37 °C). The conversion of 4·NaBr to 4·KBr is essentially instantaneous on the human time scale.

Figures 1-6 illustrate the different kinds of plots obtained (wavelength vs absorbance) involving various forms of **1-4**. Figure 1 corresponds to Table I run numbers 13-15, Figure 3, to run numbers 16-20, Figure 4, to run numbers 40-42, Figure 5, to run numbers 43-45, and Figure 6, to run numbers 46-50.

The solutions used for determining the ΔA values of Table III were prepared as follows. Aqueous 0.10 M buffer solutions were prepared to provide pH values of 6.0 (MES), 6.6 (MES), 7.0 (HEPES), 8.0 (CHES), and 9.0 (CHES) at 25 °C. To each of these was added an amount of 4·NaBr dissolved in dioxane to provide a final concentration of 0.10 mM of 4·NaBr. The volumes of dioxane were varied to achieve concentrations of 1%, 25%, and 50% by volume of dioxane. Thus three sets of reagent solutions were prepared, all being 0.10 mM in 4·NaBr. Each set comprised the five pH levels with buffer at 0.10 M concentration, but each set varied from one another only in dioxane concentrations. To 2 mL of each sample of reagent was added 0.10 mL of 1.0 M NaCl or 1.0 M KCl in water in an optical cuvette to give a final salt concentration of 50 mM. After mixing, the absorbance of each solution was measured on a Beckman DU-8 spectrophotometer at 300-700 nm. The absorbances at λ = 450 nm were read and are recorded in Table III.

Scavenging of Radicals by Vitamin E in the Membranes As Studied by Spin Labeling

Mareyuki Takahashi, Jyunichi Tsuchiya, and Etsuo Niki*

Contribution from the Department of Reaction Chemistry, Faculty of Engineering, The University of Tokyo, Hongo, Tokyo 113, Japan. *Received August 18, 1988*

Abstract: The scavenging of radicals by α-tocopherol in the liposomal membranes was studied by using a spin label technique. It has been shown experimentally that α-tocopherol can scavenge lipophilic radicals that are closer to the membrane surface more efficiently than those that reside deep in the lipid region of the bilayer membrane. It was also found that α-tocopherol effectively scavenges aqueous oxygen radicals attacking from oustide of the membrane.

It is now generally accepted that the autoxidation of lipids in biological membranes is associated with a variety of important pathological events and aging, and the generation, reactions, and scavenging of radicals in the membranes have received great interest.[1] Vitamin E (tocopherols) is known to function as a lipophilic, chain-breaking antioxidant.[2] The chemistry of the mode of action of tocopherols in the homogeneous solution is now fairly well understood,[3,4] but the detailed mechanism for the inhibition of oxidation by tocopherols in the membrane has not been clearly elucidated. It has been shown that α-tocopherol, the most potent tocopherol, is retained in the membrane[5] and suppresses the oxidations of phospholipid liposomal membranes[6-9] and erythrocyte membranes,[10-12] although the antioxidant activity of α-tocopherol is considerably smaller in the membranes than in the homogeneous solution.[13] One of the important questions is how the phenolic hydrogen of α-tocopherol, which is accepted to be located at the surface of the membrane,[14,15] can interact with lipid peroxyl radical, which must reside deep in the bilayer membranes. The objective of the present study is to study the scavenging of radicals by α-tocoperhol in the phosphatidylcholine liposomal membranes by using a spin label technique.[16,17]

Experimental Section

Materials. Commercial soybean phosphatidylcholine (PC) purchased from Daigo Chemical Co. (Osaka) was purified with alumina and silica gel columns as previously.[18] Dimyristoylphosphatidylcholine (14:0 PC) obtained from Sigma Chemical Co. (St. Louis, MO) and dilinoleoylphosphatidylcholine (18:2 PC) obtained from Nihon Seika Co. (Osaka) were used without further purification. (*R,R,R*)-α-tocopherol (α-Toc), 2,2,5,7,8-pentamethyl-6-chromanol (PMC), (*R,R,R*)-δ-tocopherol (δ-Toc), and tocol (Toc) were kindly supplied from Eisai Co. (Tokyo) and used as received. A water-soluble radical initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and oil-soluble radical initiator, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), were obtained from Wako Pure Chemical Co. (Osaka).

Five kinds of *N*-oxyl-4,4′-dimethyloxazolidine derivatives of stearic acid, 5-NS, 7-NS, 10-NS, 12-NS, and 16-NS were used as spin probes, where the nitroxide group is attached at various positions along the fatty acid chain to situate the nitroxide groups at different depths in the hydrophobic interior of the phospholipid bilayer. The spin probes were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Methods. The liposomal membranes were prepared as follows as reported previously.[18] PC and lipid-soluble additives such as antioxidants, spin probes, or AMVN, when necessary, were dissolved in chloroform, and the solution was transferred to a round-bottom flask. Chloroform was removed on a water aspirator by using a rotary vacuum evaporator to obtain a thin film on the flask wall. An appropriate amount of 0.1 M NaCl aqueous solution was added, and the film was slowly peeled off by shaking to obtain white, milky, multilamellar liposome suspensions. They were sonicated to obtain unilamellar liposomal membranes and subjected to oxidation when AAPH was used as a radical initiator. The

(1) Many books, reviews, and proceedings have been published on these subjects, for example: (a) *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1976-1984; Vol. I-VI. (b) *Methods in Enzymology*; Packer, L., Ed.; Academic Press: Orlando, FL, 1984; Vol. 105. (c) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*; Clarendon Press: Oxford, 1985. (d) *Oxidative Stress*; Sies, H., Ed.; Academic Press: London, 1985. (e) *Oxygen Radicals in Biology and Medicine*; Simic, M. G., Ed.; Plenum Press: New York, 1989. (f) *Medical, Biochemical and Chemical Aspects of Free Radicals*; Hayaishi, O., Niki, E., Kondo, M., Yoshikawa, T., Eds.; Elsevier: Amsterdam, 1989.

(2) See, e.g.: (a) *Vitamin E, A Comprehensive Treatise*; Machlin, L. J., Ed.; Marcel Dekker: New York, 1980. (b) *Biology of Vitamin E*; Ciba Foundation Symposium 101: Pitman: London, 1983. (c) *Clinical and Nutritional Aspects of Vitamin E*; Hayaishi, O., Mino, M., Eds.; Elsevier: Amsterdam, 1987.

(3) Burton, G. W.; Ingold, K. U. *Acc. Chem. Res.* **1986**, *19*, 194-201.

(4) Niki, E. *Chem. Phys. Lipids* **1987**, *44*, 227-253.

(5) Niki, E.; Kawakami, A.; Saito, M.; Yamamoto, Y.; Tsuchiya, J.; Kamiya, Y. *J. Biol. Chem.* **1985**, *260*, 2191-2196.

(6) Weenen, H.; Porter, N. A. *J. Am. Chem. Soc.* **1986**, *19*, 262-268.

(7) Niki, E.; Kawakami, A.; Yamamoto, Y.; Kamiya, Y. *Bull. Chem. Soc. Jpn.* **1985**, *58*, 1971-1975.

(8) Doba, T.; Burton, G. W.; Ingold, K. U. *Biochim. Biophys. Acta* **1985**, *835*, 298-303.

(9) Barclay, L. R. C. *J. Biol. Chem.* **1988**, *263*, 16138-16142.

(10) Yamamoto, Y.; Niki, E.; Eguchi, J.; Kamiya, Y.; Shimasaki, H. *Biochim. Biophys. Acta* **1985**, *819*, 29-36.

(11) Yamamoto, Y.; Niki, E.; Kamiya, Y.; Miki, M.; Tamai, H.; Mino, M. *J. Nutr. Sci. Vitaminol.* **1986**, *32*, 475-479.

(12) Miki, M.; Tamai, H.; Mino, M.; Yamamoto, Y.; Niki, E. *Arch. Biochem. Biophys.* **1987**, *258*, 373-380.

(13) Niki, E.; Takahashi, M.; Komuro, E. *Chem. Lett.* **1986**, 1573-1576.

(14) Perly, B.; Smith, I. C. P.; Hughes, L.; Burton, G. W.; Ingold, K. U. *Biochim. Biophys. Acta* **1985**, *819*, 131-135.

(15) Kagan, V. E.; Quinn, P. J. *Eur. J. Biochem.* **1988**, *171*, 661-667.

(16) Ohnishi, S.; McConnell, H. M. *J. Am. Chem. Soc.* **1965**, *87*, 2293.

(17) Smith, I. C. P.; Schreier-Muccillo, S.; Marsh, D. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1976; Vol. I, pp 149-197.

(18) Yamamoto, Y.; Niki, E.; Kamiya, Y.; Shimasaki, H. *Biochim. Biophys. Acta* **1984**, *795*, 332-340.

0002-7863/89/1511-6350$01.50/0

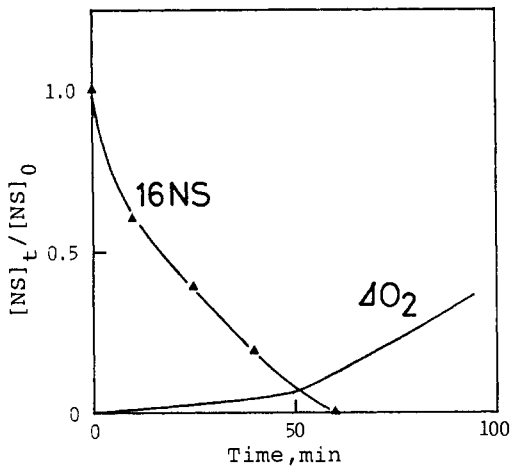

Figure 1. Decrease of 16-NS incorporated into 18:2 PC liposomes and oxygen uptake during the oxidation induced by AAPH at 37 °C under air. [18:2 PC] = 5.15 mM, [AAPH] = 40 mM, [16-NS] = 100 μM.

spin probes were incorporated into the liposomal membranes as follows. The chloroform solution containing an appropriate amount of spin probe was transferred to round-bottom flask, and solvent was slowly removed under reduced pressure to obtain a thin film on the flask wall. The aqueous dispersions of unilamellar PC liposomes were then added to the above flask, and the mixture was gently shaken at the room temperature. In the experiment using AMVN, spin probes were dissolved in chloroform simultaneously with PC and incorporated into the liposomal membranes as described above.

In the experiments using AAPH, an appropriate amount of AAPH was added to the liposomal suspensions at the beginning of the measurement, and oxidation was carried out at 37 °C under air and under laboratory light. When AMVN was used as a radical initiator, liposomal suspensions containing AMVN were incubated at 50 °C under air and under laboratory light. The reaction solution was taken into the quartz flat cell for ESR measurement with some intervals of time. The ESR spectra were recorded on an X-band JEOL FE1X spectrometer using a quartz flat cell at 37 °C.

The concentration of spin probes in the liposomal membranes was measured from their ESR signals by using standard calibration chart.

The concentration of α-tocopherol in liposomal membranes was determined with a high-pressure liquid chromatographer equipped with an electrochemical detector set at +800 mV vs Ag/AgCl. An ODS column (Senshu pak, particle size 5 μm) was used, and methanol containing 0.05 M $NaClO_4$ was delivered as an eluent at 1 mL/min.

Autoxidation of liposomes were conducted in an automatic recording gas absorption apparatus equipped with a sensitive pressure transducer (Toyoda Model PWA-5). In some experiments oxygen uptake was measured by following the dissolved oxygen concentration in the reaction solutions automatically with an oxygen electrode (Yellow Springs Instrument Co., OH, Model YSI 53).

Results

When AAPH was added to the aqueous suspensions of soybean or 18:2 PC liposomes, it induced oxidation, and a constant rate of oxygen uptake was observed without any appreciable induction period. The spin probe suppressed the oxidation of soybean and 18:2 PC liposomal membranes. As shown in Figure 1, 16-NS incorporated into 18:2 PC liposomal membranes suppressed the AAPH-induced oxidation and produced a clear induction period. The 16-NS was consumed with time, and when it was depleted the induction period was over and a fast oxidation proceeded at the similar rate as that without 16-NS. Substantially the same results were observed for all the spin probes used in this study. The results of oxidation of 18:2 PC liposomal membranes in the aqueous dispersions induced by AAPH are summarized in Table I. As observed for α-tocopherol, the length of the induction period was proportional to the spin probe concentration and, as shown in Figure 2, NS gave the same induction period as α-tocopherol, suggesting that each molecule of NS scavenges two molecules of radicals.[3,5,19-21] However, the rate of oxidation during the induction period inhibited by NS was much larger than that inhibited by α-tocopherol, suggesting that α-tocopherol scavenges radicals much faster than the spin probe.[5] The kinetic chain length during the induction period was larger than 1, suggesting that some, though not long, chain propagation sequences take place even during the induction period and that NS scavenges lipid or lipid peroxyl radicals predominantly rather than the initiating radicals derived from AAPH.

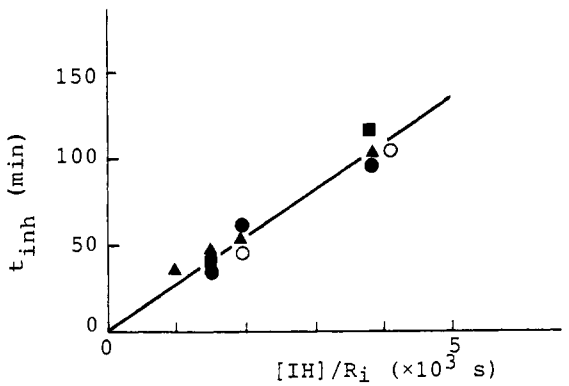

Figure 2. Plot of the induction period (t_{inh}) against $[IH]/R_i$ in the oxidation of 5.15 mM 18:2 PC liposome initiated with 40 mM AAPH: ● 5-NS; ■, 12-NS; ▲, 16-NS; ○, α-Toc.

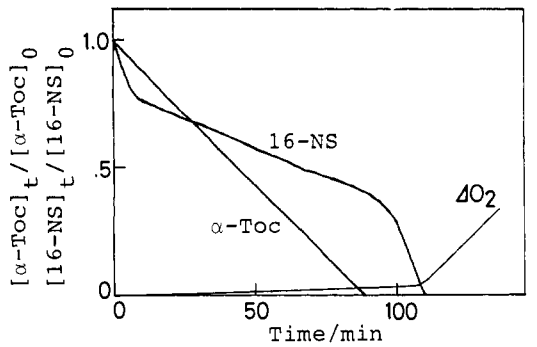

Figure 3. Decrease of 16-NS incorporated into 5.15 mM soybean PC liposomes during the oxidation initiated with 300 mM AAPH at 37 °C under air. [α-Toc] = 1.0 mM, [16-NS] = 86.7 μM.

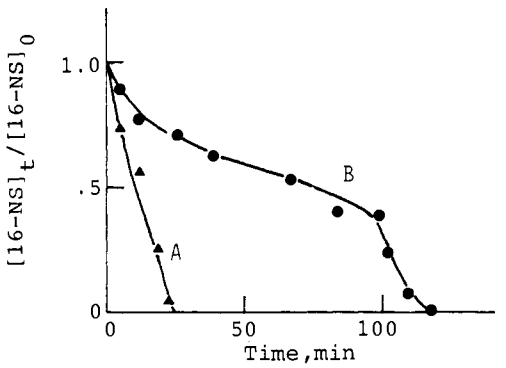

Figure 4. Consumption of 16-NS incorporated into soybean PC liposomes during the oxidation initiated with 300 mM AAPH in the absence (A) and presence (B) of 1.0 mM α-tocopherol at 37 °C under air. [soy PC] = 5.15 mM, [16-NS] = 86.7 μM.

Figure 3 shows the results of oxidation of soybean PC liposomes induced by AAPH in the presence of both 16-NS and α-tocopherol incorporated simultaneously into the PC liposomal membranes. α-Tocopherol was consumed at a constant rate, while 16-NS decreased slowly when α-tocopherol was present and then decayed rapidly after α-tocopherol was depleted. Figure 4 compares the

(19) Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6472-6477.

(20) Niki, E.; Saito, T.; Kawakami, A.; Kamiya, Y. *J. Biol. Chem.* **1984**, *259*, 4177-4182.

(21) Pryor, W. A.; Strickland, T.; Church, D. F. *J. Am. Chem. Soc.* **1988**, *110*, 2224-2229.

Table I. Oxidation of 5.15 mM 18:2 PC Liposomal Membranes in Aqueous Dispersions Induced by AAPH in the Presence of NS and α-Tocopherol at 37 °C

[AAPH], mM	NS	[NS], μM	t_{inh}, min	$10^{-7}R_i$(aq),[a] M/s	$10^{-7}R_{inh}$, M/s	$(kcl)_{inh}$[b]	$10^{-7}R_p$, M/s	$(kcl)_p$[c]
20	5-	100	100	0.26	4.4	16.9	11.7	45.0
20	12-	100	115	0.26	6.9	26.5	13.3	51.2
20	16-	100	101	0.26	2.8	10.7	11.9	45.8
40	5-	010	58	0.52	5.3	10.2	13.5	26.0
40	16-	100	56	0.52	4.7	9.0	15.7	30.2
50	5-	100	36	0.65	10.0	15.4	17.6	27.1
50	12-	100	42	0.65	2.8	4.3	12.3	18.9
40	16-	50	39	0.52	7.1	13.7	15.7	30.2
40	16-	200	145	0.52	3.6	6.9	13.7	26.3
40	α-Toc	100	45	0.52	1.0	1.9	14.1	27.1

[a] Rate of free-radical generation in aqueous phase; $R_i(aq) = 1.3 \times 10^{-6}$[AAPH] s^{-1}. See ref 23. [b] Kinetic chain length during the induction period. [c] Kinetic chain length after the induction period.

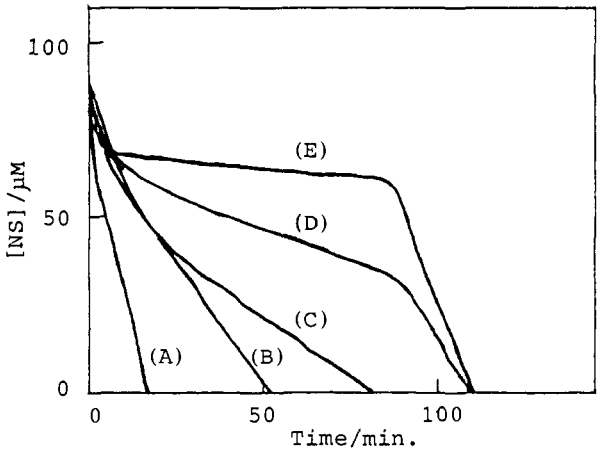

Figure 5. Consumption of 16-NS incorporated into 5.15 mM soybean PC liposomes during the oxidation initiated with 300 mM AAPH at 37 °C under air in the absence (A) and presence of tocol (B), δ-tocopherol (C), α-tocopherol (D), or PMC (E).

rates of consumption of 16-NS during the oxidation of soybean PC liposomes induced by AAPH in the presence and absence of α-tocopherol. It shows clearly that α-tocopherol spares 16-NS at the expense of itself. Similar sparing effect of α-tocopherol was observed for other NSs and also when these spin probes were incorporated into 14:0 PC liposomal membranes, which are not oxidized.

Figure 5 shows the rate of consumption of 16-NS incorporated into soybean PC liposomes during the oxidation initiated with AAPH in the absence and presence of several kinds of antioxidants. It shows that every antioxidant suppressed the consumption of 16-NS, but the sparing efficiency varies considerably among the antioxidants.

Although α-tocopherol suppressed the consumption of the spin probes very efficiently during the oxidation of PC liposomes induced by AAPH, the sparing action was less efficient when the oxidation was initiated by a lipid-soluble AMVN which was incorporated into the liposomal membranes simultaneously with the spin probe, that is, the spin probes were consumed at considerable rates even in the presence of α-tocopherol when the radicals were generated initially within the liposomal membranes. Interestingly, the sparing efficiency depended on NS and, as shown in Figure 6, α-tocopherol reduced the rate of consumption of 5-NS more efficiently than that of 16-NS.

Discussion

The above results show that the spin probes NS function as antioxidants in the oxidations of PC liposomal membranes induced by free-radical initiator when they are incorporated into the same membranes. Furthermore, α-tocopherol incorporated into the liposomes simultaneously with the spin probe acts as a stronger antioxidant than the spin probe and spares the consumption of the spin probe. It is not clear at present how the nitroxide radical is lost.

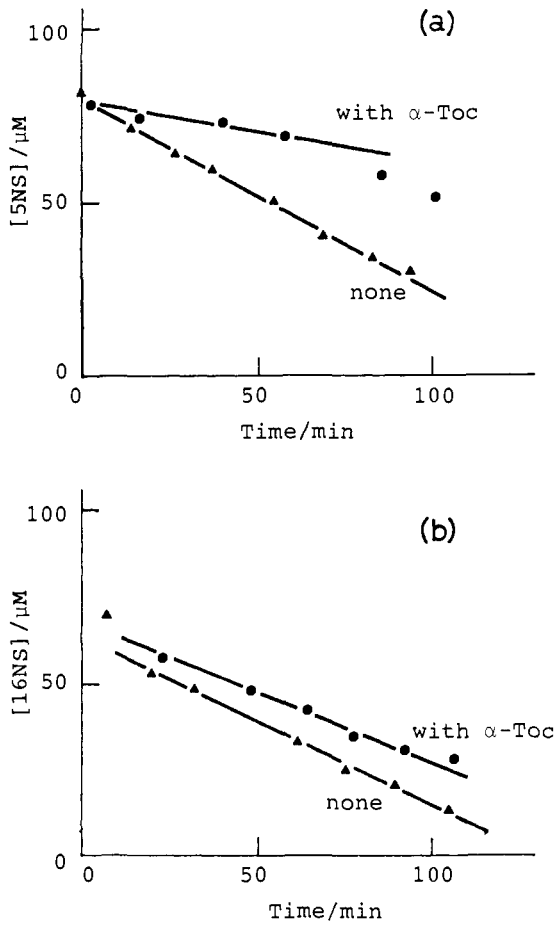

Figure 6. Consumption of spin probes 5-NS (a) and 16-NS (b) incorporated into 10.3 mM 14:0 PC liposomes in the presence (●) and absence (▲) of α-tocopherol during the oxidation initiated with 4.0 mM AMVN at 50 °C under air. [α-Toc] = 100 μM, [NS] = 86.7 μM.

The efficiency of α-tocopherol for sparing the consumption of spin probe was estimated from the eq 1, where R_0 and R_t represent

$$P = \frac{R_0 - R_t}{R_0} \qquad (I)$$

the rates of consumption of the spin probe in the absence and presence of α-tocopherol, respectively. The rate of consumption of the spin probe was measured at its steady state when α-tocopherol was consumed at a constant rate. Figure 7 shows the sparing efficiency of α-tocopherol for the AMVN-induced consumption of different NS incorporated into soybean PC and 14:0 PC liposomal membranes. It shows that the sparing efficiency decreases as the nitroxide group goes deeper into the interior of the phospholipid bilayer. Figure 7 also shows that the spin probes were consumed even when they were incorporated into saturated

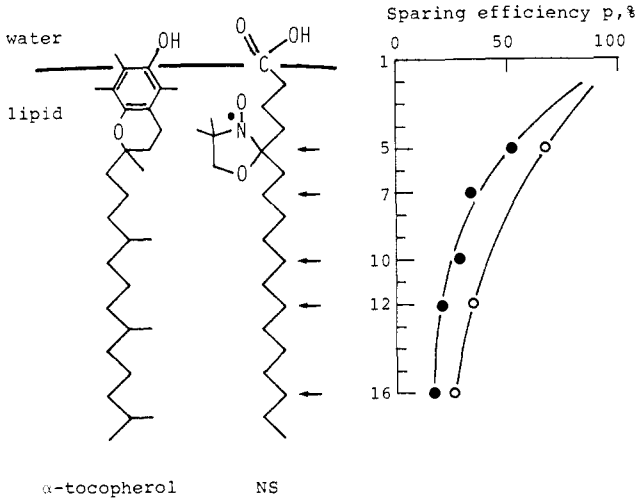

Figure 7. Probable location of α-tocopherol and NS in the liposomal membranes and the sparing efficiency by α-tocopherol for the consumption of NS induced by AMVN in soybean PC (○) and 14:0 PC (●) liposomal membranes at 50 °C.

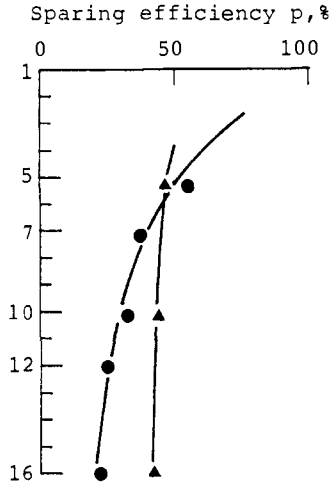

Figure 8. Sparing efficiency by α-tocopherol (●) and PMC (▲) for the consumption of NS induced by AMVN in 14:0 PC liposomal membranes at 50 °C. [14:0 PC] = 10.3 mM, [AMVN] = 4.0 mM, [IH] = 1.0 mM, [NS] = 86.7 μM.

14:0 PC liposomal membranes which do not undergo oxidation. The sparing efficiency was lower in 14:0 PC liposomes than in soybean PC liposomes. Figure 8 shows a similar plot for α-tocopherol and PMC.

α-Tocopherol scavenges the peroxyl radicals by donating a phenolic hydrogen atom at the 6-position, and it is accepted that the phenolic hydrogen of α-tocopherol is located at or near the interface of the membranes as illustrated in Figure 7. On the other hand, the nitroxide groups of the spin probes NSs must be located within the interior of bilayer. Therefore, α-tocopherol must spare the spin probes more efficiently against the aqueous radicals attacking from outside of the membranes than against the lipophilic radicals generated within the membranes. However, as mentioned above, the kinetic chain length of the oxidation in the presence of α-tocopherol during the induction period was considerably longer than 1, suggesting that not all of the aqueous radicals generated from AAPH are scavenged by α-tocopherol at the membrane surface and that α-tocopherol scavenges lipophilic radicals as well within the membranes. This is not surprising since α-tocopherol suppresses the oxidation of PC liposomes induced by AMVN which generates free radicals initially within the interior of the membranes. It may be noteworthy that hydrophilic antioxidants such as ascorbic acid (vitamin C) and uric acid are not able to suppress the AMVN-initiated oxidation of liposomes although they are effective antioxidants for the AAPH-induced oxidation.[7]

The results shown in Figure 7 suggest that α-tocopherol can scavenge lipophilic radicals that are closer to the surface of the membrane more efficiently than those that reside deep in the lipid region of the bilayer membrane. Figure 7 also suggests that α-tocopherol scavenges the phospholipid peroxyl radicals easier than the peroxyl radicals derived from AMVN, probably because lipophilic AMVN resides largely in the more fluid, central region of the PC membranes,[22] while 18:2 PC and soybean PC produce the peroxyl radicals at 9- and 13-positions.

One of the structural characteristics of α-tocopherol is that it has a long phytyl side chain.[3,5] The effect and role of the phytyl side chain have received much attention, and it is known that the phytyl side chain is required for the incorporation and retainment of α-tocopherol within the membranes.[2] Figures 5 and 8 show that the phytyl side chain of α-tocopherol decreases the antioxidant activity probably by decreasing the mobility of α-tocopherol within the membranes.

The ratio of α-tocopherol to phospholipid used in the present study is about 1 to 5, which is much higher than the physiological condition. There is no direct experimental evidence if α-tocopherol is incorporated evenly into the liposomal membranes. However, the experimental findings that α-tocopherol used suppressed the AMVN-initiated oxidations of liposomal membranes and decreased the membrane fluidity[22] dose dependently and monotonously suggest that most of α-tocopherol is incorporated into the liposomal membranes evenly.

It has been argued extensively how the phenolic hydrogen of α-tocopherol and phospholipid fatty acid peroxyl radicals which must reside at the surface and interior of the membranes, respectively, interact with each other. This study does not answer the question whether the phospholipid peroxyl radicals float up or α-tocopherol goes in or both. However, the results given above show, for the first time, that α-tocopherol scavenges radicals less effectively as the radicals go deeper into the interior of the membranes and that α-tocopherol is better prepared against the radicals attacking from outside of the membranes.

Acknowledgment. We gratefully acknowledge the gift of tocopherols and PMC from Eisai Co., Tokyo. This study was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 62470078 and 62607003).

Registry No. 14:0 PC, 18194-24-6; 18:2 PC, 6542-05-8; α-TOC, 59-02-9.

(22) Takahashi, M.; Tsuchiya, J.; Niki, E. *J. Nutr. Sci. Vitaminol.* **1988**, *34*, 25–34.
(23) Niki, E.; Yoshikawa, Y.; Yamamoto, Y.; Kamiya, Y. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 471–477.