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Redox Cycles of Vitamin E: Hydrolysis and Ascorbic Acid Dependent Reduction of 8a-(Alkyldioxy)tocopherones†

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ABSTRACT: Oxidation of the biological antioxidant α-tocopherol (vitamin E; TH) by peroxyl radicals yields 8a-(alkyldioxy)tocopherones, which either may hydrolyze to α-tocopheryl quinone (TQ) or may be reduced by ascorbic acid to regenerate TH. To define the chemistry of this putative two-electron TH redox cycle, we studied the hydrolysis and reduction of 8a-[(2,4-dimethyl-1-nitrilopent-2-yl)dioxy]tocopherone (**1**) in acetonitrile/buffer mixtures and in phospholipid liposomes. TQ formation in acetonitrile/buffer mixtures, which was monitored spectrophotometrically, declined with increasing pH and could not be detected above pH 4. The rate of TQ formation from **1** first increased with time and then decreased in a first-order terminal phase. Rearrangement of 8a-hydroxy-α-tocopherone (**2**) to TQ displayed first-order kinetics identical with the terminal phase for TQ formation from **1**. Both rate constants increased with decreasing pH. Hydrolysis of **1** in acetonitrile/$H_2{}^{18}O$ yielded [^{18}O]TQ. These observations suggest that **1** loses the 8a-(alkyldioxy) moiety to produce the tocopherone cation (T^+), which hydrolyzes to **2**, the TQ-forming intermediate. Incubation of either **1** or **2** with ascorbic acid in acetonitrile/buffer yielded TH. Reduction of both **1** and **2** decreased with increasing pH. In phosphatidylcholine liposomes at pH 7, approximately 10% of the T^+ generated from **1** was reduced to TH by 5 mM ascorbic acid. The results collectively demonstrate that T^+ is the ascorbic acid reducible intermediate in a two-electron TH redox cycle, a process that probably would require biocatalysis to proceed in biological membranes.

The lipophilic antioxidant vitamin TH[1] (vitamin E) is the principal nonenzymatic defense against cellular membrane damage by reactive free radicals (Machlin & Bendich, 1987). TH interrupts radical chains by reacting with peroxyl radicals to form the relatively stable tocopheroxyl radical (Burton &

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[1] Abbreviations: AMVN, azobis(2,4-dimethylvaleronitrile); HPLC, high-performance liquid chromatography; TH, *d*-α-tocopherol; TQ, *d*-α-tocopheryl quinone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride. The term "ascorbic acid" refers to both the un-ionized and anionic forms of the molecule.

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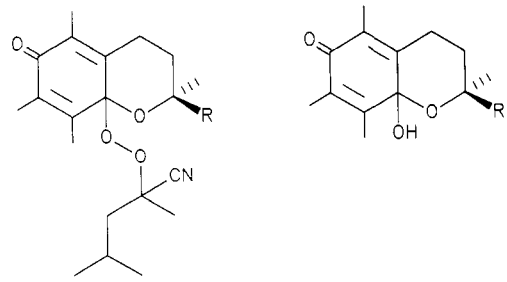

1 2

FIGURE 1: Structures of **1** and **2** (R- = phytyl, $-C_{16}H_{33}$).

Ingold, 1986). Tappel et al. (1961) proposed that the tocopheroxyl radical could be reduced by the water-soluble antioxidant ascorbic acid to regenerate TH. This reaction would make the reducing power of much larger water-soluble cellular antioxidant pools available for the reduction of peroxyl radicals in membranes. The chemical feasibility of a one-electron TH redox cycle was confirmed when Packer et al. (1979) directly observed tocopheroxyl radical reduction by ascorbic acid in homogeneous solution. TH and ascorbic acid also display a synergistic antioxidant effect in phosphatidylcholine liposome suspensions (Leung et al., 1981; Doba et al., 1985; Niki et al., 1985; Liebler et al., 1986). This finding suggests that electron transfer from the aqueous phase into the phospholipid bilayer can complete a one-electron TH redox cycle in biological membranes.

Tocopheroxyl radicals that do not undergo reduction may react with a second peroxyl radical to produce nonradical products. Among these are 8a-(alkyldioxy)tocopherones, which are adducts formed between peroxyl radicals and the tocopheroxyl radical 8a-position. 8a-(Alkyldioxy)tocopherones account for approximately half of the TH consumed by peroxyl radicals in homogeneous solution (Winterle et al., 1984). TH oxidation in other systems yields analogous 8a-alkoxytocopherones (Durckheimer & Cohen, 1964; Goodhue & Risley, 1965), which decompose to TQ in aqueous acid but are easily reduced to TH by aqueous ascorbic acid. McCay (1985) recently suggested that their facile reduction to TH could permit tocopherones to complete a two-electron TH redox cycle in cellular membranes. To investigate this hypothesis further, we synthesized the model 8a-(alkyldioxy)tocopherone 8a-[(2,4-dimethyl-1-nitrilopent-2-yl)dioxy]tocopherone (**1**, Figure 1) and studied its hydrolysis and reduction in acetonitrile/buffer mixtures and in phosphatidylcholine liposomes. We found that hydrolysis of 8a-(alkyldioxy)tocopherones is an acid-catalyzed reaction that yields the tocopherone cation (T^+), which hydrolyzes to 8a-hydroxy-α-tocopherone (**2**, Figure 1), the TQ-forming intermediate. Ascorbic acid reduces neither **1** nor **2** directly but reacts instead with T^+. The slow rates of these reactions at neutral pH suggest that biochemical catalysis would be required to complete a two-electron TH redox cycle in biological membranes.

Experimental Procedures

Chemicals and Instrumentation. Ascorbic acid and L-α-phosphatidylcholine (from soybean, type III-S) were purchased from Sigma. AMVN from Polysciences, Warrington, PA, was used as supplied. TH (99+% *d*-α-tocopherol) was from Henkel Corp., Fine Chemicals Division, LaGrange, IL. Acetonitrile (Omni-Solv) from EM Science was used without further purification. Mass spectral analyses were done with a Finnegan MAT-90 instrument equipped with a Micro PDP-11/73 computer (U.S. Design, Palo Alto, CA) and a Finnegan ICIS data system. Samples were introduced by direct probe insertion. For chemical ionization analysis, ammonia was used as the reagent gas. HPLC analyses were done with a Spectra Physics 8800 solvent delivery system and either a Kratos SF757 variable-wavelength UV–vis detector or an ESA Coulochem electrochemical detector with standard ESA guard and analytical cells. Samples were injected with a Spectra Physics 8775 autosampler.

Synthesis of **1** *and* **2**. TH (10 mg, 0.023 mmol) and AMVN (43.3 mg, 0.175 mmol) were dissolved in 5 mL of oxygen-saturated acetonitrile, and the mixture was heated in a screw-capped test tube at 50 °C for 3 h. UV spectroscopy of the reaction mixture indicated that the TH had been completely consumed. **1** was separated from other components of the product mixture by reverse-phase HPLC on a Hamilton PRP-1 10-μm, 4.6 × 250 mm analytical column, which was eluted with methanol/2-propanol (75:25 v/v) at a flow rate of 2 mL min^{-1}. Products were detected by UV absorbance at 240 nm. Removal of the solvent in vacuo afforded **1** as a clear oil in approximately 50% yield. The UV spectrum (acetonitrile) indicated the presence of the tocopherone chromophore with an absorbance maximum at 240 nm (ϵ = 13 700 M^{-1} cm^{-1}). An exact mass measurement for **1** was obtained by chemical ionization analysis with tetrakis(trimethylsilyl)adenosine (MH^+ = 556.263) as the mass reference standard. The measured mass for **1** (MH^+) was 572.469 ± 0.004 (calculated MH^+ = 572.479). Analysis in the electron impact mode produced fragments consistent with the proposed structure: *m/z* 571 [M^+], 1.4%; 461 [M^+ – $-C(CN)(CH_3)CH_2CH(CH_3)_2$], 2.5%; 445 [$M^+$ – $-OC(CN)(CH_3)CH_2CH(CH_3)_2$], 44.2%; 430 [$M^+$ – $-OOC(CN)(CH_3)CH_2CH(CH_3)_2$], 100%; 417 [445 – CO], 68.9%; 401, 15.8%. Analysis of this material by reverse-phase HPLC on a Spherisorb ODS-2 5-μm, 4.6 × 250 mm analytical column, which was eluted with methanol/1 N sodium acetate, pH 4.25 (93:7 v/v), at 1.5 mL min^{-1}, indicated that the product was a mixture of four diastereomers due to chiral centers at C-8a and C-2′ of the AMVN-derived alkyldioxy group. The mixture was used for all the experiments described here. **2** was synthesized by the *N*-bromosuccinimide oxidation of TH as described previously (Durckheimer & Cohen, 1964). This procedure consistently yielded a mixture of **2** (70–80%) and TQ (20–30%). Attempts to purify the mixture by reverse-phase HPLC as described above yielded only TQ, apparently because **2** rearranged to TQ on the column. Quantification of **2** in freshly prepared solutions by UV absorbance at 240 nm was subject to up to 25% error due to some absorbance from TQ (UV_{max} = 266 nm). The significance of this limitation is discussed under Results.

Incubation Conditions and Product Analyses. Formation of TQ was monitored spectrophotometrically with a Hewlett-Packard 8452A diode array UV–vis instrument operated with a Zenith 248 microcomputer and Hewlett-Packard 85930A MS-DOS UV–vis operating software. **1** or **2** was dissolved in 0.6 mL of acetonitrile added to 0.4 mL of 50 mM sodium formate buffer in a 1-mL quartz cuvette. The absorbance at 266 nm versus that at a reference wavelength of 350 nm then was monitored with time. All experiments were done at 25 °C. For studies of ascorbic acid dependent reduction, 20 nmol of **1** or **2** was diluted in 0.6 mL of acetonitrile, and then 0.4 mL of buffer containing ascorbic acid was added to begin the incubation. Samples (0.05 mL) of the mixture were quickly transferred to silanized tubes, which contained 0.5 mL of water, 0.5 mL of ethanol, 2 mL of hexane, and 0.5 nmol of γ-tocopherol and γ-tocopheryl quinone internal standards. After mixing, the organic layer was removed to

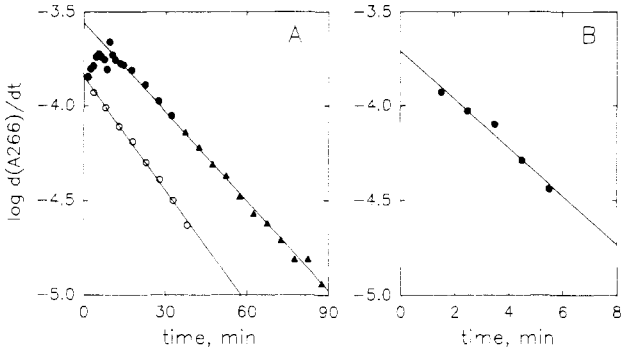

FIGURE 2: (A) Rates of TQ formation from **1** (solid symbols) and **2** (open symbols) versus time in acetonitrile/50 mM sodium formate, pH 3.6 (6:4 v/v). Data points used for linear regression analysis of the terminal phase are represented by solid triangles. (B) Residual plot of differences between extrapolated terminal-phase line and measured rates between 1 and 5 min in (A). See text for discussion.

another silanized tube, the solvent evaporated under a stream of nitrogen, and the residue dissolved in 1 mL of methanol for HPLC analysis. Despite the use of γ-tocopherol as an internal standard, analyses after a single extraction underestimated the TH concentration by about 15%; an additional extraction with hexane was necessary to approach 100% recovery. In experiments where the time course of TH generation by ascorbic acid from **1** or **2** was measured, only single extractions were possible and the TH levels were underestimated by about 15% (vide infra). TH and TQ were separated by reverse-phase HPLC on Spherisorb ODS-2 as described above. TH was detected by oxidation at a potential of +0.3 V and TQ by reduction at −0.5 V. For studies of the incorporation of ^{18}O into TQ, 100 nmol of **1** was dissolved in 0.75 mL of acetonitrile. To this was added 0.5 mL of H_2O or $H_2{}^{18}O$ acidified to pH 2.9 with 3.6 μL of acetyl chloride. After 15 min, the TQ product was extracted with hexane and the extract was analyzed by chemical ionization mass spectrometry as described above.

Liposome Incubations. A stock solution of soybean phosphatidylcholine (0.5 μmol) in chloroform was evaporated to dryness under a nitrogen stream, and then 0.1 mL of methanol and 0.01 mL of methanol/concentrated NH_4OH (9:1 v/v) were added to dissolve the residue. The mixture was immediately evaporated again to dryness. This ammonia treatment was used to remove traces of HCl from the phospholipid that arise from its storage in chloroform. Failure to remove the acid caused partial hydrolysis of **1** to TQ during liposome preparation. A solution of **1** (15 nmol in 0.017 mL of hexane) was added to the lipid residue, and the hexane was evaporated under nitrogen. The lipid mixture was then dissolved in 0.1 mL of ethanol, and unilamellar liposomes were prepared in 10 mL of 50 mM Tris-HCl and 50 mM NaCl, pH 7.0, by the ethanol injection method (Kremer et al., 1977). Liposome suspensions were incubated at 37 °C under an air atmosphere. Aliquots of the liposome suspension (0.5 mL) were removed for analysis of TH as described above.

RESULTS

Kinetics of TQ Formation from **1**. Incubation of **1** in acetonitrile/buffer mixtures yielded TQ, which was monitored by its absorbance at 266 nm. The overall rate of TQ formation decreased with increasing pH. Above pH 4, no increase in absorbance due to TQ could be detected. The reaction at pH 3.6 was then studied in detail. The rate of TQ formation initially increased and then declined in an apparent first-order terminal phase with a rate constant k_{term} = 0.036 min^{-1} (Figure 2A). We hypothesized that this kinetic profile reflected the

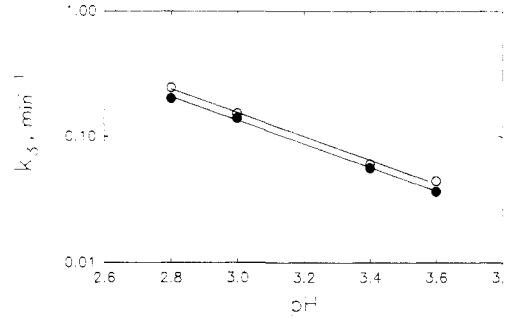

FIGURE 3: Dependence on pH of the terminal-phase rate constant for TQ formation from **1** (k_{term}) (solid circles) and the first-order rate constant for rearrangement of **2** to TQ (k_3) (open circles).

formation of TQ via three sequential reactions, which are the first-order dissociation of **1** to T^+ (eq 1), the fast pseudo-first-order hydrolysis of T^+ to **2** (eq 2), and the first-order rearrangement of **2** to TQ (eq 3). The apparent first-order

$$\mathbf{1} \xrightarrow{k_1} T^+ \quad (1)$$

$$T^+ + H_2O \xrightarrow{k_2} \mathbf{2} + H^+ \quad (2)$$

$$\mathbf{2} \xrightarrow{k_3} TQ \quad (3)$$

rate constant for hydrolysis of **1** to **2**, k_{obs}, was estimated with the method of residuals, in which the terminal-phase decline in TQ formation from **1** was extrapolated to $t = 0$. A semilog plot of the difference between the measured and extrapolated rates at early time points between 1 and 5 min was linear and yielded an estimate of k_{obs} = 0.296 min^{-1} (Figure 2B). Although this rate constant describes the two-step conversion of **1** to **2**, the linear residual plot indicates that one step is rate-limiting. Since eq 1 and 2 describe an S_N1 hydrolysis reaction, in which $k_1 \ll k_2$, k_{obs} should closely approximate k_1.

To test the hypothesis that **1** yields TQ via an obligatory intermediate **2**, authentic **2** was incubated under the same conditions and TQ formation was monitored (Figure 2A). The rate of TQ formation declined in a log-linear fashion, which indicates a first-order rearrangement of **2** to TQ with an apparent rate constant k_3 = 0.046 min^{-1}. This value is close to that found for k_{term}, the terminal-phase rate constant for the formation of TQ from **1**. Rate constants for the rearrangement of **2** to TQ were compared with those for the terminal phase of TQ formation from **1** as a function of pH (Figure 3). The two rate constants were virtually identical and displayed an identical dependence on pH. These observations collectively suggest that the formation of TQ proceeds via the intermediates T^+ and **2** as indicated in eq 1–3.

Hydrolysis of **1** *in* $H_2{}^{18}O$. The mechanism outlined above for the conversion of **1** to TQ requires that an intermediate tocopherone cation T^+ hydrolyze to yield **2**, which then rearranges to TQ. One oxygen atom from water is thereby incorporated into TQ. To further test this hypothesis, **1** was incubated in mixtures of acetonitrile and either acidified H_2O or $H_2{}^{18}O$. The TQ formed then was analyzed by chemical ionization mass spectrometry (Figure 4). TQ isolated from incubations with H_2O exhibited an M^+ ion at m/z 446, an $M^+NH_4^+$ adduct ion at m/z 464, and a base peak at m/z 429, which corresponds to the dehydration product of MH^+. The corresponding incubation in $H_2{}^{18}O$ yielded TQ in which one atom of ^{18}O had been incorporated as indicated by the analogous signals at m/z 448, 466, and 431. This result reinforces the view that T^+ is an obligatory intermediate in the formation of **2** and TQ. Although a molecular ion and

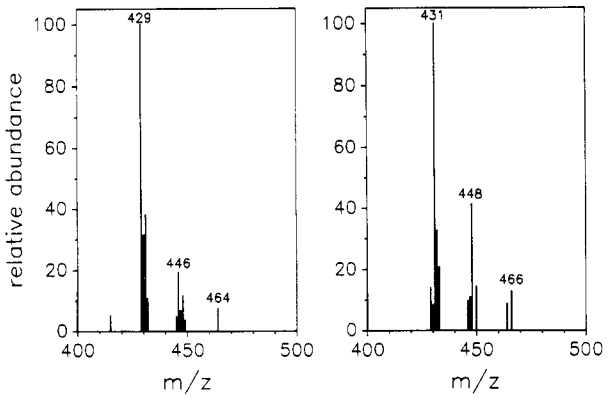

FIGURE 4: Ammonia chemical ionization mass spectra of TQ formed by hydrolysis of **1** in acetonitrile/$H_2{}^{16}O$ (left) or acetonitrile/$H_2{}^{18}O$ (right).

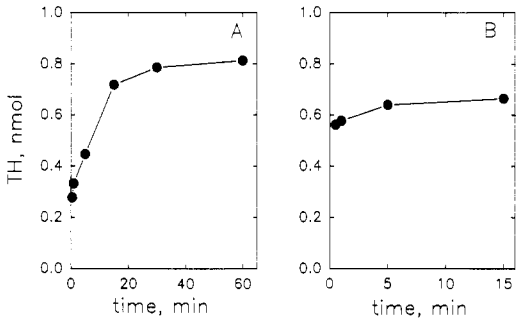

FIGURE 5: Formation of TH versus time from **1** (panel A) and **2** (panel B) in acetonitrile/50 mM sodium formate, pH 3.5 (6:4 v/v) in the presence of 50 mM ascorbic acid. TH formation is expressed as nmol/nmol of precursor.

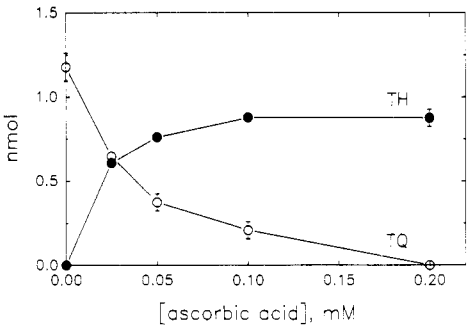

FIGURE 6: Formation of TH and TQ from **1** versus ascorbic acid concentration in acetonitrile/50 mM sodium formate, pH 3.5 (6:4 v/v), during a 60-min incubation. Product amounts are expressed as nmol/nmol of **1**.

ammonia adduct ions were observed, the MH^+ ion for TQ was very weak, yet the corresponding dehydration ion (m/z 429/431) was most prominent. This dehydration of MH^+ ions is a noteworthy characteristic of several TH oxidation products.[2]

Reduction of **1** *and* **2** *by Ascorbic Acid.* Incubation of **1** with 50 mM ascorbic acid in acetonitrile/buffer at pH 3.0 resulted in rapid generation of TH that was essentially complete at 30 min (Figure 5A). In an identical experiment with **2**, TH generation was essentially complete by 5 min (Figure 5B). At pH 5 and 7, the reduction of **2** was slower and less extensive (not shown). The yield of TH from both **1** and **2** at pH 3.5 increased with increasing ascorbic acid concentration (Figures 6 and 7). At a given ascorbic acid concentration, **1** and **2** were reduced to a similar extent. A 50% reduction of **1** to TH was achieved at an ascorbic acid:**1** ratio of approximately 1.25 (Figure 6), whereas the ratio for 50% reduction of **2** was approximately 2.5 (Figure 7). The fraction of **1** or **2** that was not reduced to TH was recovered as TQ.

[2] D. C. Liebler, unpublished observations.

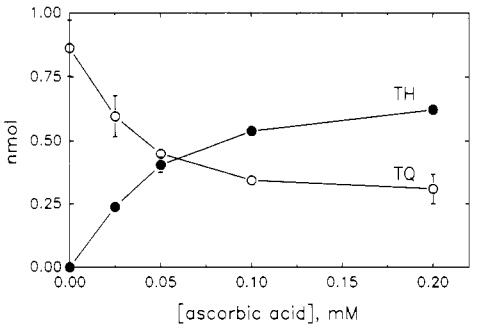

FIGURE 7: Formation of TH and TQ from **2** versus ascorbic acid concentration in acetonitrile/50 mM sodium formate, pH 3.5 (6:4 v/v), during a 60-min incubation. Product amounts are expressed as nmol/nmol of **2**.

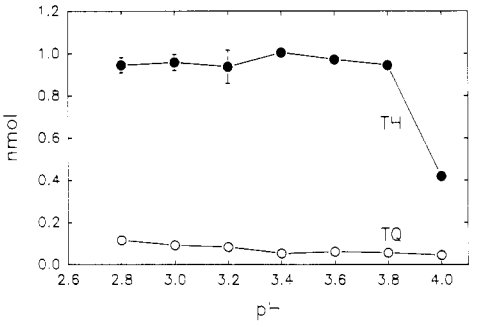

FIGURE 8: Yield of TH and TQ from **1** during a 60-min incubation with 0.5 mM ascorbic acid in acetonitrile/50 mM sodium formate (6:4 v/v). Yields are expressed as nmol/nmol of **1**.

It appears from Figure 5 that "complete" reduction of **1** or **2** yielded less than 1 nmol of TH/nmol of **1** or **2** used. This is because the rapid single-extraction procedure used to measure TH in time course studies consistently underestimated the TH level by about 15–20% (see Experimental Procedures). TH yield thus appeared to be about 0.8 nmol/nmol of **1** at the completion of the reaction (Figure 5A). In experiments where TH production was measured as a function of ascorbic acid concentration, two extractions were used to recover all of the product after 60 min, and the yield approached 1 nmol of TH/nmol of **1** (Figure 6). An additional source of error in determining the TH yield from experiments with **2** is that fresh stock solutions of **2** also contained 10–25% TQ, which interfered with the UV quantitation of **2**. This consistently led to an overestimation of the amount of **2** added to the incubation mixture and made the yield of TH appear 10–25% less than expected (Figure 7). The TQ present after a 60-min incubation with 0.2 mM ascorbic acid in Figure 6 therefore represents TQ formed during the preparation of **2** rather than TQ formed during the experiment.

As observed for the hydrolysis of **1** to TQ, the reduction of **1** by ascorbic acid was pH-dependent (Figure 8). Ascorbic acid completely reduced **1** to TH within 60 min below pH 3.8. Above pH 3.8, the yield of TH fell off, but no additional TQ was formed. The optimal pH range for reduction thus coincided with that for the conversion of **1** to TQ. This suggests that ascorbic acid reduced either T^+ or **2** rather than **1** itself. The pH dependence of ascorbic acid mediated reduction of **1** and **2** was then examined. The TH yield from both **1** and **2** declined as pH increased to neutrality (Table I). Since **2** exhibited a pH dependence for reduction identical with that of **1**, it appears that T^+, rather than **2**, is the ascorbic acid reducible intermediate.

Table I: Formation of TH from **1** and **2** as a Function of pH[a]

pH	TH [nmol/(60 min·nmol of precursor)]	
	1	**2**
3.0	0.982 ± 0.015	0.616 ± 0.053
5.0	0.394 ± 0.016	0.334 ± 0.009
7.0	0.143 ± 0.012	0.034 ± 0.001

[a] **1** and **2** were incubated for 60 min in acetonitrile/buffer (6:4 v/v) with 50 mM ascorbic acid. The buffers used were 50 mM sodium formate, pH 3.0; 50 mM sodium acetate, pH 5.0; and 50 mM Tris-HCl, pH 7.0. At the end of the incubation, triplicate analyses were performed for TH as described under Experimental Procedures.

The preceding results do not suggest that the ionization state of ascorbic acid significantly affected the reduction of **1** or **2** to TH in the pH range studied. The pK_{a1} of ascorbic acid in the acetonitrile/buffer mixtures was 3.8 as determined by spectrophotometric titration. The fraction of ascorbic acid present as the ascorbate anion therefore ranged from approximately 10% at pH 2.8 to near 100% at pH 7.

Because **1** was reduced to TH at a small but measurable rate in homogeneous solution (Table I), the reaction was studied in a liposome system. Soybean phosphatidylcholine liposomes were prepared in buffer with ascorbic acid so that both the inner and outer leaflets of the vesicles were exposed to ascorbic acid. The ascorbic acid concentrations in these experiments ranged from 0.1 to 5 mM ascorbic acid, which produced a 67- to 3333-fold molar ratio of ascorbic acid to **1**. During 1 h of incubation with 5 mM ascorbic acid, 0.2 nmol of TH was formed from 15 nmol of **1**, which corresponded to a conversion of 1.3%. No TH was detected in incubations at lower ascorbic acid concentrations. Without ascorbic acid, 13.7% of **1** hydrolyzed to TQ. At 5 mM, ascorbic acid thus reacted with 9.5% of the T^+ that was generated from **1** under these conditions.

Discussion

Cellular antioxidant protection relies to a great extent on redox cycles, which funnel reducing equivalents to specialized antioxidant molecules and provide a buffer against sudden antioxidant depletion. This mechanism has been proposed to explain the antioxidant synergism between TH and ascorbic acid and the apparent resistance of tissue TH to depletion despite its rapid reaction with free radicals (Tappel, 1962; McCay, 1985). Several studies in vitro provide evidence for a one-electron TH redox cycle involving the tocopheroxyl radical [reviewed by McCay (1985)]. Nevertheless, tocopherone products of TH oxidation also are chemically reducible to TH (Durckheimer & Cohen, 1964; Goodhue & Risley, 1965). Consequently, a two-electron redox cycle also must be considered. The findings presented here show that the completion of an ascorbic acid dependent two-electron cycle requires acid catalysis to generate T^+, which is the ascorbic acid reducible intermediate.

The reactions involved in the hydrolysis of **1** to **2**, the rearrangement of **2** to TQ, and the reduction of **1** and **2** to TH are depicted in Figure 9. The first step in both the reduction of **1** to TH and in its hydrolysis to TQ is the dissociation of the 8a-(alkyldioxy) moiety to generate the tocopherone cation T^+ (eq 1). This step appears to be acid-catalyzed based on two observations. First, the initial phase of TQ formation from **1**, during which the rate of TQ formation increased, became progressively shorter and less distinct as the pH of the reaction medium decreased (not shown). Second, both the hydrolysis of **1** to TQ and the ascorbic acid dependent reduction of **1** to TH decreased with increasing pH. An intermediate common

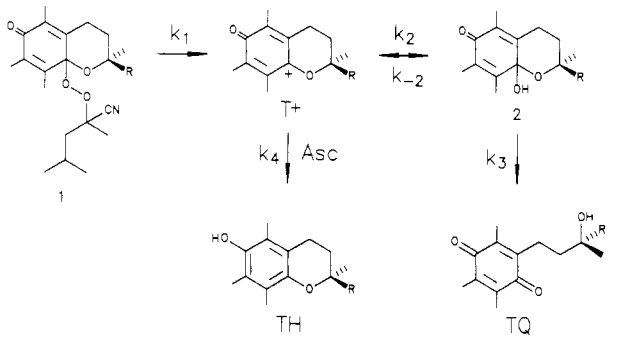

FIGURE 9: Reactions involved in the hydrolysis of **1** to **2**, the rearrangement of **2** to TQ, and in the reduction of **1** and **2** to TH (R– = phytyl, $-C_{16}H_{33}$).

to both processes therefore is formed preferentially at low pH. Nucleophilic displacement of the 8a-(alkyldioxy) moiety is unlikely because the tocopherone 8a-position is sterically hindered. The 8a-(alkyldioxy) moiety is probably lost instead as its conjugate acid following protonation (eq 4 and 5). The

$$\text{TOOR (1)} + H^+ \xrightarrow{\text{fast}} T(H^+)OOR \quad (4)$$

$$T(H^+)OOR \xrightarrow{\text{slow}} T^+ + \text{ROOH} \quad (5)$$

rearrangement of **2** to TQ also appears to be acid-catalyzed based on the increase in k_3 with decreasing pH (Figure 3). In the presence of ascorbic acid, however, acidic conditions increase the conversion of **2** to TH. This is presumably due to a shift in the T^+/**2** equilibrium toward T^+.

The basic requirement of an efficient two-electron TH redox cycle is that the rate of T^+ reduction by ascorbic acid (eq 6)

$$T^+ + \text{Asc} + H^+ \xrightarrow{k_4} \text{TH} + \text{Asc}^\bullet \quad (6)$$

must exceed that of T^+ hydrolysis to **2** (eq 2). Although we have measured neither k_2 nor k_4, approximate values for these rate constants can be estimated. The hydrolysis of T^+ to **2** is apparently a fast reaction. Marcus and Hawley (1970) generated the chromanone cation of the TH analogue 2,2,5,7,8-pentamethyl-6-hydroxychroman electrochemically in aqueous acetonitrile under near-neutral conditions. The second-order rate constant for hydrolysis of the chromanone cation to the 8a-hydroxychromanone was 150 M^{-1} min^{-1} at 23 °C. Application of this estimate to our system, where the H_2O concentration is 37 M, yields a pseudo-first-order rate constant k_2' of approximately 5×10^3 min^{-1}. The competing reaction, in which T^+ is reduced to TH (eq 6), must be even faster than reaction 2 because complete reduction of **1** to TH required only a 5-fold molar excess of ascorbic acid under the conditions described in Figure 6. If it is assumed that complete T^+ reduction requires a rate of T^+ reduction that is at least 100 times that of T^+ hydrolysis, then the second-order rate constant for T^+ reduction, k_4, would be at least 10^5 min^{-1}.

Although TH resides in the phospholipid bilayer, location of the chromanol portion of the molecule in the hydrophilic surface region exposes TH to components of the aqueous phase (Perly et al., 1985). Nevertheless, **1** was not efficiently reduced to TH in liposomes by aqueous-phase ascorbic acid at physiologic concentrations. At 5 mM, the highest ascorbic acid concentration tested, only about 10% of the T^+ generated from **1** was reduced to TH. This could reflect a relatively low effective ascorbic acid concentration in the bilayer surface region compared to the bulk aqueous phase or a reduced rate constant for the reaction. It is interesting that aqueous-phase ascorbic acid in the high micromolar–low millimolar concentration range apparently regenerates TH from the tocopheroxyl

radical in liposomes (Scarpa et al., 1984; Doba et al., 1985; Niki et al., 1985; Liebler et al., 1986). Recycling of the tocopheroxyl radical may proceed more efficiently because there is no competing hydrolysis reaction as there is for T^+. Some form of biochemical catalysis would almost certainly be required for a two-electron redox cycle to efficiently regenerate TH from 8a-(alkyldioxy)tocopherones in living tissues. Since 8a-(alkyldioxy)tocopherones account for about half of the TH consumed by peroxyl radicals in lipid bilayers,[3] it seems plausible that their enzyme-catalyzed reduction to TH may occur.

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Registry No. 1, 123438-35-7; TH, 59-02-9; TQ, 62726-91-4; AMVN, 4419-11-8; ascorbic acid, 50-81-7; 8a-hydroxy-α-tocopherol, 3626-81-1.

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Conformational Change of the Heme Moiety of the Ferrous Cytochrome P-450$_{scc}$–Phenyl Isocyanide Complex upon Binding of Reduced Adrenodoxin†

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ABSTRACT: Reduction of cytochrome P-450$_{scc}$(SF) (SF, substrate free) purified from bovine adrenocortical mitochondria with sodium dithionite ($Na_2S_2O_4$) or with β-NADPH mediated by catalytic amounts of adrenodoxin and adrenodoxin reductase in the presence of phenyl isocyanide produced a ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex with Soret absorbance maximum at 455 nm having a shoulder at 425 nm. On the other hand, when a preformed cytochrome P-450$_{scc}$(SF)–adrenodoxin complex was reduced chemically or enzymatically under the same conditions, the absorbance spectrum showed drastic changes, i.e., an increase in intensity at 425 nm and a concomitant decrease in intensity at 455 nm. Similar spectral changes could be produced by addition of the same amount of reduced adrenodoxin afterward to the ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide complex. Titration experiments with adrenodoxin showed that (1) a 1:1 stoichiometric saturation of the spectral change was obtained for both the absorbance increase at 425 nm and the absorbance decrease at 455 nm, (2) there was no spectral change in the presence of 0.35 M NaCl, and (3) there was no spectral change for cytochrome P-450$_{scc}$(SF) whose Lys residue(s) essential to the interaction with adrenodoxin had been covalently modified with PLP. These results suggest that ternary complex formation of ferrous cytochrome P-450$_{scc}$(SF)–phenyl isocyanide with reduced adrenodoxin caused a conformational change around the ferrous heme moiety. By analysis of temperature and pH dependencies of the spectral change of the ternary complex, it was suggested that this conformational change may reflect the essential step for electron transfer from reduced adrenodoxin to the ferrous–dioxygen complex of cytochrome P-450$_{scc}$.

Cytochrome P-450$_{scc}$ is an iron protoporphyrin IX containing monooxygenase, which is responsible for the side-chain cleavage reaction of cholesterol to produce pregnenolone, the first and rate-limiting step of steroid hormone biosynthesis (Burstein & Gut, 1971). The side-chain cleavage reaction consists of three consecutive monooxygenase reactions; each reaction can be envisioned as a cyclic process composed of five major steps and requiring two electrons and a dioxygen molecule. The steps comprising the first of this series of

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