  Bull. Chem. Soc. Jpn., 65, 679—684 (1992)

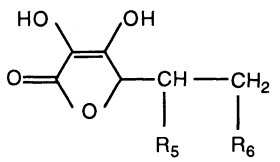
Action of Fatty Acid Esters of L-Ascorbic Acid as Antioxidants in Phosphatidylcholine Liposomal Membranes

Mareyuki TAKAHASHI, Erika KOMURO, Etsuo NIKI,*,† and Kunihiko TANAKA††
Department of Reaction Chemistry, Faculty of Engineering,
The University of Tokyo, Hongo, Tokyo 113
† Research Center for Advanced Science and Technology,
The University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153
†† Shinto Paint Co., Ltd., Minami Tsukaguchi, Amagasaki 661
(Received October 9, 1991)

The antioxidant activities of saturated fatty acid esters of L-ascorbic acid were studied in the oxidations of soybean phosphatidylcholine liposomes in aqueous dispersions aiming at elucidating their action as antioxidants in the liposomal membranes, especially the effects of number and length of the fatty acid ester side chains at either 6- or both 5- and 6-positions. Ascorbic acid esters were found to act as radical-scavenging antioxidants in the oxidations initiated by either aqueous free radical attacking from outside of the membrane or by lipophilic radical generated within the membranes. They inhibited the oxidation cooperatively with α-tocopherol in the membranes and reduced the rate of α-tocopherol consumption, their sparing effects on α-tocopherol being greatest for the monoesters and for the esters with the shortest fatty acid chains within the diester group. It was concluded that the intramembrane and intermembrane mobilities of ascorbic acid esters decreased with increasing number and length of fatty acid chains.

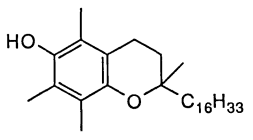
It has been known that the free radical-mediated oxidations by molecular oxygen induce the degradation and deterioration of plastics, rubber, oil, and foods.[1] The oxidations of lipids in biological systems induced by free radicals and active oxygens have also received much attention recently with increasing number of evidence which shows that such oxidations are important as the primary events in a variety of pathological events, cancer and aging.[2-6] Various kinds of antioxidants have been used practically for plastics, rubber, oil, and foods, while the aerobic organisms are protected by an array of defense systems. Among others, the oxidation of membranes and its inhibition are important from both practical and fundamental points of view: Practically, the membranes are often the primary target in the oxidative damage in biological systems and the stabilization of artificial membranes is an important problem in, for example, the use of phospholipid liposomal membranes as a drug carrier, while fundamentally the chemistry for actions and scavenging of radicals in the membranes have not been fully elucidated yet.

Vitamin E (tocopherols)[7,8] and vitamin C (ascorbic acid)[9,10] are known as lipophilic and hydrophilic radical-scavenging antioxidants respectively. Their actions as antioxidants in the homogeneous solution are now fairly well established, but those in membranes are not fully understood yet. Vitamin E is a strong radical scavenger, but its antioxidant activity is much smaller in membranes than in homogeneous solution, primarily due to a decrease in its mobility in the membranes.[11,12] We have shown that the rate of scavenging of oxygen radicals by α-tocopherol decreases as the radicals go deeper into the interior of the membranes and suggested that the phytyl side chain, which is essential for its biopotency, reduces the mobility of α-tocopherol in the membranes.[11] We have also reported previously that the intermembrane mobilities of chromanols decrease as the length of the side chain at the 2-position of chromanol increases.[13] Ascorbic acid is a hydrophilic antioxidant, but a few fatty acid esters of ascorbic acid have been also used as lipophilic antioxidants.[14] We have previously prepared various fatty acid esters of L-ascorbic acid at either 6- or both 5- and 6-positions and found that they functioned as antioxidants in the oxidation of methyl linoleate in homogeneous solution.[15] In this work, we have studied the antioxidant activities of the fatty acid esters of ascorbic acid **1** having side chains of different length and number for the oxidation of phosphatidylcholine liposomes aiming at elucidating their action as antioxidant in the membranes, especially the effect of length and number of side chain of ascorbic acid esters on their mobilities and antioxidant activities.

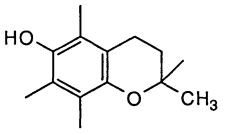


1

R_5, R_6 : OH or $OC(O)(CH_2)_nCH_3$



2



3

Experimental

Commercial soybean phosphatidylcholine (PC) purchased from Daigo Chemical Co. was purified with alumina and silica-gel columns as reported previously.[16] Dimyristoyl PC (14:0 PC) and cholesterol were obtained from Sigma Chemical Co. and used without further purification. Various kinds of fatty acid esters of L-ascorbic acid were synthesized by the esterification of L-ascorbic acid with corresponding fatty acids as reported previously.[15] Table 1 shows the ascorbic acid esters used in this study and their abbreviations. 5,6-Di-*O*-palmitoylerythorbic acid was also synthesizied similarly and used. Natural (2*R*,4′*R*,8′*R*)-α-tocopherol (*d*-α-tocopherol) **2** and 2,2,5,7,8-pentamethyl-6-chromanol (PMC) **3** were kindly provided by Eisai Co. 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) used as water-soluble and lipid-soluble radical initiator[17] respectively, were obtained from Wako Pure Chemical Co. and used as received.

The liposomal membranes were prepared as follows. PC and oil-soluble additives, when necessary, were dissolved in chloroform and the solution was taken into a pear-shaped flask. Chloroform was removed by evaporation to obtain a thin film of the lipids on a flask wall. An appropriate amount of 0.1 M NaCl aqueous solution (1 M=1 mol dm^{-3}) was added and the film was slowly peeled off by shaking to obtain a white, milky aqueous suspensions of multilamellar liposomal membranes. In some cases, they were sonicated with Branson sonifier Model 185 to obtain unilamellar vesicles. The liposomal membranes were subjected to oxidation immediately after preparations.

The oxidation was carried out at 37 °C under air, though, as described later, a few experiments were carried out at 50 °C. The rate of oxidation was measured by following the rate of oxygen uptake with either a pressure transducer or an oxygen electrode.[15,16] In some experiments, the rate of oxidation was followed by measuring the accumulation of conjugated diene PC hydroperoxides with an HPLC at 234 nm equippped with a UV detector set.[17] An aliquot of the reaction mixture was directly injected into HPLC. JASCO LC-18 column (4.6 mm×25 cm) was used and methanol–water (90:10 by v/v) was delivered as an eluent at 1.20 ml min^{-1}. It should be noted that soybean PC contained about 70% linoleic acid as a predominant polyunsaturated fatty acid and that its oxidation gives conjugated diene hydroperoxides quantitatively.[16,18,19] The rate of consumption of α-tocopherol was also followed with an HPLC using an absorption at 294 nm. A Finepak SIL-CN 4.6×250 mm column was used and samples were eluted with hexane–isopropyl alcohol–acetic acid (1000:15:1, v/v/v) at a flow rate of 1 ml min^{-1}. The rate of disappearance of ascorbic acid esters was measured similarly with an HPLC with ODS column equipped with an electrochemical detector, which was set at +800 mV vs. Ag/AgCl. Samples were eluted with methanol containing 0.05 M $NaClO_4$.

Table 1. Ascorbic Acid Esters Used in This Study

Abbreviation	R_2	R_5	R_6
AsA	H	H	H
5,6-C_3	H	COC_2H_5	COC_2H_5
5,6-C_8	H	COC_7H_{15}	COC_7H_{15}
2,5,6-C_8	COC_7H_{15}	COC_7H_{15}	COC_7H_{15}
5,6-C_{14}	H	$COC_{13}H_{27}$	$COC_{13}H_{27}$
6-C_{16}	H	H	$COC_{15}H_{31}$
2,6-C_{16}	$COC_{15}H_{31}$	H	$COC_{15}H_{31}$
5,6-C_{16}	H	$COC_{15}H_{31}$	$COC_{15}H_{31}$
2,5,6-C_{16}	$COC_{15}H_{31}$	$COC_{15}H_{31}$	$COC_{15}H_{31}$
6-C_{18}	H	H	$COC_{17}H_{35}$
5,6-C_{18}	H	$COC_{17}H_{35}$	$COC_{17}H_{35}$
5,6-C_{22}	H	$COC_{21}H_{43}$	$COC_{21}H_{43}$
5,6-C_{16}-EA[a]	H	$COC_{15}H_{31}$	$COC_{15}H_{31}$

a) 5,6-Di-*O*-palmitoylerythorbic acid.

Results and Discussion

Inhibition of Oxidation of Soybean PC Liposomes by Ascorbic Acid Esters. The rate of spontaneous oxidation of soybean PC liposomes in the absence of a radical initiator was quite small at 37 °C, but the incorporation of AMVN into liposomal membranes or the addition of AAPH into the aqueous region induced the oxidations of PC membranes at a constant rate without any noticeable induction period. Figure 1 shows the rates of oxygen uptake in the oxidations of soybean PC liposomes initiated with AAPH in the absence or presence of fatty acid esters of ascorbic acid. The rate of consumption of 5,6-di-*O*-stearoylascorbic acid is also shown in Fig. 1. Figure 2 shows the examples for the accumulation of PC hydroperoxides induced by AMVN. Ascorbic acid esters incorporated into PC liposomes suppressed the oxidation markedly and produced a clear induction period. It was consumed at a constant rate until it was depleted, then a fast oxidation took place at a rate similar to that in the absence of ascorbic acid ester. Figure 1 also shows the effect of α-tocopherol as an antioxidant. It suppressed the oxidation somewhat more efficiently than ascorbic acid esters and produced a clear induction period. These results show that the fatty acid esters of ascorbic

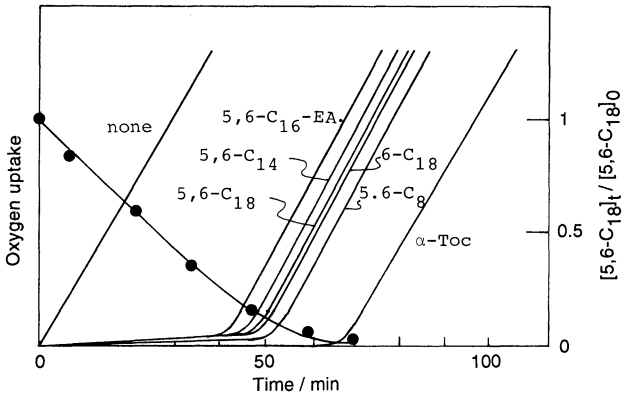


Fig. 1. Rates of oxygen uptake in the oxidations of soybean PC liposome initiated with AAPH at 37 °C under air in the absence or presence of ascorbic acid esters or α-tocopherol. The rate of consumption of 5,6-di-*O*-stearoylascorbic acid is also shown (●). [Soybean PC]=5.15 mM; [AAPH]=20 mM; [antioxidant]=100 μM.

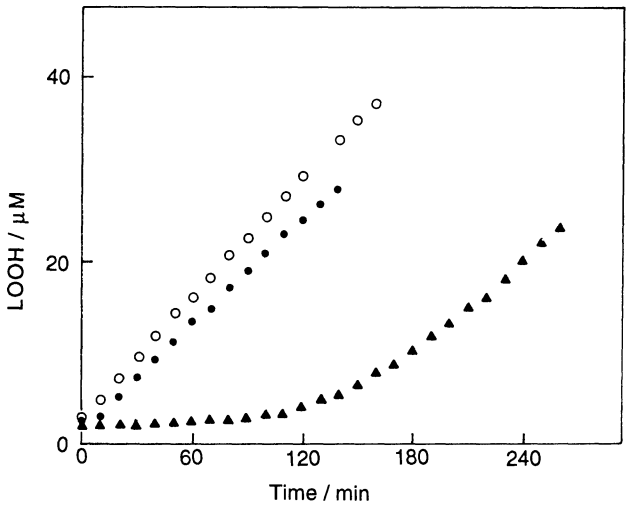


Fig. 2. Accumulation of conjugated diene hydroperoxides during the oxidation of 5.15 mM soybean PC liposome initiated with 1.02 mM AMVN in the absence and presence of 5,6-di-*O*-stearoylascorbic acid. [5,6-C_{18}]=(○) none; (●) 3 μM; (▲) 30 μM.

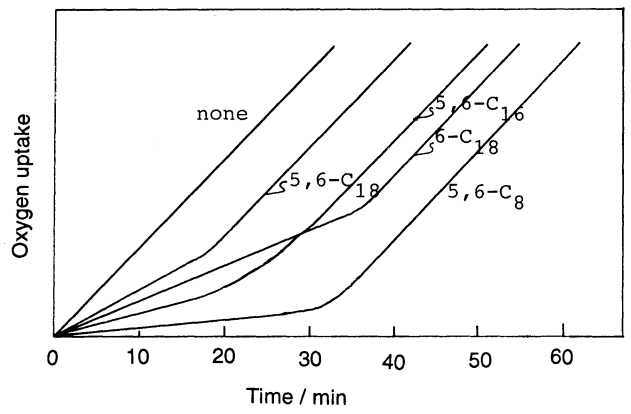


Fig. 3. Inhibition of AAPH-initiated oxidation of soybean PC liposome by 100 μM fatty acid esters of ascorbic acid incorporated into dimyristoyl PC liposome in the same aqueous suspensions. [Soybean PC]=5.15 mM; [14:0 PC]=5.90 mM; [AAPH]=20 mM.

acid function as radical-scavenging antioxidants as observed in the homogeneous solution.[15]

Similar results were obtained for ascorbic acid esters at either 6- or both 5- and 6-positions independent of the length of fatty acid moiety. 5,6-Di-*O*-palmitoylerythorbic acid also functioned as an antioxidant. The stoichiometric number *n* of the peroxyl radicals trapped by each ascorbic acid ester in the membranes calculated from the induction period by comparison with that produced by α-tocopherol whose *n* is 2[20,21] ranged from 1.4 to 1.6. On the other hand, those having ester groups at 2-position, 2,5,6-C_8, 2,6-C_{16}, and 2,5,6-C_{16} did not suppress the oxidation (data not shown).

Figure 3 shows how the fatty acid esters of ascorbic

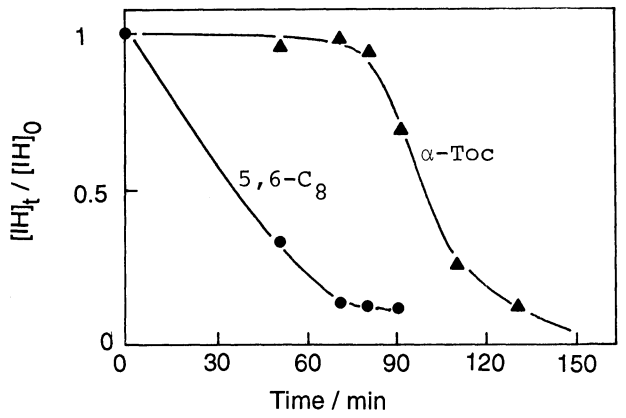


Fig. 4. Consumption of α-tocopherol and 5,6-di-*O*-octanoylascorbic acid in the oxidation of soybean PC liposome initiated with AAPH at 37°C under air. [Soybean PC]=12.9 mM; [AAPH]=30 mM; [α-Toc]=50 μM; [5,6-C_8]=300 μM.

acid incorporated into dimyristoyl PC liposomes suppressed the oxidations of different soybean PC liposomes in the same aqueous suspensions. It shows that the antioxidant activities of ascorbic acid esters decreased as the length of ester groups at 5- and 6-positions became longer.

Interaction of Ascorbic Acid Esters with α-Tocopherol in the Membranes. It has been known that ascorbic acid and tocopherols act cooperatively or even synergistically under certain circumstances to suppress lipid peroxidation.[7-10,21-24] Such an effect has been observed experimentally not only in homogeneous solution but also in aqueous dispersions of liposomes where the hydrophilic ascorbic acid and lipophilic tocopherol reside separately in water phase and lipid phase respectively.[25-28] This is attributed to the regeneration of tocopherol by ascorbic acid; that is, tocopheryloxyl radical formed when tocopherol scavenges peroxyl radical is reduced back to tocopherol by ascorbic acid.

Figure 4 shows the rates of consumptions of α-tocopherol and 5,6-di-*O*-octanoylascorbic acid during the oxidation of soybean PC liposomes initiated with AAPH in the presence of both antioxidants in the same membranes. When either of the antioxidants was present alone, it was consumed linearly with time without any time lag at the similar rate (data not shown). On the other hand, when both were present, the consumption of α-tocopherol was suppressed almost completely and ascorbic acid ester was consumed preferentially, and α-tocopherol began to decrease after the ascorbic acid ester was almost depleted. These results suggest that ascorbic acid ester regenerated α-tocopherol from α-tocopheryloxy radical and/or ascorbic acid ester scavenged the radicals derived from AAPH and PC more rapidly than α-tocopherol.

Figure 5 shows the rate of consumption of α-tocopherol during the oxidation of soybean PC

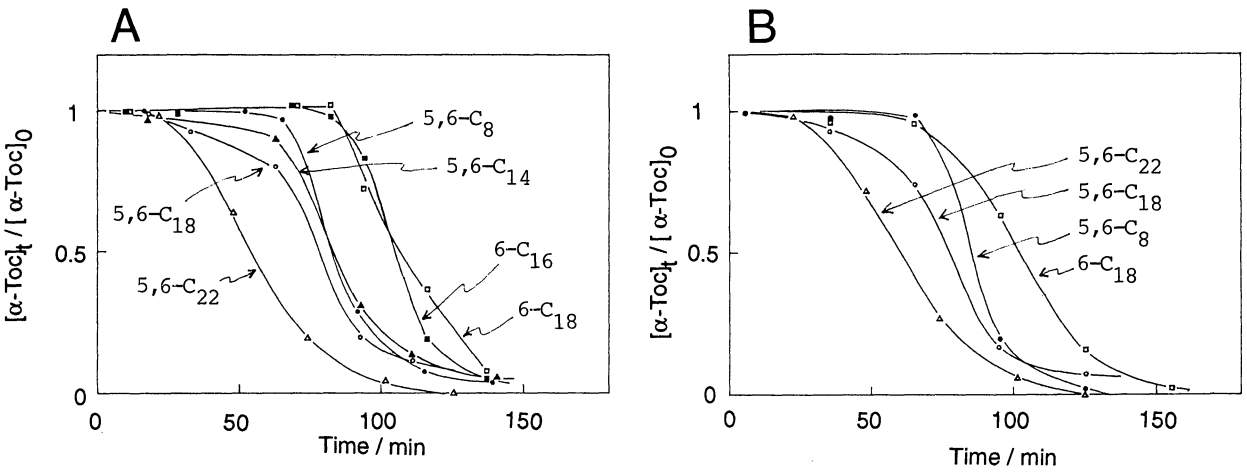


Fig. 5. Effect of side chain of ascorbic acid esters on the rate of consumption of α-tocopherol in the oxidation of soybean PC liposomes at 37°C under air. In Fig. 5B, the liposomes contained cholesterol (2.00 mM) as well as soybean PC (5.15 mM). [Soybean PC]=5.15 mM; [AAPH]=30 mM; [α-Toc]=50 μM; [ascorbic acid ester]=300 μM.

liposomes induced by AAPH in the presence of both α-tocopherol and one of the ascorbic acid esters. Figure 5B shows the results for soybean PC liposomes containing 28 mol% of cholesterol which reduces the fluidity of the liposomal membranes.[29] In the absence of ascorbic acid ester, α-tocopherol decreased lineary with time without any time lag. In every experiment, ascorbic acid esters delayed the consumption of α-tocopherol, but their sparing efficiencies were dependent on the number and length of fatty acid residues. The fatty acid esters only at 6 position spared α-tocopherol more efficiently, whereas the fatty acid esters at both 5 and 6 positions were less efficient and this effect was enhanced as the length of fatty acid portion became longer. These results, together with the facts that the side chain of the ascorbic acid esters incorporated into liposomal membranes had little effect on the inhibition of their oxidations and that α-tocopherol suppressed the oxidation more efficiently than ascorbic acid esters (Fig. 1), suggest that the fatty acid esters of ascorbic acid spared α-tocopherol primarily by reducing α-tocopherol radical to regenerate α-tocopherol but that it became less efficient as the number and length of the fatty acid side chain increase, probably because their mobilities within the liposomal membranes become slower. Figure 5B shows that cholesterol has little substantial effect on the mobility of fatty acid esters of ascorbic acid.

To obtain more information on the relative importance of regeneration of α-tocopherol by ascorbic acid ester, the effect of side chain of α-tocopherol was studied. The oxidations of soybean PC liposomes induced by AMVN and inhibited by α-tocopherol or PMC were carried out at 50 °C in the presence or absence of 6-*O*-palmitoylascorbic acid. The results are shown in Fig. 6. In the absence of 6-*O*-palmitoylascorbic acid, α-tocopherol and PMC were consumed linearly at the

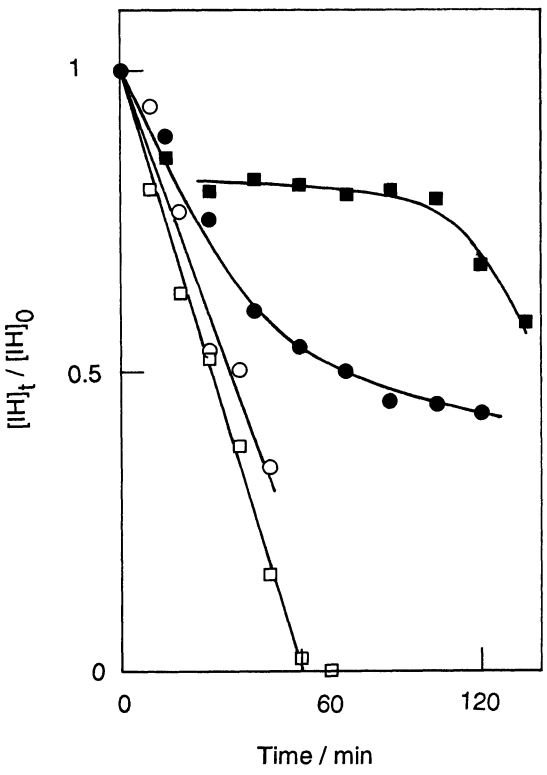


Fig. 6. Rates of consumption of α-tocopherol (○, ●) or PMC (□, ■) in the oxidations of soybean PC liposomes in the absence (open mark) and presence (solid mark) of 6-*O*-palmitoylascorbic acid at 50°C under air. 6-*O*-Palmitoylascorbic acid, when used, was incorporated into the soybean PC liposomes together with either α-tocopherol or PMC. [soybean PC]=5.0 mM; [AMVN]=2.0 mM; [α-Toc]=50 μM; [PMC]=50 μM; [6-C_{16}AsA]=150 μM.

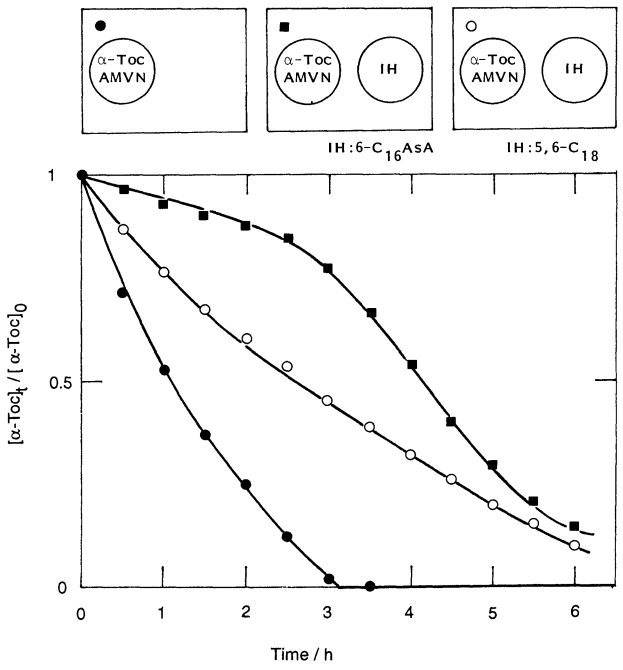


Fig. 7. Consumption of α-tocopherol during the oxidation of soybean PC liposomes (multilamellar vesicles) initiated with AMVN in the absence and presence of ascorbic acid esters incorporated into the different dimyristoyl PC liposomes, 37 °C. [Soybean PC]=5.15 mM; [AMVN]=2.0 mM; [α-Toc]=50 μM; [ascorbic acid ester]=150 μM.

similar rate, whereas in its presence, PMC was spared more efficiently than α-tocopherol. These results strongly suggest that α-tocopherol is spared primarily by the regeneration from α-tocopheryloxyl radical by ascorbic acid esters, not by preferential scavenging of peroxyl radicals by ascorbic acid esters. The second-order rate constants for the reactions of fatty acid esters of ascorbic acid with 5,7-diisopropyltocopheryloxyl radical in solution were obtained previously as 310 (5,6-C_{16}-AsA)—690 (6-C_{16}-AsA) $M^{-1}s^{-1}$ at 25 °C.[30]

Figure 7 shows the effect of number of side chain of fatty acid esters of ascorbic acid on their mobilities between the membranes. α-Tocopherol was incorporated into soybean PC liposomes together with AMVN and ascorbic acid ester, 6-*O*-palmitoylascorbic acid or 5,6-di-*O*-palmitoylascorbic acid, was incorporated into different dimyristoyl PC liposomes. In the absence of ascorbic acid ester, α-tocopherol was consumed linearly with time, but it was spared by ascorbic acid ester located into different liposomes. Figure 7 shows that the diester of ascorbic acid was less efficient than monoester in sparing α-tocopherol in the different liposomal membranes. Ascorbic acid ester located in the same liposomal membrane as α-tocopherol was more efficient in sparing α-tocopherol than that located in the different membranes.

In conclusion, the fatty acid esters of ascorbic acid incorporated into PC liposomal membranes acted as radical scavengers and suppressed the peroxidation of PC. Probably, the fatty acid groups are burried parallel to the phospholipid molecules and the hydroxyl group at the 2 and 3-positions must be located at the water–lipid interface. Their lateral diffusion becomes less facile as the fatty acid groups, which act as an anchor, increase in number and length. Similarly, the mobilities of ascorbic acid esters between the membranes become less efficient as the number and length of fatty acid group increase.

This study was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, the Asahi Glass Foundation for Industrial Technology, and the Nagase Science and Technology Foundation.

References

1) G. Scott, "Atmospheric Oxidation and Antioxidants," Elsevier, Amsterdam (1965).
2) B. Halliwell and J. M. C. Gutteridge, "Free Radicals in Biology and Medicine," Clarendon Press, Oxford (1985).
3) "Free Radicals in Liver Injury," ed by G. Poli, K. H. Cheeseman, M. U. Dianzani, and T. F. Slater, IRL Press, Oxford (1985).
4) "Oxidative Stress," ed by H. Sies, Academic Press, London (1987).
5) "Oxygen Radicals in Biology and Medicine," ed by M. G. Simic, K. A. Taylor, J. F. Ward, and C. von Sonntag, Plenum Pub. Corp., New York (1989).
6) "Medical, Biochemical, and Chemical Aspects of Free Radicals," ed by O. Hayaishi, E. Niki, M. Kondo, and T. Yoshikawa, Elsevier, Amsterdam (1989).
7) G. W. Burton and K. U. Ingold, *Acc. Chem. Res.*, **19**, 194 (1986).
8) E. Niki, *Chem. Phys. Lipids*, **44**, 227 (1987).
9) A. Bendich, L. J. Machlin, O. Scandurra, G. W. Burton, and D. D. M. Wayner, *Adv. Free Rad. Biol. Med.*, **2**, 419 (1986).
10) E. Niki, *World Rev. Nutr. Diet.*, **64**, 1 (1991).
11) M. Takahashi, J. Tsuchiya, and E. Niki, *J. Am. Chem. Soc.*, **111**, 6350 (1989).
12) L. R. C. Barclay, K. A. Baskin, K. A. Dakin, S. F. Locke, and M. R. Vingvist, *Can. J. Chem.*, **68**, 2258 (1990).
13) E. Niki, A. Kawakami, M. Saito, Y. Yamamoto, J. Tsuchiya, and Y. Kamiya, *J. Biol. Chem.*, **260**, 2191 (1985).
14) W. M. Cort, *Adv. Chem. Ser.*, **200**, 533 (1982).
15) M. Takahashi, E. Niki, A. Kawakami, A. Kumasaka, Y. Yamamoto, Y. Kamiya, and K. Tanaka, *Bull. Chem. Soc. Jpn.*, **59**, 3179 (1986).
16) Y. Yamamoto, E. Niki, Y. Kamiya, and H. Shimasaki, *Biochim. Biophys. Acta*, **795**, 332 (1984).
17) E. Niki, "Methods in Enzymology," ed by L. Packer, Academic Press, New York (1990), Vol. 186, pp. 100—108.
18) N. A. Porter, *Acc. Chem. Res.*, **19**, 262; *Adv. Free Rad. Biol. Med.*, **2**, 283 (1986).
19) H. W. Gardner, *Free Rad. Biol. Med.*, **7**, 65 (1989).
20) G. W. Burton and K. U. Ingold, *J. Am. Chem. Soc.*, **103**, 6472 (1981).

21) E. Niki, T. Saito, A. Kawakami, and Y. Kamiya, *J. Biol. Chem.*, **259**, 4177 (1984).
22) P. B. McCay, *Annu. Rev. Nutr.*, **5**, 323 (1985), and references cited therin.
23) E. Niki, *Ann. N. Y. Acad. Sci.*, **498**, 186 (1987).
24) H. Sies, *Int. J. Vitam. Nutr. Res., Suppl.*, **30**, 215 (1989).
25) E. Niki, A. Kawakami, Y. Yamamoto, and Y. Kamiya, *Bull. Chem. Soc. Jpn.*, **58**, 1971 (1985).
26) T. Doba, G. W. Burton, and K. U. Ingold, *Biochim. Biophys. Acta*, **835**, 298 (1985).
27) R. J. Mehlhorn, S. Sumida, and L. Packer, *J. Biol. Chem.*, **264**, 13448 (1989).
28) D. C. Liebler, K. L. Kaysen, and T. A. Kennedy, *Biochemistry*, **28**, 9772 (1989).
29) M. Takahashi, J. Tsuchiya, E. Niki, and S. Urano, *J. Nutr. Sci. Vitaminol.*, **34**, 25 (1988).
30) K. Mukai, M. Nishimura, A. Nagano, K. Tanaka, and E. Niki, *Biochim. Biophys. Acta*, **993**, 168 (1989).