow-up</th>
<th>Expected completion</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAVE(^{16})</td>
<td>Multicenter</td>
<td>Cardiovascular</td>
<td>420 women, ≥ 38 y old</td>
<td>III</td>
<td>Prevention, treatment, randomized, double-blind, 2 × 2 factorial</td>
<td>800 IU/d vitamin E and 1000 mg/d vitamin C, or placebo, and/or hormone replacement therapy</td>
<td>3 y</td>
<td>2002</td>
</tr>
<tr>
<td>CLIPS(^{17})</td>
<td>Multicenter</td>
<td>Leg ischemia; cardiovascular</td>
<td>350</td>
<td>III</td>
<td>Prevention, treatment, randomized, double-blind, 2 × 2 factorial</td>
<td>Antioxidants (600 mg/d vitamin E, 250 mg/d vitamin C, 20 mg/d (\beta)-carotene) and/or aspirin, or placebo</td>
<td>Up to 4 y</td>
<td>2001</td>
</tr>
</tbody>
</table>

\(^a\)Data for studies 1–12, 14, and 16 were obtained from [www.clinicaltrials.gov](http://www.clinicaltrials.gov); for study 13, from Ref. 112; for study 15, [www.suvimax.org](http://www.suvimax.org); and for study 17, from [www.vas-int.org](http://www.vas-int.org).

\(^b\)The official titles of the studies are as follows:

1. Randomized Masked Study to Evaluate the Use of Vitamin E in the Treatment of Uveitis-Associated Macular Edema.
3. A Randomized, Double-Blind, Placebo-Controlled Trial to Evaluate the Safety and Efficacy of Vitamin E and Donepezil HCl (Aricept) to Delay Clinical Progression from Mild Cognitive Impairment (MCI) to Alzheimer’s Disease (AD).
4. Phase III Randomized Study of Selenium and Vitamin E for the Prevention of Prostate Cancer.
5. Phase II Randomized Study of Vitamin E and Pentoxifylline in Women with Lymphedema After Radiotherapy for Breast Cancer.
6. Phase II Randomized Study of Isotretinoin with or Without Vitamin E for the Chemoprevention of Lung Cancer.
7. Double-Blind, Placebo-Controlled Trial of Vitamin E as Add-On Therapy for Children with Epilepsy.
8. Effect of High Dose Vitamin E on Carotid Atherosclerosis.
11. Age-Related Eye Disease Study.
12. Women’s Health Study—I Trial of Aspirin and Vitamin E in Women.
13. Physicians’ Health Study.
14. Women’s Antioxidant and Cardiovascular Study.
15. Supplementation en Vitamines et Minéraux Antioxydants.
16. Women’s Angiographic Vitamin and Estrogen Study (completed but the results have not yet been published).
17. Critical Leg Ischemia Prevention Study (completed but the results have not yet been published).
Experimental studies document a beneficial role of vitamin E, and several small clinical trials and some large human intervention studies are ongoing (Table 9.1). The following chapters on vitamin E in this book highlight recent findings and discuss biological functions, health benefits, and the potential therapeutic role of vitamin E in humans.

Acknowledgments

The authors are very grateful to Dr. Estibaliz Olano-Martin, Department of Respiratory and Critical Care Medicine, University of California, Davis for helpful discussions and for summarizing ongoing clinical trials.

References


Chapter 10

Bioavailability and Biopotency of Vitamin E in Humans: An Ongoing Controversy

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Introduction

Vitamin E (α-tocopherol) occurs in nature as a single compound in which carbon atoms 2, 4′ and 8′ are in the R position and the phytol side chain is straight (RRR, Fig. 10.1). Synthetic vitamin E (all-rac-α-tocopherol) is a mixture of eight stereoisomers including RRR. Half of the material is in the 2R-conformation, half in the 2S. It is believed that biopotency depends largely on the 2R-position; this is evident from the relative potencies in Fig. 10.1 (in brackets) as determined in the rat gestation-resorption test (1).

The bioavailability and biopotency of natural (RRR) and synthetic (all-rac) α-tocopherol have been discussed controversially for decades. Studies in humans that tried to assess potency of vitamin E are rare and the methods used do not appear to be sensitive enough to allow measurement of graded dose responses. Therefore bioavailability studies have been used in lieu of potency studies. The terms bioavailability and biopotency have often been used interchangeably, resulting in potentially misleading and invalid conclusions. The confusion in terminology is widespread in the literature and has hampered progress in determining potency. The aim of this presentation is a critical review of the studies on potency and bioavailability.

Definitions of Bioavailability and Potency

Bioavailability is defined as the rate and extent of a drug’s appearance in blood. The purpose of conducting bioavailability studies comparing two drugs is to determine whether they are bioequivalent. Bioequivalence has been defined as follows: “Two medicinal products are bioequivalent if they are pharmaceutical equivalents (containing the same amount of the same active substance in the same form) or pharmaceutical alternatives (containing the same active moiety but in a different chemical form, e.g., salt or ester) and if their bioavailabilities following the same molar dose are similar to such a degree that their effects, with respect to efficacy and safety, will be essentially the same” (2). Essentially the same has been defined as follows: “The 90% confidence interval for the ratio of means [e.g., \( C_{\text{max}} \), area under the curve (AUC) or steady-state concentration] should lie within an acceptance interval of 0.80 to 1.25”
The acceptance range was established to allow for the variation in response between subjects and because the clinical effects from dosages within the range cannot be distinguished. Consequently, comparative bioavailability studies are accepted as a surrogate method for potency studies, provided that the compounds to be compared are pharmaceutical equivalents or alternatives. It is obvious, that this proviso does not apply to the comparison of RRR and all-rac, because RRR as a single compound and all-rac as a mixture of eight compounds are neither pharmaceutical equivalents nor alternatives.

Potency is a measure of the effects of a compound. Typical vitamin E effects are the prevention of fetal resorption (death) in the rat, the prevention or cure of myopathy in laboratory animals, and the prevention of erythrocyte hemolysis. The relative potencies of RRR vs. all-rac that are presently accepted for humans were derived from the gestation-resorption test in rats. They are as follows: (i) 1 mg all-rac-α-tocopheryl acetate = 1 USP unit; and (ii) 1 mg RRR-α-tocopheryl acetate = 1.36 USP units (3).

**Novel Definition of Vitamin E Activity by Food and Nutrition Board**

Recently, in establishing Dietary Reference Intakes, the Food and Nutrition Board (FNB) suggested a redefinition of vitamin E activity (4). It was suggested that the vit-

![Fig. 10.1. The eight stereoisomers of all-rac-α-tocopherol. Given in brackets are the potencies relative to RRR α-tocopherol as determined by the rat-gestation test. Source: Ref. 1.](image-url)
amin E activity of α-tocopherol is limited to the natural \( RRR \)-form and the three other 2\( R \)-stereoisomeric forms in synthetic α-tocopherol, excluding the four 2\( S \)-stereoisomers. This would result in a theoretical potency ratio (\( RRR \) vs. \( all-rac \)) of 2:1. The natural (\( RRR \)) β-, γ- and δ- homologues and the respective tocotrienols were not considered to have vitamin E activity in humans. This contrasts with activities of 25–40, 1–11, 1, 28 and 5% reported for β-, γ- and δ- tocopherols, and α- and β-tocotrienols, respectively, relative to α-tocopherol determined in the rat gestation-resorption test (5). The redefinition is a marked change from the present definition of vitamin E that includes all tocopherols and tocotrienols that exhibit qualitatively the biological activity of α-tocopherol (5). The FNB argued that hepatic α-tocopherol transfer protein (α-TTP) has a preference for \( RRR \) compared with the other forms, resulting in preferential secretion into and “maintenance in blood,” whereas the stereoisomers with 2\( S \)-configuration and the non-α-tocopherol homologues are “not maintained” in plasma and tissues. Note that the term “maintenance” is not defined unlike appropriate bioavailability terminology such as mean residence time or half-life. It is a vague term because γ-tocopherol and \( SRR-\alpha \)-tocopherol (one of the stereoisomers that is considered by FNB as not having vitamin E activity) are also absorbed into blood (6) and are present in blood, albeit with an apparent shorter half-life than \( RRR \) (7). γ-Tocopherol constitutes as much as 30–50% of total vitamin E in human skin, muscle, vein, and adipose tissue (8). As recently reviewed, it is a more effective trap for lipophilic electrophiles than α-tocopherol, and as its urinary metabolite, γ-carboxyethyl-hydroxychroman (γ-CEHC) also has natriuretic and anti-inflammatory activities. Thus, it may be more important to human health than previously appreciated (9).

**The Challenge to Determine Vitamin E Potency in Humans**

Designing a true potency study for humans is a challenge for several reasons. Overt vitamin E deficiency is virtually nonexistent, and healthy subjects with normal plasma α-tocopherol (~25 \( \mu \)mol/L) cannot be used. Vitamin E depletion would require >1 y and would be unethical. Myopathy that can be monitored by plasma creatine kinase does not normally occur in human vitamin E deficiency. Finally, vitamin E deficiency presents as subtle neurological symptoms (peripheral neuropathy) that are not useful for establishing a graded dose-response relationship, unlike myopathy in animals. At present there are no clinical end points that appear suitable to assay potency in humans.

Biochemical end points reflecting antioxidant potency such as breath pentane (10) and peroxide-induced hemolysis *ex vivo* in vitamin E–depleted subjects have been used (11). Data obtained by the latter method were taken by FNB to draw a line between vitamin E deficiency and adequacy at 12 \( \mu \)mol/L to determine the requirement (4). α-Tocopherol in plasma low density lipoprotein (LDL; a bioavailability measure) and the rate and lag time of conjugated diene formation, thiobarbituric acid-reactive substances, and macrophage degradation of LDL (measures of potency) after copper-oxidation *in vitro* were measured in a study comparing \( RRR \) and \( all-rac \) at supplement doses of 1600 mg/d for 8 wk (12). α-Tocopherol in LDL increased and the
susceptibility to oxidation decreased at similar rates for both groups, indicating that either form of $\alpha$-tocopherol provided equal antioxidant protection at this high dose. However, the sensitivity of this method to detect minor differences in potency is not sufficient for a precise assessment of the ratio.

**Bioavailability Studies**

*Comparing RRR and all-rac in Parallel Groups*

A total of nine studies were published that compared RRR and all-rac in parallel groups of subjects, three using a single dose and six using repetitive dosing ranging from 10 to 56 d [for a review, see (13)]. The doses ranged from 100 to 1600 mg/d. Plasma concentration at 24 h postdosing, $C_{\text{max}}$, the AUC for plasma or red blood cells, the plasma steady-state concentration, and the concentration in LDL were variously used as parameters of bioavailability. An overview of the ratios found for RRR:all-rac in these studies is shown in Figure 10.2. The majority of the ratios are clustered around the line indicating the presently accepted potency ratio, 1.36, and within the range of acceptance. A single value outside the range (2.62) was reported by Horwitt (14) based on the percentage increase at 24 h, a one-time point measurement. If the AUC$_{6-48h}$ is calculated from the author’s data, a ratio of 1.56 is obtained. Figure 10.2 is in striking contrast to the assumption that all-rac should be allotted half the potency of RRR (14). Despite the fact that, as outlined above, the bioavailability factor does not reflect the biopotency factor, it is interesting to note that the ratio is consistently below 2:1. This indicates that, contrary to the opinion of the FNB (4), more than one half of all-rac may be retained in plasma. In tissues, the ratio tends to be even lower than in plasma (8).

*Intraindividual Biokinetic Comparisons of d3-RRR and d6-all-rac*

In these studies, differentially deuterated forms ($d_3$-RRR and $d_6$-all-rac) were dosed simultaneously at a 1:1 ratio by weight (15). The technique was developed to obtain a deeper insight into the kinetics of absorption, plasma transport, and tissue distribution (15) and to elucidate the sites of biodiscrimination between vitamin E homologues and stereoisomers. Using this approach, intraindividual comparisons were made of plasma and tissue kinetics (8,16) including plasma kinetics in smokers and nonsmokers (17), hepatic VLDL secretion (6), metabolic breakdown to $\alpha$-CEHC (18), maternal-fetal transfer (19), and of biodiscrimination between vitamin E homologues and stereoisomers (20). Studies measuring the plasma response to differentially labeled RRR-$\alpha$-tocopherol, SRR-$\alpha$-tocopherol (one of the eight stereoisomers of all-rac), and $\gamma$-tocopherol have led to the hypothesis that absorption takes place with roughly equal efficiencies indicating little or no discrimination among isomers in the digestive tract (6). In contrast, biodiscrimination occurs in the liver, resulting in preferential resorption of RRR via nascent very low density lipoproteins (VLDL) into blood (6), and this
has been attributed to a higher affinity of \( \alpha \)-TTP for \( \text{RRR} \) (21). It is not known whether discrimination by \( \alpha \)-TTP also occurs after high (supplemental) doses. The main sites and potential events leading to discrimination are given in Table 10.1.

From these studies, the ratios (\( d_3 \)-\( \text{RRR} : d_6 \)-all-rac) of concentrations in plasma and various tissues (“bioavailability ratios”) were also reported. The ratios obtained in all published studies are shown in Figure 10.3, as reviewed recently (13). Included in Figure 10.3 are data reported by the authors and data calculated from the authors’ data. Not included is the ratio of 3.42 found for umbilical cord plasma (19). Figure 10.3 indicates that roughly one half of the data fall into the range of acceptance as defined above, whereas the remainder lies between the upper limit of the range and the value of 2. This is at variance with the statements of the authors who reported ratios of \( \sim 2 \) (17,19,21). Traber \textit{et al}. (17) claimed that the ratio was roughly 2, while specifying in the same sentence, that the average value for all subjects and time points was 1.7 \( \pm \) 0.2. It appears that reiteration of this claim that was originally raised by Horwitt (14), one of the leading vitamin E researchers of his time, has superceded subsequent attempts at estimating the ratio precisely.

In total, the ratios obtained with the competitive uptake method are somewhat higher than those derived from the parallel group studies with unlabeled material. This...
may accrue from experimental bias. Cohn (22) pointed out that, because the total dose contains 50% \( \delta_3 \)-2R-stereoisomers, 25% \( \delta_6 \)-2R-stereoisomers, and 25% \( \delta_6 \)-2S-stereoisomers, 75% \( \alpha \)-forms compete with 25% \( \alpha \)-forms. Because of the threefold higher abundance of the 2-R-form and because of the preference of \( \alpha \)-TTP for 2-R-stereoisomers, an overestimation of RRR and discrimination against all-rac result.

How to explain the differences between the ratios shown in Figure 10.3 and the purported ratio of 2? One explanation is that some authors reported maximum ratios (8,18,23). These were found at time points during the elimination phase rather than at established time points such as \( C_{\text{max}} \) or during steady state. Because elimination rates of RRR and all-rac are different, the ratio of concentrations is not stable but becomes wider with increasing distance from the last dose (13). This results in variable ratios depending on the time point. Furthermore, as shown by kinetic modeling (13), the ratio also depends on the dose, with supplement doses resulting in lower discrimination than dietary doses. Thus, because the bioavailability ratio is affected by the time point and the dose, it is arbitrary and not a true reflection of the ratio of potency.

There is one example of a comparative bioavailability study using differentially labeled vitamin E forms that truly reflects their relative potencies (24). In that study, the free phenolic form and the acetate ester of natural-source vitamin E were given.

<table>
<thead>
<tr>
<th>Site</th>
<th>Event</th>
<th>Discrimination</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas</td>
<td>Carboxylester hydrolase</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Small intestinal chyme</td>
<td>Release from matrix</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tocopherylester hydrolysis</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Entry into mixed micelles</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Micelle diffusion</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Enterocyte</td>
<td>Intracellular transport</td>
<td>No</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td>Incorporation into chylomicrons</td>
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<td></td>
<td>Secretion of chylomicrons</td>
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</tr>
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<td>Plasma chylomicrons</td>
<td>Endothelial lipoprotein lipase</td>
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<td></td>
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<tr>
<td></td>
<td>Formation of chylomicron remnants</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kinetics</td>
<td>No</td>
<td>(6)</td>
</tr>
<tr>
<td>Plasma</td>
<td>Biokinetics</td>
<td>Yes</td>
<td>(6,8,16,20)</td>
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<tr>
<td>Liver</td>
<td>Binding to remnant receptor</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Binding to ( \alpha )-TTP</td>
<td>Dietary doses: Yes</td>
<td>(21)</td>
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<td></td>
<td></td>
<td>Supplements (( \geq 50 ) mg): ?</td>
<td>(33)</td>
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<td></td>
<td>VLDL secretion</td>
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<td>(6)</td>
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<tr>
<td></td>
<td>Biliary secretion</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \alpha )-CEHC formation</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td>( \alpha )-CEHC excretion</td>
<td>Yes</td>
<td>(18)</td>
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<td>Other tissues</td>
<td>Rates of uptake, release and metabolism</td>
<td>No (e.g., CNS)</td>
<td>(8)</td>
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<tr>
<td></td>
<td></td>
<td>No (e.g., liver, muscle)</td>
<td>(8)</td>
</tr>
<tr>
<td>Placenta</td>
<td>Maternal/fetal transfer</td>
<td>Yes</td>
<td>(19)</td>
</tr>
</tbody>
</table>

Abbreviations: \( \alpha \)-TTP, \( \alpha \)-tocopherol transfer protein; VLDL, very low density lipoprotein; \( \alpha \)-CEHC, \( \alpha \)-carboxyethyl-hydroxychroman; CNS, central nervous system.
jointly as \( d_3 \)-RRR-tocopherol and \( d_6 \)-RRR-\( \alpha \)-tocopheryl acetate, respectively, at the same molar dose. The mean ratio \( d_3/d_6 \) for plasma and the AUC was 1.0 and the ratio was constant at all time points. Because in this case the compounds were pharmacologic alternatives containing the same active moiety, RRR-\( \alpha \)-tocopherol, the bioavailability ratio truly reflects the potency ratio.

Conclusions

Rat studies have shown that RRR has 1.36-fold the potency of all-rac. In humans, a lack of sensitive clinical end points or biomarkers has precluded biopotency comparisons. Bioavailability studies were conducted in lieu of potency studies and these have resulted in estimates of the ratio of bioavailability of up to 2. This has resulted in the inference, literally or indirectly, that this ratio reflects the ratio of potency. This inference is challenged on the basis of the differences between RRR and all-rac in stereochemistry and hence, kinetics. Because of these differences, potency studies that measure functional end points must be conducted. At present there is clearly a lack of adequate methods to conduct such studies in humans. Until sensitive and specific clinical end points or biomarkers have been developed, the discussion about the potency of all-rac relative to RRR will be ongoing.

References


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Vitamin E: Evidence for the 2:1 Preference for RRR-Compared with all-rac-α-Tocopherols

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Vitamin E Structures vs. Activities

Almost since the discovery of vitamin E in 1922 (1), there have been disagreements concerning the biologic activities of the various tocopherols and tocotrienols. α-Tocopherol is found in highest concentrations in animal plasma and tissues. Currently, only α-tocopherol has been demonstrated to reverse human vitamin E deficiency symptoms; it is the only form of vitamin E that meets the year 2000 vitamin E recommended dietary allowance (RDA) (2). However, in the plant kingdom, a variety of compounds that have vitamin E antioxidant activity have been described. The latest of these was described in plankton and cold water fishes that consume plankton (3).

Vitamin E was originally defined as a required nutrient because it is essential for maintaining the fetus during pregnancy (1). Thus, tests to evaluate biologic activities of the naturally occurring vitamin E forms were based on amounts required to protect the fetus (4). Unfortunately, most of the studies (5,6) were conducted long before modern methods of chromatography along with sensitive detection methods were available. It is therefore impossible to assess how much contaminating α-tocopherol might have been in the preparations used to test the biologic activity of other vitamin E forms. The presence of contaminating α-tocopherol is important because the α-tocopherol transfer protein (TTP) is expressed during pregnancy in the uterus and placenta (7). This protein selectively transfers α-tocopherol compared with other tocopherols and tocotrienols (8) and may be critical for maintaining the maternal/fetal unit α-tocopherol concentrations.

The rationale for possible differences in biologic activities between various naturally occurring vitamin E forms has been based on slight differences in antioxidant activities. However, antioxidant activities cannot be the explanation for differences in the biologic activities between naturally occurring and synthetic α-tocopherol. When α-tocopherol is chemically synthesized, eight stereoisomers are formed arising from the three chiral centers in the tail, which can be R or S. The naturally occurring α-tocopherol is in the RRR-form, whereas the synthetic contains equimolar concentrations of RRR, RSR, RRS, RSS, SSR, SSR, SRS, and SSS. These forms have identical chromanol rings and thus identical antioxidant activities.
Lack of Bioequivalence of \textit{RRR}- and all-rac-\textit{\alpha}-Tocopherols

Bioequivalence implies comparable bioavailability and biopotency (9). Different formulations are \textit{bioequivalent} if the rate and extent of absorption of the active ingredients are not significantly different when administered under similar conditions, or result in the same concentration vs. time curves, or if all of their effects are identical (10). \textit{RRR}- and all-rac-\textit{\alpha}-tocopherols have not been proven to meet any of these criteria for bioequivalence; thus, they must be presumed to be different compounds, not different formulations of the same compound. Because the active moieties (stereoisomers) differ in \textit{RRR}- and all-rac-\textit{\alpha}-tocopherols, and have different or unknown potencies for each physiologic effect, bioavailability and biopotency ratios cannot be assumed to be equal.

Determination of Biological Activities Using Deuterium-Labeled Vitamin E

\textit{Bioavailability of \alpha-Tocopherol}

Plasma concentrations of \alpha-tocopherols reflect the sum of the four primary pharmacokinetic processes: absorption, distribution, metabolism, and excretion. An individual molecule goes through these processes sequentially, but all four processes occur simultaneously. Clearance from plasma occurs by both distribution and elimination. Distribution and elimination (metabolism and excretion) begin as soon as the first molecule is absorbed. Slower absorption allows more distribution and elimination to occur before absorption is complete. Thus, the area under the curve (AUC) of plasma concentrations vs. time is related to the rate and extent of absorption and distribution and to the rate of elimination (9).

Bioavailability is a measure of an individual’s total exposure to a substance. The ratio of steady-state plasma concentrations of a substance after oral compared with intravenous administration is proportional to its fractional absorption. \textit{Bioavailability} is the rate and extent to which the active moiety is absorbed, enters the systemic circulation, and is available at the site of action (9,10).

Calculation of deuterated \alpha-tocopherol (AUC) may be the best method for determining vitamin bioavailability E. When healthy adults ingested 15, 75, or 150 mg deuterated \textit{RRR}-\alpha-tocopheryl acetate (11), the AUC calculated from plasma d\textsubscript{3}-\textit{RRR}-\alpha-tocopherol concentrations increased linearly with dose (i.e., a 10-fold increase in dose resulted in a 10-fold increase in AUC). Linear increases in the AUC with dose reflect linear increases in the sum of absorption, distribution, metabolism, and excretion; this suggests that these processes were not saturated.

However, there was little change in plasma total (labeled plus unlabeled) \alpha-tocopherol concentrations, which were 12 \mu mol/L at baseline and averaged 13.3, 15.4, and 16.7 over 96 h after the 15, 75, and 150 mg doses, respectively. Thus, the newly absorbed labeled vitamin E was preferentially used to replace the “old” circulating vit-
This observation also makes it clear that interpretation of vitamin E bioavailability cannot be carried out using unlabeled vitamin E. Thus, studies comparing the efficacy of supplements containing either natural or synthetic vitamin E are invalid because the amount of newly absorbed vitamin E from the supplement cannot be assessed.

**Mechanisms of Transport and Discrimination in Humans**

The use of stable isotope–labeled vitamin E has been instrumental in describing how differences in the absorption and plasma transport of vitamin E forms lead to differences in their biological activities. Because these topics have been reviewed extensively elsewhere (12,13), they will be discussed only briefly here. Studies using \( RRR \) and \( SRR-\alpha \)-tocopherols and \( RRR-\gamma \)-tocopherol labeled with different amounts of deuterium demonstrated that all of these forms were equally absorbed and secreted in chylomicrons into the plasma (14,15). Subsequently, there was a preferential secretion of \( RRR-\alpha \)-tocopherol from the liver into the plasma in very low density lipoproteins (VLDL). This preference for \( \alpha \)-tocopherol is dependent upon the function of TTP (16) because patients with a genetic defect in TTP become spontaneously vitamin E deficient when consuming diets adequate in vitamin E (17).

**Studies in Mice Lacking the \( \alpha \)-Tocopherol Transfer Protein**

Although studies in humans with defects in the TTP gene have suggested that TTP was required for maintenance of plasma \( \alpha \)-tocopherol concentrations (17), only limited information is available on these vitamin E–deficient subjects’ tissue \( \alpha \)-tocopherol concentrations (18). Mice in which the gene for TTP was deleted have extraordinarily low vitamin E plasma and tissue concentrations (19–21) and express vitamin E deficiency symptoms (7,19). To determine whether TTP knockout mice were unable to discriminate between natural and synthetic vitamin E (21), adult TTP knockout (\( Ttpa^{-/-} \), \( n = 5 \)), heterozygous (\( Ttpa^{+/+} \), \( n = 7 \)), and control (\( Ttpa^{+/+} \), \( n = 3 \)) mice consumed equimolar \( d_6 \) \( RRR \)- and \( d_3 \) all-rac-\( \alpha \)-tocopheryl acetates (30 mg each/kg diet) for 3 mo; labeled and unlabeled \( \alpha \)-tocopherols in plasma and 17 tissues were then measured by liquid chromatography/mass spectrometry (LC/MS) (21). Although deuterium-labeled \( \alpha \)-tocopherols represented >85% of the plasma \( \alpha \)-tocopherol in all groups, \( Ttpa^{-/-} \) mice had plasma total \( \alpha \)-tocopherol concentrations that were only 5.4% of \( Ttpa^{+/+} \) and 7.7% of \( Ttpa^{+/-} \) mice. \( Ttpa^{-/-} \) tissue (except liver) total \( \alpha \)-tocopherol concentrations were 2–20% of those in \( Ttpa^{+/+} \) mice. These data are consistent with the concept that vitamin E is absorbed and transported to the liver, and only if TTP is expressed is \( \alpha \)-tocopherol exported from the liver into the plasma for tissue delivery. Clearly, TTP is required not only to maintain plasma \( \alpha \)-tocopherol, but also tissue \( \alpha \)-tocopherol.

The other proposed function of TTP is the preferential secretion of \( RRR-\alpha \)-tocopherol from the liver into the plasma. In mice fed 1:1 \( d_6 \) \( RRR \)- and \( d_3 \) all-rac-\( \alpha \)-tocopheryl acetates for 3 mo, the \( d_6:d_3 \) ratios in plasma and 16 tissues from
$Ttpa^{+/+}$ and $Ttpa^{+/−}$ mice were double those of $Ttpa^{−/−}$ mice (Fig. 11.1) (21). In TTP-expressing mice, tissue enrichment of natural over synthetic α-tocopherol appears to be due to nonspecific uptake of α-tocopherol from the plasma, which contained 2:1 $d_6:d_3$ α-tocopherols. $Ttpa^{+/−}$ mice that expressed half the amount of hepatic TTP (20) also had $d_6:d_3$ ratios of nearly 2. These data suggest that plasma α-tocopherol concentrations are highly dependent upon the function of TTP and that this protein preferentially selects only the 2R-α-tocopherol forms from all-rac-α-tocopherol for secretion into plasma. Importantly, patients who were α-TTP heterozygotes (expressing two different mutations) were also able to discriminate between RRR- and SRR-α-tocopherols (17,22). These 2:1 ratios are also consistent with findings in normal humans administered labeled RRR- and all-rac-α-tocopheryl acetates (23–25).

Fig. 11.1. α-Tocopherol transfer protein (TTP)-knockout mice do not discriminate between natural and synthetic vitamin E. Shown are the $d_6:d_3$ α-tocopherol ratios in adult α-TTP knockout ($Ttpa^{−/−}$, n = 5), heterozygous ($Ttpa^{+/−}$, n = 7), and wild-type ($Ttpa^{+/+}$, n = 3) mice that consumed a 1:1 $d_6$-RRR- and $d_3$-all-rac-α-tocopheryl acetate–containing diet (30 mg each/kg diet) for 3 mo. Labeled and unlabeled α-tocopherols in plasma and 17 tissues were measured. The $d_6:d_3$ α-tocopherol ratios in plasma and 16 tissues (excluding liver) from $Ttpa^{+/+}$, $Ttpa^{+/−}$ and $Ttpa^{−/−}$ mice were $1.8 \pm 0.2$, $1.9 \pm 0.2$, and $1.1 \pm 0.1$, respectively ($P < 0.0001$, $Ttpa^{−/−}$ vs. $Ttpa^{+/+}$ or $Ttpa^{+/−}$). Source: Adapted from (21).
Vitamin E Kinetics in Pigs

Vitamin E supplements are often provided to farm animals; therefore, the relative bioavailabilities of RRR- and all-rac-α-tocopherols were evaluated in swine (26). Deuterium-labeled vitamin E (150 mg each of d3-RRR- and d6-all rac-α-tocopheryl acetates) was administered orally to female pigs (n = 3) with the morning feed. Blood samples were obtained at 0, 3, 6, 9, 12, 24, 36, 48, and 72 h. The maximum observed plasma concentration of d3-RRR-α-tocopherol was achieved at 12 h (1.12 µmol/L), whereas d6-α-tocopherol peaked earlier (at 9 h) at a lower concentration (0.66 µmol/L, P < 0.05, Fig. 11.2). The d3/d6 ratios were 1.35 ± 0.73 at 3 h and increased to 2.00 ± 0.14 at 72 h (P < 0.03). The d3-α- and d6-α-tocopherol disappearance rates were similar for both tocopherols and estimated to be 0.029 µmol/L per hour. The AUC for d3-α- and d6-α-tocopherol were 38.2 and 18.5, respectively. These studies suggest that pigs rapidly discriminate between forms of vitamin E and selectively retain 2R-forms in the plasma.

![Graph showing discrimination between natural and synthetic vitamin E in swine.](Fig. 11.2. Discrimination between natural and synthetic vitamin E in swine. The plasma d3- and d6-α-tocopherol concentrations after deuterium-labeled vitamin E (150 mg each d3-RRR- and d6-all rac-α-tocopheryl acetates) was administered orally to adult female pigs (n = 3) with the morning feed are shown. The ratio of d3/d6-α-tocopherols increased from 1.35 ± 0.73 at h 3 to 2.0 ± 0.14 at h 72 (P < 0.03). Source: Adapted from (26).)
Vitamin E Kinetics During Pregnancy and Lactation in Sows

The biological activities of RRR- and all-rac-α-tocopherols were also evaluated during pregnancy and lactation, using pigs as a model system (27). Vitamin E delivery to fetuses and to suckling piglets was monitored by feeding 150 mg each of d₃-RRR-α- and d₆-all-rac-α-tocopheryl acetates daily to 3 pregnant sows from 7 d before to 7 d after birth. Blood from fasting sows was obtained in the morning of d −7, −4, 1, and 4 (during supplementation, d 1 was delivery day) and on d 7, 14, 21. Sow’s milk, as well as piglet (n = 9) plasma and tissues were obtained at birth, 7 and 21 d. Labeled and unlabeled vitamin E concentrations were measured by LC/MS.

At birth, despite markedly elevated sow plasma deuterated α-tocopherol concentrations, no labeled α-tocopherol was detected in piglet plasma or tissues. After initiation of suckling by the piglets, a dramatic increase in plasma and tissue α-tocopherol concentrations was observed. These data emphasize the limited placental vitamin E transfer and the importance of milk as a delivery system for enhancing the vitamin E status of the newborn. At d 7 compared with birth, total α-tocopherol concentrations in most piglet tissues had increased 10-fold. The highest deuterated vitamin E concentrations in piglet tissues were found in liver, followed by lung, heart, kidney, muscle, intestine, and brain. Plasma d₃-α-tocopherol concentrations were approximately double those of d₆-α-tocopherol in both sows and piglets.

Biologic Activity of the Tocotrienols: Importance of Metabolism

Because tocotrienols have both antioxidant activities and reportedly decrease serum cholesterol synthesis, the serum cholesterol response and LDL oxidation after α-, γ-, or δ-tocotrienyl acetate supplementation was studied in hypercholesterolemic subjects (28). The subjects consumed a low-fat diet for 4 wk, then were randomly assigned to placebo (n = 13), α- (n = 13), γ- (n = 12), or δ- (n = 13) tocotrienyl acetate supplements (250 mg/d). Supplements were eaten with dinner for 8 wk while subjects continued to consume the low-fat diet.

No differences were observed in plasma cholesterol levels, LDL cholesterol levels, or LDL apolipoprotein B concentrations, suggesting that the tocotrienols were ineffective in modulating cholesterol synthesis. Importantly, tocotrienyl supplements did not affect plasma α- or γ-tocopherol concentrations. Twelve hours after the last dose of supplemental tocotrienyl acetates, fasting plasma concentrations of α-, γ-, and δ-tocotrienols were ~1, 0.5, and 0.1 µmol/L, respectively. Apparently, tocotrienyl acetate supplements are hydrolyzed and absorbed, but plasma tocotrienol concentrations remain barely detectable. The reason for these relatively low concentrations compared with the ~20 µmol/L α-tocopherol concentration is the very fast clearance of the plasma tocotrienols, as demonstrated by Yap et al. (29).

The mode of tocotrienol elimination from the body is not known. Hypothetically, the rapid disappearance of the tocotrienols could be a result of metabolism.
Carboxyethyl-hydroxychromans (α- and γ-CEHC) are human urinary metabolites of α- and γ-tocopherols, respectively (30). Lodge et al. (31) demonstrated that α- or γ-tocotrienols are metabolized to the same carboxyethyl-hydroxychromans (α- and γ-CEHC) as are α- and γ-tocopherols, respectively, and that these CEHC are detectable in urine. The subjects (n = 5 men, 1 woman) were healthy, nonsmokers, who did not take antioxidant supplements. A single supplement was consumed with breakfast each week for 4 wk in the following order: 125 mg γ-tocotrienyl acetate, 500 mg γ-tocotrienyl acetate, 125 mg α-tocotrienyl acetate, then 500 mg α-tocotrienyl acetate. Complete urine collections were obtained 24 h before, as well as on the day of supplementation, and on the subsequent 2 d. The fraction of the dose excreted as CEHC, as shown in Figure 11.3, was ~1 and 5% for α- and γ-tocotrienols, respectively. These relatively low percentages suggest that tocotrienols (and perhaps other vitamin E forms) that are rapidly cleared from plasma are eliminated apparently by a mechanism other than metabolism, or the metabolites are excreted by other routes.

Conclusions

The use of stable isotope–labeled vitamin E has allowed the estimation of vitamin E pharmacokinetic parameters. However, unlike drugs that are generally absorbed,
transported, and excreted by nonspecific mechanisms, the use of labeled vitamin E forms has demonstrated how differences in the plasma transport of vitamin E forms lead to differences in their biologic activities. It is quite apparent that TTP activity determines that only 2R-α-tocopherols are specifically maintained in the plasma. This leads to the question concerning what function α-tocopherol plays specifically and why α-tocopherol if other tocopherols and tocotrienols have potent antioxidant activities.

References


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Introduction

Vitamin E is a term that includes 4 tocopherols (α, β, γ, δ) and 4 tocotrienols (α, β, γ, δ) (1). Naturally occurring tocopherols have an RRR stereochemistry, whereas the synthetic all-rac-tocopherols comprise all 8 possible stereoisomers. Tocotrienols have an unsaturated side chain and thus contain only one chiral center, which in the natural form has the R configuration. RRR-α-tocopherol is preferentially retained in the human body indicating that it may have functions that cannot be exerted by other forms of vitamin E. The special aspects of handling α-tocopherol include sorting, degradation, and possibly also absorption.

Absorption

The human diet contains the range of vitamin E types, with γ-tocopherol as the most abundant form in the typical American diet (2). Despite the higher γ-tocopherol intake, its concentration in human plasma is 5–10 times less than that of α-tocopherol. Administration of 250 mg α-, γ-, or δ-tocotrienol/d for 8 wk did not lead to plasma levels >1 µmol/L (3). It is generally believed that all forms of vitamin E are equally absorbed in the intestine [for review see (4)] and that the preferential treatment of α-tocopherol occurs in the liver by means of the α-tocopherol transfer protein (α-TTP; see below). Studies with children with cholestatic liver disease (5) and with patients suffering from cystic fibrosis (6) demonstrated that uptake of vitamin E into intestinal mucosa cells requires bile acids and pancreatic enzymes. Absorption is believed to be a passive process facilitated by fat intake. Release of vitamin E into the circulation occurs via chylomicrons. The mechanism of chylomicron formation and lipid loading is relatively well known (7), whereas it remains unclear how chylomicrons are loaded with different forms of vitamin E. In cultured hepatocytes, fibroblasts, and macrophages, an α-TTP–dependent but lipoprotein assembly–independent process was reported (8). Similar to the cholesterol reverse transport, it appears to be mediated by the ABCA1 transporter (9). Whether such a pathway is also functioning in the intestine requires investigation.
Sorting

In the liver, \(\alpha\)-TTP selects the \(\alpha\)-tocopherols with \(R\)-configuration at C-atom 2 and delivers them to very low density lipoproteins by a mechanism that is not yet known. The affinities of \(\alpha\)-TTP for \(\beta\)-, \(\gamma\)-, \(\delta\)-tocopherols and \(\alpha\)-tocotrienol are 38, 9, 2, and 11% of that for \(\alpha\)-tocopherol, respectively (10), explaining the high levels of \(\alpha\)-tocopherol in plasma. The vital function of \(\alpha\)-TTP is evident from patients with mutations in the gene for \(\alpha\)-TTP. These patients have extremely low \(\alpha\)-tocopherol plasma levels and suffer from neurological symptoms typical of vitamin E deficiency, e.g., cerebellar ataxia (11,12). \(\alpha\)-TTP is expressed in Bergmann glia cells surrounding and alimenting Purkinje cells (13), which are involved in the coordination of intentional movements. \(\alpha\)-TTP is obviously needed in the Purkinje layer to regulate the transfer of \(\alpha\)-tocopherol, a role that other tocopherols cannot perform.

In 1922, Evans and Bishop (14) reported on the indispensability of vitamin E for the reproduction of female rats. The detection of \(\alpha\)-TTP in the mouse uterus links \(\alpha\)-TTP to \(\alpha\)-tocopherol–dependent reproduction (15). In \(\alpha\)-TTP knockout mice, the embryos die between d 9.5 and 10.5 of gestation and are resorbed (15). Circumstantial evidence suggests that \(\alpha\)-TTP is also operative in the human placenta. Pregnant women were given equal amounts of \(d_3\)-\(RRR\)-\(\alpha\)- and \(d_6\)-all-\(rac\)-\(\alpha\)-tocopheryl acetate 5 d before delivery (16). The ratio of \(d_3/d_6\) was found to be 1.86 in the mothers’ plasma and 3.42 in the cord blood. This means that \(\alpha\)-tocopherol has been sorted twice, first in the mothers’ liver and obviously again at the maternal-fetal interface.

Degradation

Tocopherols and tocotrienols are metabolized by side-chain degradation. The final products, carboxyethyl hydroxychromans (CEHC), are conjugated with glucuronic acid or sulfate and excreted in the urine (17–21). Irrespective of the dosage, only 1–3% of the ingested \(RRR\)-\(\alpha\)-tocopherol appears in the urine as \(\alpha\)-CEHC (22). In contrast, at least 50% of \(\gamma\)-tocopherol is degraded and eliminated this way (23). Up to 2 and 6% of \(\alpha\)- and \(\gamma\)-tocotrienol administered orally have been found as urinary \(\alpha\)- and \(\gamma\)-CEHC, respectively (21). Thus, different forms of vitamin E are metabolized at different metabolic rates. Pertinent degradation pathways were therefore studied in cultured HepG2 cells.

\(\beta\)-Oxidation

The structures of the final degradation products, CEHC, suggest that side-chain degradation must have occurred via a \(\beta\)-oxidation pathway (Scheme 12.1). The identification of immediate precursors of \(\alpha\)- and \(\gamma\)-CEHC, \(\alpha\)- and \(\gamma\)-carboxymethylbutyl hydroxychroman (CMBHC) in human urine (22,24–26) and of \(\alpha\)-carboxymethylhexyl hydroxychroman (\(\alpha\)-CMMHC) in HepG2 cells (27) established this mechanism. For \(\gamma\)-tocopherol, all theoretical intermediates of the \(\beta\)-oxidation pathway were confirmed recently (28).
Although the \( \beta \)-oxidation scheme is straightforward for the tocopherols, auxiliary enzymes are required for the degradation of tocotrienols because of their unsaturated side chain (Scheme 12.2). Formation of the \( \alpha,\beta \)-unsaturated fatty acid in the second and fourth round in the \( \beta \)-oxidation of tocotrienols leads to compounds with two conjugated
SCHEME 12.2. Mechanism of \( \gamma \) -tocotrienol side-chain degradation. \( \beta \)-Oxidation intermediates of \( \gamma \)-tocotrienol, \( \gamma \)-carboxymethylbutyl hydroxychroman (\( \gamma \)-CMBHC), the \( \gamma \)-carboxymethylhexyl hydroxychroman (CMHHC) precursor \( \gamma \)-carboxymethylhexenyl hydroxychroman [\( \gamma \)-CMH(en)HC], and \( \gamma \)-carboxydimethyloctenyl [\( \gamma \)-CDMO(en)HC] as well as the final product \( \gamma \)-carboxyethyl hydroxychroman (\( \gamma \)-CEHC) were identified in the culture medium of HepG2 cells. The cells were incubated with 50 \( \mu \)mol/L \( \gamma \)-tocotrienol for 2 d. Then, medium was extracted, metabolites separated by high-performance liquid chromatography (HPLC) with electrochemical detection and identified by gas chromatography (GC)/mass spectrometry (MS) (27). All MS spectra showed an \( m/z \) typical of the fragmented chroman structure, which is 223 in case of \( \gamma \)-substitution, and an \( m/z \) of 73 characteristic for the cleaved trimethylsilyl group. Molecular ion \( m/z \) was 408, 450, 476, and 518 indicating \( \gamma \)-CEHC, \( \gamma \)-CMBHC, \( \gamma \)-CMH(en)HC, and \( \gamma \)-CDMO(en)HC. Compounds (a) and (b) are hypothetical intermediates produced by 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase, respectively.
double bonds that are not accepted by enoyl-CoA hydratase that usually forms the β-hydroxyacyl-CoA. In linoleic acid metabolism, this impasse is resolved by the action of two auxiliary enzymes, 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase (see biochemistry texts). The identification of γ-carboxydimethyloctenyl hydroxychroman [γ-CDMO(en)HC] and γ-CMBHC lacking the double bond originally present in the tocotrienol (Scheme 12.2) confirms that the unsaturated side chain of tocotrienols is indeed metabolized like unsaturated fatty acids. Production of the CMBHC precursor, γ-carboxymethylhexenyl hydroxychroman [γ-CMH(en)HC], does not require auxiliary steps and has been found to be released as such from HepG2 cells incubated with α- or γ-tocotrienol. In principle, the enzymatic steps implicated are possible in peroxisomes and in mitochondria. At present, however, it is not clear where the side-chain degradation of tocopherols and tocotrienols is performed. The release of CEHC precursors by HepG2 cells indicates that the formation of the final product CEHC might involve rate-limiting steps. Whether precursors accumulate to relevant concentrations also in vivo and to which compartment they are released remains to be investigated.

**ω-Oxidation**

β-Oxidation of tocopherol and tocotrienol depends on initial ω-hydroxylation and subsequent oxidation of the hydroxyl group to an aldehyde and carboxylic acid function. The involvement of cytochrome P450 (CYP) enzymes appears plausible and has been deduced from inhibition and induction studies. Ketoconazole and sesamin, then believed to affect CYP3A-type enzymes specifically, have been shown to inhibit the release of γ- and δ-CEHC and γ- and δ-CMBHC from HepG2 cells (29). Rifampicin, known as a CYP3A inducer, stimulated the release of α-CEHC from HepG2 cells when treated with all-rac-α-tocopherol but not when treated with RRR-α-tocopherol (27). Recently, however, microsomes from insect cells transfected with human CYP4F2 have been shown to be most active in oxidizing the ω-methyl group of RRR-α- and γ-tocopherol, whereas a variety of other cytochromes including CYP3A4 were inactive (28). In this system, CYP4F2 preferentially degraded γ-tocopherol, whereas α-tocopherol was bound slightly better but metabolized more slowly. This was interpreted to explain the comparatively slow turnover of RRR-α-tocopherol in general. Difficulties in detecting α-CEHC release from HepG2 cells might, however, equally result from other reasons. We have shown that RRR-α-tocopherol degrades only after a long incubation time or in cells already adapted to all-rac-α-tocopherol (27). The inability of rifampicin to up-regulate RRR-α-tocopherol degradation could point to the involvement of a different CYP in the metabolism of this stereoisomer. In addition, the experimental results shown in Figure 12.1 may be interpreted along these lines. The yield of degradation products from HepG2 cells was substantially higher with γ-tocopherol and could not be further increased by rifampicin treatment, whereas rifampicin stimulated all-rac-α-tocopherol-derived α-CEHC and α-CMBHC release. If not due to different CYP, the differential rates and inducibilities of the tocopherol metabolism may result from the following: (i) highly different affinities or metabolizing rates of the hydroxylation system for α- and γ-tocopherol as suggested (28); (ii)
Rifampicin stimulates the release of metabolites from all-rac-α-tocopherol but not from γ-tocopherol from HepG2 cells into the culture medium. Tocopherol-adapted HepG2 cells (27) were deprived of α-tocopherol by incubation in tocopherol-free medium for 4 d (wash-out). After that, no metabolites were detectable in the cell culture medium. During the last 2 d of the wash-out period, 50 µmol/L rifampicin was added to the culture medium. Control cells did not receive rifampicin. After the complete wash-out phase, 100 µmol/L all-rac-α- or γ-tocopherol was added. After 72 h, the medium was collected and 1-naphthol added as internal standard (final concentration 0.033 µmol/L). Thereafter, the medium was acidified to pH 4.5 and extracted 3 times with 10 mL t-butylmethylether. Solvent was removed by evaporation; the resulting residue was dissolved in high-performance liquid chromatography (HPLC) solvent and analyzed by HPLC-electrochemical detection as described (27). To obtain faster elution, acetonitrile was mixed with the solvent from min 32 to reach 40% acetonitrile after 52 min. Because an authentic standard was not available for γ-carboxymethylbutyl hydroxychroman (γ-CMBHC), data are expressed as 1-naphthol equivalents (NE). Response factors were 1.46 and 1.48 for α- and γ-carboxyethyl hydroxychromans (CEHC), respectively, and 2.44 for α-CMBHC. Actual concentrations (pmol/mg protein) were: 29.6 ± 16.2 for α-CEHC (control); 91.3 ± 42.5 for α-CEHC (rifampicin); 602 ± 176 for α-CMBHC (control); 1471.5 ± 397.5 for α-CMBHC (rifampicin). The concentrations of γ-CEHC (nmol/mg protein) were: 3.89 ± 0.71 (control) and 4.08 ± 1.32 (rifampicin). Thus, concentrations of α-CMBHC are somewhat underestimated in the figure and probably also those of γ-CMBHC. Values are means ± SD (n = 3). For better clarity, the α-CEHC is shown again in the insert on a different scale.
effective binding of α-tocopherol to α-TTP or to one of the tocopherol-associated proteins (TAP) (30,31), thereby preventing degradation; or (iii) upregulation of the metabolizing system by γ-tocopherol itself. Which of the possibilities finally turns out to be true remains to be determined.

**Relevance of Side-Chain Degradation In Vivo**

Although only 1–3% of the administered dose of RRR-α-tocopherol is found as α-CEHC in human urine, the percentage of all-rac-α-tocopherol–derived CEHC is 3–4 times higher (32). In rats administered a single dose of equal amounts of radioactively labeled SRR- and RRR-α-[5-methyl-14C] tocopherol, 87.6 and 83% of the respective radioactivity was recovered in feces after 96 h (33). Of the administered dose, 7.8% was observed in the urine as α-CEHC from SRR- and 1.3% from RRR-α-tocopherol. This means that <10% of RRR-α-tocopherol that was not excreted via the feces was eliminated in the urine, whereas this fraction was up to 60% for SRR-α-tocopherol. This corresponds to the calculation made by Swanson et al. (23) who reported that 50% of the γ-tocopherol consumed may be converted to CEHC. Both studies demonstrated that urinary excretion of CEHC appears to be more relevant for tocopherols distinct from RRR-α-tocopherol. A detailed study of tocotrienol degradation has not yet been undertaken. First results show a higher CEHC excretion from γ- than from α-tocotrienol (21). However, accumulated precursors as observed in HepG2 cells and probably excreted via the bile in vivo should be considered for balancing. In addition, fecal elimination may be important.

Taken together, our knowledge of the mechanism of vitamin E metabolism has increased tremendously during the last few years. These studies further demonstrate the unique and preferential role played by α-tocopherol. Different metabolic rates certainly will influence biopotency and bioequivalence of individual forms of vitamin E. The possibility that CYP, i.e., drug or xenobiotic metabolizing enzymes, are involved in the degradation of vitamin E opens the possibility of an interference of vitamin E with the drug metabolism that should be investigated thoroughly in view of the large dosages of vitamin E taken for self-supplementation.

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**References**


Chapter 13

γ-Tocopherol Metabolism and Its Relationship with α-Tocopherol in Humans

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Introduction

Vitamin E is widely associated with lipids in nature, especially monounsaturated and polyunsaturated fatty acids. In this context, it plays an important role in minimizing and, in some cases, preventing the oxidation of susceptible lipid molecules. Although this antioxidant function has been considered to be the primary role of vitamin E for several decades, it is becoming increasingly clear that this antioxidant vitamin also has a range of other important functions in cell biology. This multiplicity of function is due, in part, to the range of molecules that comprise the vitamin E family, namely, α, β, δ, and γ-tocopherols and α, β, δ, and γ-tocotrienols. These eight isoforms and some of their respective metabolites perform a wide range of roles within the body.

Humans have lost the ability to make vitamin E; as a consequence, they must acquire it from their diet. In contrast to other antioxidant vitamins such as vitamin C and β-carotene that are abundant in fruit and vegetables, members of the vitamin E family are more prevalent in plant seeds and oils. Even though α-tocopherol is by far the most prevalent form of vitamin E in humans, this is not the case in the plant kingdom. In many instances such as with soybean, corn, and sesame oil, γ-tocopherol is the predominant form of vitamin E (1). Indeed, γ-tocopherol predominates so much in our natural food chain that it represents ~70% of all vitamin E consumed in the typical American diet. Equivalent information is not available for Europe but there also, γ-tocopherol is likely to dominate vitamin E intake in most countries.

Given the large intakes of γ-tocopherol by humans, it is somewhat surprising that blood contains a relatively small proportion in comparison to α-tocopherol. Typically, the ratio is ~10:1 in favor of α-tocopherol (2). This intriguing observation was eventually explained by a series of interesting studies by Traber and colleagues (3–5). They showed that intestinal cells take up all forms of vitamin E equally and then release them into the circulation with chylomicrons. The isoforms then reach the liver in association with chylomicron remnants, although there is some opportunity for direct transfer to tissues during this transfer process. This may explain why certain tissues such as muscle and adipose tissue have higher than expected concentrations of γ-tocopherol. On reaching the liver, the chylomicron remnants are taken up and a specific protein, the α-tocopherol transfer protein...
(α-TTP), preferentially selects α-tocopherol from other incoming tocopherols for incorporation into very low density lipoproteins (6). Non-α-tocopherol forms are much less well retained; as a consequence, they are metabolized and either excreted via the bile or transported via the circulation to the kidney for subsequent elimination in urine (7,8).

**γ-Tocopherol and Disease**

A low γ-tocopherol concentration and a high α- to γ-tocopherol ratio have been reported in patients with coronary heart disease (CHD) compared with controls (9,10) and in a population with a high incidence of CHD (11). These observations suggest that a serum tocopherol profile with low γ-tocopherol levels and a high α- to γ-tocopherol ratio may be an indicator of an increased risk for CHD. In support of such a conclusion, Kushi et al. (12) reported that intake of dietary-derived vitamin E (i.e., mainly γ-tocopherol), but not supplemental vitamin E (α-tocopherol) was beneficial in reducing cardiovascular disease. Moreover, the same group recently arrived at a similar conclusion for postmenopausal women and death from stroke (13).

In response to these and other positive observations about γ-tocopherol, there is renewed interest in approaches to improve γ-tocopherol status by dietary change. For example, it has been suggested that rapeseed oil–rich diets help to increase the levels of γ-tocopherol in the body. In the Lyons Diet Heart Study it was found that a Mediterranean diet rich in α-linolenic acid (high in rapeseed oil) was beneficial in secondary prevention of CHD (14). Similarly, Lemcke-Norojarvi et al. (15) reported that substitution of corn and sesame oils in the diet (which contain substantial quantities of γ-tocopherol) leads to an increase in serum γ-tocopherol in healthy women without affecting the serum α-tocopherol concentrations. Similar conclusions were reached by Clooney and colleagues (16) who demonstrated that the source of dietary γ-tocopherol is an important determinant of its subsequent plasma concentrations. They found that the consumption of γ-tocopherol in the form of sesame seeds is the most efficient way of increasing blood γ-tocopherol concentrations. For example, consumption or 5 mg of γ-tocopherol/d for 3 d in the form of sesame seeds, but not walnut or soybean oil significantly elevated serum γ-tocopherol levels (19%) while decreasing β-tocopherol concentrations (34%).

**Specific Beneficial Effects of γ-Tocopherol**

Recently, Saldeen and colleagues (17) examined the differential effects of α- and γ-tocopherol on thrombogenesis. In a rat model, they found that γ-tocopherol was significantly more potent than α-tocopherol in inhibiting platelet aggregation and thrombogenesis. Moreover, they also report that γ-tocopherol reduced superoxide anion generation, lipid peroxidation, and low density lipoprotein oxidation and increased superoxide dismutase activity to a much greater extent than α-tocopherol.
γ-Tocopherol Metabolism

A number of studies have demonstrated that α-2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (α-CEHC) is the main metabolite of the α-tocopherol in both plasma (18,19) and urine (20,21). Swanson et al. (22) suggested that the formation of γ-CEHC and its consequent excretion in urine represent a primary route of elimination of γ-tocopherol in humans. They also identified a putative metabolite analog to γ-CEHC, namely, γ-CMBHC (γ-carboxymethylbutyl hydroxychroman), which is excreted in urine as a minor form (1–4% of the concentration of γ-CEHC) and secreted in vitro by hepatoma cells (23). In the same study, the authors described in one subject, the time course of γ-CEHC and γ-CMBHC excretion after RRR-γ-tocopherol supplementation (150 mg/d for 2 d). The increase in the urinary concentration of γ-CEHC and γ-CMBHC 14 h after supplementation was approximately eight- and threefold, respectively. In general, studies undertaken in North America record high baseline levels of urinary γ-CEHC excretion (2.5–31.5 µmol/L), reflecting the higher intake of γ-tocopherol in the U.S. diet compared with that of Europe.

Interestingly, γ-CEHC was first identified by Wechter and colleagues in 1996 (24) when they were searching for molecules that elicit natriuretic activity. γ-CEHC, or LLU-α as it was named by Wechter, was shown to inhibit the 7O pS ATP-sensitive K+ channel in the thick ascending limb cells of the rat kidney (24). In this respect, γ-CEHC activity may be unique in its ability to regulate extracellular fluid volume. Recently, however, Hatton and colleagues (25), reported that administration of either γ-tocopherol or γ-tocotrienol to male rats gave rise to increased plasma concentrations of γ-CEHC. Moreover, γ-tocotrienol supplementation in humans has been reported to increase urinary γ-CEHC concentrations (26). These findings weaken the case for a single class of nutrients being the immediate precursor of an important regulatory factor. Tocotrienols have a structure similar to that of tocopherol except that they have an unsaturated side chain. Previously, tocotrienols have been reported to lead to cholesterol suppression (27), tumor suppression (28), and a blockage of increased blood pressure (29). But studies using purified tocotrienols in hypercholesterolemic subjects did not confirm these findings (30).

Use of Deuterated γ-Tocopherol to Examine Its Biokinetics in Normal Subjects

Given the potentially unique qualities of γ-tocopherol and its metabolites and the fact that supplementation with α-tocopherol has a profound influence on body γ-tocopherol levels (31), we thought it worthwhile to examine further the bioavailability and metabolism in human subjects.

The objective of the study was to establish the biokinetics of γ-tocopherol within the body. We approached this problem by supplementing healthy subjects with deuterium-labeled γ-tocopherol and followed its appearance in the plasma pool and subsequent urinary excretion (32). Twenty-one subjects were recruited, (11 men/10 women) between 22 and 47 y of age (mean 32.6 ± 8.3 y). Subject characteristics are

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provided in Table 13.1. All were healthy nonsmoking individuals who were not taking any medication or vitamin supplements for the duration of this study or 4 wk before the start. The study was approved by the St. Thomas’ Hospital Research Ethics Committee.

**Protocol**

Subjects were asked to make five visits to the laboratory when early morning urine and blood samples were collected. On the first visit when baseline samples were collected, subjects were given a capsule containing 100 mg of RRR d2-γ-tocopherol acetate. This was taken with a supplied breakfast (a cake or chocolate bar containing a similar amount of fat). Subsequent visits were made on d 1, 3, 7, and 10 when early morning urine and blood samples were also collected. In addition to this, two subjects provided samples 6, 9 and 12 h postsupplementation. Plasma was obtained by centrifugation (13,000 × g, 15 min at 4°C) and immediately stored at −80°C until processing. Urine samples were stored at −20°C.
**HPLC Analysis of Plasma α- and γ-Tocopherol**

The plasma concentration of α- and γ-tocopherol were measured by high-performance liquid chromatography (HPLC) as described previously (33). Briefly, an internal standard of α-tocopherol acetate (Sigma, Dorset, UK) was added to 200 µL of plasma and vortexed. This was then mixed with cold hexane (500 µL) and vortexed again. The hexane layer was removed and evaporated to dryness under nitrogen. The dry extract was resuspended in methanol (400 µL) for analysis on a Gilson HPLC system (Anachem, Beds, UK) using reverse-phase HPLC. The extracted sample (100 µL) was then injected onto an Apex II Octadecyl 5-µm 10-cm column preceded by an Apex Bio300 Guard column 4.6-mm i.d. cartridge (Jones Chromatography, Glamorgan, UK). The separation was achieved using a mobile phase of 98% methanol/2% water set at a flow rate of 1 mL/min. The α-tocopherol, γ-tocopherol, and tocopherol acetate were measured using ultraviolet light detection at 292 nm. External standards of α-tocopherol (Sigma) and γ-tocopherol (Roche, Herts, UK) were also prepared. The final α- and γ-tocopherol plasma concentrations were corrected for cholesterol. Plasma cholesterol levels were determined by enzymatic colorimetric test using CHOD/PAP methods and Unimate 5 Chol kits, (Roche Diagnostic). Analysis was carried out by the Chemical Pathology Department, St. Thomas’ Hospital.

**Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Urinary Metabolites QL and CEHC**

Internal standards d³ quinone lactone (QL), d² CEHC and d⁹ CEHC (4 nmol in 25 µL of acetonitrile or ethanol) were added to an aliquot of urine (4 mL). Enzymatic hydrolysis of the glucuronide conjugate of the metabolites was carried out using *Escherichia coli* β-glucuronidase (Sigma, EC: 3.2.1.31) 650 U in 200 µL of 0.25 mol/L sodium acetate buffer (pH 6.2). The samples were flushed with nitrogen and incubated at 30°C in the dark for 14 h. The resulting solution was acidified to pH 1.5 and extracted with hexane/tert-butyl methyl ether (10 mL, 99:1). After centrifugation (13,000 × g at 4°C for 15 min), the organic layer was removed and evaporated under nitrogen. The residue was dissolved in anhydrous pyridine (100 µL) and silylated at 65°C for 1 h with 50 µL N,O-bis (trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (Pierce Chemical, Rockford, IL), after flushing the tube head space with nitrogen. The solvents were evaporated under nitrogen and the residue dissolved in hexane for GC-MS analysis.

The disilyl ester of CEHC metabolites and unsilylated QL metabolite were quantified by GC-MS using a Hewlett-Packard 6990 GC (Agilent Technologies, Stockport, UK) coupled to a Hewlett-Packard 5973 Mass Selective Detector. The carrier gas was helium. The separation of the compounds was achieved using a HP-1 cross-linked methylsioxane column (25 m × 0.2-mm i.d. and film thickness 0.33 µm). The oven temperature was set to 50°C for 2 min followed by ramp of 50°C/min to 240°C; this was held for 4 min before a further increase of 25°C/min to 285°C, which
was maintained for 6 min. The injection volume was 1 µL injected in splitless mode. The transfer line temperature was 290°C and the MS source temperature was 230°C.

In selected ion mode, the following ions corresponding to the molecular ion of the metabolites were monitored: m/z 276 (d⁰ QL), 279 (d³ QL), 285 (d⁹ QL), 408 (d⁰ γ-CEHC), 410 (d² γ-CEHC), 422 (d⁰ α-CEHC), 425 (d³ α-CEHC), and 431 (d⁹ α-CEHC). Concentrations of d⁰ QL, γ- and α-CEHC were calculated from the peak area relative to the corresponding internal standard (d³ QL, d² CEHC and d⁹ CEHC, respectively). The concentration of the d⁰ γ-CEHC was calculated using a response factor and the d⁹ α-CEHC as internal standard (Fig. 13.1).

**Fig. 13.1.** Gas chromatography-mass spectrometry trace of a typical urine sample. Ion 276 represents d⁰ α-QL and the corresponding internal standard ion 279 d³ α-QL; ion 408 represents d⁰ γ-2,7,8-trimethyl-2-((β-carboxyethyl)-6-hydroxychroman (γ-CEHC) and the corresponding internal standard ion 410 d² γ-CEHC; and ion 422 represents d⁰ α-CEHC and the corresponding internal standard ion 431 d⁹ α-CEHC.
The final urinary CEHC and QL concentrations were adjusted for creatinine concentrations. The concentration of creatinine was determined spectrophotometrically using a Sigma Diagnostics creatinine kit and following manufacturer’s instructions (555A, Sigma Chemical, St. Louis, MO). Regression analysis was carried out with robust standard errors. The nonparametric data were log transformed to allow for parametric analysis.

Results

Plasma responses. Baseline \( \alpha \)-tocopherol, \( \gamma \)-tocopherol, cholesterol, and cholesterol-corrected tocopherol data are shown in Table 13.2. As expected, \( \gamma \)-tocopherol at baseline was <10% of the \( \alpha \)-tocopherol concentration. Moreover, considerable interindividual differences in both \( \alpha \)- and \( \gamma \)-tocopherol existed among the subjects (Fig. 13.2; d 0 data). Ingestion of 100 mg of \( RRR \) \( \gamma \)-tocopherol acetate increased plasma \( \gamma \)-tocopherol concentrations 2.6-fold on d 1 compared with d 0 (0.37 ± 0.15 to 0.98 ± 0.42 µmol/mmol cholesterol, \( P < 0.0001 \)). However, this response disappeared by d 3

<table>
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<th>( \gamma )-Toc (µmol)</th>
<th>Chol (mmol)</th>
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when plasma γ-tocopherol concentrations had returned to baseline (Fig. 13.2). Ingestion of 100 mg of RRR γ-tocopherol acetate did not alter the plasma α-tocopherol concentration (Fig. 13.2).

**Urinary metabolite responses.** After γ-tocopherol supplementation, urinary d²-γ-CEHC increased markedly, rising to 2.24 ± 1.96 μmol/g creatinine on d 1 (Fig. 13.3). As seen with the plasma γ-tocopherol response, d²-γ-CEHC concentrations returned to

![Figure 13.2](image)

**Fig. 13.2.** Plasma α- and γ-tocopherol concentrations after ingestion of 100 mg d²-γ-tocopherol at d 0. Data are provided both as medians and interquartile ranges as individual data points.

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In contrast, the urinary \( \gamma \)-CEHC concentrations changed only marginally, increasing from 0.80 ± 0.55 to 1.07 ± 0.68 \( \mu \text{mol/g creatinine} \) on d 1. Similarly, the \( \alpha \)-tocopherol metabolites in urine showed very little response to \( \gamma \)-tocopherol supplementation (Fig. 13.3). There was a small increase in \( \alpha \)-CEHC on d 1 (2.21 ± 2.12 to 3.19 ± 2.78 \( \mu \text{mol/g creatinine} \)), whereas \( \alpha \)-QL was unaffected or slightly decreased after supplementation with \( \gamma \)-tocopherol.

**Interindividual responses.** A wide range in response to \( \gamma \)-tocopherol supplementation was seen in these healthy individuals (Fig. 13.4). Plasma responses over the 24-h postsupplementation period ranged from no change to a threefold increase. Such differences were even more marked in the urinary excretion of the \( d^2 \) \( \gamma \)-CEHC metabo-

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**Fig. 13.3.** Mean urinary metabolite concentrations given as \( \mu \text{mol/g creatinine} \) (the error bars show SD) after a 100-mg dose of \( d^2 \)-\( \gamma \)-tocopherol at d 0. \( \alpha \)-QL excretion rate did not change significantly from baseline. In contrast, \( \alpha \)-2,7,8-trimethyl-2-(\( \beta \)-carboxyethyl)-6-hydroxycromon (\( \alpha \)-CEHC) increased significantly (\( P < 0.05 \)) at d 1 compared with baseline but then returned to baseline concentrations by d 3. The same pattern of increase was seen in the \( d^0 \)-\( \gamma \)-CEHC metabolite (\( P < 0.05 \)) at d 1. The \( d^2 \)-\( \gamma \)-CEHC metabolite increased significantly at d 1 (\( P < 0.0001 \)) and remained significantly increased at d 3 (\( P < 0.005 \)) but then returned to baseline concentrations by the next sampling point at d 5.
Early responses to \( \gamma \)-tocopherol supplementation. In the two subjects examined, the major plasma responses to \( \gamma \)-tocopherol supplementation occurred within the first 24 h (Fig. 13.5). In both, peak plasma responses occurred at or before 6 h postsupplementation; by 24 h, these levels had fallen to approximately the same as the unlabeled, endogenous \( \gamma \)-tocopherol concentrations. Examination of the appearance of \( \gamma \)-CEHC in plasma revealed that this also occurs, rapidly building to a peak

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**Fig. 13.4.** Interindividual responses to supplementation with 100 mg of \( d^2-\gamma \)-tocopherol acetate. The plasma concentrations of \( \gamma \)-tocopherol and its urinary metabolite, \( \gamma \)-2,7,8-trimethyl-2-(\( \beta \)-carboxyethyl)-6-hydroxychroman (\( \gamma \)-CEHC) are shown for 21 healthy volunteers before and 24 h postsupplementation with 100 mg of \( d^2-\gamma \)-tocopherol acetate.
plasma level by 8 h postsupplementation. A similar pattern of $\gamma$-CEHC appearance occurred in urine (Fig. 13.5).

**Discussion**

Supplementation with 100 mg of $\gamma$-tocopherol resulted in a transient increase in plasma $\gamma$-tocopherol and urinary $\gamma$-CEHC concentrations. In most subjects, the plasma response was rapid and complete within 72 h. The majority of the changes in the plasma pool likely occurred within the first 24 hours. At the peak level, plasma $\gamma$-toco-
pherol concentration increased approximately threefold, whereas urinary \(\gamma\)-CEHC concentrations increased >20-fold. Importantly, however, ingestion of this large bolus of \(\gamma\)-tocopherol had no apparent effect on the plasma \(\alpha\)-tocopherol concentration and produced only a slight increase in urinary \(\alpha\)-CEHC. Thus, supplementation with \(\gamma\)-tocopherol, even at this large dose, does not displace \(\alpha\)-tocopherol from the circulation. This is most likely due to the high specificity that \(\alpha\)-TTP has for binding \(\alpha\)-tocopherol over any other vitamin E homolog (34).

The considerably greater increase in urinary \(\gamma\)-metabolite excretion compared with plasma \(\gamma\)-tocopherol increase suggests that the metabolic clearance of \(\gamma\)-tocopherol increased considerably after ingestion of the bolus dose. From the combined excretion curves, it was possible to calculate that >90% of the \(\gamma\)-CEHC was eliminated in the first 72 h after \(^{d2}\)-\(\gamma\)-tocopherol administration. From a more detailed examination of the early response in two subjects, it is likely that the majority of this occurred in the first 24 h.

Considerable interindividual variability was observed in the response to \(\gamma\)-tocopherol loading (Fig. 13.4). For example, there was little difference in plasma \(\gamma\)-tocopherol values between 0 and 24 h in some subjects, whereas others showed marked increase. This was not simply due to a difference in clearance rates among subjects because those subjects with the greatest urinary excretion rates were not those with the lowest plasma values at 24 h. Similarly, these sizable differences in \(\gamma\)-tocopherol bioavailability were not due to dietary differences at the time of the study because the fat content of the food taken with the supplement was the same for each subject. Similar differences have been reported previously for \(\alpha\)-tocopherol bioavailability (35) and the response appears to be specific to the individual.

In the United States, the average daily intake of \(\gamma\)-tocopherol has been estimated to be 18 mg (22). Based on this and a measure of \(\gamma\)-CEHC metabolite excretion, Swanson and colleagues could account for ~50% of the dietary intake. In the present study, ~38% was accounted for as this metabolite.

In conclusion, this stable isotope study illustrates the rapid metabolism and loss of ingested \(\gamma\)-tocopherol in humans. Urinary \(\gamma\)-CEHC appears to represent a main, but not exclusive route for excretion of \(\gamma\)-tocopherol metabolites. Furthermore, it is of interest to note that the concentration and metabolism of \(\alpha\)-tocopherol in plasma are not influenced by \(\gamma\)-tocopherol supplementation.

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References


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