Reaction of 8*a*-Hydroperoxy Tocopherones with Ascorbic Acid

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8*a*-Hydroperoxy tocopherones, prepared from the photooxidation of α -, γ - and δ -tocopherols, were allowed to react with ascorbic acid in ethanol. The hydroperoxy tocopherones were reduced to 8*a*-hydroxy and 8*a*-ethoxy tocopherones which in turn were reduced to tocopherols by ascorbic acid. The tendency of the hydroperoxy tocopherones to form tocopherols during the reaction correlated with vitamin E activities of tocopherols. The results indicate that α -tocopherol and ascorbic acid can act synergistically on the quenching of $^1\text{O}_2$. The inhibitory effects of α -tocopherol and ascorbic acid were examined on the chlorophyll-sensitized photooxidation of methyl linoleate and soybean oil. At the initial stage of the oxidation, the inhibitory effect of α -tocopherol was lengthened in the presence of ascorbic acid.

The role of tocopherols in the biological system has been recognized as an antioxidant to inhibit the free radical chain reaction of lipid peroxidation.^{1,2)} The initiation process of lipid peroxidation is caused by singlet molecular oxygen ($^1\text{O}_2$).^{3~6)} Tocopherols are capable of highly efficient $^1\text{O}_2$ quenching, both by a physical process and by an irreversible reaction.^{7~10)} α -Tocopherol which possesses the highest biological activity as vitamin E is indicated to be the most effective quencher of $^1\text{O}_2$ among the tocopherols from the data of $^1\text{O}_2$ quenching rate.^{7,10)} However, α -tocopherol had a poor inhibitory effect on the $^1\text{O}_2$ -initiated deterioration of vegetable oils.¹¹⁾ This poor inhibitory effect of α -tocopherol was explained by its high reactivity toward $^1\text{O}_2$.¹²⁾

Nishikimi *et al.*^{13,14)} have reported that 8*a*-hydroxy α -tocopherone, formed as a labile intermediate in the oxidation of α -tocopherol to α -tocopheryl quinone by the superoxide radical (O_2^-), was reversibly reduced to α -tocopherol by ascorbic acid. Packer *et al.*¹⁵⁾ have reported the direct observation of a free radical interaction between α -tocopherol and ascorbic acid by pulse radiolysis technique, α -tocopherol acting as the primary free radical scavenger and the resulting phenoxy radical

then reacting with ascorbic acid to generate α -tocopherol. We have reported that the photooxidation of α -, γ - and δ -tocopherols produced two geometric isomers of 8*a*-hydroperoxy tocopherones as the primary products.¹⁶⁾ The structure of 8*a*-hydroperoxy tocopherones resembles 8*a*-hydroxy α -tocopherone. Therefore, 8*a*-hydroperoxy tocopherols may also be reversibly reduced to tocopherols by ascorbic acid.

This paper deals with the reaction of 8*a*-hydroperoxy tocopherones, the primary products formed by the photooxidation of tocopherols, with ascorbic acid. The inhibitory effect of α -tocopherol and ascorbic acid on the chlorophyll-sensitized photooxidation of methyl linoleate and soybean oil was also examined.

MATERIALS AND METHODS

Materials. *d*- α -, *d*- γ - and *d*- δ -tocopherols were supplied by Eisai Co. and were purified before use.¹⁰⁾ L-Ascorbic acid and methylene blue were purchased from Wako Pure Chem. Methyl linoleate from Tokyo Kasei Co. and soybean oil from Wako Pure Chem. were purified by Florisil column chromatography.¹²⁾ Chlorophyll *a* was obtained from spinach leaves.¹⁷⁾ Other reagents were of commercial grade and were used without further purification.

Preparation of 8*a*-hydroperoxy tocopherones. 8*a*-Hydroperoxy α -, γ - and δ -tocopherones were prepared from the methylene blue sensitized photooxidation of the corresponding tocopherols.¹⁰ Each tocopherol (50 μ mol) was dissolved in 10 ml of ethanol containing methylene blue (5 μ mol). A reaction vessel fitted with a reflux condenser was placed into an ice bath and air bubbled. The light source was a 60 W tungsten projector lamp at a distance of 30 mm from the vessel. The reactions were carried out for 1 hr (α -tocopherol), 4 hr (γ -tocopherol), and 9 hr (δ -tocopherol), respectively.

The reaction products were checked by thin layer chromatography (TLC), on a Wako pre-coated silica gel plate (0.25 mm thick), using benzen-methanol (97:3) as the solvent system. Only two peroxides for each reaction mixture were detected on the plate. The ultraviolet spectra of the reaction mixture showed absorption maxima at 242 nm for α -tocopherol ($\epsilon=10,500$), at 236 nm for γ -tocopherol ($\epsilon=12,000$), and at 230 nm for δ -tocopherol ($\epsilon=12,300$) in ethanol. Therefore, the three tocopherols reacted nearly quantitatively with $^1\text{O}_2$ to form the corresponding two geometric isomers of 8*a*-hydroperoxy tocopherones.^{16,18} The solution of 8*a*-hydroperoxy tocopherones thus prepared was labile,¹⁶ and was stored at -30°C .

Reaction of 8*a*-hydroperoxy tocopherones with ascorbic acid. *n*-Hexane (5 ml) was added to the 8*a*-hydroperoxy tocopherone solution (5 ml), and washed with distilled water to remove the methylene blue. *n*-Hexane was then removed using a vacuum rotary evaporator at below 25°C . The 8*a*-hydroperoxy tocopherone was redissolved in 5 ml of ethanol (the concentration of each 8*a*-hydroperoxy tocopherone was *ca.* 5×10^{-3} M). Each 8*a*-hydroperoxy tocopherone solution (100 μ l) was incubated at 37°C with 100 μ l of 10^{-2} M ascorbic acid. After incubation, the reaction mixture was extracted with chloroform and washed with distilled water. The chloroform layer was spotted on the TLC plate. After being developed in the solvent system of benzen-methanol (97:3), the compounds were detected by spraying with 50% H_2SO_4 followed by heating at 110°C for 10 min.

The reaction products giving large spots on the TLC plate were characterized by mass spectrometry. The reaction mixture was separated by preparative TLC, Merck silica gel PF₂₅₄ (0.5 mm thick), with the same solvent system. The compounds were detected under ultraviolet light. The bands of compounds were scraped off and then extracted with ethyl ether. Each fraction was analyzed by the mass spectrometer. Mass spectra were recorded with a Hitachi RMU-6M spectrometer operating at 70 eV.

The quantities of tocopherols in the reaction mixture were determined by gas liquid chromatography (GLC).¹⁰

Photooxidation of methyl linoleate and soybean oil. The method of photooxidation of methyl linoleate and soybean oil was the same as described by Terao *et al.*¹² The light source was a 60 W tungsten projector lamp

at a distance of 200 mm from the vessel. The temperature was kept at 37°C and the reaction mixture was shaken mechanically during irradiation. The conditions for each experiment are given in the legends accompanying the figure and table. The contents of α -tocopherol and methyl linoleate hydroperoxide were determined by GLC.¹⁰ The peroxide values were measured by the iodometric method.⁶

RESULTS

Reaction of 8*a*-hydroperoxy tocopherones with ascorbic acid

The mixture of each 8*a*-hydroperoxy tocopherone and ascorbic acid was incubated at 37°C . At intervals, the reaction products of 8*a*-hydroperoxy tocopherones were analyzed by TLC. Figure 1 shows the reaction products of 8*a*-hydroperoxy α -tocopherone with ascorbic acid during incubation time. After 5 min incubation, two spots corresponding to 8*a*-hydroperoxy α -tocopherone isomers almost disappeared and two spots (α -tocopherol and compound **a**) were observed. Then, compound **a** disappeared and only α -tocopherol was ob-

FIG. 1. Thin Layer Chromatogram of the Reaction Products of 8*a*-Hydroperoxy α -Tocopherone with Ascorbic Acid.

The reaction mixture containing 100 μ l of the 8*a*-hydroperoxy α -tocopherone solution prepared from 5×10^{-3} M α -tocopherol and 100 μ l of 10^{-2} M ascorbic acid in ethanol was incubated for 0 min (1), 5 min (2), 15 min (3), 30 min (4) and 180 min (5). The 8*a*-hydroperoxy α -tocopherone solution was incubated for 180 min (6). The reaction products, (1)~(6), and α -tocopherol (7) were charged on the TLC plate with benzene-methanol (97:3). The compounds were detected by spraying with 50% H_2SO_4 followed by heating at 110°C for 10 min.

FIG. 2. Mass Spectrum of Compound **a**. FIG. 3. Thin Layer Chromatogram of the Reaction Products of 8*a*-Hydroperoxy γ -Tocopherone with Ascorbic Acid.

The reaction mixture containing 100 μ l of the 8*a*-hydroperoxy γ -tocopherone solution prepared from 5×10^{-3} M γ -tocopherol and 100 μ l of 10^{-2} M ascorbic acid in ethanol was incubated for 0 min (1), 15 min (2), 30 min (3), 60 min (4) and 180 min (5). The 8*a*-hydroperoxy γ -tocopherone solution was incubated for 180 min (6). The reaction products, (1)~(6), and γ -tocopherol (7) were charged on the TLC plate. The development and the detection were the same as described in Fig. 1.

served at 30 min. On the other hand, two spots of 8*a*-hydroperoxy α -tocopherone isomers and a spot corresponding to α -tocopheryl quinone¹⁶⁾ were observed when the hydroperoxy tocopherone alone was incubated for 180 min. Compound **a** was separated by preparative TLC. The ultraviolet spectrum of compound **a** showed an absorption maximum at 242 nm in ethyl ether which was consistent with the assignment as a cross conjugated dienone.¹⁸⁾ From the mass spectrum shown in Fig. 2, compound **a** was identified to be 8*a*-hydroxy α -tocopherone; m/z 446 (M^+), 430 ($[M-O]^+$), 221 ($[M-C_{16}H_{33}]^+$), 205 ($[M-C_{16}H_{33}O]^+$) and 164 ($[M-C_{19}H_{37}OH]^+$).

The reaction products of 8*a*-hydroperoxy γ - and δ -tocopherones with ascorbic acid were also analyzed by TLC (Figs. 3 and 5). 8*a*-Hydroperoxy γ -tocopherone reacted with ascorbic acid to produce compound **b** and then to produce γ -tocopherol and compound **c**. Compound **b** was separated by TLC. The

FIG. 4. Mass Spectrum of Compound **b**.

FIG. 5. Thin Layer chromatogram of the Reaction Products of 8*a*-Hydroperoxy δ -Tocopherone with Ascorbic Acid.

The reaction mixture containing 100 μ l of the 8*a*-hydroperoxy δ -tocopherone solution prepared from 5×10^{-3} M δ -tocopherol and 100 μ l of 10^{-2} M ascorbic acid in ethanol was incubated for 0 min (1), 30 min (2), 60 min (3), 120 min (4) and 180 min (5). The 8*a*-hydroperoxy δ -tocopherone solution was incubated for 180 min (6). The reaction products, (1)~(6), and δ -tocopherol (7) were charged on the TLC plate. The development and the detection were the same as described in Fig. 1.

ultraviolet spectrum of compound **b** showed an absorption maximum at 236 nm in ethyl ether. From the mass spectrum shown in Fig. 4, compound **b** was identified to be a mixture of 8*a*-ethoxy γ -tocopherone; m/z 460 (M^+), 445 ($[M-CH_3]^+$), 431 ($[M-C_2H_5]^+$), 416 ($[M-C_2H_4O]^+$), 235 ($[M-C_{16}H_{33}]^+$), 191 ($[M-C_{18}H_{37}O]^+$) and 151 ($[M-C_{21}H_{41}O]^+$), and 8*a*-hydroxy γ -tocopherone; m/z 432 (M^+), 416 ($[M-O]^+$), 207 ($[M-C_{16}H_{33}]^+$), 191 ($[M-C_{16}H_{33}O]^+$) and 151 ($[M-C_{19}H_{37}O]^+$). Compound **c**, which showed a red-brown spot on the TLC plate, yielded too small an amount to be identified. The reaction products of 8*a*-hydroperoxy δ -tocopherone were the same as that of 8*a*-hydroperoxy γ -tocopherone (Fig. 5). However, only a small amount of δ -tocopherol was produced after 180 min incubation. The primary product corresponding to spot **d** on the plate showed an absorption maximum at 227 nm in ethyl ether, and was identified to be a mixture of 8*a*-ethoxy and 8*a*-hydroxy δ -tocopherones from the mass spectrum; 8*a*-ethoxy δ -tocopherone: m/z 446 (M^+), 431 ($[M-CH_3]^+$), 417 ($[M-C_2H_5]^+$), 402

FIG. 6. Time Course of Tocopherol Formation.

An equal volume of the 8*a*-hydroperoxy tocopherone solution prepared from 5×10^{-3} M tocopherol and 10^{-2} M ascorbic acid in ethanol was incubated at 37°C. The amounts of tocopherols were determined by GLC.

○—○, α -tocopherol; $\triangle$ — $\triangle$, γ -tocopherol; $\square$ — $\square$, δ -tocopherol.

TABLE I. REDUCTION OF 8*a*-HYDROPEROXY α -TOCOPHERONE BY SOME REDUCTANTS

The reaction mixture contained 100 μ l of 8*a*-hydroperoxy α -tocopherone prepared from 5×10^{-3} M α -tocopherol and 100 μ l of 10^{-2} M reactant. Incubation was carried out for 30 min at 37°C.

Reductants	α -Tocopherol, formed (μ g/200 μ l)
Ascorbic acid	106
Ascorbyl stearate	103
Hydroquinone	3
Butylated hydroxytoluene	0
Butylated hydroxyanisole	19
Diphenylamine	0
Glutathione, reduced	0
Sodium borohydride	0

($[M-C_2H_4O]^+$), 401 ($[M-C_2H_5O]^+$), 221 ($[M-C_{16}H_{33}]^+$), 177 ($[M-C_{18}H_{37}O]^+$) and 137 ($[M-C_{21}H_{41}O]^+$), 8*a*-hydroxy δ -tocopherone: m/z 418 (M^+), 402 ($[M+O]^+$), 401 ($[M-OH]^+$), 193 ($[M-C_{16}H_{33}]^+$), 177 ($[M-C_{16}H_{33}O]^+$) and 137 ($[M-C_{19}H_{37}O]^+$).

The formation rates of tocopherols by the reaction of 8*a*-hydroperoxy tocopherones with ascorbic acid were determined by GLC (Fig.

6). 8*a*-Hydroperoxy α -tocopherone was most rapidly reduced to α -tocopherol by ascorbic acid among the three hydroperoxides. Then, the reducing activities of some reductants were tested (Table I). Only ascorbic acid and its derivative, ascorbyl stearate, reduced the hydroperoxide to α -tocopherol. Other reductants had little effect upon the reduction of hydroperoxide to α -tocopherol.

Inhibitory effects of α -tocopherol and ascorbic acid on the chlorophyll-sensitized photooxidation of methyl linoleate and soybean oil

The photooxidation product of α -tocopherol, 8*a*-hydroperoxy α -tocopherone, could be reduced to α -tocopherol by ascorbic acid. Therefore, ascorbic acid was expected to enhance the inhibitory effect of α -tocopherol on the $^1\text{O}_2$ -initiated lipid peroxidation. Figure 7 shows the formation of methyl linoleate hydroperoxide which is the photooxidation

product of methyl linoleate⁶⁾ and the decrease in α -tocopherol on the chlorophyll-sensitized photooxidation of methyl linoleate. At the initial stage of the photooxidation, the coexistence of α -tocopherol and ascorbic acid in methyl linoleate had an appreciable effect on the inhibition by α -tocopherol alone. But the effect decreased rapidly with the illumination time. Conversely, ascorbic acid had an accelerative effect on this oxidation. The amount of α -tocopherol decreased with the illumination time. The decrease of α -tocopherol was retarded slightly by the addition of ascorbic acid.

Table II shows the changes in the peroxide value on the chlorophyll-sensitized photooxidation of soybean oil. Similarly with the result shown in Fig. 7, ascorbic acid or ascorbyl stearate enhanced the inhibitory effect of α -tocopherol at the initial stage of the photooxidation. However, the effect decreased with the illumination time.

DISCUSSION

The regeneration of α -tocopherol by ascorbic acid has been demonstrated on the free radical reactions.^{14,15,19)} This study showed that 8*a*-hydroperoxy tocopherones reacted

FIG. 7. Time Courses of Methyl Linoleate Hydroperoxide Formation (A) and α -Tocopherol Decay (B).

The reaction mixture containing methyl linoleate (2×10^{-4} mol) and chlorophyll *a* ($38 \mu\text{g}$) was exposed to irradiation at 37°C . The contents of methyl linoleate hydroperoxide and α -tocopherol were determined by GLC. The amount of methyl linoleate hydroperoxide is represented as the molar ratio to methyl linoleate.

●—●, no addition; ○—○, 1 mol% α -tocopherol; Δ — Δ , 1 mol% α -tocopherol + 2 mol% ascorbic acid; □—□, 2 mol% ascorbic acid.

TABLE II. PEROXIDE VALUES OF PHOTO-OXIDIZED SOYBEAN OILS

Soybean oil was decolorized by Florisil column chromatography with elution by *n*-hexane. The tocopherols in the oil were not detected by this treatment. α -Tocopherol (0.2 wt%), ascorbic acid (0.2 wt%) and ascorbyl stearate (0.2 wt%) were added to soybean oil (containing 0.07 wt% of chlorophyll *a* as the photosensitizer) which was exposed to irradiation at 37°C for 2, 4 and 6 hr.

Additive	Peroxide value (meq/kg)		
	2 hr	4 hr	6 hr
None	31	55	71
α -Tocopherol	23	39	48
α -Tocopherol + ascorbic acid	21	41	57
α -Tocopherol + ascorbyl stearate	17	34	54
Ascorbic acid	32	60	92
Ascorbyl stearate	25	57	77

FIG. 8. Reaction Sequence of the Reduction of 8 α -Hydroperoxy Tocopherones.

with ascorbic acid to produce the corresponding tocopherols (Figs. 1, 3 and 5). The primary reaction products of 8 α -hydroperoxy tocopherones (2) were 8 α -hydroxy tocopherones (3a) as shown in Fig. 8. When 8 α -hydroperoxy γ - and δ -tocopherones were used as the reactants, 8 α -ethoxy tocopherones (3b) were also produced. The reaction rate of γ - and δ -tocopherones with ascorbic acid was much slower than that of α -tocopherone. Therefore, the 8 α -hydroxy γ - and δ -tocopherones may be attacked by ethanol as the solvent to give the ethoxy compounds. 8 α -Hydroxy α -tocopherone has been previously reported to be involved in the free radical reaction of α -tocopherol¹⁹⁾ or the O_2^- reaction of α -tocopherol.¹⁴⁾ A good correlation was obtained between the formation rates of tocopherols from 8 α -hydroperoxy tocopherones and the vitamin E activities of the corresponding tocopherols (Fig. 6). Moreover, the reaction to α -tocopherol was achieved only by ascorbic acid (Table I). The results indicate that the primary products formed by the photosensitized oxidation of α -tocopherol is also reduced back to α -tocopherol by ascorbic acid (Fig. 8). Therefore, α -tocopherol and ascorbic acid can act synergistically on the quenching of $^1\text{O}_2$, α -tocopherol acting as the $^1\text{O}_2$ quencher and the resulting 8 α -hydroperoxy α -tocopherone then reacting with ascorbic acid to regenerate α -tocopherol.

While α -tocopherol could be expected to have a high inhibitory effect on the $^1\text{O}_2$ -

initiated lipid peroxidation, its effect decreased rapidly for its high reactivity toward $^1\text{O}_2$.^{10~12)} The data in Fig. 7 and Table II show that the coexistence of α -tocopherol and ascorbic acid has an appreciable effect on the inhibition of the photooxidation by α -tocopherol alone and that the decrease of α -tocopherol is retarded slightly. The results indicate the possibility that α -tocopherol and ascorbic acid act synergistically on the photooxidation of oils. However, the inhibitory effect of the two vitamins decreased rapidly with the illumination time. The oxidation product of ascorbic acid may have a pro-oxidant effect on the oxidation.

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