Ann. Rev. Nutr. 1985. 5:323–40

VITAMIN E: Interactions With Free Radicals and Ascorbate

Paul B. McCay

Biomembrane Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

CONTENTS

INTRODUCTION

The association of the tocopherols with lipid peroxidation in biological systems began in the early days of investigations on the chemical nature of these substances (25). Since then a great deal of work has attempted to relate the symptoms of vitamin E deficiency with peroxidative degradation of lipids, primarily those associated with membranous organelles. The signs of vitamin E deficiency in various species of animals are diverse, involving different tissues with different manifestations and different degrees of severity. Thus, it seems clear that the biological function of this vitamin is not specific in the sense of its being a cofactor for an enzymic reaction (as is the case for the B-complex vitamins). Given the appropriate species and certain dietary and environmental conditions, essentially any tissue can be made to develop characteristic signs as a result of a deficiency of this vitamin. In some human disease states, supplementation of vitamin E at levels far exceeding those normally required ameliorates or improves the condition. Because of this multiplicity of effects, a common denominator that might explain the effects of a deficiency or excess of this vitamin is not obvious. A common denominator to consider, however, is

0199-9885/85/0715-0323$02.00

one suggested by the chemical properties of the vitamin, i.e. its capacity to function as an antioxidant or a free radical scavenger in chemical systems in vitro. Because there have been reports that vitamin E is a poor antioxidant, a closer look at its properties is in order.

Interest in the chemical nature of vitamin E developed soon after the observation by Evans & Bishop (19) that most foods contained a substance that prevented sterility in rats when the latter were fed a diet formulated to contain only the nutrients that were known to be required at the time. Within a short period following that discovery, Evans and co-workers determined that the factor was, in reality, a family of closely related tocopherols (18), the richest source of which were vegetable oils (17, 20, 21). The most active of this group of compounds (in terms of preventing sterility in male rats) was determined to be α-tocopherol (19). Since the biological activity of the different tocopherols is proportional to their proficiency as antioxidants (12), a compelling rationale exists for considering that the biological function of these compounds is to suppress undesirable oxidative processes in the membranous structures of cells. Indeed, one could find a basis for explaining the diverse effects of vitamin E deficiency in various animals if species differences in the molecular organization of the membranous structures in various tissues resulted in differing capacities to take up and retain tocopherol at sites susceptible to oxidative damage. For example, the cerebellum of the chick develops necrotic lesions causing severe ataxia and death after several weeks on a vitamin E–deficient diet (29); other species show no such effect on the cerebellum even though they manifest damage in other tissues (32). The capacity of certain membranous structures in the cerebellum of the chick to take up and retain tocopherol may be limited compared to the same structures in other species. Burton et al (10) provided evidence suggesting that the composition and structure of tissue lipid components may determine tissue levels of α-tocopherol.

THE ANTIOXIDANT PROPERTIES OF α-TOCOPHEROL

As recently as 1980 the question of whether or not α-tocopherol was truly an effective antioxidant compound was still being raised. This doubt persisted to some extent from earlier work reported by Chipault (13) that vitamin E was a rather mediocre antioxidant in vitro compared to other phenolic compounds of both natural and synthetic origin. As stated by Burton et al (10), the view of Chipault and co-workers had been accepted rather generally even though one would predict from the chemical structure of this antioxidant that it should be an extremely efficient antioxidant, capable of terminating free-radical chain reactions very effectively (26). Ingold and co-workers clearly demonstrated that α-tocopherol was a superb chain terminator of free-radical chain reactions by measuring the inhibition rate constant k_{inh} for vitamin E of the radical-mediated

chain autoxidation of styrene, in which the abstraction of the phenolic hydrogen is the controlling factor in the reaction. This chemical activity is typical of all phenolic antioxidants according to the following reactions:

$$\mathrm{ROO\cdot} + \mathrm{ArOH} \xrightarrow{k_{inn}} \mathrm{ROOH} + \mathrm{ArO\cdot} \qquad 1.$$

$$\mathrm{ROO\cdot} + \mathrm{ArO\cdot} \xrightarrow{\text{fast}} \text{unreactive products,} \qquad 2.$$

where Reaction 1 is the controlling step, and the rate of Reaction 1 is increased by methyl group substituents on the phenolic ring. Furthermore, Ingold and colleagues demonstrated that the special radical-scavenging properties of vitamin E must reside in the fused chroman ring system, and that in vitro at least, the phytyl side chain does not influence the inhibitory properties of this chromanol (11, 27). The chemical nature of the side chain does determine, however, whether or not the particular chromanol possesses vitamin E activity in vivo (11, 47). The highly effective antioxidant properties of the fused ring structure of the chromanols is believed to be due to the positioning of the pair of pi electrons of the ethereal oxygen moiety in the ring 90° from the plane of the ring; this gives stability to the phenoxyl radical formed in Reaction 1 above (11). Tocopheroxy radicals produced in chloroform by reaction with diphenylpicrylhydrazyl are stable for hours at room temperature (7).

The nature of the side chain of the chromanol is important in determining the effectiveness of the antioxidant in vivo, but not necessarily in vitro, a fact that is highly significant. Because vitamin E is associated primarily with membranous organelles (an inevitable consequence of its insolubility in water and its total miscibility with the lipids of biological membranes), the phytyl chain appears to provide the tocopherol molecule with an affinity for the hydrophobic environment of the membrane. Replacement of the phytyl group with a methyl group results in a loss of vitamin E activity in vivo even though the antioxidant activity of the methyl-substituted compound in vitro is essentially the same as that of α-tocopherol in vitro.

In considering the biological function of vitamin E, the requirement for lipid solubility of the compound is interpreted by many as support for prevention of lipid peroxidation being its primary role. However, there is evidence that scavenging of lipid radicals in membranes may not be its only form of activity. Bisby, Ahmed & Cundall (6) recently reported that a vitamin E analogue, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox C) (III), which has more solubility in water than α-tocopherol, can perform a repair-like function on free radicals of several amino acids that are formed by one-electron

oxidations caused by pulse radiolysis of solution containing amino acids. Radicals of tryptophan, tyrosine, methionine, and histidine are rapidly reduced to their original structure in the presence of this antioxidant (6). The antioxidant is oxidized to the phenoxy radical, which, because of the chroman ring structure discussed above, is relatively stable. The rate constants for the repair reactions were determined by the decay of the amino acid radicals and the formation of Trolox C radicals (6). The enzyme lysozyme was also subjected to the same treatment and formed a radical that was repaired by Trolox C (6). These findings suggest that hydrophobic proteins that undergo radical attack in biological membranes may also be repaired by vitamin E present in the lipid environment of those proteins. Indeed, it was reported that vitamin E (and other antioxidants) protect cytochrome P-450 from alteration by free radicals produced by the cytochrome's own catalytic activity in the metabolism of carbon tetrachloride (33).

A recent study by Burton, Joyce & Ingold (10) showed that vitamin E is the only lipid-soluble antioxidant present in plasma and in erythrocyte membranes. Their findings also indicate that this vitamin is the only radical chain-breaking substance of any significance present in these biological materials. These investigators state that of all the known phenolic antioxidants, the chain-breaking free-radical-scavenging activity of α-tocopherol is the most effective (9). Even when an equilibrium existed in the distribution of vitamin E between the plasma and the red cells, there was a 3-to-1 difference in favor of the plasma (10). As mentioned above, this striking finding suggests that membrane structure is critical in determining how much tocopherol those membranes will be able to absorb when an excess of the vitamin is available. The implications are that, regardless of the amount of vitamin E an animal may consume, the amount that may be taken up in the critical sites in membranes (where protection against initiation of free-radical chain reactions is needed) is inherently limited for a particular type of membranous structure. A further implication is that intake of excess α-tocopherol would not result in deposition at critical sites in membranes in excess of that dictated by the structure of a particular membrane.

Although it has been suggested that the only function of vitamin E is to prevent fortuitous, potentially damaging peroxidation of lipids from occurring (8), it is by no means certain that this is its only action. From the standpoint of the nutritional significance of this vitamin in health and disease, this is an extremely important question to resolve. There is a mystique held by the public at large about the enhancement of health by vitamin E supplements. A more pressing question is whether or not the tocopherols are actually beneficial in the treatment of a number of disease states for which they are recommended! In fact, elevation of vitamin E intake in laboratory animals has been shown to increase the number of observable enzyme-generated toxic free-radicals produced in the liver of animals exposed to toxic compounds compared to those

formed in similarly exposed animals with normal vitamin E intakes (34). Many individuals are consuming considerable quantities not only of vitamin E but also vitamin C on a daily basis, yet there is no biological data supporting the belief that excessive supplementation is beneficial. Recently, a number of reports appeared on the interaction of ascorbic acid and vitamin E in biological systems. In view of the supplemental human consumption of these two vitamins, often in large quantities, a review of these reports is appropriate; there may be unknown, long-term effects of excess intake of these two nutrients.

INTERACTION OF VITAMINS E AND C IN BIOLOGICAL SYSTEMS

In 1968 Tappel published a report (48) in which he suggested that ascorbic acid might reduce tocopheroxyl radicals formed during the scavenging of free radicals formed in vivo during metabolism, which permits a single molecule of tocopherol to scavenge many radicals and also links vitamin C to the protection of membranes against free-radical damage. This was an attractive idea, and if true, would have considerable significance for nutritionists and membrane biochemists. Since that time, a number of investigators have explored this possibility. Most of the work has been performed with chemical systems or with in vitro reactions involving tissue homogenates or subcellular fractions thereof. Some of the investigations support such a role for ascorbic acid and some do not. O'Connor et al (38) reported a radioprotective effect for vitamin C, but observed that it was very weak compared to sulfhydryl compounds such as cysteamine. But this is not a strong argument against such a role for ascorbic acid, because only a few sulfhydryl compounds are capable of protecting mice against radiation injury (1). On the other hand, how substantial is the evidence supporting this role for ascorbate? The concept of repair of free radicals generated by radiation or highly reactive chemical species was very well reviewed by R. L. Willson (49). Among the various studies attempting to demonstrate the chemical occurrence of radical repair, values for the absolute rate constants for repair of the isopropanol radical range from 54×10^7 mol^{-1} sec^{-1} for dithiothreitol (42) and 18×10^7 mol^{-1} sec^{-1} for glutathione (49) for the reaction

$$CH_3\dot{C}OHCH_3 + RSH \rightarrow CH_3CHOHCH_3 + RS\cdot \qquad 3.$$

to only 0.12×10^7 mol^{-1} sec^{-1} for the reaction of the isopropanol radical with ascorbate (49). According to this information, at the concentrations of glutathione found in most tissues, the reaction with glutathione would be favored over ascorbate, at least for this type of aliphatic radical.

Willson and colleagues performed a number of interesting studies on the

reactions of vitamins C and E following pulse radiolysis. They reported a direct reaction between the vitamin E radical and ascorbate in a system composed of an aerated, aqueous solution containing isopropanol (50%), acetone (10%), carbon tetrachloride (0.04 M), and vitamin E (3.4×10^{-3} M). Radiolysis of this solution produced a transient species with an absorption spectrum that they assigned to the vitamin E phenolic radical (30), and that they believed to be due to the following sequence of reactions (40):

$$H_2O \xrightarrow{h\nu} H\cdot + HO\cdot + e^-_{aq} \quad 4.$$

$$H\cdot \text{ or } HO\cdot + (CH_3)_2CHOH \longrightarrow (CH_3)_2\dot{C}OH + H_2 \text{ or } H_2O \quad 5.$$

$$e^-_{aq} + (CH_3)_2CO \xrightarrow{H_2O} (CH_3)_2CO^- \longrightarrow (CH_3)_2\dot{C}OH \quad 6.$$

$$(CH_3)_2\dot{C}OH + CCL_4 \longrightarrow (CH_3)_2CO + Cl_3C\cdot + H^+ + Cl^- \quad 7.$$

$$Cl_3C\cdot + O_2 \longrightarrow Cl_3COO\cdot \quad 8.$$

$$Cl_3COO\cdot + \text{Vitamin E} \longrightarrow \text{Vitamin E}\cdot + Cl_3COOH. \quad 9.$$

The transient species observed had an absorption maximum at 425 nm and disappeared within 3 msec. In the presence of ascorbate at a concentration of 1.6×10^{-3} M, the transient species disappeared within 1.6 msec (40). The rate constant for Reaction 9 was determined to be 1.5×10^6 mol^{-1} sec^{-1}. The authors concluded that ascorbate functions to recycle vitamin E in vivo using ascorbate to reduce the tocopherol radical back to tocopherol. Further work from that laboratory has shown that ascorbate also reacts with the isopropanol radical at an appreciable rate (0.12×10^7 mol^{-1} sec^{-1}) (43); therefore, it is likely that ascorbate can spare tocopherol in that system by reacting with isopropanol radicals.

Packer et al (39, 41) presented evidence that although vitamin E appears to react readily with $Cl_3COO\cdot$ radicals, it apparently does not react with carbon-centered radicals such as $Cl_3C\cdot$ and aliphatic organic radicals during radiolysis. Cited as evidence are the pulse radiolysis studies performed with their system in which oxygen had been excluded, or in which CCl_4 was omitted. The utilization of vitamin E (as measured by the absorbance at 425 nm) did not occur and these workers concluded that a molecular repair by tocopherol of any radicals formed under those conditions did not take place (49).

On the other hand, Simic & Hunter (47) published data showing that $Cl_3C\cdot$ radicals and other carbon- and oxygen-centered radicals react with vitamin E at a significant rate (see Table 1, which is taken from their publication). If this is the case, their findings must be reconciled with the finding by Packer et al (40)

that no absorbance attributable to the phenolic tocopherol radical was observed in their anaerobic water–isopropanol–carbon tetrachloride–tocopherol system subjected to pulse radiolysis. Table 1 also shows the reaction rate constants for several other types of radicals with vitamin E and certain other phenolic antioxidants, as well as with ascorbate.

Simic & Hunter also reported that the carbon-centered cyclohexyl radical ($\cdot C_6H_{11}$) reacts rapidly with vitamin E, while the same radical reacts inefficiently with butylated hydroxytoluene (BHT) (45) (see Table 1). Differential reaction rates with various radicals may partially explain the differential biological effectiveness of nontocopherol antioxidants as substitutes for vitamin E. In order for ascorbate to be effective in regenerating tocopherol from the tocopheroxyl radical before the vitamin is depleted by a free-radical reaction, the rate of regeneration for that process would have to be appreciable.

How the tocopherol radical is measured is critical to the conclusions being made about its reaction with ascorbate. Bascetta et al (5) investigated the electron spin resonance (ESR) spectra obtained when vitamin E and partially peroxidized methyl linoleate were thermally oxidized in a monolayer coated on silica gel. The coated material was placed in a quartz container and the air evacuated. This quartz tube was placed in the cavity of an ESR spectrometer and heated to 90°C. A strong ESR signal developed. This was a seven-line spectrum (Figure 1) that the authors concluded was due to the tocopherol radical, and that they believed to be formed from the reaction of tocopherol with alkoxy and other radicals produced in the decomposition of hydroperoxides of methyl linoleate on the silica gel. According to these investigators, the tocopherol radical was not observed when neat tocopherol was placed on the silica gel and treated in the same manner. The partially peroxidized ethyl linoleate alone heated to 90°C did produce an ESR signal, but it was not well defined and was a weak, featureless signal.

When the silica gel was first coated with vitamin C and then was coated with the tocopherol and methyl linoleate mixture, the thermal treatment generated only a very weak signal (see Figure 1). The authors concluded that ascorbic acid was reducing the tocopherol radical to tocopherol, and that the resulting

Table 1 Rate constants (in mol^{-1} sec^{-1}) for reaction of some radicals with antioxidants[a]

Antioxidant	R·	ROO·	·CCl_3	$CCl_3OO\cdot$	·OH[c]
Vitamin E	$<10^5$	7.9×10^6	4.5×10^6	1.8×10^8	10^{10}
Ascorbate	—	2.2×10^6	—	2.2×10^8	10^{10}
BHA	—	3.4×10^6	7.8×10^6	3.9×10^7	6×10^9
BHT	$<10^2$	$\sim 10^4$	$\sim 10^4$	1×10^5	6×10^9
Phenol	—	$<10^6$	—	2.3×10^8	6×10^9

[a]From Simic & Hunter (47).

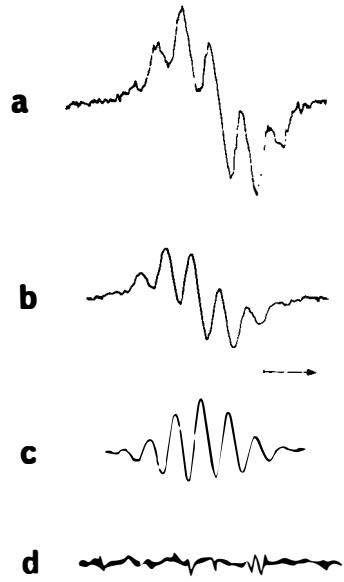


Figure 1 Effect of vitamin C (ascorbic acid) on the lifetime of α-tocopheroxyl radicals on a model membrane. (*a*) First-derivative ESR spectrum of α-tocopherol in di-*t*-butyl peroxide and hexadecane solution obtained by photolysis at 320 K. Spectrum obtained with a gain of 2.5×10^4 and field modulation of 6.3 Gpp. (*b*) First-derivative ESR spectrum of a monolayer containing autoxidized linoleate and α-tocopherol only adsorbed on silica gel. Spectrum obtained at 363 K with a gain of 3.2×10^5 and a modulation of 1.0 Gpp. (*c*) Second-derivative representation of (*b*). (*d*) First-derivative ESR spectrum of a monolayer containing autoxidized linoleate, α-tocopherol, and vitamin C adsorbed on silica gel. Spectrum obtained at 363 K with a gain of 10×10^5 and modulation of 1.0 Gpp. [From Bascetta et al (5).]

ascorbate radical decayed too rapidly to be observed by the electron paramagnetic resonance (EPR) technique (5). Measurement of the amount of tocopherol remaining on the silica gel after this treatment was not reported. If the tocopherol was preserved by the presence of the ascorbic acid in the silica gel system, their data does not exclude the possibility that the ascorbate itself would react with the radicals produced by the decomposition of the methyl linoleate hydroperoxides. Insofar as the EPR data are concerned, that possibility would not be eliminated. It may depend on the relative concentrations of ascorbate and tocopherol on the silica gel. These investigators used about three times as much ascorbate (10 mg) as tocopherol (3.1 mg) in their system (5).

According to Simic & Hunter, the reaction rates for ascorbate and tocopherol with alkoxy radicals (under the conditions they employed) are quite similar (47). The influence of factors governed by the surface properties of the silica gel may be involved in the investigations of Bascetta et al. The data do not exclude the possibility of tocopherol sparing by the presence of ascorbate in their system.

Another aspect of the vitamin E–vitamin C interaction quandry is the question of phases. Although dehydroascorbate has some lipid solubility, ascorbate itself has little such solubility. Since vitamin E is totally localized within the membranous organelles in cells (apart from that portion bound by transporting proteins) and is essentially insoluble in aqueous systems, the mechanism through which these substances could interact to reduce vitamin E radicals is not known. The membrane-cytosol interface may have unusual properties that facilitate reduction of tocopherol radicals in the membrane by an external source of reducing equivalents such as ascorbate, but this has not been demonstrated experimentally. In addition, cytosolic concentrations would favor glutathione over ascorbate as the donor of electrons in most tissues. The studies most frequently cited as having demonstrated that ascorbate functions as a reducing agent for tocopherol radicals were done in single-phase systems (39). Ginter et al (23) expressed the idea that these two vitamins are functioning in different phases, performing different functions that have a synergistic effect in controlling free-radical-mediated reactions that damage cellular membranes. This is an appealing idea and worthy of further consideration.

Barclay et al (3) investigated the peroxidation of the methy esters of linolenic and linoleic acids using the thermal initiator di-*tert*-butylhyponitrite (DBHN). These esters were in sodium dodecyl sulfate micelles. When DBHN was introduced into the micellar system, oxidation of the unsaturated fatty acid esters began and proceeded according to the kinetic rate law, which was compatible with that established for autoxidation of organic compounds in homogeneous solutions (3). When α-tocopherol was present, there was a substantial lag period in the oxygen uptake of the system (Figure 2). Addition of ascorbate instead of tocopherol resulted in a much shorter delay in O_2 uptake. However, when both tocopherol and ascorbate were present, there was a substantial lengthening of the delay in O_2 uptake (Figure 2). The authors attributed this delay to the regeneration of tocopherol from its radical form at the expense of reducing equivalents from ascorbate. They proposed that DBHN initiates the formation of lipid radicals (R·), which immediately react with O_2 to form peroxy radicals (ROO·). These radicals are scavenged by tocopherol to form the tocopherol radical (T·), which is then reduced to tocopherol by ascorbate (3).

The data shown in Figure 2 are certainly compatible with that argument, but it would seem not to eliminate other possibilities. If, as they propose, ascorbate can interact with the tocopherol radical because the phenolic head group is located near the aqueous interface, it means that a large entropy change would have to occur in order to bring about the reaction of tocopherol with a lipid peroxy radical in the hydrophobic part of the membrane; such a change would greatly reduce the efficiency of the reaction (46). Tocopherol would not be an effective antioxidant in that situation. Simic (46) states that the presence of the

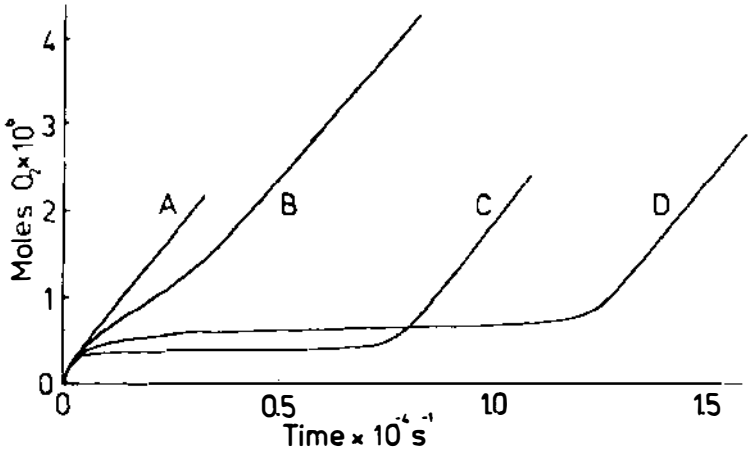


Figure 2 Autoxidation of linoleic acid (6.4×10^{-5} M) in 0.50-M SDS (2.0 ml) at pH 7.0 initiated with DBHN (7.2×10^{-6} M): *A*, uninhibited reaction; *B*, inhibited with ascorbic acid (1.95×10^{-7} M); *C*, inhibited with α-tocopherol (5.14×10^{-8} mol); *D*, inhibited with α-tocopherol (5.14×10^{-8} M) and ascorbic acid (1.95×10^{-7} M). [From Barclay et al (3).]

hydroxyl group of tocopherol at the surface of the membrane would enable it to act as an interceptor of free radicals formed in the aqueous environment of the membrane, in which case it would have the high efficiency as an antioxidant that it actually exhibits in micellar systems ($10^7 - 10^8$ mol^{-1} sec^{-1}), and it would facilitate the reaction of the tocopheroxyl radical with ascorbate. If physicochemical factors in membranes caused radical moieties on LOO· molecules to migrate to the surface of the membrane in order to position themselves near the tocopherol phenolic group, ascorbate may be able to react with the radical moiety of LOO· molecules, but only those that reach the surface. Hence the inhibition of the rate of peroxidation caused by DBHN is not very great (Figure 2). According to the concept of Barclay et al (3), when tocopherol is present in the system, it must be able to react with the LOO· radical anywhere in the micelle in order to account for the marked inhibition of oxygen uptake it causes as shown in Figure 2. But the entropy change predicted by Simic (46) makes such freedom of action of tocopherol in the membrane unlikely. However, the combined radical scavenging activity of ascorbate and tocopherol at the surface of the micelle, essentially acting in the aqueous phase to intercept radicals, could prolong the lag period before rapid peroxidation of the fatty acid esters due to DBHN radicals in the polar phase attacking at the surface of the micelles. Reduction of tocopherol radicals by ascorbate could also be occurring; high pressure liquid chromatography (HPLC) analyses of tocopherol in the system indicate that the tocopherol content of the system remains constant during most of the period of inhibition (3). However, the possible sparing

action of ascorbate on tocopherol in this system cannot be excluded by these results. Detection of any oxidation products of tocopherol that might have been produced in the system was not reported.

Niki et al (35) investigated a similar system with the exception that their reactions took place in a single phase (*tert*-butyl alcohol : methanol, 3 : 1). The peroxidation of methyl linoleate was initiated by either 2,2'-azobisisobutyronitrile (AIBN) or 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN). These investigators found that ascorbic acid was a reasonably good inhibitor of the peroxidation of methyl linoleate and appeared to react in a stoichiometric ratio of 1 : 1 with the peroxy radical of methyl linoleate (35). When vitamins E and C were both present in the peroxidizing methyl linoleate system, they observed that essentially all of the vitamin C was consumed first before the vitamin E began to disappear (see Figure 3 taken from their publication).

These are highly interesting results and they do support an interpretation that tocopherol reacts with the methyl linoleate peroxy radical (LOO·). The resulting tocopherol radical then reacts with vitamin C to regenerate tocopherol. Because of the excess of methyl linoleate in the system, the initiating radicals formed by the azobis compounds must react primarily with the methyl linoleate molecules. It would be pertinent, however, to determine whether or not vitamins E and C, either separately or together, react with the azobis initiating radicals in this solvent in the absence of any methyl linoleate. If either of these vitamins do react with the azobis radicals, it would be important to compare their reaction rates with the reaction rate of the azobis radicals with methyl linoleate.

These investigators noted that for the peroxidation of methyl linoleate, the apparent inhibition rate constant (k_{inh}) for ascorbate was 7.5×10^{-4} mol^{-1} sec^{-1}, whereas that for vitamin E was 5.1×10^{5} mol^{-1} sec^{-1}. It is interesting to speculate why ascorbate should react with the vitamin E radical (which is a relatively stable radical) much more rapidly than it apparently does with the peroxy radical of methyl linoleate, which is a relatively reactive radical. If ascorbate were reacting with both the tocopherol radical and the peroxy radical, it would be difficult to explain why the *rate* of oxygen consumption does not increase more rapidly after the ascorbate is depleted since the concentration of tocopherol would decrease rapidly after that. But the oxygen consumption data shown in Figure 3 show no increase until essentially all of the tocopherol is depleted. This is what one might expect if both ascorbate and tocopherol were reacting with the azobis radicals before the latter begin to initiate a significant attack on the methyl linoleate molecules. The reaction rates for ascorbate and tocopherol with the azobis radicals were not reported in the paper by Niki et al, but if the rates are appreciable, additional interpretations of the overall reaction may exist.

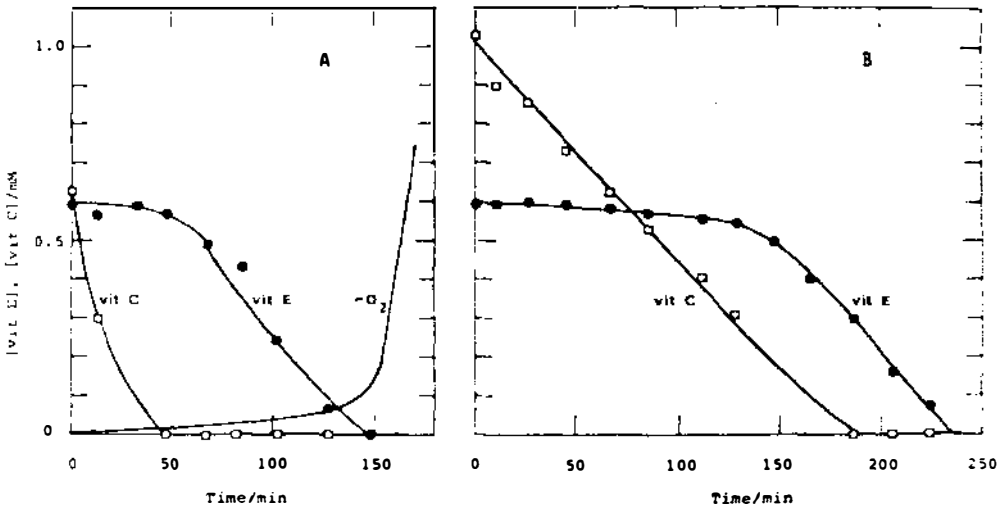
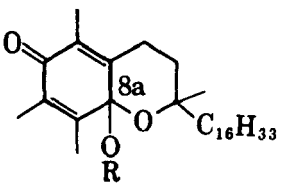


Figure 3 Disappearance of vitamin E and vitamin C in the oxidation of methyl linoleate at 37°C in *tert*-butyl alcohol : methanol (3 : 1) by volume. The concentrations of components were methyl linoleate, 0.60 M; AMVN, 0.01 M; α-tocopherol, 0.595 mM; vitamin C, 0.620 mM (*A*) and 1.03 mM (*B*). [From Niki et al (35).]

Another interesting investigation into the relationship between ascorbate and tocopherol concerning the inhibition of lipid peroxidation was reported by Leung et al (31). Using phospholipid liposomes as model membranes, they demonstrated that when vitamins E and C were both present in a system in which the liposomes were subjected to peroxidation because of the addition of Fe^{2+}, the time during which peroxidation was suppressed was generally equal to the sum of the suppression times caused by the addition of each vitamin alone. At higher concentrations of the vitamins, however, which caused longer delays in peroxidation, they observed that these delays were more extensive than the sum of the inhibition times caused by either vitamin added alone (31). Because no measurements of tocopherol were made during or after the reaction, it is not possible to assess the behavior of tocopherol in the system. Other possibilities still exist, including one in which both compounds are scavenging the same initiating chemical species. In the system they were using, the initiating chemical species was unknown. Their studies were conducted at pH 5.0, which could influence the free-radical chemistry involved, but since the details of the chemistry of iron-catalyzed lipid peroxidation have not been established for any pH, that aspect cannot be evaluated.

The enhancement of the antioxidant activity of tocopherol by ascorbate may be due to some property other than perturbation of free-radical chemistry in the system. For example, Fukuzawa et al (22) reported that the inhibition by tocopherol of lecithin liposome peroxidation (catalyzed by iron and ascorbate) was markedly enhanced by the addition of cholesterol in amounts equimolar to the tocopherol; yet cholesterol itself exhibited no antioxidant properties in that system. For steric reasons, it is unlikely that cholesterol could be involved in the formation of an 8-α-tocopherone derivative (24) (Figure 4).

Tocopherones, however, might play a key role in the prolongation of α-tocopherol's ability to inhibit lipid peroxidation. Formation of tocopherones may extend the free-radical-scavenging capacity of α-tocopherol by virtue of the degree of tocopherone stability. The latter may provide sufficient time for reduction of the tocopherone by ascorbate before the quinone is formed. It has been established that 8a-alkoxy-tocopherones are formed when α-tocopherol is subjected to a variety of oxidizing conditions such as reaction with (*a*) benzoyl peroxide in dry alcohols; (*b*) ferric chloride and 2-2'-bipyridine in dry alcohols;



Ia, R = n-C_nH_{2n+1}
Ib, R = H
Ic, R = $CH(CH_3)_2$
Id, R = $C(CH_3)_3$

Figure 4 Structures of 8a-alkoxy-α-tocopherones. [From Goodhue & Risley (24).]

and (*c*) cyclohexylamine and bromine in dry alcohols. Tocopherones are also formed in systems in which water is present. The reaction of α-tocopherol with tetrachloro-*o*-quinone or *n*-bromosuccinimide in aqueous acetonitrile forms 8a-hydroxy-α-tocopherol (15, 16). In addition, the photooxidation of α-tocopherol in methyl linoleate or soybean oil results in the formation of 8a-hydroperoxy-tocopherone (50).

The sequence of events in the formation of tocopherones appears to be as follows. The properties of α-tocopherol indicate that the molecule has electron deficiencies at the 8a, 5-methyl, and 6-oxygen positions of the ring structure (24). In the presence of reactive free radicals (or some ionic species), this property facilitates an attack by the radicals on tocopherol at the 6-oxygen to form the tocopheroxyl radical. This radical species then undergoes an addition reaction (also involving either another radical or ionic species) at the 8a-position, which rearranges a double bond in the ring system and forms of the tocopherone. This reaction probably has biological significance since oxidation of tocopherol in systems containing nucleophilic sulfhydryl or hydroxy groups should yield tocopherone derivatives (24). In fact, such reactions have been indicated to be involved in the protection of essential sulfhydryl groups in enzymes by α-tocopherol (2, 14).

Durckheimer & Cohen (16) showed that the 8a-hydroxy-α-tocopherone can be formed in aprotic media where only a trace of water may exist, and that the stability of tocopherones in lipid regions of membranes may be enhanced (a half-life of hours). In addition, they showed that tocopherones degrade to the tocopheryl quinone, a relatively stable oxidation product of tocopherol. Because the latter is not normally found as a significant metabolite of tocopherol in animal tissues, there must be an efficient cellular mechanism for preventing conversion of tocopherol to the quinone. Tocopherones may provide the metastable oxidation product of radical reactions that enable its conversion back to tocopherol. For example, the tocopherones, including the 8a-hydroperoxy tocopherone, are known to be easily reduced to tocopherol by ascorbic acid (15, 16, 36, 37). Therefore it is reasonable to propose that tocopherones are formed in biological membranes during the oxidation of α-tocopherol as the latter scavenges reactive species in inhibiting the initiation phase of lipid peroxidation. The tocopherones, being relatively stable, would facilitate the reduction of the partially oxidized form of tocopherol, which otherwise would be converted to tocopheryl quinone. The latter is an oxidized form of tocopherol that is not reduced by ascorbic acid. Although tocopherones do not appear to have been detected in animal tissues, the procedures used to extract tocopherol and its metabolites from biological membranes as well as the procedures for fractionating these compounds may cause the tocopherones to convert to the quinone form and thereby escape detection (24).

Scarpa et al (44) took a more direct approach to studying the biological

importance of the reduction of the tocopheroxyl radical by ascorbic acid. They reported some highly interesting studies on the behavior of the EPR signal of the tocopheroxyl radical formed during the peroxidation of phospholipid liposomes catalyzed by chelated iron. These workers showed that the tocopheroxyl radical was not observed in this system when ascorbate was present. Only when all the ascorbate was oxidized did the tocopherol radical appear. Several factors need to be considered. First, the phospholipid used for preparing the liposomes was already partially peroxidized so that events associated with iron-catalyzed degradation of preformed lipid peroxides. In addition, in the absence of tocopherol, ascorbate was consumed in this liposomal system even in the absence of oxygen. This indicates that ascorbate can react directly with the iron-catalyzed breakdown products of the lipid peroxides, which promote further peroxidation; this would account then for the inhibition of lipid peroxidation observed by these workers upon addition of ascorbate alone. If that is the case, in the liposomal systems in which tocopherol was present, it becomes important to know to what extent tocopherol is being spared by the competition of ascorbate for the reactive products of lipid peroxide breakdown as opposed to the reduction of tocopheroxyl radicals by ascorbate per se. Both reactions may be occurring, but the degree to which either reaction predominates would be a function of their respective reaction rates. These reaction rates are not known. The studies of Scarpa et al, however, are very significant in that they show that ascorbate can inhibit a free-radical-mediated process in a lipid bilayer whether tocopherol is present or not.

The various findings discussed above indicate that ascorbic acid can react with intermediates of α-tocopherol oxidation to effect a reduction of the intermediate to its initial state. However, some definitive studies are needed to resolve alternative interpretations of some of the data obtained. In addition, it remains to be determined if the type of interaction between ascorbate and α-tocopherol that occurs in in vitro systems reflects what is occurring in vivo. If an efficient reaction between ascorbate and tocopherol radicals does take place in vivo, it might seem that it would be very difficult to produce a severe antioxidant deficiency in animals without a concomitant deficiency of ascorbate. A deficiency of ascorbate would not occur in most laboratory animals other than the guinea pig and primates and a few other species that cannot synthesize ascorbic acid. A survey of the literature on vitamin E does not reveal a sparing action of ascorbate on the vitamin E requirements of intact animals. However, some very interesting investigations by Bendich, D'Apolito, Gabriel & Machlin (4) showed that the level of vitamin E found in plasma and in lung tissue is higher in guinea pigs supplemented with ascorbic acid than in guinea pigs fed the same diet without ascorbic acid. Bendich and co-workers (A. Bendich, P. D'Apolito, E. Gabriel, L. J. Machlin, personal communication) did not, however, find that the lower levels of α-tocopherol in the scorbutic

guinea pigs was accompanied by a measureable increase in tocopherol oxidation products such as tocopheryl quinone. Hruba et al (28) also demonstrated that a marginal deficiency of vitamin C resulted in lower tocopherol levels in guinea pigs in some tissues (lung and liver) but not others compared to controls fed the same diet supplemented with adequate vitamin C. These results indicate that vitamin C helps maintain tissue levels of vitamin E, but there could be several mechanisms through which this might occur. One possible mechanism is the presence of vitamin C in the diet helping to prevent autoxidative loss of α-tocopherol in food and during the digestive process. Also, ascorbate could conceivably affect the distribution of available tocopherol within the animal body.

Ascorbic acid may play a dual role with respect to free-radical processes in animal tissues, depending on localized concentrations that may occur in individual cells. Low levels of ascorbate (below 1.0 mM) are known to promote lipid peroxidation in vitro, while higher levels of the vitamin inhibit peroxidation. Interesting studies of importance to the nutritional roles of these two vitamins and their possible interactions still remain to be done.

Literature Cited

1. Alexander, P., Charlesby, A. 1955. Physico-chemical methods of protection against ionizing radiations. In *Radiobiology Symposium 1954*, ed. Z. M. Bacq, P. Alexander, pp. 49–59. New York: Academic
2. Ames, S. R., Risley, H. A. 1949. Effects of the tocopherols and their phosphates on enzyme systems. *Ann. NY Acad. Sci.* 52:149–55
3. Barclay, L. R. C., Locke, S. J., MacNeil, J. M. 1983. The autoxidation of unsaturated lipids in micelles. Synergism of inhibitor vitamins C and E. *Can. J. Chem.* 61:1288–90
4. Bendich, A., D'Apolito, P., Gabriel, E., Machlin, L. J. 1984. Interaction of dietary vitamin C and vitamin E on guinea pig immune responses to mitogens. *J. Nutr.* 114:1588–93
5. Bascetta, E., Gunstone, F. D., Walton, J. C. 1983. Electron spin resonance study of the role of vitamin E and vitamin C in the inhibition of fatty acid oxidation in a model membrane. *Chem. Phys. Lipids* 33:207–10
6. Bisby, R. H., Ahmed, S., Cundall, R. B. 1984. Repair of amino acid radicals by a vitamin E analogue. *Biochem. Biophys. Res. Commun.* 119:245–51
7. Boguth, W., Niemann, H. 1971. Electron spin resonance of chromanoxy free radicals from tocopherol and tocol. *Biochim. Biophys. Acta* 248:121–30
8. Burton, G. W., Cheeseman, K. H., Doba, D., Ingold, K. U., Slater, T. F. 1983. Vitamin E as an antioxidant *in vitro* and *in vivo* In *Ciba Found. Symp., Biology of Vitamin E* ed. R. Porter, J. Whelan, 101:4–18. London: Putnam
9. Burton, G. W., Ingold, K. U. 1981. Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. *J. Am. Chem. Soc.* 103:6472–77
10. Burton, G. W., Joyce, A., Ingold, K. U. 1983. Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? *Arch. Biochem. Biophys.* 221:281–90
11. Burton, G. W., Le Page, Y., Gabe, E. J., Ingold, K. U. 1980. Antioxidant activity of vitamin E and related phenols. Importance of stereoelectronic factors. *J. Am. Chem. Soc.* 102:7791–92
12. Century, B., Horwitt, M. K. 1965. Biological availability of various forms of vitamin E with respect to different indexes of deficiency. *Proc. Soc. Exp. Biol.* 24:906–11
13. Chipault, J. R. 1962. In *Autoxidation and Antioxidants*, ed. W. O. Lundberg, 2:477–542. New York: Interscience
14. Corwin, L. M., Schwarz, K. 1963. Relation of tocopherol to enzyme sulfhydryl sites. *Arch. Biochem. Biophys.* 100:385–92
15. Durckheimer, W., Cohen, L. A. 1962.

Mechanisms of α-tocopherol oxidation: synthesis of the highly labile 9-hydroxy-α-tocopherone. *Biochem. Biophys. Res. Commun.* 9:262–65
16. Durckheimer, W., Cohen, L. A. 1964. The chemistry of 9-hydroxy-α-tocopherone, a quinone hemiacetal. *J. Am. Chem. Soc.* 86:4388–93
17. Eggitt, P. W. R., Ward, L. D. 1953. *J. Sci. Food Agric.* 4:469
18. Emerson, O. H., Emerson, G. A., Mohammad, A., Evans, H. M. 1937. The chemistry of vitamin E. Tocopherols from various sources. *J. Biol. Chem.* 122:99–107
19. Evans, H. M., Bishop, K. S. 1922. On the existence of a hitherto unrecognized dietary factor essential for reproduction. *Science* 56:650–51
20. Evans, H. M., Burr, G. O. 1925. The antisterility vitamin fat-soluble E. *Proc. Natl. Acad. Sci. USA* 11:334–41
21. Evans, H. M., Burr, G. O. 1928. Development of paralysis in the suckling young of mothers deprived of vitamin E. *J. Biol. Chem.* 76:273–97
22. Fukuzawa, K., Chida, H., Tokumura, A., Tsukatani, H. 1981. Antioxidative effect of α-tocopherol incorporation into lecithin liposomes on ascorbic acid-Fe^{2+}-induced lipid peroxidation. *Arch. Biochem. Biophys.* 206:173–80
23. Ginter, E., Kosinova, A., Hudecova, A., Vejmolova, J. 1982. Effect of vitamins C and E on bioreactive oxygen products and on the cytochrome P_{450} cycle. *Biologia* 37:1195–1202
24. Goodhue, C. T., Risley, H. A. 1965. Reactions of vitamin E with peroxides. II. Reaction of benzoyl peroxide with *d*-α-tocopherol in alcohols. *Biochemistry* 4:854–58
25. Harris, P. L., Mason, K. E. 1956. Vitamin E and metabolic processes. In *Vitamina E, Atti del Terzo Congresso Internazionale, Venezia,* pp. 1–29. Verona: Edizione Valdonega
26. Howard, J. A., Ingold, K. U. 1963. The inhibited autoxidation of styrene. III. The relative inhibiting efficiencies of *O*-alkylphenols. *Can. J. Chem.* 41:2800–6
27. Howard, J. A., Ingold, K. U. 1963. The inhibited autoxidation of styrene. II. The relative inhibiting efficiencies of meta- and para-substituted phenols. *Can. J. Chem.* 41:1744–51
28. Hruba, F., Novakova, V., Ginter, E. 1982. The effect of chronic marginal vitamin C deficiency on the α-tocopherol content of the organs and plasma of guinea pigs. *Experientia* 38:1454–55
29. Jungherr, E. L., Singsen, E. P., Matterson, L. D. 1956. Nutritional encephalomalacia of chickens. *Lab. Invest.* 5:120–25
30. Land, E. J., Porter, G., Strachan, E. 1961. Primary photochemical processes in aromatic molecules. Part 6. The absorption spectra and acidity constants of phenoxyl radicals. *Trans. Faraday Soc.* 57:1885–93
31. Leung, H.-W., Vang, M. J., Mavis, R. D. 1981. The cooperative interaction between vitamin E and vitamin C in suppression of peroxidation of membrane phospholipids. *Biochim. Biophys. Acta* 664:266–72
32. McCay, P. B., King, M. M. 1980. Vitamin E: Its role as a biological free radical scavenger and its relationship to the microsomal mixed function oxidase system. In *Vitamin E—A Comprehensive Treatise,* ed. L. J. Machlin, pp. 289–317. New York: Dekker
33. McCay, P. B., King, M. M., Fong, K.-L., Poyer, J. L., Brueggemann, G., et al. 1983. Nutritional factors *in vivo:* The effect of antioxidants on the formation of trichloromethyl radicals from CCl_4 in liver of intact rats. In *Radioprotectors and Anticarcinogens,* ed. O. F. Nygaard, M. G. Simic, pp. 585–604. New York: Academic
34. McCay, P. B., King, M. M., Lai, E. K., Poyer, J. L. 1983. The effect of antioxidants on free radical production during in vivo metabolism of carbon tetrachloride. *J. Am. Coll. Toxicol.* 3:195–206
35. Niki, E., Saito, T., Kawakami, A., Kamiya, Y. 1984. Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. *J. Biol. Chem.* 259:4177–82
36. Nishikimi, M., Machlin, L. J. 1975. Oxidation of α-tocopherol model compound by superoxide anions. *Arch. Biochem. Biophys. Acta* 170:684–89
37. Nishikimi, M., Yamada, H., Yagi, K. 1980. Oxidation by superoxide of tocopherols dispersed in aqueous media with deoxycholate. *Biochim. Biophys. Acta* 627:101–8
38. O'Connor, M. K., Malone, J. F., Moriarty, M., Mulgrew, S. 1977. A radioprotective effect of vitamin C observed in Chinese hamster ovary cells. *Br. J. Radiol.* 50:587–91
39. Packer, J. E., Slater, T. F., Willson, R. L. 1978. Reactions of the CCl_4-related peroxy free radical with amino acids: pulse radiolysis evidence. *Life Sci.* 23:2617–20
40. Packer, J. E., Slater, T. F., Willson, R. F. 1979. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 278:737–39
41. Packer, J. E., Willson, R. L., Behne-

mann, D., Asmus, K. D. 1980. Electron transfer reactions of halogenated aliphatic peroxyl radicals: measurement of absolute rate constants by pulse radiolysis. *J. Chem. Soc. Perkin Trans.* 2:296–99

42. Redpath, J. L. 1973. Pulse radiolysis of dithiothreitol. *Radiat. Res.* 54:364–74
43. Redpath, J. L., Willson, R. L. 1973. Reducing compounds in radioprotection and radiosensitization: model experiments using ascorbic acid. *Int. J. Radiat. Biol.* 23:51–65
44. Scarpa, M., Rigo, K. A., Maiorino, M., Ursini, F., Gregolin, C. 1984. Formation of α-tocopherol radical and recycling of α-tocopherol by ascorbate during peroxidation of phosphatidyl choline liposomes. *Biochim. Biophys. Acta* 801:215–19
45. Simic, M. G. 1980. The kinetics of peroxy radical reactions with α-tocopherol. In *Autoxidation in Food and Biochemical Systems*, ed. M. G. Simic, M. Karel, p. 17. New York: Plenum
46. Simic, M. 1981. *J. Chem. Educ.* 58:125–31
47. Simic, M. G., Hunter, E. P. L. 1983. Interaction of free radicals and antioxidants. See Ref. 33, pp. 449–60
48. Tappel, A. L. 1968. Will antioxidant nutrients slow aging processes? *Geriatrics* 23:97–105
49. Willson, R. L. 1983. Free radical repair mechanisms and the interactions of glutathione and vitamins C and E. See Ref. 33, pp. 1–22
50. Yamauchi, R., Kato, K., Ueno, Y. 1981. Reaction of 8a-hydroperoxy tocopherones with ascorbic acid. *Agric. Biol. Chem.* 45:2855–61