Oxidation of Vitamin E during Iron-Catalyzed Lipid Peroxidation: Evidence for Electron-Transfer Reactions of the Tocopheroxyl Radical†

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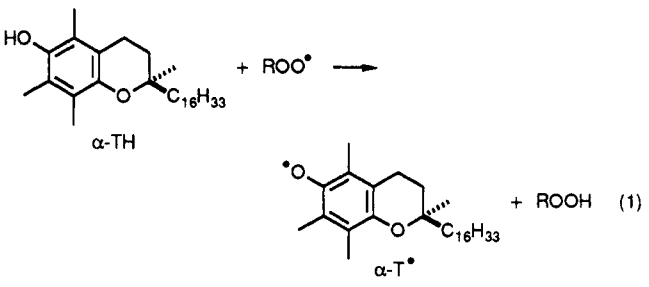
Received May 13, 1992; Revised Manuscript Received June 16, 1992

ABSTRACT: Incubation of phosphatidylcholine liposomes containing the biological antioxidant α-tocopherol (α-TH) with xanthine, xanthine oxidase, and $FeCl_2$ caused α-TH oxidation to α-tocopherol quinone (α-TQ) and 8a-hydroperoxytocopherone (**2**). In addition, 4a,5-epoxy-8a-hydroperoxytocopherone (**3**), 7,8-epoxy-8a-hydroperoxytocopherone (**4**), and their respective hydrolysis products 2,3-epoxy-α-tocopherol quinone (**6**) and 5,6-epoxy-α-tocopherol quinone (**7**) also were formed. α-TQ was the major product at less than 20% α-TH oxidation, whereas epoxides were the predominant products when α-TH was more extensively oxidized. 8a-(Alkyldioxy)tocopherones **1**, which are formed when peroxyl radicals oxidize α-TH in other systems and which are precursors to α-TQ, were not found. 8a-Hydroxytocopherone (**5**), rather than 8a-(alkyldioxy)tocopherones **1**, appeared to be the precursor to α-TQ. Approximately 30% of the α-TH consumed was regenerated by treatment of samples with ascorbic acid or nordehydroguaiaretic acid (NDGA) at pH 3, but not at pH 7. The stability of the ascorbic acid- and NDGA-reducible species and pH dependence for regeneration matched those of 8a-hydroxytocopherone (**5**) and contrasted with the properties of the tocopheroxyl radical (α-T•). Incubation of liposomes containing α-TH with the diphenylpicrylhydrazyl (DPPH) radical, which oxidizes α-TH to α-T• in high yield, formed an ascorbic acid-reducible species with properties identical to those of compound **5**. The results indicate that phospholipid peroxyl radicals oxidize α-T• to epoxides, 8a-hydroperoxytocopherone (**2**), and the tocopherone cation (α-T^+), which hydrolyzes to **5**, the immediate precursor to α-TQ. 8a-Hydroxytocopherone (**5**) and α-TQ also may be formed by disproportionation of α-T• to α-TH and α-T^+. Antioxidant reactions that form α-T^+ and **5** may contribute to α-TH redox cycles in membranes.

The lipid-soluble antioxidant α-TH[1] (vitamin E) is the principal inhibitor of lipid autoxidation in biological membranes [for a recent review, see Machlin (1991)]. α-TH reacts with peroxyl radicals by hydrogen atom transfer to form a hydroperoxide and α-T•, a resonance-stabilized phenoxyl radical that does not readily participate in radical propagation reactions (eq 1). Several studies indicate that cellular reductants, particularly ascorbic acid, can reduce α-T• back to α-TH in biological media (Scarpa et al., 1984; Melhorn et al., 1989; Motoyama et al., 1989; Kalayanaraman et al., 1992; Kagan et al., 1992). α-T• that do not complete redox cycles may themselves react with peroxyl radicals. Nonradical products of α-T•–peroxyl radical reactions have been isolated from systems in which radicals were generated by thermolysis of organic peroxides or azo compounds such as AMVN (Winterle et al., 1984; Yamauchi et al., 1989b, 1990; Liebler et al., 1990). Some of the α-T• generated in these systems react with peroxyl radicals to form 8a-(alkyldioxy)tocopherones **1** (eq 2). This reaction is analogous to the radical trapping reactions of other antioxidant phenoxyl radicals and is an *antioxidant* (i.e., radical-consuming) reaction of α-T•. Two other reactions compete with this antioxidant reaction. One relatively minor reaction forms 8a-hydroperoxytocopherone (**2**) (Matsuo et al., 1989; Liebler et al., 1990). A more significant competing reaction forms epoxyhydroperoxytocopherone products **3**/**4** (Matsuo et al., 1989; Liebler et al., 1990). The presence of 8a-hydroperoxy groups in epoxides **3**/**4** and compound **2** suggests that these products are formed by *autoxidation* reactions (i.e., reactions in which the antioxidant is consumed without producing an antioxidant effect) (Matsuo et al., 1989; Liebler et al., 1990). Consequently, α-TH antioxidant efficiency may depend on characteristics



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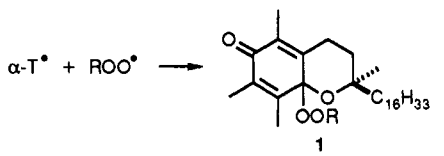

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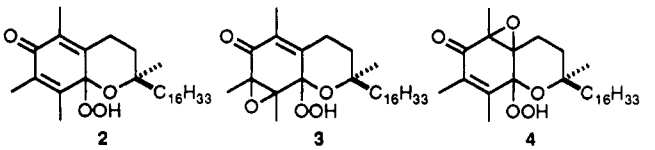

† Supported in part by USPHS Grant CA47943.

[1] Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); α-TH, α-tocopherol; α-TQ, α-tocopherol quinone; α-T•, tocopheroxyl radical; α-T^+, tocopherone cation; BHT, 2,6-di-*tert*-butyl-4-methylphenol; DPPH, diphenylpicrylhydrazyl radical; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; NDGA, nordehydroguaiaretic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride. (2*R*,4′*R*,8′*R*)-α-Tocopherol [(*RRR*)-α-tocopherol] was used exclusively in these experiments.

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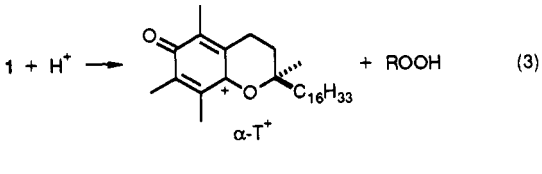
of the experimental system that control the partitioning of α-T• between antioxidant and autoxidation reactions.

Oxidations initiated by AMVN and similar initiators probably are inadequate models for biological α-TH oxidation. First, even in lipid bilayers, α-TH reacts primarily with AMVN-derived peroxyl radicals (Liebler et al., 1991), rather than with phospholipid peroxyl radicals, which presumably are the principal oxidants of α-TH in biological systems. Second, AMVN-initiated oxidations do not adequately mimic biological lipid peroxidation, which is driven by metal-catalyzed reactions of superoxide and hydrogen peroxide and which forms phospholipid peroxyl and alkoxyl radicals [reviewed by Halliwell and Gutteridge (1990)]. Surprisingly little is known of the oxidative fate of α-TH during metal-catalyzed lipid peroxidation in biological membranes. Although McCay et al. (1971) and Pesh-Imam and Recknagel (1977) found that α-TH oxidation during microsomal lipid peroxidation produced small, variable amounts of α-TQ, no other products have been identified.

Some of the α-TH consumed during membrane lipid peroxidation in vitro can be regenerated from product mixtures with reducing agents. For example, Leedle and Aust (1990) reported that α-TH recovery was increased when peroxidized microsomal lipids were extracted in the presence of ascorbic acid. Chan et al. (1991) reported that α-TH consumed during peroxidation of human platelet homogenates was partially regenerated by ascorbic acid, NDGA, or glutathione addition to the oxidized homogenates. Regeneration also occurred when ascorbic acid or NDGA, but not glutathione, was added to ethanol-denatured homogenates.

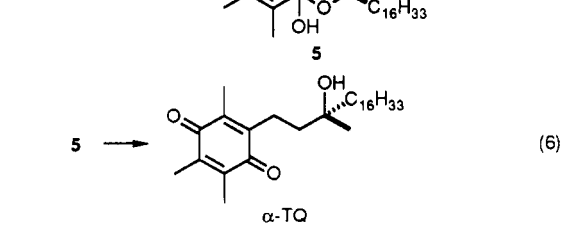
We have described previously the reduction of 8a-(alkyldioxy)tocopherones **1** to α-TH by ascorbic acid (Liebler et al., 1989). Acid-catalyzed loss of the 8a-(alkyldioxy) moiety yields α-T$^+$ (eq 3), which is reduced by ascorbic acid to α-TH (eq 4). α-T$^+$ also may reversibly hydrolyze to 8a-(hydroxy)-



(3)

α-T$^+$ + ascorbic acid → α-TH + dehydroascorbic acid (4)

tocopherone (**5**) (eq 5), which then rearranges to α-TQ (eq 6). Thus, compounds **1**, **2**, and **5** are reduced by ascorbic acid



(5)

(6)

to α-TH, whereas hydrolysis and rearrangement convert the same compounds to α-TQ. This raises the possibility that α-TH was regenerated from tocopherones in previous studies.

The first objective of this investigation was to identify the products of α-TH oxidation formed during iron- and superoxide-dependent lipid peroxidation. The second objective of the work was to determine whether tocopherone products could account for the ascorbic acid-reducible pool of α-TH oxidation products. Here we report that iron-catalyzed lipid peroxidation oxidizes α-TH to epoxide products, α-TQ and 8a-(hydroperoxy)tocopherone (**2**). However, phospholipid-derived 8a-(alkyldioxy)tocopherones analogous to **1** were not formed. We present evidence that 8a-hydroxytocopherone (**5**) is formed directly from α-T• rather than via 8a-(alkyldioxy)tocopherones **1** and that **5** is the ascorbic acid-reducible product of α-TH oxidation. These findings suggest that electron-transfer reactions are the principal mechanism by which α-T• scavenges phospholipid peroxyl radicals.

EXPERIMENTAL PROCEDURES

Chemicals. α-TH [(*RRR*)-α-tocopherol] was a gift of Henkel Fine Chemicals (LaGrange, IL). Soybean phosphatidylcholine (type III-S), xanthine oxidase, xanthine, ascorbic acid, BHT, DPPH, and NDGA were from Sigma (St. Louis, MO). $FeCl_2$ was from Aldrich (Milwaukee, WI). Deferoxamine was a gift of CIBA-GEIGY Corp. (Suffern, NY). *N*-(Trimethylsilyl)imidazole was from Pierce Chemical Co. (Rockford, IL). [^{14}C]-α-TH (1 mCi mmol^{-1}) was synthesized as described (Urano et al., 1980; Liebler et al., 1990). 8a-Hydroxytocopherone (**5**) and [^{14}C]-8a-(alkyldioxy)tocopherone **8** (1 mCi mmol^{-1}) were synthesized as described previously (Durckheimer & Cohen, 1964; Liebler et al., 1989).

Liposome Incubations. Small, unilammelar liposomes containing [^{14}C]-α-TH were prepared by ethanol injection (Kremer et al., 1977). Liposomes contained 0.1 mol % [^{14}C]-α-TH (mol of α-TH/mol of phospholipid) and were suspended in in 10 mL of 50 mM Tris-HCl, pH 7, at a phospholipid concentration of 0.5 mM. Liposome suspensions were incubated under air in stoppered Erlenmeyer flasks at 37 °C. Oxidations were initiated by adding xanthine (0.3 mM) and xanthine oxidase (0.01 unit mL^{-1}), followed immediately by $FeCl_2$ (0.1 mM). Incubations were terminated by extraction twice with 2 volumes of hexane/2-propanol (3:2 v/v), and the extracts were evaporated in vacuo. The lipid residue was dissolved in methanol and analyzed by reverse-phase HPLC on a 4.6- × 250-mm Spherisorb ODS-2 5-μm column (Alltech, Deerfield, IL) eluted with methanol/1 N sodium acetate, pH 4.25, at 1.5 mL min^{-1}. For analysis of [^{14}C]-α-TH oxidation products, fractions were collected at 0.8-min intervals and assayed by liquid scintillation counting.

Liposomes containing 0.1 mol % [^{14}C]-8a-(alkyldioxy)tocopherone **8** were prepared as described above except that the phosphatidylcholine used was first treated with NH_3/methanol to remove contaminating HCl from phospholipid storage that causes tocopherone hydrolysis (Liebler et al., 1989). Liposomes containing [^{14}C]-**8** were then incubated with xanthine, xanthine oxidase, and $FeCl_2$ as described above, and the mixture was sampled at 15, 30, and 60 min. The samples were extracted and [^{14}C]-**8**, and its products were analyzed by reverse-phase HPLC as described above. Liposomes containing 0.1 mol % 8a-hydroxytocopherone (**5**) were prepared as described for experiments with [^{14}C]-**8**. Oxidations with DPPH were done by adding DPPH in 0.05 mL of ethanol to a final concentration of 0.2 or 5 μM to liposomes containing 0.1 mol % unlabeled α-TH or [^{14}C]-α-TH, which were prepared as described above.

Analysis of α-TH Oxidation Products. A liposome suspension containing 50 μmol of phosphatidylcholine and 0.1 mol % α-TH in 100 mL of 50 mM Tris-HCl, pH 7, was prepared and incubated with xanthine, xanthine oxidase, and $FeCl_2$ as described above for 60 min at 37 °C. The mixture then was extracted with hexane/2-propanol (3:2 v/v) and evaporated in vacuo. α-TH oxidation products were separated from phospholipids in the lipid extracts by solid-phase extraction on silica (Juaneda & Rocquelin, 1985). The lipid

residue was dissolved in 10 mL of $CHCl_3$, and 2-mL portions of this solution were each applied to a 6-mL Bakerbond silica solid-phase extraction column (J. T. Baker, Phillipsburg, NJ) prewetted with $CHCl_3$ and α-TH, and its oxidation products then were eluted with 4 mL of $CHCl_3$. The eluent fractions were pooled and evaporated under N_2 and then redissolved in 2 mL of ethanol and 1 mL of 0.1 N HCl. After 30 min at room temperature, the mixture was extracted with 3 × 3 mL of hexane, and the extracts were evaporated under N_2. The residue was dissolved in methanol and α-TQ, and epoxides **6**/**7** were separated by preparative reverse-phase HPLC on a 10- × 300-mm Spherisorb ODS-2 5-μm column (Alltech, Deerfield, IL) eluted with methanol at 7 mL min^{-1}. Products were detected by UV absorbance at 270 nm. Fractions containing α-TQ and epoxides **6**/**7** were collected and evaporated under N_2. The product residues were treated with 0.1 mL of *N*-(trimethylsilyl)imidazole at 60 °C for 1 h and then analyzed by GC–MS on a Varian 3400 gas chromatograph interfaced with a Finnegan MAT 700 ion trap detector. Samples were injected in the splitless mode, and products were separated isothermally on a 30-m SPB-5 capillary column (Suppelco, Bellefonte, PA) at 270 °C. Mass spectra were obtained in the electron ionization mode at an ionizing voltage of 70 eV.

Ascorbic Acid-Dependent Reduction of 8a-Hydroxytocopherone (**5**) *and α-TH Oxidation Products.* Samples of authentic 8a-hydroxytocopherone (**5**) (0.5 nmol) or liposomes (equivalent to 0.5 nmol initial α-TH content) were added to tubes containing 1.125 mL of ethanol, 20 nmol of BHT, and 187.5 μmol of ascorbic acid in 0.5 mL of either 300 mM sodium formate, pH 3, or 50 mM Tris-HCl, pH 7. In some experiments, 16.5 μmol of NDGA was substituted for ascorbic acid. After 30 min at room temperature, 0.5 nmol of δ-tocopherol (an internal standard for HPLC) and 1 mL of ethanol/water (1:1 v/v) were added, the samples were extracted with three 1-mL portions of hexane, and the extracts were evaporated under N_2. The samples were redissolved in 1 mL of methanol and analyzed by reverse-phase HPLC on a 4.6- × 150-mm Spherisorb ODS-2 5-μm column eluted with methanol/1 N sodium acetate, pH 4.25 (98:2 v/v), at 1.5 mL min^{-1}. Tocopherols were detected with an ESA Coulochem electrochemical detector equipped with a Model 5011 analytical cell operated at +0.35 V.

For measurement of ascorbic acid- or NDGA-reducible products of iron/superoxide- or DPPH-dependent α-TH oxidation, liposomes containing 0.1 mol % α-TH were prepared and three 0.5-mL samples were removed, diluted with 1.125 mL of ethanol containing 0.5 nmol of δ-tocopherol internal standard and 20 nmol of BHT, and then extracted with hexane and analyzed for α-TH by HPLC as described above. The remainder of the liposome mixture was incubated either with xanthine, xanthine oxidase, and $FeCl_2$ or with DPPH as described above. Incubations with $FeCl_2$ were terminated by addition of the iron chelator deferoxamine to a concentration of 1 mM. Samples (0.5 mL) were removed immediately, extracted, and analyzed directly for α-TH as described above. Other 0.5-mL samples of the incubation mixture were then treated immediately or after a delay with either ascorbic acid or NDGA at pH 3 or pH 7 for 30 min, extracted, and analyzed for α-TH as described above.

RESULTS

Superoxide- and Iron-Dependent α-TH Oxidation in Liposomes. Incubation of phosphatidylcholine liposomes containing α-TH with $FeCl_2$, xanthine, and xanthine oxidase caused α-TH oxidation. In similar systems, the major chain-initiating events are (a) the formation of alkoxyl radicals by Fe^{2+}-catalyzed reductive cleavage of phospholipid hydroperoxides (eq 7), (b) the reduction of superoxide-derived hydrogen peroxide to hydroxyl radicals by Fe^{2+} (i.e., the Fenton reaction) (eq 8), and (c) the generation of other oxidants by reactions of Fe^{2+}, Fe^{3+}, and superoxide [reviewed by Minotti and Aust (1989) and Halliwell and Gutteridge (1990)].

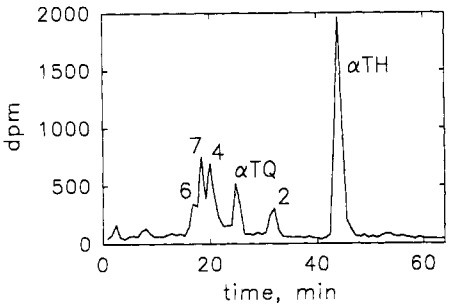


FIGURE 1: Reverse-phase HPLC analysis of products of [^{14}C]-α-TH oxidation in phosphatidylcholine liposomes incubated with xanthine, xanthine oxidase, and $FeCl_2$ for 45 min at 37 °C. Liposome preparation, incubation, and chromatography conditions are described under Experimental Procedures.

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+} \quad (7)$$

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + OH^{-} + Fe^{3+} \quad (8)$$

Oxidation in this system probably is initiated largely via reaction 7, which is favored by the levels of lipid hydroperoxide (approximately 0.1–0.2%) present in the phospholipid used. However, reaction 7 slows as Fe^{2+} is consumed by oxidation (Liebler et al., 1986). Superoxide sustains the reaction by regenerating Fe^{2+} from Fe^{3+} (eq 9). Both alkoxyl

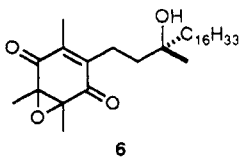
$$O_2^{-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (9)$$

and hydroxyl radicals react primarily with phosphatidylcholine polyunsaturated fatty acyl chains, which are present in 1000-fold excess over α-TH (eq 10). The resulting carbon-centered radicals add oxygen to form phospholipid peroxyl radicals (eq 11), which react with α-TH.

$$LO^{\bullet} \text{ or } HO^{\bullet} + LH \rightarrow LOH \text{ or } H_2O + L^{\bullet} \quad (10)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (11)$$

Products of α-TH Oxidation. Liposomes containing 0.1 mol % [^{14}C]-α-TH were treated with $FeCl_2$, xanthine, and xanthine oxidase, and [^{14}C]-α-TH and its oxidation products were analyzed by reverse-phase HPLC. Analysis of a typical incubation mixture is depicted in Figure 1. In addition to [^{14}C]-α-TH, which eluted at 44 min, product peaks coeluted with authentic standards of 5,6-epoxy-α-tocopherol quinone (**6**), 2,3-epoxy-α-tocopherol quinone (**7**), epoxytocopherone **4**, α-TQ, and 8a-hydroperoxytocopherone (**2**). Although epoxytocopherone **3** was not observed directly, its formation was inferred from the presence of its hydrolysis product **6**. To confirm the identities of the major products, incubations with



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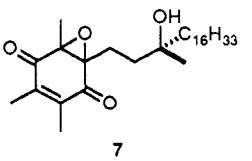

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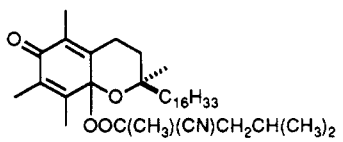
Table I: Distribution of [^{14}C]-α-TH and Its Oxidation Products after Iron- and Superoxide-Dependent Oxidation in Liposomes[a]

expt	incubation time, min	α-TH depletion, %	% of total radioactivity						% of products	
			α-TH	4	6	7	α-TQ	2	α-TQ	epoxides[b]
4	20	5.4	94.6	nd[c]	nd	nd	2.0	nd	100	0
3	30	8.7	91.3	nd	0.3	0.4	1.9	0.8	56	21
6	10	12.3	87.7	1.1	nd	0.5[d]	5.0	nd	76	24
7	10	16.5	83.5	0.9	nd	0.9[d]	6.7	nd	79	21
2	20	24.1	75.9	3.5	3.5	4.6	5.6	2.2	29	60
1	10	29.0	71.0	nd	1.4	6.8	2.0	2.9	15	63
4	60	41.7	58.3	7.7	4.6	8.5	7.4	2.1	24	69
3	60	51.2	48.8	9.2	nd	15.7[d]	7.7	3.3	21	69
5	30	55.0	45.0	7.4	5.5	8.9	9.4	5.2	26	60
5	45	61.3	38.7	13.0	6.7	10.5	8.7	5.9	19	67
5	90	79.5	20.5	13.5	9.2	15.7	8.5	4.8	16	74

[a] Phosphatidylcholine liposomes containing [^{14}C]-α-TH were incubated with xanthine, xanthine oxidase, and $FeCl_2$ as described under Experimental Procedures. After the incubation for the times indicated, lipids were extracted from the incubation mixtures and [^{14}C]-α-TH and its products were separated and quantified by reverse-phase HPLC and liquid scintilation counting as described. [b] Percent of products present as compounds **4**, **6**, and **7**. [c] Not detected. [d] Compounds **6** and **7** were incompletely resolved, and the combined amount is listed.

unlabeled α-TH were done and the products were then extracted and subjected to mild acid hydrolysis. Hydrolysis converts epoxytocopherones **3**/**4** to their corresponding epoxyquinones **6**/**7** and 8a-hydroperoxytocopherone (**2**) to α-TQ (Liebler et al., 1989, 1990). The hydrolysis products were silylated and analyzed by capillary GC–MS. The silylated epoxyquinones **6**/**7** and α-TQ each displayed retention times and mass spectra identical to those of authentic standards (data not shown). Diagnostic fragment ions observed for epoxyquinones **6**/**7** at *m*/*z* 309 and for α-TQ at *m*/*z* 293 corresponded to loss of the phytyl chain (i.e., $C_{16}H_{33}$) from the molecular ions. These HPLC and mass spectrometry data indicate that superoxide- and iron-dependent oxidation of α-TH forms epoxytocopherones **3**/**4**, their hydrolysis products **6**/**7**, α-TQ and 8a-hydroperoxytocopherone (**2**).

The distribution of α-TH oxidation products was similar to that in AMVN-initiated liposome oxidations (Liebler et al., 1991). However, AMVN-initiated oxidation also yielded 8a-(alkyldioxy)tocopherones **8** derived from addition of AMVN-derived peroxyl radicals to α-T•. In reverse-phase HPLC, the AMVN-derived 8a-(alkyldioxy)tocopherones eluted immediately after α-TH. Phospholipid-derived 8a-(alkyldioxy)tocopherones **9**, which would contain a phosphatidylcholine-dioxy substituent in the 8a-position, have not been synthesized, and their elution characteristics in this reverse-phase HPLC system are unknown. However, phosphatidylcholine itself elutes near α-TH, whereas phosphatidylcholine oxidation products elute prior to α-TH. No additional radioactivity was eluted with methanol/ethyl acetate (1:1 v/v), which elutes very nonpolar spiro dimer and trimer oxidation products of α-TH (Yamauchi et al., 1989a). Since essentially all of the applied radioactivity eluted as α-TH or more polar oxidation products, we conclude that phospholipid-derived 8a-(alkyldioxy)tocopherones were not present in the product extracts.



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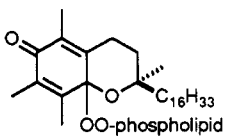

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The time-consuming nature of the product analyses precluded detailed kinetic studies of α-TH oxidation and product formation. Moreover, the rates of α-TH consumption varied somewhat between experiments. Nevertheless, the data from several similar experiments document the relationship between the extent of α-TH oxidation and product yield (Table I). The extent of α-TH oxidation varied between 5.4% and 79.5%. Individual product yields all increased with increasing α-TH oxidation. However, the relative proportions of products shifted as α-TH oxidation progressed. At low (<20%) α-TH oxidation, α-TQ appeared to be the principal product and accounted for 80–100% of the α-TH consumed. At higher degrees of α-TH oxidation, epoxides were formed in higher yield. At 79.5% α-TH oxidation, epoxides accounted for approximately 75% of the products. In similar experiments where liposomes containing 0.1 mol % [^{14}C]-α-TQ were incubated with $FeCl_2$, xanthine, and xanthine oxidase, α-TQ was converted to epoxyquinones **6**/**7** in about 5% yield (data not shown). However, this conversion was suppressed when liposomes also contained 0.1 mol % unlabeled α-TH. The increase in epoxide yield with increasing α-TH oxidation therefore does not reflect secondary oxidation of α-TQ to epoxide products. The yield of 8a-hydroperoxytocopherone (**2**) ranged from 0% to 20% and did not exhibit a clear dependence on the extent of α-TH oxidation.

Stability of 8a-(Alkyldioxy)tocopherones in Liposome Incubations. Phospholipid-derived 8a-(alkyldioxy)tocopherones **1** were not detected as α-TH oxidation products, yet α-TQ, a tocopherone hydrolysis product, was found. This result might indicate rapid hydrolysis of phospholipid-derived 8a-(alkyldioxy)tocopherones under the incubation conditions. Because phospholipid-derived 8a-(alkyldioxy)tocopherones have not been successfully synthesized, this hypothesis could not be tested directly. Instead, we evaluated the stability of 8a-(alkyldioxy)tocopherone **8**, a product of AMVN-dependent α-TH oxidation. Compound [^{14}C]-**8** was incorporated into liposomes, which were incubated with $FeCl_2$, xanthine, and xanthine oxidase. Amounts of [^{14}C]-**8** and [^{1}14C]-α-TQ then were measured by HPLC. Although approximately 15% of the [^{14}C]-**8** hydrolyzed to α-TQ during liposome preparation, less than 10% of the remaining [^{14}C]-**8** hydrolyzed during a 60-min incubation (data not shown). This stability was similar to that observed previously in liposomes containing AMVN (Liebler et al., 1991) and suggests that the iron/superoxide system does not accelerate tocopherone decomposition. Thus, phospholipid-derived 8a-(alkyldioxy)tocopherones **9** should be relatively resistant to hydrolysis in liposomes, and failure to observe these compounds suggests that they were not formed by α-TH oxidation.

Role of 8a-Hydroxytocopherone (**5**) *in α-TH Oxidation.* Because phospholipid-derived 8a-(alkyldioxy)tocopherones **1** were not formed in the liposome system, α-TQ may have been formed by other reactions that yield its precursor, 8a-hydroxytocopherone (**5**). In one possible mechanism, a phospholipid peroxyl radical may abstract an electron from α-T• to form a phospholipid hydroperoxide and α-T$^+$ (eq 12). The

$$\alpha\text{-T}^{\bullet} + \text{LOO}^{\bullet} + \text{H}^{+} \rightarrow \alpha\text{-T}^{+} + \text{LOOH} \quad (12)$$

α-T^+ would hydrolyze to 8a-hydroxytocopherone (**5**) as described in eq 5. Although 8a-hydroxytocopherone (**5**) is too unstable to analyze directly by HPLC, it may be measured indirectly by ascorbic acid-dependent reduction to α-TH (Liebler et al., 1989). In ethanol/sodium formate, pH 3 mixtures, **5** was quantitatively reduced to α-TH at ambient temperature (eqs 4 and 5). Authentic **5** was incorporated into liposomes, and then samples were taken for ascorbic acid reduction immediately or after 15 or 30 min at 37 °C. Ascorbic acid treatment of liposome samples immediately after liposome preparation resulted in only 54% recovery of **5** as α-TH. This apparently was due to rearrangement of approximately half of the compound **5** to α-TQ during liposome preparation. Thereafter, **5** decomposed relatively slowly in the bilayer; ascorbic acid treatment of samples taken after 15 and 30 min resulted in 31% and 25% recoveries of **5** as α-TH (data not shown).

To determine whether α-TH was oxidized to 8a-hydroxytocopherone (**5**) during iron- and superoxide-dependent lipid peroxidation, liposome samples from incubations with $FeCl_2$, xanthine, and xanthine oxidase were treated with ascorbic acid in ethanol/sodium formate, pH 3, and then analyzed for α-TH (Table II). α-TH depletion in these incubations ranged from approximately 50% to 100%. The oxidations were quenched with deferoxamine, and then samples were taken either immediately or after a delay of 15 or 30 min. In samples treated immediately with ascorbic acid, 40% and 32%, respectively, of the α-TH consumed during the incubation was recovered as α-TH following ascorbic acid treatment (Table II). Samples treated with ascorbic acid after a 15- or 30-min delay yielded α-TH in similar amounts. The stability of the ascorbic acid-reducible species formed by α-TH oxidation in liposomes therefore was similar to that of 8a-hydroxytocopherone (**5**). Moreover, the fraction of oxidized α-TH converted back to α-TH by ascorbic acid treatment was similar to the fraction accounted for as α-TQ in radiochromatographic product analyses (Figure 1 and Table I).

pH-Dependent Regeneration of α-TH by Ascorbic Acid and NDGA. The above results suggest that 8a-hydroxytocopherone (**5**) is the ascorbic acid-reducible intermediate. We tested this hypothesis further by examining the pH dependence of regeneration and the effectiveness of NDGA, which has been reported to regenerate α-TH oxidized in human platelet homogenates (Chan et al., 1991). We had observed previously that in acetonitrile/buffer mixtures, 8a-hydroxytocopherone (**5**) was reduced more efficiently at pH 3 than at pH 7 (Liebler et al., 1989). We first examined how pH affects the reduction of **5** to α-TH by ascorbic acid and NDGA (Table III). Ascorbic acid reduced **5** to α-TH in about 20-fold higher yield at pH 3 than at pH 7. The apparent failure of ascorbic acid to produce greater than 70–80% reduction of **5** to α-TH was observed previously and may be due to the presence of approximately 20–30% α-TQ in freshly synthesized preparations of **5** (Liebler et al., 1989). The contaminating α-TQ, which cannot easily be removed, interferes with the quantitation of stock solutions of compound **5** by UV spectroscopy and causes the content of **5** to be overestimated by 15–30%. Consequently, the actual yield of α-TH from **5** in ascorbic acid-dependent reductions (e.g., in Tables II and IV, see below) probably exceeds 90%. Reduction by NDGA also exhibited the same pH dependence, although α-TH yields appeared slightly lower than with ascorbic acid (Table III).

The ability of ascorbic acid and NDGA to produce a pH-dependent regeneration of α-TH consumed by iron- and superoxide-dependent liposomal oxidation was then studied. Liposomes containing α-TH were incubated with $FeCl_2$, xanthine, and xanthine oxidase, and then the incubation was quenched with deferoxamine. Immediately thereafter, samples were removed for treatment with ascorbic acid or NDGA at pH 3 or 7 (Table IV). α-TH was completely consumed during the iron- and superoxide-dependent incubations. Treatment of samples with ascorbic acid at pH 3 regenerated α-TH in approximately 30–40% yield, whereas ascorbic acid treatment at pH 7 yielded little or no α-TH. Similarly, NDGA treatment at pH 3 formed α-TH in about 25% yield, whereas NDGA treatment at pH 7 yielded almost no α-TH. The pH dependence of α-TH regeneration by ascorbic acid and NDGA was identical to that observed for 8a-hydroxytocopherone (**5**).

Oxidation of α-TH in Liposomes by DPPH. A possible alternative reaction of α-$T^{\bullet}$ is disproportionation to form α-T^+ and α-TH (eq 13). α-T^+ then may form α-TQ via reactions

$$\alpha\text{-T}^{\bullet} + \alpha\text{-T}^{\bullet} + \text{H}^{+} \rightarrow \alpha\text{-T}^{+} + \alpha\text{-TH} \quad (13)$$

5 and 6. To determine whether α-$T^{\bullet}$ reacts this way in the lipid bilayer, we employed DPPH, a stable radical that oxidizes phenols to phenoxyl radicals in high yield (Rousseau-Richard et al., 1988). Addition of DPPH to liposome suspensions caused rapid α-TH oxidation. α-TH was maximally depleted within 1 min after DPPH addition. Radiochromatographic analysis of incubations with [^{14}C]-α-TH indicated the formation of a single product with a retention time identical to that for authentic α-TQ (data not shown). Liposomes containing α-TH were incubated with 1 or 5 μM DPPH for 1 min, and then samples were treated with ascorbic acid in ethanol/sodium formate, pH 3 (Table V). Ascorbic acid treatment resulted in 60–80% recovery of the consumed α-TH.

Table II: Ascorbate-Dependent Regeneration of α-TH Oxidized in Liposomes[a]

expt	α-TH_0	time, min	α-TH_f	delay, min	α-TH_{asc}[b]	α-TH_{rec}[c]	α-TH_{rec}, %[d]
1	301 ± 1	20	152 ± 6	0	212 ± 9	60 ± 9	40
				15	210 ± 4	58 ± 6	39
				30	207 ± 19	55 ± 19	37
2	359 ± 14	30	126 ± 3	0	201 ± 3	75 ± 3	32
				15	204 ± 3	78 ± 3	33
				30	197 ± 6	71 ± 6	30

[a] Liposomes containing α-TH were incubated with xanthine, xanthine oxidase, and $FeCl_2$ for the indicated times. The incubation was terminated with deferoxamine, and samples were analyzed for α-TH or were treated with ascorbic acid to regenerate oxidized α-TH after the delay period indicated. All product amounts are expressed as pmol of α-TH per 0.5-mL sample of liposome suspension and are reported as mean ± SD of triplicate analyses. [b] α-TH present in 0.5-mL sample after treatment with ascorbic acid. [c] α-TH recovered by ascorbic acid treatment. [d] Recovery expressed as percent of α-TH consumed during incubation.

Table III: Effect of pH on the Reduction of 8a-Hydroxytocopherone (**5**) to α-TH by Ascorbic Acid and NDGA[a]

reductant	pH	α-TH regenerated[b]	% reduction
ascorbic acid	3	360 ± 12	72 ± 2
	7	16 ± 4	3 ± 1
NDGA	3	269 ± 31	54 ± 6
	7	4 ± 7	1 ± 1

[a] Freshly-synthesized 8a-hydroxytocopherone (**5**) (0.5 nmol) was treated with ascorbic acid or NDGA at pH 3 or 7, and the α-TH produced was measured as described under Experimental Procedures. [b] α-TH yields are in pmol and are reported as mean ± SD (n = 3).

DISCUSSION

An effective membrane antioxidant traps oxidants rapidly without generating intermediates that lead to further oxidative

Table IV: pH-Dependent Regeneration by Ascorbic Acid and NDGA of α-TH Oxidized in Liposomes[a]

expt	α-TH$_0$	incubation time, min	α-TH$_t$	reductant, pH	α-TH$_{red}$[b]	α-TH$_{rec}$[c]	α-TH$_{rec}$, %[d]
1	460 ± 10	15	0	asc, 3	151 ± 5	151 ± 5	33 ± 1
				asc, 7	0	0	0
				NDGA, 3	107 ± 6	107 ± 6	23 ± 1
				NDGA, 7	0	0	0
2	401 ± 25	5	0	asc, 3	169 ± 9	169 ± 9	42 ± 2
				asc, 7	19 ± 1	19 ± 1	5 ± 0
				NDGA, 3	105 ± 8	105 ± 8	26 ± 2
				NDGA, 7	13 ± 0	13 ± 0	3 ± 0

[a] Liposomes containing α-TH were incubated with xanthine, xanthine oxidase, and $FeCl_2$ for the indicated times. The incubation was terminated with deferoxamine, and samples were analyzed for α-TH or were treated immediately with ascorbic acid or NDGA at the indicated pH and then analyzed for α-TH. All product amounts are expressed as pmol of α-TH per 0.5-mL sample of liposome suspension and are reported as mean ± SD of triplicate analyses. [b] α-TH present in 0.5-mL sample after treatment with reductant. [c] α-TH recovered by reductant treatment. [d] Recovery expressed as percent of α-TH consumed during incubation.

Table V: pH-Dependent Regeneration of α-TH Oxidized in Liposomes by DPPH[a]

expt	α-TH$_0$	DPPH, μM	α-TH$_t$	pH	α-TH$_{asc}$[b]	α-TH$_{rec}$[c]	α-TH$_{rec}$, %[d]
1	377 ± 128	1	64 ± 5	3	252 ± 1	188 ± 5	60
				7	69 ± 1	5 ± 5	2
2	430 ± 150	5	0	3	323 ± 6	323 ± 6	75
				7	19 ± 1	19 ± 1	4
3	577 ± 19	5	0	3	346 ± 21	346 ± 21	60
				7	0	0	0

[a] Liposomes containing α-TH were incubated with DPPH at the indicated concentrations for 1 min, and then samples were analyzed for α-TH or treated immediately with ascorbic acid at the indicated pH and then analyzed for α-TH. α-TH amounts are reported in pmol per 0.5-mL liposome incubation sample and are mean ± SD of triplicate analyses. [b] α-TH present in 0.5-mL sample after treatment with ascorbic acid. [c] α-TH recovered by ascorbic acid treatment. [d] Recovery expressed as percent of α-TH consumed during incubation.

damage. Although α-TH itself is one of Nature's best peroxyl radical traps, its one-electron oxidation product α-T$^\bullet$ is not necessarily as effective. α-T$^\bullet$ can undergo several competing reactions that either consume or generate radicals. Antioxidant protection is enhanced and sustained by reactions of α-T$^\bullet$ with reductants that regenerate α-TH (Packer et al., 1979; Scarpa et al., 1984; Mehlhorn et al., 1989) and by reactions in which α-T$^\bullet$ traps peroxyl radicals to form 8a-(alkyldioxy)tocopherones (Winterle et al., 1984; Yamauchi et al., 1989b; Liebler et al., 1990). Other reactions of α-T$^\bullet$ that form epoxytocopherones **3**/**4** and 8a-hydroperoxytocopherone (**2**) probably involve peroxyl radical intermediates (Matsuo et al., 1989; Liebler et al., 1990) that lead to α-TH autoxidation and diminished antioxidant protection. Because different reactions of α-T$^\bullet$ yield different products, the distribution of α-TH oxidation products reflects the balance between competing reactions of α-T$^\bullet$. Here we report the first quantitative study of the oxidative fate of α-TH during iron- and superoxide-catalyzed lipid peroxidation. The results indicate that antioxidant reactions of α-T$^\bullet$ yield a single, reversibly oxidized product, 8a-hydroxytocopherone (**5**).

The initial goal of this study was to identify the products of α-TH oxidation during iron- and superoxide-catalyzed lipid peroxidation. This system more accurately models biological lipid peroxidation than AMVN-initiated oxidations studied previously. Nevertheless, iron- and superoxide-dependent α-TH oxidation yielded epoxides **3**–**7**, α-TQ, and 8a-hydroperoxytocopherone (**2**), all of which were formed in AMVN-initiated oxidations (Liebler et al., 1991). Moreover, epoxyquinones **6**/**7** and one of their precursors, epoxytocopherone **7** (Figure 1), were detected together in proportions similar to those observed in AMVN-initiated liposome oxidations. The similarity of the α-TH product distribution to that in AMVN-initiated oxidations, where peroxyl radicals are the principal oxidants, indicates that phospholipid peroxyl radicals oxidize α-TH in iron- and superoxide-dependent oxidations.

A notable difference between the two systems is that phospholipid peroxyl radicals do not react with α-T$^\bullet$ to form 8a-(alkyldioxy)tocopherones like those formed in AMVN-initiated oxidations. Our failure to detect phospholipid-derived 8a-(alkyldioxy)tocopherones **9** apparently does not reflect tocopherone instability in the liposomes, since authentic, AMVN-derived 8a-(alkyldioxy)tocopherones **8** were stable in liposomes during iron/superoxide-initiated oxidations. Although 8a-(alkyldioxy)tocopherones are precursors to 8a-hydroxytocopherone **5** and α-TQ under certain conditions (Liebler et al., 1989), the absence of phospholipid-derived 8a-(alkyldioxy)tocopherones in the present system indicates that compound **5** also is formed by another mechanism. Here we propose that α-T$^\bullet$ reacts with phospholipid peroxyl radicals by electron transfer to form α-T$^+$ (eq 12) or that two α-T$^\bullet$ disproportionate to α-T$^+$ and α-TH (eq 13). The α-T$^+$ formed by either reaction would readily hydrolyze to 8a-hydroxytocopherone (**5**), which would rearrange to α-TQ (eqs 5 and 6).

We have been unable to analyze compound **5** directly, as this intermediate accounts for a small fraction of the lipid mixture and readily rearranges to α-TQ during HPLC purification. Nevertheless, several lines of evidence indicate that 8a-hydroxytocopherone (**5**) was present in product mixtures. First, treatment of product mixtures with ascorbic acid or NDGA in ethanol/sodium formate, pH 3, regenerates α-TH. Authentic 8a-hydroxytocopherone (**5**) is also reduced to α-TH under these conditions. Second, the pH profile for regeneration of α-TH from product mixtures matches that for reduction of authentic 8a-hydroxytocopherone (**5**) to α-TH. α-TH is regenerated from product mixtures or authentic compound **5** at pH 3, but not at pH 7. Third, the stability of the ascorbic acid-reducible species in liposomes is similar to that of authentic 8a-hydroxytocopherone (**5**) (Tables II and III). Finally, the fraction of α-TH regenerated by ascorbic acid corresponded approximately to the sum of the α-TQ and 8a-hydroperoxytocopherone (**2**) observed in HPLC analyses of [^{14}C]-α-TH oxidation products (compare Tables I, II, and IV). Like compound **5**, compound **2** is reduced to α-TH easily by ascorbic acid. In principle, the fraction of oxidized α-TH regenerated should equal the fraction observed in HPLC

analyses as α-TQ plus **2** (Table I). In practice, somewhat less was regenerated, as some of the **2** and **5** may have hydrolyzed and rearranged to α-TQ before samples were treated with ascorbic acid. α-TQ is not reduced to α-TH by ascorbic acid under the conditions we employed.

Although 8a-hydroxytocopherone appeared to be the ascorbic acid-reducible intermediate in our experiments, Chan et al. (1991) recently suggested that α-T• was the ascorbic acid- and NDGA-reducible product formed by α-TH oxidation in platelet homogenates. However, α-T• and the reducible product in our experiments exhibt quite different properties. Photolytically-generated α-T• decays in liposomes almost completely within 1 min (Mehlhorn et al., 1989), whereas the reducible product in our system was relatively stable for up to 30 min (Table II). Moreover, in Triton X-100 micelles, an α-T• analog was reduced by ascorbic acid most efficiently at pH 7, but very slowly at pH 3 (Mukai et al., 1991). This pH profile for reduction is opposite that for 8a-hydroxytocopherone (**5**) and for the ascorbic acid-reducible product in our liposome system (Table III).

Experiments with DPPH, a one-electron oxidant that generates α-T•, provide evidence that 8a-hydroxytocopherone (**5**) may be formed by rapid disproportionation of α-T• in the lipid bilayer. Treatment of liposomes with DPPH maximally depleted α-TH within 1 min. However, in samples taken at 1 min, α-TH was regenerated by ascorbic acid at pH 3, but not at pH 7 (Table V). The pH dependence for reduction argues that 8a-hydroxytocopherone (**5**), rather than α-T• was the ascorbic acid-reducible species as early as 1 min after oxidation of α-TH by DPPH. As noted above, the decay of α-T• to other products in a similar liposome system is sufficiently rapid to consume virtually all of the α-T• within 1 min (Mehlhorn et al., 1989).

Competition between antioxidant reactions of α-T•, which form 8a-hydroxytocopherone (**5**), and autoxidation reactions, which form epoxides and 8a-hydroperoxytocopherone (**2**), may determine how effectively α-TH inhibits membrane oxidation. Product analyses indicate that, at less than 15% α-TH consumption, α-TQ was the principal product (Table I). Thus, most of the α-T• was converted to α-T$^+$, which was detected as α-TQ after hydrolysis and rearrangement. At α-TH oxidation of greater than 20%, epoxides accounted for 60–70% of the products. This shift in product distribution is not due to secondary oxidation of α-TQ to epoxides. Instead, it may reflect a shift of α-T• from reactions with peroxyl radicals (i.e., reaction 12 and epoxidation) to disproportionation (reaction 13). Under conditions where peroxyl radical flux is relatively low, α-TH, rather than α-T•, would be the principal radical scavenging species. α-T• then may be consumed primarily by disproportionation. Conversely, when peroxyl radical flux is relatively high, α-T• may react increasingly with peroxyl radicals.

These considerations underscore the potential importance of recycling reactions that regenerate α-TH. Regeneration of α-TH from α-T• would prevent the autoxidative loss of α-TH as epoxides **3**/**4** and 8a-hydroperoxytocopherone (**2**). On the other hand, disproportionation of α-T• that escape reduction would yield α-T$^+$ and 8a-hydroxytocopherone (**5**). Regeneration of α-TH from α-T$^+$ or **5** would complete a two-electron redox cycle. Recycling reactions therefore may prevent α-TH depletion most effectively under conditions where the peroxyl radical flux is relatively low. More rapid radical generation, on the other hand, may lead to formation of α-TH-derived epoxides and to an irreversible loss of antioxidant protection.

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