Tocopheroxyl Radical Persistence and Tocopherol Consumption in Liposomes and in Vitamin E-enriched Rat Liver Mitochondria and Microsomes*

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Substantial loading of rat liver mitochondrial and microsomal membranes with d-α-tocopherol was achieved by dietary supplementation with no adverse effects of this loading being apparent, e.g., on treadmill exercise or uric acid levels. The tocopheroxyl radical was readily detected by ESR in the enriched microsomes and mitochondria. Continuous enzymatic oxidation with horseradish peroxidase and a hydrophilic phenol, to favor selective oxidation of tocopherol without the involvement of lipoperoxidation, allowed the tocopheroxyl radical to be observed for up to 1 h in liposomes of dioleoylphosphatidylcholine and for about 15 min in the subcellular membranes. Total α-tocopherol decreased throughout this period, but a significant residual fraction remained after all the ESR signal of tocopheroxyl had disappeared. Decay kinetics of the tocopheroxyl radical ESR signal produced by a burst of intense UV irradiation consisted of a rapid initial phase and a slower exponential decay. A more narrow and more persistent ESR signal, not yet chemically identified, was observed after the tocopheroxyl radical had disappeared under prolonged oxidation. Ascobic acid prevented formation of the tocopheroxyl radical until the ascorbyl radical ESR signal had decayed, whereas uric acid, up to saturating concentration in phosphate buffer, had no effect.

Tocopherols are the principal antioxidants protecting membranes from free radical chain reactions (1). Their effectiveness is considered to result from a facile reduction of polyunsaturated fatty acid peroxyl radicals by tocopherol, which leads to the relatively unreactive tocopheroxyl radical. Direct evidence for the stability of the tocopheroxyl radical has been found by ESR, which has demonstrated that the tocopheroxyl radical can be accumulated to sufficient steady state concentrations under continuous oxidation to be directly detectable in solvents and aqueous liposome suspensions (2, 3). However, the stability of the tocopheroxyl radical is limited and, like all free radicals, tocopheroxyl is subject to numerous decay processes. Chemical studies, mostly in homogeneous solvents, have identified dimers, trimers, and tocopherol quinone among the decomposition products of tocopherol (4–6). However, reaction products and mechanisms may be quite different in membranes, where there may be specific orientation and chemical effects not found in organic liquids. Moreover, in the presence of proteins and redox-active compounds like quinones, tocopherol may behave very differently than in pure lipid membranes. To investigate this possibility, we have now compared the oxidation of tocopherol in liposomes and natural membranes.

Failing to detect tocopheroxyl radicals in mitochondria and microsomes isolated from normal rats, we pursued nutritional supplementation of rats with high doses of α-tocopherol to provide sufficient enrichment of this vitamin in liver membranes for the detection of the tocopheroxyl radical with appropriate oxidizing systems. We also investigated the interaction of the aqueous antioxidants ascorbate and urate with the α-tocopheroxyl radical. Previous work had demonstrated a very efficient sparing of tocopherol by ascorbate (estimated rate constant of $2 \times 10^8$ M$^{-1}$ s$^{-1}$ for the reaction of ascorbic acid with the tocopheroxyl radical in soybean phosphatidylcholine liposomes, 3); we have now studied the interaction of ascorbate as well as uric acid with the tocopheroxyl radical in dioleoylphosphatidylcholine liposomes and in subcellular rat liver membranes. Dioleoylphosphatidylcholine was chosen to simulate the fluidity of biological membranes without the complication of the high autooxidizability of polyunsaturated lipids of less homogeneous lipid preparations (e.g., egg or soybean phospholipids).

MATERIALS AND METHODS

Animals—Female Sprague-Dawley rats, 10–12 weeks old, were purchased from Bantin and Kingman, Fremont, CA. The animals were fed ad libitum a diet (7) enriched with 10,000 IU d-α-tocopherol/kg of diet. They were maintained on this diet for 5 weeks prior to killing by CO$_2$ inhalation and decapitation. Environmental conditions: 25 °C room temperature, light cycles 12 h on/off.

Treadmill Exercise—Rats were exercised on a motorized rodent treadmill at a speed of 27 m/min and a grade of 15%. Endurance time was determined as published (8).

Membranes—Mitochondria and submitochondrial particle (SMP) membranes were prepared using methods previously described (9, 10). Microsomes were prepared initially by perfusing the liver with ice-cold 1.15% KCl. The liver was then removed and homogenized followed by a 10-min 10,000 x g centrifugation. The supernatant from this fraction was centrifuged at 105,000 x g for 60 min. This pellet was resuspended as the microsomal preparation. Protein was measured by the method of Lowry (11). Membranes were finally resuspended in 10 mM sodium phosphate, pH 7.0.

Liposome Preparation—Lipids were combined with α-tocopherol in chloroform and the solvent was removed under a stream of nitrogen. Small unilamellar liposomes were prepared by hydrating phos-

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* The abbreviations used are: SMP, submitochondrial particle; HPLC, high performance liquid chromatography.
phospholipids (dioleoylphosphatidylcholine from Sigma or asolectin from Associated Concentrates, woodworking, NY) in 10 mM sodium phosphate buffer, pH 7.0 (100 mg/ml). Subsequently the liposome preparation was sonicated with a Branson Sonicator until it achieved an opalescent appearance which could not be altered by additional sonication, using a micropipet at an output level of 5. The tocopherol concentration in the final liposome suspension was 400 μM.

Chemicals—TOLH (2,2,6,6-tetramethyl-1-hydroxypiperidin-1-ol) was synthesized by reduction of Tempol and Ternpol, purchased from Aldrich with excess ascorbate, extraction into diethyl ether, and removal of the ether under reduced pressure. D-α-Tocopherol was a gift from Henkel Co. Stripped corn oil was from Teklid Co. All other chemicals and enzymes were purchased from Sigma.

Quantitation of d-α-Tocopherol—This utilized HPLC with electrochemical detection, as published (12). Briefly, membranes, including liposomes, are extracted with hexane, the hexane is removed under a stream of nitrogen, and the extract is dissolved in ethanol/methanol, 1:1 (v/v), and injected into the HPLC column. The tocopherol peak intensities are newly calibrated for each day experiments.

ESR Spectroscopy—First derivative ESR spectra (100 kHz modulation) were recorded at ambient temperature (22°C) on a Bruker ER 260 D-SCR spectrometer (X-band). Instrument settings were: modulation amplitude 2.5 G, scan range 100 G, central field 3450 G, microwave power 10 mW. For kinetic studies, the height of the tocopheroxyl peak adjacent to and on the low-field side of the center line was used as a measure of the radical concentration. Samples were placed in gas-permeable tubing (Zeus Industrial Products, Ranjan, NJ) under air to provide oxygen for the glucose oxidase system and a photo-oxidant for the UV photolysis of α-tocopherol.

Light Source—Samples were illuminated within the cavity of the ESR spectrometer with a 100 W Universal Arc lamp, Model 6140 (Oriel Corp., CT), cutting off wavelengths below 240 nm.

RESULTS

Enzymic Formation of Tocopheroxyl Radicals—For studies of natural membranes in an enzyme system for generating phenoxyl radicals was designed to specifically generate the tocopheroxyl radical, while minimizing the involvement of other free radicals, e.g. lipid radical species. An appropriate enzyme system proved to be horseradish peroxidase coupled to a hydrogen peroxide generating system. The reactions of this system are:

Glucose + O2 → gluconolactone + H2O2
H2O2 + 2 PhOH → 2 H2O + 2 PhO•

where GO refers to glucose oxidase, HRP, horseradish peroxidase, PhOH, arbutin (4-hydroxyphenyl-β-D-glucopyranoside), and α-TocOH, α-tocopherol. Treatment of tocopherol-containing dioleoylphosphatidylcholine liposomes with this system yielded the ESR spectrum of the tocopheroxyl radical (Fig. 1A). Similar spectra were observed in asolectin and egg lecithin liposomes (data not shown), and rat liver subcellular mitochondrial and microsomal membranes (Fig. 1, B and C). After prolonged treatment with the enzyme system, the ESR spectrum of tocopheroxyl was replaced by another radical (designated here as the secondary E radical) shown as the bottom spectrum in the figures, which was even more persistent than tocopheroxyl. For example, in the liposomes this radical could be observed for at least 3 h. This secondary radical is only observed in the presence of tocopherol and has very similar ESR line shapes in all of the tested liposomes and in SMPs and microsomes.

Kinetics of Tocopheroxyl Radical Decay—After the tocopheroxyl radical had been generated photochemically, its post-illumination decay kinetics could be determined in the liposomes and liver subcellular membranes. The decay kinetics are at least biphasic, as illustrated by a semilogarithmic plot of the ESR signal intensities in dioleoyl liposomes (Fig. 2). After an initial rapid decay period (there is insufficient data to resolve whether this phase is exponential), a subsequent exponential decay phase is observed, with a decay time of t1/2 = 5 s. Oleic acid is expected to be unreactive with the UV irradiation, thus ensuring that photochemical activation was specific for tocopherol and did not involve other free radical species. UV irradiation of SMP and microsomes exhibited similar decay kinetics to those observed with the liposomes (data not shown).

Persistence of the Tocopheroxyl Radical and Depletion of Total Tocopherol in a Continuous Radical Generating System—The ESR signal of tocopheroxyl in liposomes was quite persistent if the radical was generated continuously by the peroxidase system (Fig. 3). There was some variability in the rate of decay among different samples (the time for complete disappearance of the tocopheroxyl radical varied from 30 to 60 min), probably because of the flexibility of the gas-permeable tubing, which did not allow for reproducible sample geometries (and therefore mean oxygen tensions). The tocopherol concentration decreased during the same time period (Fig. 3), but significant amounts remained after all of the ESR signal had vanished (the figure is representative of 6 separate experiments). UV irradiation of the sample after the ESR signal had disappeared, to check whether some of the tocopherol might be inaccessible to the enzyme system, e.g. due to lack of transmembrane mobility, did not elicit an ESR signal.

Studies of SMP and microsomes required a higher concentration of peroxidase to overcome the catalase contamination present in these membrane fractions. At 5 units/ml of horseradish peroxidase a tocopheroxyl radical signal was readily observed in both mitochondria and microsomes (Fig. 1, B and C). The persistence of the ESR signal was about half that observed with the liposomes (Figs. 3 and 4). However, the observation of a significant residual tocopherol concentration in the membranes after all of the ESR signal had decayed was common to both liposomes and the liver membrane fractions. The high degree of enrichment in tocopherol achieved in the liver membranes is noteworthy. Mitochondria from normal rats contain 0.08-0.20 nmol of tocopherol/mg of protein (11). The rats on the vitamin E-supplemented diets appeared normal to the eye and, in a preliminary experiment, one of these exhibited exercise endurance capacity on a treadmill similar to rats on normal diets.

Confirmation of Sustained Activity of the Peroxidase System—The activity of the glucose oxidase/peroxidase system was checked in terms of the oxidation of an ESR probe of phenoxyl radicals. The probe is TOLH, which is efficiently oxidized by phenoxyl radicals to the stable nitroxide-free radical Tempol, observable by ESR. In liposomes containing the oxidation system of Fig. 1A, the signal of Tempol increases nearly linearly with time throughout the period that was used to generate tocopheroxyl radicals, reaching a signal intensity corresponding to 500 μM Tempol after 30 min and continuing to increase nearly linearly thereafter, i.e. after the period required for the complete disappearance of the tocopheroxyl radical (data not shown). This observation, taken together with the progressive depletion of α-tocopherol from both liposomes and liver membranes, indicates that exhaustion of the enzyme system is not responsible for the disappearance of the tocopheroxyl radical when tocopherol still remains.

Transient Suppression of Tocopheroxyl Radical Formation with Ascorbate but Not Urate—Treatment of dioleoylphosphatidylcholine liposomes with 200 μM ascorbic acid prior to
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Addition of the peroxidase enzyme system initially yielded only the ascorbyl radical signal, with no tocopheroxyl radical signal being apparent in the ESR spectra (Fig. 5). Immediately prior to the complete disappearance of the ascorbyl radical signal the tocopheroxyl radical signal could be detected, and this latter signal increased when all the ascorbyl radical had disappeared. It should be noted that because of the existence of oxygen gradients in the gas-permeable tubing, different portions of the sample may have reacted at different rates and the observation of both ascorbyl and tocopheroxyl radical signals is not necessarily indicative of these two species co-existing at ESR-detectable levels. When the liposomes were treated with 5 mM sodium dodecyl sulfate to give them a negative surface potential this did not prevent the suppression of the tocopheroxyl ESR signal by ascorbate. Such charging of neutral liposomes with sodium dodecyl sulfate substantially decreases the rate of reduction of membrane-bound nitroxide-free radicals by ascorbate due to Coulombic repulsion of the ascorbate anion from the membrane (data not shown).

Addition of uric acid to the liposomes did not diminish the tocopheroxyl radical signal, even when the urate concentration was raised to 1 mM, approaching its solubility limit in the phosphate buffer (data not shown). This indicates that urate does not significantly affect the production of tocopheroxyl or arbutin radicals.

Fig. 1. ESR spectra of tocopherol-derived radicals. Numbers refer to minutes of elapsed time between sample mixing and initiation of the ESR scans. Glucose oxidase added last to start the enzymic oxidation. A, liposomes, 100 mg/ml dioleoylphosphatidylcholine; 230 μM D-α-tocopherol. Oxidation system: 5 mM glucose, 0.4 unit/ml glucose oxidase, 100 μM arbutin, 0.2 unit/ml horseradish peroxidase. B, SMP, vitamin E-enriched SMPs at 26.6 mg of protein/ml. Oxidation system: 10 mM glucose, 0.4 unit/ml glucose oxidase, 200 μM arbutin, 5 units/ml horseradish peroxidase. C, microsomes, vitamin E-enriched microsomes at 24 mg of protein/ml. Same oxidation system as for SMPs.

Fig. 2. Tocopheroxyl radical decay in liposomes after UV irradiation. 100 mg/ml dioleoylphosphatidylcholine; 230 μM D-α-tocopherol. Inset: data replotted on semilogarithmic scale.

Fig. 3. Tocopheroxyl and tocopherol decay in liposomes. Conditions as in Fig. 1A. O, ESR signal; Δ, D-α-tocopherol. Parallel samples for HPLC analysis were incubated under identical conditions to those used for the ESR experiment.
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**DISCUSSION**

It has been demonstrated that: 1) high levels of vitamin E can be incorporated into liver membranes without any evidence of short-term adverse effects, ruling out significant prooxidant activity of vitamin E or its oxidation products on membranes that carry out electron transport activity, 2) tocopheroxyl radicals are detectable in isolated mitochondrial and microsomal membranes and their persistence is comparable to that observed in liposomes composed of oxidation-resistant lipids, 3) substantially different mechanisms of tocopheroxyl radical decay exist at low and high concentrations in both natural and synthetic membranes, and 4) uric acid is not an effective reductant for the tocopheroxyl radical under conditions where ascorbate is extremely effective.

**High Tocopherol Enrichment and ESR Detection of Tocopheroxyl Radicals**—The detection of the tocopheroxyl radical by ESR in subcellular liver membranes was made possible by isolating these membranes from rats that had been fed vitamin E-enriched diets. After 5 weeks of tocopherol enrichment, mitochondrial tocopherol concentrations exceeded those of rats on normal diets by a factor of more than 50. The microsomal content of $\alpha$-tocopherol relative to membrane lipids was significantly smaller than that of mitochondria, especially when considering the greater protein-to-lipid ratio of mitochondria relative to microsomes. Previous studies had suggested that excess tocopherol might impair mitochondrial function; our results suggest that tocopherol concentrations of nearly 100 nmol/mg protein in the inner mitochondrial membrane can be tolerated without any apparent adverse effects on rats.

Because of the multiplicity of potential free radical species in subcellular membranes, it seemed important to select an oxidation system for tocopherol that would be as specific as possible. Arbutin was chosen as the phenol for two reasons: 1) the polar glucopyranosyl residue implies very limited partitioning into the lipid phase of liposomes, consistent with substantially lower concentrations required to generate the tocopheroxyl radical than when normal phenol was used (data not shown), and 2) little likelihood of direct reaction of the resulting phenoxy radicals with the double bonds of polyunsaturated lipids of natural membranes. The high polarity of arbutin and the membrane impermeability of horseradish peroxidase also suggests that this enzyme system will react exclusively with tocopherol molecules facing only external membrane surfaces.

**Analysis of Tocopheroxyl Radicals in Liposomes, SMP, and Microsomes**—The tocopheroxyl radical was observed to be highly persistent in both model and subcellular membranes. In liposomes composed of unreactive dioleoylphosphatidylcholine the possible reactions of the tocopheroxyl radical are very limited. Moreover, the feasibility of working with very high rapid concentrations made it possible to detect the tocopheroxyl radical when tocopherol-to-lipid ratios were in the physiological range (12), i.e. about 1 tocopherol/2,000-10,000 lipids. The similar decay rates of tocopheroxyl radicals of tocopherol in liposomes and in subcellular membranes is compatible with the interpretation that mechanisms of tocopherol depletion in all these membranes are the same. In all membranes studied the persistence of both $\alpha$-tocopherol and the tocopheroxyl radical is strongly dependent on the steady state concentration of the radical.

**Kinetics of Tocopheroxyl Radical Decay**—The formation of a large ESR signal by brief irradiation with UV light provided evidence of different mechanisms of decay at low and high concentrations of the tocopheroxyl radical. The rapid initial phase suggests that radical-radical collisions at high concentrations of tocopheroxyl in the membrane are responsible for the decay, whereas some spontaneous decomposition process occurs at low radical concentrations. Consistent with this interpretation, the crossover concentration between these two decay phases is of the order of magnitude expected for one tocopheroxyl radical per liposome as estimated from the radical concentration and the average liposome sizes under our

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**Fig. 4.** Tocopheroxyl and tocopherol decay in mitochondria and microsomes isolated from vitamin E-enriched rats. Conditions as in Fig. 1, C, ESR signal, D, $\alpha$-tocopherol.

**Fig. 5.** Effect of ascorbic acid on tocopheroxyl radical formation. Conditions as in Fig. 1A, except that 200 $\mu$M ascorbate was present. The numbers refer to elapsed time in minutes between mixing of the reaction system and beginning the ESR scans.
sonication conditions. The similar decay kinetics seen in liver and liposome membranes suggests that the considerably greater structural complexity of the former does not have much of an effect on the stability of the tocopheroxyl radical. In particular, free radicals observed in metabolically active tissues by ESR (15) and redox-active compounds like ubiquinone, appear to have little effect on the combined processes of generation and decomposition of the tocopheroxyl radical, at least in the absence of reducing substrates.

Taking into account the rapid decay of tocopheroxyl radicals at high concentrations, it seems plausible that the high persistence of both tocopherol and tocopherol in the continuous generating system is due to a low rate of tocopherol oxidation, which ensures sufficiently low steady state concentrations of the radicals to minimize reactions between them. This interpretation is compatible with the similar magnitudes of the ESR signal seen with continuous radical generation and in the exponential phase of the post-UV decay experiment. For higher rates of continuous tocopherol oxidation a relatively high initial ESR signal is observed to rapidly decay to a signal of similar magnitude to that observed with lower oxidation rates (data not shown), suggesting that radical concentrations in excess of 1 liposome are rapidly decomposed, perhaps via disproportion or dimer/trimer formation (16).

The rate of tocopherol disappearance by HPLC was about an order of magnitude less than the rate of phenoxyl radical production by the peroxidase system, estimated from the rate of TOLH oxidation. This suggests that the conversion of phenoxyl to tocopheroxyl radicals was incomplete or that the activity of the enzyme system was diminished because of limiting reductant for the arbutin radicals under these conditions. The rate of ascorbate consumption, as judged by the time course for the disappearance of the ascorbyl radical signal, was more than twice the rate expected from the TOLH oxidation data (200 μM ascorbate versus 160 μM TOLH oxidized in 12 min, data not shown), possibly indicating that ascorbate may have undergone autooxidation, perhaps transition metal-mediated, concurrently with its reduction to tocopheroxyl radicals.

The rapid decay of the α-tocopheroxyl radical at high concentrations suggests that there will be an accelerated loss of vitamin E from tissues under intense oxidative stress, similar to the very rapid disappearance of α-tocopherol in both chemical and photolytic systems, when we used very active systems to attempt to increase the magnitude of the ESR signal.

The midpoint potential of tocopherol/tocopheroxyl has been estimated to be less than 0.53 V (17), suggesting that a diversity of biological reductants other than ascorbate have the capacity to reduce the tocopheroxyl radical. However, our data suggest that uric acid is not an effective reductant for tocopheroxyl. This interpretation is consistent with the failure of urate to prolong the tocopherol-dependent induction period for lipid peroxidation (18). Redox considerations imply that components of the mitochondrial respiratory chain, including ubiquinol and ubiquinone, would also be capable of reducing tocopherol. The high persistence of the tocopheroxyl radical observed in mitochondria and microsomes suggests that such reducing systems would be highly efficient in preventing irreversible loss of vitamin E from the membranes of these organelles, particularly under mild oxidant stress.

Finally, the phenomenon of a residual fraction of tocopherol that seems resistant to oxidation to the radical has not been elucidated. We speculate that this may be related to the chemistry of tocopherol decomposition. Perhaps products of tocopherol decomposition can act as reductants, and a sufficient accumulation of these products could lead to an efficient quenching of the tocopheroxyl radical. The decomposition products would be oxidized in the process, so they would not prevent net tocopherol consumption, consistent with the sustained consumption of tocopherol that is observed after the radical signal can no longer be observed.

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