α-Tocopherol Oxidation Mediated by Superoxide Anion (O_2^-). I. Reactions in Aprotic and Protic Conditions

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The reaction of α-tocopherol (α-T) with superoxide anion (O_2^-) in both dry acetonitrile and in aqueous acetonitrile solution is described. The O_2^- was generated by the electrochemical reduction of molecular oxygen in acetonitrile, using tetrabutylammonium bromide as an electrolyte. α-T was reacted with O_2^- either in dry acetonitrile or in a 10% aqueous acetonitrile solution. In dry acetonitrile, α-T was oxidized to a very unstable primary intermediate, which was further oxidized to a secondary, more stable intermediate. The formation of the secondary intermediate depended upon the presence of molecular oxygen. This intermediate readily converted into two compounds in equimolar amounts (designated A and B). The primary, very unstable intermediate was readily reduced again to α-T by treatment with $LiAlH_4$ or ascorbic acid. However, the secondary intermediate or the stable oxidation products could not be reduced to α-T. In the 10% aqueous acetonitrile, α-T was oxidized to α-tocopheryl quinone, α-tocopherol dimer and α-tocopherol dihydroxy dimer, and an unknown compound. In the aqueous medium, no intermediates were formed by the action of O_2^-. The results of this study indicate that the reaction of α-T with O_2^- under aprotic conditions is different from that observed under protic conditions.
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The superoxide anion (O_2^-) is produced in biological systems and foods by enzymatic (1,2) or non-enzymatic reactions (3,4). It is relatively stable under anhydrous conditions, but not in aqueous media. O_2^- has been found to have detrimental effects on cells and cell constituents (5). Several studies have shown α-tocopherol (α-T) involvement in the prevention of membrane lipid peroxidation caused by O_2^-. The protective effect of α-T is thought to be due to the scavenging action of α-T which results in its oxidation by free radicals. Inconsistencies in α-tocopherol oxidation products formed under protic (aqueous) conditions and aprotic (anhydrous) conditions have previously been reported (6–10). These inconsistencies might be attributed to the instability of O_2^- under aqueous conditions and suggest that there may be differences in the reaction mechanism of α-T with O_2^- under anhydrous and aqueous conditions.

The objectives of this study were to compare the oxidation of α-T with O_2^- under protic and aprotic conditions, and to isolate the oxidation products arising under both conditions.

MATERIALS AND METHODS

Reagents. Acetonitrile (spectral grade, 0.006% water; Burdick and Jackson, Muskegon, MI), mercury (electro grade, 99.9999%; Alfa Products, Danvers, MA), ethyl acetate high-performance liquid chromatography (HPLC) grade; Fisher Scientific Company, Itasca, IL), hexane (HPLC grade; Fisher Scientific Company), isopropanol (HPLC grade; Fisher Scientific Company), tetrabutylammonium bromide (Fisher Scientific Company), α-tocopherol (Eastman Kodak Company, Rochester, NY), and oxygen (99.999%; Air Products Inc., Minneapolis, MN) were used in this study. In all experiments, doubly distilled water was used. α-Tocopheryl quinone (TQ) was synthesized and purified by the method of Eggitt and Norris (11). α-Tocopherol dihydroxy dimer (DHD) and α-tocopherol dimer (D) were synthesized according to Csallany *et al.* (12). Purification of the above compounds was carried out by the method of Ha and Csallany (13). All other chemicals used were reagent grade.

Generation of O_2^-. The O_2^- was generated by electrochemical reduction of molecular oxygen (O_2), as described by Fee and Hildenbrand (14), with slight modifications. One-tenth M tetrabutylammonium bromide (TBAB) in acetonitrile was used as a supporting electrolyte. The TBAB was recrystallized from ethyl acetate and dried overnight in a vacuum desiccator. The electrolytic cell (H-shape) contained 60 mL of the electrolyte and the compartments were separated by a sintered glass disc 1 cm in diameter. The anode was a coil of platinum wire and the cathode was a pool of mercury. O_2 was dried by passing it through both concentrated H_2SO_4 and $CaCl_2$, and bubbled continuously through the cathode compartment for 30 min with a velocity of one bubble/sec. The O_2^- was generated using a 2 mA current for 20 min. The concentration of O_2^- was determined by measuring the optical density at 250 nm, as described by Fee and Hildenbrand (14). Electrolysis was carried out in a dry-glove box (Prospect, Kansas City, MO) that was continuously purged with dry nitrogen.

Reaction conditions. Unless otherwise specified, the following conditions were used for the reaction of α-T with O_2^- in both dry acetonitrile and aqueous acetonitrile solutions. One μmole of α-T was dissolved in 100 μL acetonitrile, and was reacted with 3 mL O_2^- solution (5.2×10^{-6} M) for 1 min. The reaction was carried out in screw-capped test tubes (20 cm × 1.5 cm) in a dry-glove box. Each reaction was repeated four times. After completing each reaction, the unreacted α-T and its oxidation products were extracted 3 times with 3 mL of hexane. The combined extracts were dried over anhydrous sodium sulfate and concentrated to 3 mL under N_2 at room temperature for HPLC analysis. The HPLC analysis of α-T and its oxidation products was performed on a normal-phase column (5 μm Ultrasphere-Si, Beckman Instruments Inc., Berkeley, CA) using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) (13). The HPLC equipment used was a model 110A solvent metering pump (Beckman Instruments, Inc.) and an Altex Model 210 solvent injector equipped with a 100-μL loop (Beckman Instruments, Inc.).

Stability of O_2^-. The stability of O_2^- in the presence of water was determined. Water was added to quartz cuvet-

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Abbreviations: α-T, α-tocopherol; D, α-T dimer; DHD, α-T dihydroxy dimer; HPLC, high-performance liquid chromatography; O_2^-, superoxide anion; O.D., optical density; TBAB, tetrabutylammonium bromide; TQ, α-tocopheryl quinone.

tes (1 cm × 1 cm) containing 3 mL O_2^- solutions to produce 0, 1, 5, and 10% per volume water concentrations. Dismutation of O_2^- was determined by measuring the rate of change of optical density (O.D.) at 250 nm, using a Beckman model DU-8 Spectrophotometer equipped with a DU-8 kinetic Compuset module (Berkeley, CA).

Formation of intermediates in dry acetonitrile. To determine the formation of the very unstable primary intermediate product, α-T was reacted with the O_2^- solution. The primary intermediate and the secondary intermediate products exhibited maximum absorbance at 350 and 235 nm, respectively. The optical density of the reaction mixture was monitored at 350 nm for the formation of the primary oxidation product and 235 nm for the formation of the secondary, more stable oxidation product of α-T. The disappearance of α-tocopherol (α-T) was monitored at 292 nm as a function of time (1/3, 1, 2, 3, 4, 5, and 10 min) using a Beckman DU-8 Spectrophotometer equipped with a kinetic Compuset module. The primary intermediate product reached a maximum concentration at 20 sec. A one-minute reaction time was used for the production of the secondary intermediate.

Reduction of the primary intermediate. One-μmole samples of α-T were each reacted with 3 mL O_2^- solution for 20 sec. The reaction mixtures were reduced immediately with $LiAlH_4$ reagent according to the method of Csallany *et al.* (15) or with 0.6 g ascorbic acid for 5 min. After reduction, the reaction mixtures were extracted with hexane, the extracts were washed with distilled water and analyzed by HPLC for α-T and its oxidation products. Control samples were treated identically, except that they were not treated with the reducing agents.

Reduction of the secondary intermediate. Four one-μmole samples of α-T were each reacted with 3 mL O_2^- solution for one min and extracted with hexane. One sample was used as a control without further treatment, and the remaining samples in hexane were treated with either $LiAlH_4$, (15) ascorbic acid or 1 mL of 0.1 M HCl. The mixtures then were extracted with hexane, which was washed with distilled water, and the samples were analyzed for α-T and its oxidation products by HPLC.

Formation of stable α-T oxidation products (A and B). The formation of two stable α-T oxidation products (compounds A and B) with O_2^- in dry acetonitrile was carried out in the same way as that of the secondary intermediate product (reaction time 1 min). The unstable secondary oxidation product was converted quantitatively to compounds A and B by evaporating the solvent hexane or by washing the solvent with water. Compounds A and B were then separated by HPLC.

Reduction of the compounds A and B. Compounds A and B were treated with $LiAlH_4$ or ascorbic acid identically as described for the primary and secondary intermediates and were analyzed by HPLC for possible conversion into α-T.

Effect of molecular oxygen. A three-mL aliquot of the O_2^- solution, was reacted with one μmole α-T for 1 min and then used as control. Other aliquots of the O_2^- solution were purged with nitrogen for 6 min to remove the remaining O_2. This solution was reacted with one μmole α-T for 1 min with or without further nitrogen purge. Each reaction mixture was assayed for α-T and its oxidation products as described above.

Reaction of α-T with O_2^- in aqueous acetonitrile solution (protic conditions). Samples of one μmole α-T were reacted with 3 mL O_2^- solution for 0, 0.5, 1, 5 and 10 min in the presence of various concentrations of water (1, 5 and 10%). The samples were extracted with hexane and then analyzed by HPLC as described above.

RESULTS

Stability of O_2^-. The relationship between the O_2^- dismutation rate and water content is shown in Figure 1. The O_2^- was quite stable in absolute acetonitrile and remained at a constant level during the test period. The O_2^- was partly dismutated by the addition of a small amount of water to the solution, with a dismutation rate proportional to the water content. With a 10% water concentration, O_2^- declined nearly to zero within one minute. These results are in agreement with those of Ozawa and Hanaki (16), who observed that O_2^- dismutation was positively correlated with the water content in the reaction medium.

Reaction of α-T with O_2^- in dry acetonitrile (aprotic conditions). A very unstable primary intermediate product was formed during the reaction of α-T with O_2^- in dry acetonitrile, which was monitored by measuring the optical density changes at 350, 292, 235 nm as a function of time (Fig. 2). The rapid increase in the primary product (350 nm) was concomitant with a dramatic fall in α-T concentration (292 nm). Maximum absorption for the primary intermediate was observed at about 20 sec, after which it slowly disappeared. Its disappearance was followed by increased absorption at 235 nm (secondary inter-

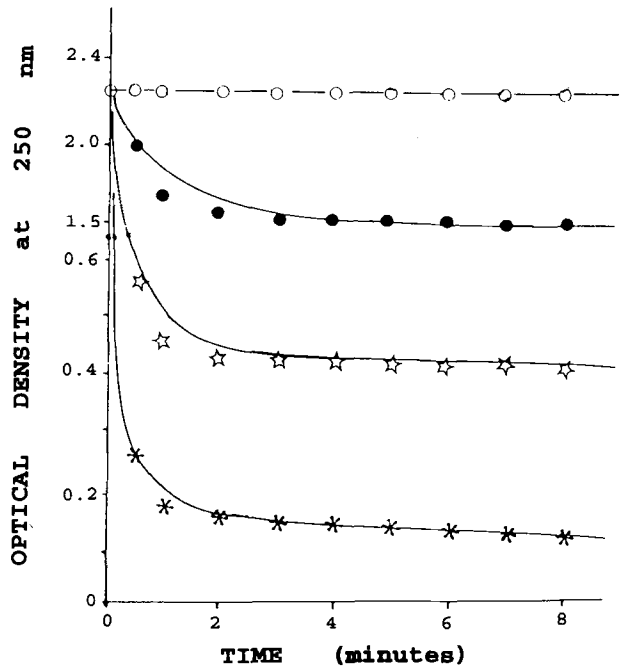

FIG. 1. Stability of O_2^- in acetonitrile containing various concentrations of water; 0 (○), 1% (●), 5% (☆), and 10% (*) water. O_2^- was generated electrochemically at 2 mA for 20 min.

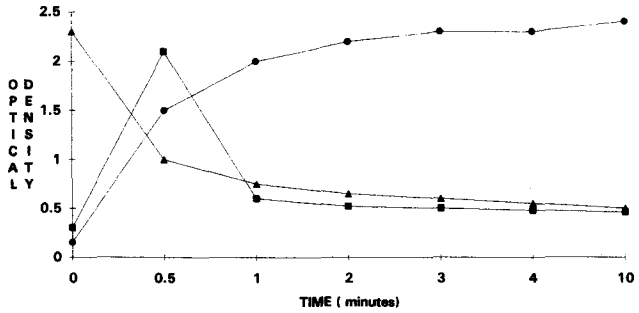

FIG. 2. Changes in optical density during the reaction of α-T with O_2^- under aprotic conditions. The primary intermediate product absorbs maximally at 350 nm (■), secondary intermediate product at 235 nm (●), and α-tocopherol at 292 nm (▲).

mediate) with a maximum intensity at 1 min. The data indicate that the reaction of α-T with O_2^- under aprotic conditions produced two distinct intermediates within 1 min. The primary product was easily reduced back to α-T by treatment with either $LiAlH_4$ or ascorbic acid, as shown in Figure 3 and Table 1. The primary intermediate product disappeared completely when the reaction mixture was treated with the reducing agents, and 91% and 58% of α-T were regenerated from the primary intermediate product when treated with $LiAlH_4$ or ascorbic acid, respectively. The limited solubility of ascorbic acid in acetonitrile may be responsible for its lower effectiveness compard to $LiAlH_4$. When no reducing agents were added, approximately 85% of the α-T converted to compounds A and B.

The α-T oxidation products produced by the reaction of α-T with O_2^- in dry acetonitrile are shown in Figure 4. Retention times were 12.8, 15.2 and 16.4 min for the α-T secondary intermediate, compound A, and compound B, respectively. The secondary product was measured at 235 nm maximum absorbance. This compound slowly converted in hexane at room temperature to compounds A and B, and also readily converted to these compounds (A and B) when washed with water or when the solvent was evaporated under nitrogen. Compounds A and B also exhibited maximum absorbance at 235 nm, and were found to be slightly more polar than the parent compound α-T. Compounds A, B, and the secondary intermediate product were not converted to α-T when treated with either $LiAlH_4$, ascorbic acid or 0.1M HCl.

The greatest conversion of α-T into the secondary intermediate product was obtained when no nitrogen was bubbled through the reaction mixture before and during the reaction of α-T with O_2^- in dry acetonitrile (Fig. 5). When the O_2^- solution was purged with nitrogen for 5 min before the reaction with α-T, considerably less secondary product formed. When the nitrogen purge was continued during the reaction of O_2^- with α-T for an additional one minute (total 6 min), the least amount of secondary product was produced. These results seem to indicate that O_2 may also be involved in α-T oxidation. Matsumoto and colleagues (17,18) observed that when O_2 was evacuated from the reaction medium, the reaction rate of an α-T model compound, 2,2,5,7,8-pentamethylchroman, with O_2^- was markedly inhibited. Nanni *et al.* (8) reported

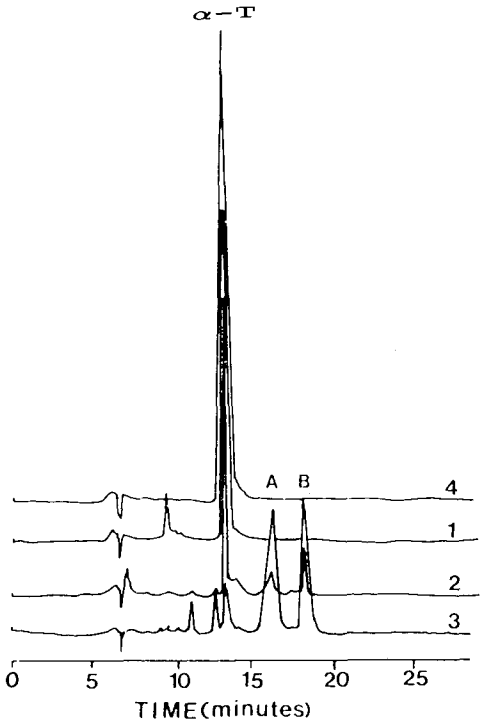

FIG. 3. HPLC tracings of the reduction products of the primary oxidation product of α-T after treatment with $LiAlH_4$ (1) or ascorbic acid (2). The primary oxidation product in the absence of reducing agent converts to compounds A and B during analysis (3). Standard α-T is shown for comparison (4). A 5 μm Ultrasphere-Si column, 250 mm × 4.6 mm i.d. was eluted with hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) at a flow rate of 0.4 mL/min. Detection was at 234 nm for compounds A and B and 292 nm for α-T; the sensitivity was 0.05 AUFS at both wavelengths.

TABLE 1

Reduction of Primary Intermediate[a]

Treatment	α-T	Compound A	Compound B
Blank[b]	9.3	0.0	0.0
Control[c]	1.4[d]	3.2	3.5
$LiAlH_4$	8.6	0.0	0.0
Ascorbic acid	6.6[d]	0.8	1.1

[a]Values are in μg and represent the average of 4 repeated experiments with triplicate injections per experiment.

[b]The blank contained α-T but no O_2^- nor reducing agent.

[c]The control contained the primary intermediate without reducing agent.

[d]Differs from blank, (Tukey's w significance test), $p < 0.05$.

that O_2 may be produced during the reaction of α-T with O_2^- and may be involved in α-T oxidation reactions as well.

Reaction of α-T with O_2^- in an aqueous acetonitrile solution (protic conditions). The disappearance of α-T was measured during the reaction with O_2^- in the presence of various amounts of water (1, 5 and 10%) in acetonitrile over a period of time and compared with a control α-T sample which contained no O_2^- and water (Fig. 6). In the presence of 1% of water (1 min reaction time), α-T quickly disappeared and produced an intermediate similar to that

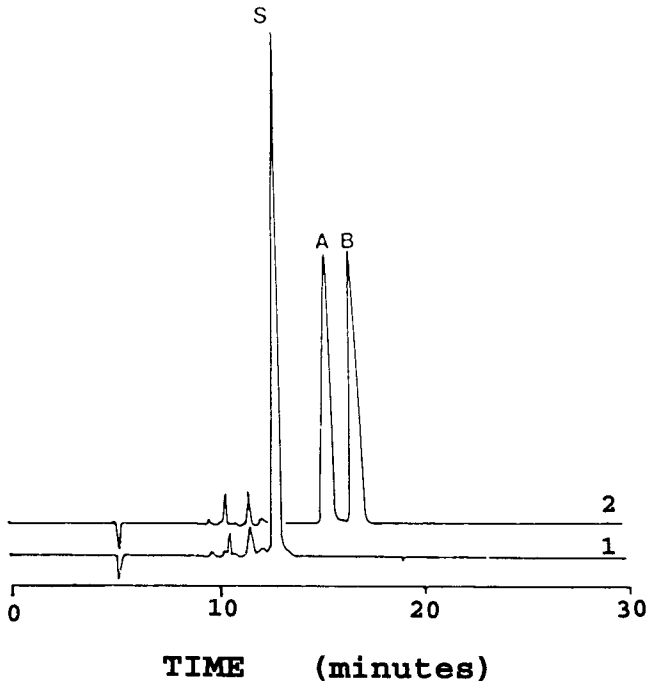

FIG. 4. Typical HPLC tracings of α-tocopherol oxidation products obtained from the reaction of α-tocopherol oxidation products obtained from the reaction of α-T with O_2^- under aprotic conditions. Chromatogram 1; secondary intermediate, S. Chromatogram 2; stable end-products compound A, A and compound B, B.

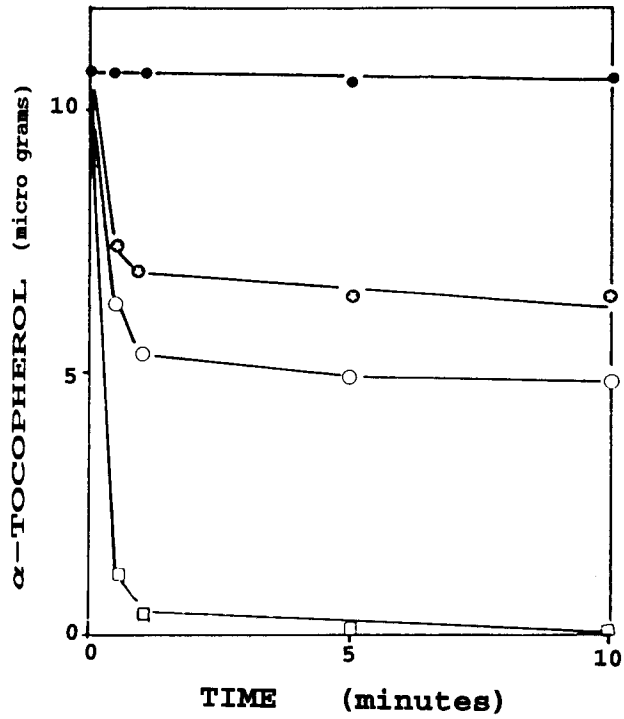

FIG. 6. Disappearance of α-T from the reaction mixture of α-T with O_2^- in the presence of various concentrations of water: 1% water (○), 5% water (○), 10% water (□) and 0% water and no O_2^- (●).

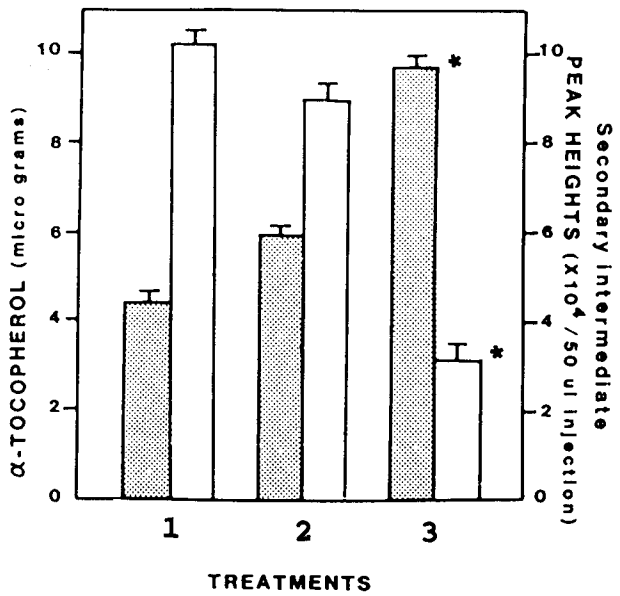

FIG. 5. Effects of N_2 purge on the reaction of α-T with O_2^- in dry acetonitrile. Treatments: 1, no nitrogen purge; 2, 5 min nitrogen purge before reaction; 3, nitrogen purge 5 min before reaction and 1 min during reaction. Shaded bars represent α-T and open bars represent secondary intermediate product. * = Differ statistically, Tukey's w significant test, $X < = 0.05$.

seen with the reaction of O_2^- itself. This result indicates that a 1% water concentration in acetonitrile is not sufficient to dismutate O_2^-. The higher reaction rate, in 10% water than in 5% water, suggests that 10% water in acetonitrile was more effective in dismutating O_2^- under the experimental conditions used. A typical HPLC separation of the products of the reaction of α-T with O_2^- in a 10% aqueous acetonitrile solution (Ultrasphere-Si column) is shown in Figure 7. Four α-T oxidation products were isolated, and three of them were identified as α-T dimer (D), α-T dihydroxy dimer (DHD) and α-T quinone (TQ) by both co-chromatography and ultraviolet spectrometry. Co-chromatography of the samples spiked with pure D, DHD and TQ did not change retention times and peak shapes when compared to the pure compounds alone. Peak heights and areas were increased proportionately to the amounts of standards added. The ultra-violet spectra of these compounds in hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) were identical to their corresponding standards (Table 2). Hence, the products of the oxidation of α-T with O_2^- were different in anhydrous acetonitrile (Fig. 4) and in the presence of 10% water in acetonitrile.

DISCUSSION

Two unstable α-T intermediate products were produced during the reaction of α-T with O_2^- in dry acetonitrile. The first intermediate was found to be very unstable (20 sec) and converted readily to the second intermediate. The

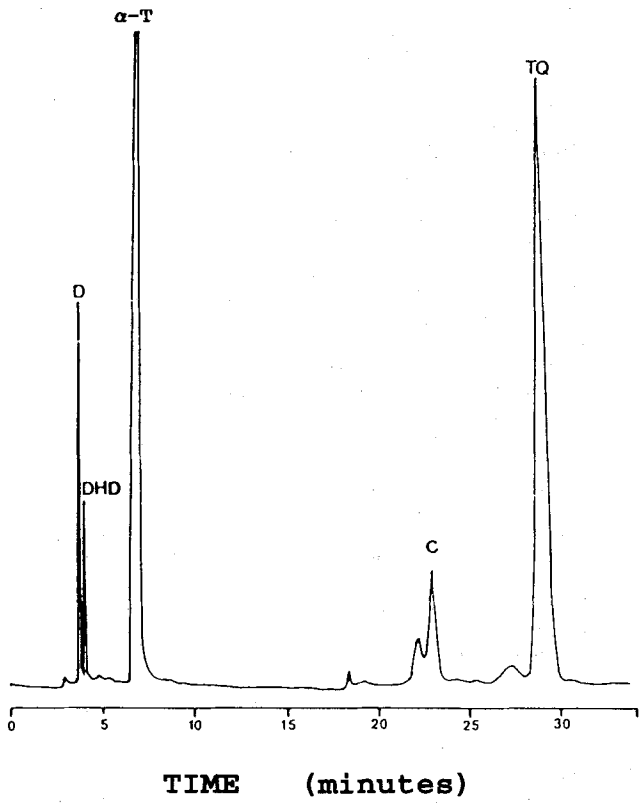

FIG. 7. A typical HPLC tracings of α-T oxidation products produced by the reaction of α-T with O_2^- in a 10% aqueous solution. The identified peaks are: D, α-T dimer; DHD, α-T dihydroxy dimer; α-T, α-tocopherol; TQ, α-tocopheryl quinone; and C, unknown compound. The HPLC conditions were as described for Figure 3.

TABLE 2

Adsorption Maxima[a] of Standards and Isolated α-Tocopherol Oxidation Products from the Reactions of α-Tocopherol with O_2^- in the Presence of 10% water

Compounds	Standards (nm)	Isolated compounds (nm)
α-Tocopherol dimer	298.5	298.6
α-Tocopherol dihydroxy dimer	291.2	291.0
α-Tocopherol	292.0	291.8
α-Tocopheryl quinone	268.0	267.8

[a]Maxima were determined in the hexane/chloroform/isopropanol (95:45:0.5, v/v/v) mixture used as mobile phase in HPLC.

secondary intermediate was more stable than the first intermediate, but also converted in about equal quantities of two stable end products, compounds A and B. The primary intermediate product was easily reconverted to α-T by treatment with $LiAlH_4$ or ascorbic acid. It may be concluded that the primary intermediate product is an α-tocopheroxy radical or an α-tocopheroxy anion. Ozawa and Hanaki (10,16,19) observed yellow color development during the reaction of an α-tocopherol model compound with O_2^-, and identified this compound as an α-chromanoxy radical by electron spin resonance (10,16,19). However, the chromanoxy radical seemed to be more stable than the primary intermediate product of α-T produced in the present experiments. According to Nanni *et al.* (8) the possibility exists for the formation of an α-tocopheroxy anion by the action of O_2^-. It has also been suggested that, in the presence of glutathione or ascorbic acid, α-T could be regenerated after being oxidized to an α-tocopheroxy radical by O_2^- (9,20). However, none of these studies provided direct evidence that α-T was the end product of the regeneration procedures. One of the interesting aspects of the present study is the demonstration of α-T regeneration from the primary intermediate product which was produced by the reaction of α-T with O_2^- in an aprotic condition. When O_2^- is produced by membrane-bound enzymes such as NADPH reductase (2,21) or by the H_2O_2 disproportionation reaction (22), α-T acts as a scavenger. If an α-tocopheroxy anion is formed by O_2^-, it could be reduced back to α-T in the presence of ascorbic acid, making α-T again available for scavenging action.

The secondary intermediate product (absorption maximum 273 nm) could not be converted to α-T by treatment with $LiAlH_4$ or ascorbic acid. It has been reported that α-tocopherone (absorption maximum 240 nm in methanol) produced from the reaction of α-T with O_2^- (20) or free radicals (3) can be reduced again to α-T by treatment with reducing agents, and can be oxidized to α-tocopheryl quinone by treatment with acid (23).

It is concluded that the secondary intermediate produced by the reaction of α-T with O_2^- under aprotic conditions is not identical to α-tocopherone because the secondary intermediate thus produced is not convertible to α-T by reducing agents nor is the secondary intermediate oxidized to α-tocopheryl quinone in the presence of acid. Production of the secondary intermediate product required O_2 in the reaction media (Fig. 5). The secondary intermediate product converted readily to equal amounts of compounds A and B in the presence of water or during sample handling. Compounds A and B were stable end products of the reaction of O_2^- under aprotic conditions.

The first step of α-T reaction with O_2^- in the aprotic media resulted in the formation of the primary intermediate product. If O_2^- in an aprotic solution acts as a Bronsted base, then phenolic compounds such as α-T (8,17,24) could be converted to α-tocopheroxy anions. The second step is the oxidation of the primary intermediate to the secondary intermediate product, mediated by the presence of O_2. The involvement of O_2 in α-T oxidation has been observed by several other investigators (8,24,25). Depending upon the reaction conditions, α-T was selectively oxidized (especially in the presence of base). The final step in the oxidation of α-T is the conversion of the secondary intermediate into the stable end products A and B.

By contrast, when α-T was reacted with O_2^- in a 10% aqueous acetonitrile solution, neither the primary intermediate product, the secondary intermediate product, nor compounds A and B were formed, rather, α-tocopherol dimer (D), α-tocopherol dihydroxy dimer (DHD), α-tocopheryl quinone (TQ) and an unknown compound were formed instead. The principal fate of O_2^- in an aqueous solution is its dismutation which results in the formation of hydroxy radical and/or singlet oxygen (5,22,26–28). Dimer, DHD, α-T trimer, and TQ are formed by the reaction of α-T with free radicals (12,23,29), in addition, TQ may be formed by reaction with singlet oxygen (30,31).

It has also been reported that TQ is produced by the reaction of α-T with O_2^- generated from the xanthine oxidase system (9). However, O_2^- dismutation must have occurred in that system in the presence of water. Therefore, TQ formation may be attributable to the formation of hydroxy free radicals. In the present experiments, α-tocopheryl quinone was not found to be produced by the direct action of O_2^-.

In summary, when α-T was reacted with O_2^- in dry acetonitrile, a very unstable intermediate product was formed. This compound could easily be converted into α-T by treatment with either $LiAlH_4$ or ascorbic acid. The primary intermediate product changed into the secondary product which was isolated by HPLC. The secondary product then converted in the presence of oxygen into two stable end products. When α-T reacted with O_2^- in a 10% aqueous acetonitrile solution, α-T dimer, α-T dihydroxy dimer, α-T quinone and an unknown compound were produced. The present experiments demonstrate that the reaction of α-T with O_2^- under aprotic conditions produces completely different oxidation products than does the reaction under protic conditions.

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