Review

Dynamics of Lipid Peroxidation and Antioxidion of \( \alpha \)-Tocopherol in Membranes

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Summary  The dynamics of initiation and inhibition of lipid peroxidation by \( \alpha \)-tocopherol (\( \alpha \)-Toc) in membranes were investigated under biological conditions using phosphatidylcholine liposomes. First, I examined how superoxide generated in the bulk water phase is able to induce lipid peroxidation in the inner hydrophobic region of the membrane. Second, I studied the localization of the antioxidant OH group of \( \alpha \)-Toc in membranes and its lipid radical-trapping dynamics. Third, I investigated how \( \alpha \)-Toc that is oxidized during radical trapping in membranes is recycled by ascorbic acid (AsA) in the bulk water phase. Finally, I studied the deactivation by \( \alpha \)-Toc of singlet oxygen (\( ^1 \text{O}_2 \)), which was generated by photoradiation at the membrane surface, in the hydrophobic membrane inner region, and in bulk water, and measured the \( ^1 \text{O}_2 \) deactivating rate constant of \( \alpha \)-Toc in membranes considering: the concentration and mobility of \( \alpha \)-Toc molecule in membranes, especially those of its active OH moiety located at the membrane domains, such as the membrane surface polar zone, inner hydrogen belt, and hydrophobic core, and the dielectric constant reflecting the reactivity of the OH moiety and \( ^1 \text{O}_2 \) in the membrane domains where they interact.

Key Words  \( \alpha \)-tocopherol, lipid peroxidation, singlet oxygen, liposomes, \( \beta \)-carotene

The superoxide anion radical (\( \text{O}_2^- \)), which is produced by aerobic organisms during metabolism and in processes involving phagocytosis or responses to xenobiotics, is widely held to be a major initiator of biological damage resulting in pathophysiological events associated with a variety of diseases. However, because \( \text{O}_2^- \) is only weakly reactive towards most organic compounds, its biological effectiveness is usually explained by the formation of more reactive species derived from it. A particular example is the oxidation of unsaturated lipids, an important event in pathophysiology, which cannot be initiated by \( \text{O}_2^- \). Conversion of \( \text{O}_2^- \) to more reactive intermediates requires the participation of metal catalysis, of which iron is the most important in biological systems. In this paper, I first describe the initiation dynamics of lipid peroxidation in membranes exposed to \( \text{O}_2^- \) in the presence of iron (1–6).

Second, because vitamin E functions as the major hydrophobic antioxidant in biological membranes by trapping the lipid peroxy radical and inactivating singlet oxygen, I describe the antioxidant dynamics of \( \alpha \)-tocopherol (\( \alpha \)-Toc) in membranes from the perspective of the location of the OH group of \( \alpha \)-Toc in the membrane and its local concentration, mobility, and reactivity (6–17).

1. Initiation Dynamics of Lipid Peroxidation in Membranes

Lipid peroxidation usually proceeds by the following free-radical chain reaction:

\[
\text{LH} \rightarrow \text{L}^\cdot \quad \text{(initiation reaction)} \]

\[
\text{L}^\cdot + \text{O}_2^- \rightarrow \text{LOO}^\cdot \quad \text{(propagation reaction)} \]

\[
\text{LOO}^\cdot + \text{LH} \rightarrow \text{LOOH} + \text{L}^\cdot \quad \text{(propagation reaction)}
\]

where \( \text{LOO}^\cdot \) is the lipid peroxy radical and \( \text{LOOH} \) is the lipid hydroperoxide. I investigated how water-soluble \( \text{O}_2^- \) and iron can initiate lipid peroxidation in the hydrophobic membrane core, where the double bonds of polyunsaturated fatty acyl chains are located, and what oxygen species derived from them directly abstract hydrogen from LH to trigger the initiation step.

To determine the active oxygen species that participate in the induction of lipid peroxidation in membranes exposed to enzymatically generated \( \text{O}_2^- \), I used egg yolk phosphatidylcholine (EYPC) liposomes as a model membrane, and xanthine and xanthine oxidase (X-XO) as an \( \text{O}_2^- \) generation system (5). As shown in Table 1, the complete system, made up of EYPC liposomes with or without dicetylphosphate (DCP) or stearylamine (SA), X-XO, and Fe\( ^{3+} \)-nitritoltriacetate (NTA) rapidly consumed oxygen, indicating the occurrence of lipid peroxidation. The involvement of \( \text{O}_2^- \) and lipophilic free radicals in this process was demonstrated by the complete inhibition of oxygen uptake by addition of superoxide dismutase (SOD) or incorporation of \( \alpha \)-tocopherol (data not shown) into the lipid membrane. Similarly, no oxygen was consumed if Fe\( ^{3+} \)-NTA

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or XO were not included, indicating that lipid peroxidation was not induced directly by either O$_2^-$ or Fe$^{3+}$-NTA. Furthermore, addition of catalase to the complete system showed no inhibitory effect, but rather a slight acceleration of lipid peroxidation, and addition of H$_2$O$_2$ to the complete system strongly lowered the rate of lipid peroxidation. These results indicate that H$_2$O$_2$ and the OH radical (HO·) derived from it by the Fenton reaction (H$_2$O$_2$+Fe$^{3+}$→OH+Fe$^{2+}$+OH$^-$) are not direct inductants of lipid peroxidation. The inability of H$_2$O$_2$ to induce membrane peroxidation even in the presence of Fe$^{3+}$-NTA and X-XO can be attributed to the decrease in Fe$^{2+}$-NTA, presumably because Fe$^{2+}$-NTA reduced by O$_2^-$ is re-oxidized by H$_2$O$_2$.

Oxygen consumption occurred in the control system with EYPC liposomes, which contained as little as 3 μM EYPC hydroperoxide (PC-OOH) in 1 mM EYPC, but not in the system with liposomes prepared from EYPC pre-treated with triphenylphosphine (TPP), which reduces endogenous PC-OOH to the corresponding alcohol (PC-OH). Incorporation of increasing amounts of PC-OOH into the liposomes led to increases in rates of peroxidation (lower tier in Table 1). This indicates that endogenous PC-OOH was indispensable for the induction of Fe$^{3+}$-NTA/X-XO-dependent lipid peroxidation.

From these results, I proposed a possible initiation mechanism and dynamics of Fe$^{3+}$-chelate-induced lipid peroxidation in liposomes exposed to generated O$_2^-$ (Fig. 1). O$_2^-$ generated by X-XO in the water phase reduces Fe$^{3+}$-chelate (Fe$^{3+}$-X) to Fe$^{2+}$-X at the membrane surface. Fe$^{2+}$-X also reacts with the OOH group of PC-OOH exposed at the membrane surface, and cleaves it in the PC-alkoxyl radical (PC-O·) by a Fenton-like reaction (PC-OOH+Fe$^{2+}$-X→PC-O+$\overset{\cdot}{Fe}^{3+}$-X+OH$^-$). The resulting alkoxyl radical moiety of PC-O· penetrates into the hydrophilic region of the membrane and triggers the initiation of lipid peroxidation by abstraction of hydrogen from the unsaturated moieties of fatty acids of EYPC.

The proposed mechanism (Fig. 1), as described above for the O$_2^-$/Fe$^{3+}$-chelate-induced PC-OOH-dependent lipid peroxidation, was confirmed by comparing the membrane charge-dependent promoting properties of four Fe$^{3+}$-chelates: Fe$^{3+}$-NTA, Fe$^{3+}$-EDTA, Fe$^{3+}$-EGTA, and Fe$^{3+}$-DTPA (Fig. 2) (5). The orders of their activities in liposomes with different charges were Fe$^{3+}$-EGTA>Fe$^{3+}$-EDTA>Fe$^{3+}$-NTA>Fe$^{3+}$-X in negatively charged DCP-EYPC liposomes, and Fe$^{3+}$-NTA>Fe$^{3+}$-EDTA>Fe$^{3+}$-EGTA>Fe$^{3+}$-DTPA in positively charged SA-EYPC liposomes (upper row in Fig. 2). Fe$^{3+}$-DTPA was essentially inactive in liposome systems with either charge.

The middle row in Fig. 2 shows the rates of degradation of PC-OOH incorporated into liposome membranes with negative and positive charges by the O$_2^-$ generation system (X-XO) and Fe$^{3+}$-chelates. The orders of the degradation rates were Fe$^{3+}$-EDTA>Fe$^{3+}$-NTA>Fe$^{3+}$-X in negatively charged DCP-DMPC liposomes, and Fe$^{3+}$-NTA>Fe$^{3+}$-EDTA>Fe$^{3+}$-EGTA>Fe$^{3+}$-DTPA in positively charged SA-DMPC liposomes. This corresponded closely with their rates of promotion of lipid peroxidation in charged membranes (upper row in Fig. 2).

The interaction of Fe$^{3+}$-chelates with liposome membranes was also examined by measuring their modifications of the zeta potentials of liposome membranes with negative and positive surface charges. As shown by the bottom row in Fig. 2, the zeta potential of negatively charged DCP-EYPC liposomes was increased by Fe$^{3+}$-NTA and Fe$^{3+}$-EGTA but not changed by Fe$^{3+}$-EDTA or Fe$^{3+}$-DTPA, and the zeta potential of positively charged
The modulating effects of Fe$_3$-SA-EYPC liposomes was decreased by Fe$_3$ around the polyunsaturated bonds of fatty acids in relation with O$_2^-$. Lipid peroxidation and PC-OOH degradation, which corresponded well with their binding to the Fe$_3$-chelates on the zeta potentials of liposomes with negative and positive charges (5, 6). The concentrations of reactants for lipid peroxidation were 1 mM EYPC liposomes containing 0.2 mM charged lipids, 30 µM xanthine, 1 mM/L of xanthine oxidase, and 30 µM Fe$_3$-chelate in 10 mM HEPES buffer (pH 7.0). The concentrations of reactants for PC-OOH degradation were 1 mM DMPC liposomes containing 0.2 mM charged lipids, 30 µM xanthine, 1 mM/L of xanthine oxidase, and 30 µM Fe$_3$-chelate in 10 mM HEPES buffer (pH 7.0). Reduction rates of Fe$_3$-chelate by O$_2^-$ were 1.7 µM/min (Fe$_3$-NTA), 0.7 µM/min (Fe$_3$-EDTA), 1.7 µM/min (Fe$_3$-EGTA), and 0.3 µM/min (Fe$_3$-DTPA). The mixtures for measurement of the zeta-potentials were 1 mM EYPC liposomes containing 0.2 mM charged lipids, and 200 µM Fe$_3$-chelate in 10 mM HEPES buffer (pH 7.4) containing 10 mM NaCl.

SA-EYPC liposomes was decreased by Fe$_3$-NTA and Fe$_3$-EDTA but not by Fe$_3$-EGTA or Fe$_3$-DTPA. The modulating effects of Fe$_3$-chelates on the zeta potentials, which corresponded well with their binding to the liposomes (data not shown), were also well correlated with their membrane charge-dependent initiations of lipid peroxidation and PC-OOH degradation.

To confirm the penetration of the alkoxy radical moiety of PC-O- derived from degraded PC-OOH by the reaction with O$_2^-$ and Fe$_3$-chelate, we labeled the region around the polyunsaturated bonds of fatty acids in EYPC liposomes with the spin probe 12-(N-oxyl-4,4'-dimethyloxasolidine-2yl)stearic acid (12-NS) and measured the effect of the decrease of its ESR spectrum on its reaction with penetrating radicals (3). As shown in Fig. 3, addition of an O$_2^-$ generation system (X-XO) or Fe$_3$-chelates individually to DCP-EYPC liposomes not treated with TPP caused rapid initial decreases of 24% and 38%, respectively, in the intensity of the ESR spectrum of the spin probe. The partial decrease observed may have been due to the reaction with 12-NS not incorporated into the deep inner region of the membranes. The decrease of the ESR spectrum due to O$_2^-$ occurred initially but not continuously, indicating that negatively charged O$_2^-$ could not penetrate into negatively charged membranes. Addition of both X-XO and Fe$_3$-chelates to liposomes prepared from EYPC not treated with TPP resulted in a continuous decrease of the spectrum. However, the spectrum decreased only initially and partially on addition of both X-XO and Fe$_3$-chelates to DCP-EYPC liposomes prepared from PC-OOH-free EYPC by treatment with TPP. These results indicate that three components, X-XO, Fe$_3$-chelates, and PC-OOH, are necessary for initiation of lipid peroxidation, and that the PC-O- generated by their reaction penetrates into the hydrophobic membrane region where polyunsaturated bonds of fatty acid are present. Iron and ascorbic acid, a well known prooxidant system, were also explained to initiate lipid peroxidation of PC-OOH dependently by the same mechanism as the PC-OOH-dependent O$_2^-/Fe$_3$-chelate system (2, 4).

Of the chelates tested, only the carcinogenic Fe$_3$-NTA was an effective catalyst of oxidation of membranes charged either negatively or positively (Fig. 2) (5). Fe$_3$-NTA forms two dimeric structures (NTA-Fe$_3^+$-NTA$\equiv$2H$^+$+NTA-Fe$_3^+-$(OH)$_2$-Fe$_3^+$-NTA) at neutral pH with reduction potentials of $-0.25$ V and $-0.48$ V, respectively (5, 18). We postulate that the
shown in Fig. 4, hydroxyl (·OH), perhydroxyl (HOO·), and O2− are included PC-OOH in DMPC liposomes, indicating that O2− did not oxidize ·Toc directly in the system which contains PC-OOH degradation in liposomes (3, 6). (©) DMPC liposomes with PC-OOH (200 μM); (●) DMPC liposomes without PC-OOH. The concentrations of reactants (complete system) were 1 mM DMPC liposomes containing 0.2 mM DCP and 50 μM α-Toc, 30 μM xanthine, 1 μU/mL of xanthine oxidase, 0.6 mM ADP, 33 μM EDTA, and 60 μM FeNH4(SO4)2 in 10 mM HEPES buffer (pH 7.0).

Fig. 4. Effect of pH on rate of oxidation of α-Toc in micelles by γ-irradiation (7). Left axis: oxidation rate by HOO·/O2− (©) and ·OH (●); Right axis: the spontaneous rate of disappearance of the superoxide radicals due to dismutation reaction (HOO·+H2O2→H2O2+O2, k1; HOO·+O2→H2O2+O2, k2; O2·−+O2·−+2H+→H2O2+O2, k3). The rate is fastest at pH 4.8 because of k2≫k1≫k3 and pk=4.8 (HOO·ÆO2·−+H+). The TTAB micellar solution containing 0.1 mM α-Toc was irradiated with 60Co source at 23°C. Rates due to O2·− were measured in the presence of formate at each selected pH, and the ·OH contribution was worked out from similar experiments carried out in formate-free solutions and from the known yields of the radicals (G values). At pH 6.4, 6.7, and 7.9 μM of ·OH and 39.5 μM of HOO· or O2·− were produced per minute in the absence and presence of formate, respectively.

unique catalytic capacity of Fe3+-NTA can be explained by its existence in two forms at neutral pH, each binding to oppositely charged membranes and initiating their peroxidation. This gives the complex the unique ability to bind any membrane, which may be a factor in its carcinogenicity.

2. Radical Scavenging Dynamics of α-Toc in Membranes (6)

α-Toc is known to act as a biological antioxidant in biomembranes. In this section, I describe the antioxidant properties of α-Toc from the following three points: reaction of α-Toc with active oxygen species in micelles and membranes, membrane location of the OH group, an antioxidant active site, and the reaction of α-Toc and its radical scavenging dynamics, and dynamics of synergistic effects of ascorbic acid and α-Toc in inhibiting lipid peroxidation in membranes.

2.1. Reaction of α-Toc with active oxygen species: comparison between micelles and liposomes

2.1.1. Reactivities of α-Toc in micelles with hydroxyl, perhydroxyl, and superoxide free radicals. The radicals hydroxyl (·OH), perhydroxyl (HOO·), and O2·− were produced in known yields by radiolysis of aqueous solutions with gamma rays using a 60Co source (7). As shown in Fig. 4, α-Toc in tetradecyltrimethylammonium bromide (TTAB) micelles was oxidized by both HOO·/O2− and ·OH if they were generated around the α-Toc molecules in water by the radiolysis. Oxidation rates due to ·OH were constant between pH 3.1 and 10.6 but the rate due to HOO·/O2− were pH-dependent, high in acid solutions, almost zero at pH 5.5, and then increasing slowly with increasing pH. Because the exact HOO·/O2− ratio is determined by the equilibrium (HOO·⇌O2·−+H+), which has a pK of 4.8, the highest rates were produced by HOO·, and the rapid fall between pH 3 and 6 indicates the increasing role of the dismutation reaction of HOO·/O2− (HOO·+O2−+H+→H2O2+O2), and the much weaker oxidizing power of O2−. The relatively higher oxidation by O2− at higher pH than at neutral may be due to the longer life of O2− at alkaline pH (right axis in Fig. 4).

The oxidation rate of α-Toc by ·OH was lower than that by HOO· in this experimental condition, although ·OH is potentially the most efficient oxidant. I supposed that ·OH reacted with the micelle substance (TTAB) due to its high reactivity to be consumed but HOO· did not, resulting in slower oxidation by ·OH.

2.1.2. Oxidation of α-Toc in liposomes exposed to enzymatically-generated O2−. α-Toc incorporated into liposomes prepared from dimyristoylphosphatidylcholine (DMPC), which is insensitive to peroxidation, was not oxidized by the addition of either or both an O2− generation system (X-XO) and Fe3+-chelates (Fig. 5). These results indicate that O2−, Fe3+-chelate, and Fe2+-chelate reduced by O2− did not oxidize α-Toc directly in the membranes. Addition of H2O2 did not oxidize α-Toc in DCP-DMPC liposomes either (data not shown). On the contrary, α-Toc was oxidized in the system which included PC-OOH in DMPC liposomes, indicating that PC-O· derived from PC-OOH, as mentioned above (Table 1), is a direct oxidant of α-Toc (Fig. 5). In liposomes prepared from peroxidation-sensitive PC with polyunsaturated fatty acids, α-Toc would be oxidized mainly by PC-
OO· generated by the radical chain reaction initiated by PC-O· because these liposomes have a higher concentration of polyunsaturated fatty acids than α-Toc.

2.2. Radical scavenging dynamics of OH group of α-Toc in membranes

The α-Toc molecule consists of two functional domains: a hydrocarbon chain that is necessary for the proper orientation of the molecule in the membranes and a chromanol nucleus with the OH group domain that is responsible for its antioxidant properties. To clarify the chain-breaking dynamics of α-Toc in membranes, we investigated the probable location of the OH group of α-Toc in phospholipid membranes and its antioxidant dynamics by scavenging lipid peroxyl radicals generated during lipid peroxidation.

2.2.1. Membrane distribution of OH group of α-Toc (8–10). The location of the OH-group of α-Toc in the phospholipid bilayer was investigated by transient-state fluorescence with the membrane spin probes 5-, 7-, 12-, and 16-NS (8, 10). These probes have a nitroxide spin group attached at different positions along a stearic acid hydrocarbon chain, and thus become situated at different depths in the hydrophobic interior of the membranes (Fig. 6) (19). The OH group of α-Toc is essential for its intrinsic fluorescence because the fluorescence is lost on acetylation of the OH group. The quenching effects of the probes on the intrinsic fluorescence of α-Toc in DMPC liposomes are shown in Fig. 7A. The linearity of these Stern-Volmer plots indicate that the quenching process followed a collisional mechanism. The quenching effects were observed in all probes indicating that the chromanol nucleus was dynamically distributed in various hydrophobic domains of the membranes at different probabilities. The quenching abilities of the probes in the order 5-NS>7-NS=12-NS>16-NS indicate that the time-averaged location of the chromanol nucleus of α-Toc in a phospholipid bilayer is highest at the positions occupied by the spin group of 5-NS, less in the region labeled with the spin groups of 7-NS-12-NS, and lowest in the membrane core labeled with the 16-NS spin groups. Aranda et al. (20) also reported a similar distribution of the chromanol of α-Toc deduced from fluorescence studies using n-(9-anthroyl)stearic acids (n-AS) as probes (19), with their efficiencies of energy transfer to the chromanol ring in the order 7-AS>2-AS>9-AS=12-AS.

Figure 7B shows Stern-Volmer plots of the quenching of α-Toc fluorescence by acrylamide, a water-soluble fluorescence quencher with a very low capacity to penetrate into phospholipid bilayers (8). Acrylamide was effective in quenching the fluorescence of α-Toc in SDS or TTAB micelles and in EtOH solution (data not shown), but had little quenching effect on the fluorescence of α-Toc in DMPC liposomes with or without the

![Fig. 6.](image6.png) Relative positions of α-Toc with respect to anthroyloxy (AO)-containing fluorescent fatty acids (n-AO stearic acid: n-AS) and nitroxide radical fatty acids (n-Doxyl stearic acid: n-NS), both of which quench tocopherol fluorescence (19).

![Fig. 7.](image7.png) Stern-Volmer plots of α-Toc fluorescence quenching by n-NS in liposomes (left) and acrylamide in liposomes or micelles (right) (6, 8, 10). (A) The concentrations of reagents were 250 μM DMPC, 25 μM α-Toc, and 0–20 μM n-NS in 10 mM HEPES buffer (pH 7.0). The fluorescence (Ex. 296 nm, Em. 325 nm) intensity of α-Toc was measured at 37°C. I and Io are the fluorescence intensity with and without quencher (n-NS). (B) DMPC liposomes (∅), DCP-DMPC liposomes (∆), SA-DMPC liposomes (○), SDS micelles (▲), TTAB micelles (■). The concentrations of reagents in liposome system were 250 μM DMPC, 50 μM DCP or SA, 25 μM α-Toc, and 0–100 mM acrylamide in 10 mM HEPES buffer (pH 7.0). The concentrations of reagents in micelle system were 50 mM SDS or TTAB, 25 μM α-Toc, and 0–100 mM acrylamide in 10 mM HEPES buffer (pH 7.0).
charged substances DCP and SA, indicating that the OH group of α-Toc is little exposed at the membrane surface.

2.2.2. Amount of OH group of α-Toc exposed at membrane surface. I investigated the amount of the OH group of α-Toc exposed at the surface by measuring the rate of reduction of Fe$^{3+}$ by α-Toc on the assumption that externally added Fe$^{3+}$ reacts with only exposed OH groups. α-Toc was rapidly oxidized by Fe$^{3+}$ in negatively charged SDS micelles but little oxidized in positively charged TTAB micelles. Positively charged Fe$^{3+}$ did not react with α-Toc in TTAB micelles because of its ionic repulsion from the surface. On the other hand, α-Toc was slowly oxidized in negatively charged DCP-DMPC liposomes and little oxidized in positively charged SA-DMPC liposomes. The reactions in SDS micelles and DCP-DMPC liposomes followed pseudo-first order kinetics with apparent rate constants of 7.14×10$^{-1}$ s$^{-1}$ and 1.41×10$^{-3}$ s$^{-1}$, respectively (10, 11). Assuming that all the OH groups of α-Toc are at the membrane surface of SDS micelles, because the oxidation rate constant of α-Toc in the DCP-DMPC liposomes is about 500 times lower than that in SDS micelles, probably only 0.2 mol% of the OH groups of α-Toc were present at the membrane surface of the liposomes.

2.2.3. Dynamics of OH group of α-Toc in scavenging lipid peroxyl radical in membranes. The findings that the OH group of α-Toc is mainly located in a hydrophilic position corresponding to 5-methylene carbons (6–7 Å) in from the membrane surface, as mentioned above, raises the question of how the OH group of α-Toc can interact with the peroxyl radical (·O$_2$) moiety of PC-OO·, which is found deep in the bilayer membrane. Barclay and Ingold (21) have suggested that the large dipole moment of the ·O$_2$-moiety of PC-OO· causes it to move up to the membrane surface. This assumption was confirmed by the following experiments (Fig. 8) (10, 11). Negatively charged AsA trapped at the membrane surface of positively charged SA-EYPC liposomes (the binding of AsA to the surface of SA-EYPC liposomes is shown in Fig. 9) was oxidized during the radical chain reaction of lipid peroxidation induced by the lipid soluble radical precursor 2,2’-azobis(2,4-dimethylvaleronitrile) (AMVN) in the hydrophobic inner region of the membranes, showing that the ·O$_2$ moiety dynamically moves from the inner region to the surface of the membrane, but AsA was not oxidized in negatively charged DCP-EYPC liposomes because of its presence in the bulk water phase. Takahashi and his co-workers (22) also supposed that α-Toc scavenge the peroxyl radicals close to the spin position of 5-NS most effectively because the decrease of n-NS in the radical generating system in the membranes was prevented by α-Toc in the order 5-NS>7-NS>10-NS>12-NS>16-NS.

Fragata and Bellemare (23) and Urano et al. (24) proposed that the OH group of α-Toc forms a hydrogen bond with the ester carbonyl group of PC at the region ~10 Å within the membranes, although I did not confirm the hydrogen bond because no changes in the P=O and C=O stretching bands (1,235 cm$^{-1}$ and 1,743 cm$^{-1}$, respectively) of DMPC liposomes were measured by Fourier transform-infrared (FT-IR) spectroscopy in the presence of α-Toc (8). Hydrogen bonds would prevent the exposure of the OH group of α-Toc to the membrane surface, where the electrostatic bond between the phosphate anion and ammonium cation of PC forms a barrier, and keeps it in the region that the spin group of 5-NS occupied for the highest time-average. The lateral diffusion coefficient of α-Toc in EYPC liposomes was reported to be 4.8×10$^{-6}$ cm$^2$ s$^{-1}$ at 25°C (10), this is about 100 times that of the EYPC molecule in a membrane bilayer, which is calculated to be 4.0×10$^{-8}$ cm$^2$ s$^{-1}$ at 31°C (25), indicating that there is no rigid interaction between α-Toc and PC even if they form a hydrogen bond, and suggesting that α-Toc diffuses not only perpendicularly but also laterally with high mobility in membranes and scavenge lipid peroxyl radicals which also move dynamically in membranes.

2.3. Dynamics of synergism of ascorbic acid and α-Toc in inhibiting lipid peroxidation in membranes

α-Toc can effectively play an antioxidant role in biological membranes, although the ratio of α-Toc to polyunsaturated fatty acids in biological membranes is 1 : 1,000–2,000. This is suggested to be the reason that some cellular reductants reduce the partially oxidized α-Toc to α-Toc and maintain its level. Both AsA and glutathione (GSH) are reported to function as reductants and regenerate α-Toc in homogeneous solutions (26, 27). However, how these water-soluble reductants can recycle the lipid-soluble α-Toc by reduction of an oxidized α-Toc intermediate such as the phenoxyl radical of α-Toc, a one-electron oxidized α-Toc, was obscure. Next, I describe the answer to this question.

2.3.1. Are AsA and GSH able to penetrate into membranes? I investigated the interaction of AsA with the membrane surface and its penetration into the mem-

![Fig. 8. Time course of AsA oxidation during lipid peroxidation induced by radical precursor AMVN in membranes with negative and positive charges (9). Solid and broken lines indicate SA-EYPC liposomes and DCP-EYPC liposomes, respectively. Mixtures of 1 mM EYPC liposomes containing 0.2 mM SA or DCP and 0.5 mM AMVN, and 100 μM AsA in 10 mM HEPES buffer (pH 7.0) were incubated at 50°C after addition of 100 μM AsA. Oxidation of AsA was measured by monitoring the decrease of absorbance at 265 nm.](image-url)
brane inner region by using 5-NS and 16-NS labeled at surface and deep inner regions of the membrane, respectively, and measuring the decrease of their ESR signals due to their interaction with externally added AsA (10, 11). As shown in Fig. 9, AsA decreased the signal of 5-NS but not that of 16-NS in SA-EYPC liposomes. These results indicate that AsA was trapped at the positively charged surface and did not penetrate into the membrane inner region. In contrast, the ESR signal of 5-NS was not affected by AsA in negatively charged DCP-EYPC or neutrally charged EYPC liposome membranes. On the other hand, GSH did not affect the ESR signals in SA-EYPC liposome (data not shown). GSH also did not reduce 5-NS in liposomes with or without DCP or SA (data not shown).

2.3.2. AsA is synergistic but GSH is not in inhibiting lipid peroxidation by α-Toc in membranes. I investigated the synergistic effects of AsA and GSH on inhibition by α-Toc of lipid peroxidation. Their synergism was studied by measuring the recycling of α-Toc from its oxidized intermediate, which formed while inhibiting lipid peroxidation in membranes due to water-soluble AsA and GSH. I used AMVN, which generated PC-OOH via a free radical chain reaction. I also used uncharged and negatively charged liposomes into which AsA and GSH could not penetrate (Fig. 9) (10, 11). Figure 10 shows that PC-OOH was generated in negatively charged DCP-EYPC liposomes without any induction period. In DCP-EYPC liposomes incorporating α-Toc, PC-OOH generation was suppressed and started after a lag period during which α-Toc almost disappeared (Figs. 10 and 11). Addition of AsA to the DCP-EYPC liposomes with α-Toc showed a sustained lack of PC-OOH generation and α-Toc oxidation during incubation for more than 5 h (Figs. 10 and 11). In contrast, addition of GSH did not elongate the suppressed lag period of lipid peroxidation by α-Toc and had little influence on the oxidation rate of α-Toc (Figs. 10 and 11), although GSH can regenerate α-Toc in organic solvents and micelles (data not shown) (26). Similar findings were obtained in EYPC liposomes without a charged substance. These results indicate that AsA regenerated α-Toc in membrane systems, resulting in synergistic inhibition of lipid peroxidation, but that GSH did not.

Considering the three findings that AsA and α-Toc had a synergistic effect on inhibiting lipid peroxidation in DCP-EYPC liposomes, although the OH group of α-Toc was not exposed at membrane surface, but AsA could penetrate into neither the inner hydrophobic region in DCP-EYPC liposomes nor the region just under the membrane surface where the OH group of α-Toc is mainly located, I propose the following mecha-
nism, which fulfills the three conditions mentioned above (9–11). The OH group of α-Toc located just under the membrane surface, which possibly forms a weak hydrogen bond (24) with the carbonyl group of phospholipids, reacts with lipid peroxyl radicals, and consequently, forms more hydrophilic intermediates of α-Toc, such as the tocopheroxyl radical and tocopheron cation, which form no hydrogen bond with carbonyl groups, and quickly move up to the membrane surface, where they react with AsA and are regenerated to α-Toc. A two-electron oxidized tocopheroxyl cation charged positively is more likely as a reversible intermediate than a one-electron oxidized tocopheroxyl radical charged neutrally as reported by Liebler and Burr (28), because negatively charged AsA showed high recycling activity, but neutrally charged GSH did not.

3. Kinetics and Dynamics of α-Toc for Singlet Oxygen Scavenging in Membranes

Attention has centered on the function of α-Toc as a radical scavenger to terminate a chain reaction, but it also functions as an effective scavenger of 1O2 by physical quenching and chemical reactions. There have been many studies on inactivation of 1O2 by α-Toc, but in most of these studies pure organic solvents, not membranes, have been used. In this review, we describe the dynamics and kinetics of α-Toc for 1O2 scavenging in membranes and compared them to those of β-carotene (β-Car) under the site-specific generation of 1O2 depending on the membrane distribution of photosensitizers (12, 14–16). The results were interpreted with respect to the generation sites of 1O2, the mobility of antioxidants and membrane phospholipids around them, the concentration of antioxidants in the membranes, especially the local concentration and mobility of their active moieties (OH group of α-Toc, the center of the conjugated polyene-chain of β-Car), and the dielectric constant (micropolarity), on which the reactivity of active OH moieties and 1O2 is dependent, at the membrane sites where they are interacted.

3.1. Comparison of oxidation of α-Toc by 1O2 in solutions and membranes

The oxidation rates of α-Toc by 1O2 in EtOH solution and DMPC liposomes were compared using pyrene-dodecanoic acid (PDA) as a hydrophobic photosensitizer (13, 16, 17). α-Toc was oxidized more rapidly in liposomes than in EtOH, although the mobility of the reactants is higher in EtOH than in liposomes (Fig. 12). The local concentrations of PDA and α-Toc should be higher in liposomes than in EtOH solution because both compounds are located in the membrane phase in the liposome system, resulting in a higher oxidation rate of α-Toc by rapid interaction with 1O2 in liposomes than in EtOH solution.

3.2. Oxidation of α-Toc by 1O2 depending on its generation sites in membranes

I investigated the oxidation rate of α-Toc by 1O2, the membrane charge of which was dependently generated at different sites by photo-irradiated hydrophilic methylene blue (MB) (12, 13, 16). I used DMPC liposomes without charged substances and with DCP or SA as negatively or positively charged liposomes, respectively. The oxidation rates differed with the membrane charge in the following order: DCP-DMPC liposomes > DMPC liposomes = SA-DMPC liposomes (Fig. 13). In DCP-DMPC liposomes, positively charged MB binds electrostatically with negatively charged membranes of DCP-DMPC liposomes and generates 1O2 at the membrane surface close to α-Toc, resulting in its rapid oxidation. In contrast, in DMPC and SA-DMPC liposomes, MB does not interact with these membranes and generates 1O2 in the bulk water phase, but α-Toc is present in the membrane, resulting in its slow oxidation. In the PDA system, however, the oxidation rate of α-Toc was independent of the membrane charge (data not shown). 1O2 is located in the inner hydrophobic region where the pyrene residue of PDA is located, and α-Toc is also located inside the membrane, resulting in no difference in the oxidation rates of α-Toc in liposomes with different charges. These results indicate that the efficiency of
1\textsuperscript{1}O\textsubscript{2} oxidation by \(\alpha\)-Toc in the membrane is due to its generation site, which depends on the locations of photosensitzers.

3.3. Effect of membrane fluidity on oxidation rate of \(\alpha\)-Toc by \(1\textsuperscript{1}O\textsubscript{2}\)

The oxidation rates of \(\alpha\)-Toc by \(1\textsuperscript{1}O\textsubscript{2}\) were compared in two membrane phases: gel in DMPC liposomes and liquid crystalline in dipalmitoylphosphatidylcholine (DPPC) liposomes at 30 \(^\circ\)C. At this temperature, DMPC liposomes and DPPC liposomes are in liquid crystalline and gel states, respectively, because their phase-transition temperatures are 24 \(^\circ\)C and 41 \(^\circ\)C, respectively. As shown in Fig. 14, \(\alpha\)-Toc was oxidized more rapidly in DMPC liposomes than in DPPC liposomes in the MB system. Similar results were observed in the PDA system (data not shown) (12, 16). These results indicate that \(\alpha\)-Toc is oxidized more quickly in the liquid crystalline phase with high PC mobility than in the gel phase with low PC mobility of membranes.

3.4. \(1\textsuperscript{1}O\textsubscript{2}\) scavenging rate constants of \(\alpha\)-Toc and \(\beta\)-Car: comparison with liposomes in EtOH solution (14–16)

The rate constant of \(1\textsuperscript{1}O\textsubscript{2}\) scavenging (\(k_s\)) was determined by the method of Young et al. (29) by measuring the inhibition by \(\alpha\)-Toc or \(\beta\)-Car of photooxidation of 1,3-diphenyl-isobenzofuran (DPBF), a specific \(1\textsuperscript{1}O\textsubscript{2}\) trap, using the following Eqs. (1) and (2):

\[
\frac{S_0}{S_{a-Toc}} = 1 + (k_s/k_d) [\alpha\text{-Toc}] \quad (1)
\]

\[
\frac{S_0}{S_{\beta-Car}} = 1 + (k_s/k_d) [\beta\text{-Car}] \quad (2)
\]

where \(S_0\) and \(S_{a-Toc}\) or \(S_{\beta-Car}\) denote the slopes of the first-order plots of disappearance of DPBF in the absence and presence of \(\alpha\)-Toc or \(\beta\)-Car. The kd value is the rate constant for the natural decay of \(1\textsuperscript{1}O\textsubscript{2}\) to \(O_2\). \(1\textsuperscript{1}O\textsubscript{2}\) was generated at the membrane surface using rose bengal (RB) in SA-DMPC liposomes or MB in DCP-DMPC liposomes because negatively charged RB interacts at the membrane surface in positively charged SA-DMPC liposomes and positively charged MB interacts at the membrane surface in negatively charged DCP-DMPC liposomes. PDA generated \(1\textsuperscript{1}O\textsubscript{2}\) in the inner region of the membrane irrespective of the membrane charge.

\(\alpha\)-Toc oxidation by \(\alpha\)-Toc in the membrane is due to its generation site, which depends on the locations of photosensitzers.

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The oxidation rates of \(\alpha\)-Toc by \(1\textsuperscript{1}O\textsubscript{2}\) were compared in two membrane phases: gel in DMPC liposomes and liquid crystalline in dipalmitoylphosphatidylcholine (DPPC) liposomes at 30 \(^\circ\)C. At this temperature, DMPC liposomes and DPPC liposomes are in liquid crystalline and gel states, respectively, because their phase-transition temperatures are 24 \(^\circ\)C and 41 \(^\circ\)C, respectively. As shown in Fig. 14, \(\alpha\)-Toc was oxidized more rapidly in DMPC liposomes than in DPPC liposomes in the MB system. Similar results were observed in the PDA system (data not shown) (12, 16). These results indicate that \(\alpha\)-Toc is oxidized more quickly in the liquid crystalline phase with high PC mobility than in the gel phase with low PC mobility of membranes.

3.4. \(1\textsuperscript{1}O\textsubscript{2}\) scavenging rate constants of \(\alpha\)-Toc and \(\beta\)-Car: comparison with liposomes in EtOH solution (14–16)

The rate constant of \(1\textsuperscript{1}O\textsubscript{2}\) scavenging (\(k_s\)) was determined by the method of Young et al. (29) by measuring the inhibition by \(\alpha\)-Toc or \(\beta\)-Car of photooxidation of 1,3-diphenyl-isobenzofuran (DPBF), a specific \(1\textsuperscript{1}O\textsubscript{2}\) trap, using the following Eqs. (1) and (2):

\[
\frac{S_0}{S_{a-Toc}} = 1 + (k_s/k_d) [\alpha\text{-Toc}] \quad (1)
\]

\[
\frac{S_0}{S_{\beta-Car}} = 1 + (k_s/k_d) [\beta\text{-Car}] \quad (2)
\]

where \(S_0\) and \(S_{a-Toc}\) or \(S_{\beta-Car}\) denote the slopes of the first-order plots of disappearance of DPBF in the absence and presence of \(\alpha\)-Toc or \(\beta\)-Car. The kd value is the rate constant for the natural decay of \(1\textsuperscript{1}O\textsubscript{2}\) to \(O_2\). \(1\textsuperscript{1}O\textsubscript{2}\) was generated at the membrane surface using rose bengal (RB) in SA-DMPC liposomes or MB in DCP-DMPC liposomes because negatively charged RB interacts at the membrane surface in positively charged SA-DMPC liposomes and positively charged MB interacts at the membrane surface in negatively charged DCP-DMPC liposomes. PDA generated \(1\textsuperscript{1}O\textsubscript{2}\) in the inner region of the membrane irrespective of the membrane charge.

Table 2. \(k_s\) values of \(\alpha\)-Toc and \(\beta\)-Car in scavenging \(1\textsuperscript{1}O\textsubscript{2}\) generated with RB in EtOH and DMPC liposomes (6, 14–17).

|          | EtOH       | Liposomes |
|----------|------------|-----------|
|          | Apparent\(^a\) | Revised\(^b\) |
| \(\alpha\)-Toc | 3.6 x 10\(^8\) | 3.4 x 10\(^9\) | 1.38 x 10\(^7\) |
| \(\beta\)-Car | 1.3 x 10\(^{10}\) | 5.2 x 10\(^9\) | 2.05 x 10\(^7\) |
| Astax | 2.4 x 10\(^{10}\) | 4.6 x 10\(^9\) | 1.81 x 10\(^7\) |

The \(k_s\) value of the antioxidant (A) was obtained from the following equation:

\[
\frac{S_0}{S_A} = 1 + \frac{k_s}{k_d}[A] \quad (3)
\]

where \(S_0\) and \(S_A\) denote the slopes of first-order plots of disappearance of diphenylisobenzofuran (DPBF), a specific \(1\textsuperscript{1}O\textsubscript{2}\) trap, in the absence and presence of antioxidant in experiments (29).

I used the kd value in liposomes considering the dielectric constant at the site where \(1\textsuperscript{1}O\textsubscript{2}\) was scavenged by antioxidants. \(^a\)Value of \(\alpha\)-Toc was calculated using the kd value of EtOH (8.3 x 10\(^4\) \(s^{-1}\)) (30). \(^b\)Values of \(\beta\)-Car and Astax were calculated using the kd value of t-BuOH (3.0 x 10\(^4\) \(s^{-1}\)) (31).

Values in liposomes were revised assuming that the concentration of antioxidants in membranes was 254 times higher in SA-DMPC liposomes than in bulk water phase. The concentrations of reactants were 25–100 \(\mu\)M \(\alpha\)-Toc or 2.5–15 \(\mu\)M \(\beta\)-Car, 5 mM DMPC liposomes with 0.5 mM SA, 0.5 mM DTPA, and 250 mM PDA in 10 mM HEPES buffer (pH 7.0). Photoirradiation was carried out at 37 \(^\circ\)C for 8 min.

Table 3. Ratios of \(k_s\) values of \(\alpha\)-Toc and \(\beta\)-Car in DMPC liposomes to those in EtOH solution in RB system (6, 13–17).

|          | Liposomes/EtOH |
|----------|----------------|
| \(\alpha\)-Toc | 1/26          |
| \(\beta\)-Car | 1/634         |
| Astax     | 1/1326        |

Table 2 shows the \(k_s\) values of \(\alpha\)-Toc and \(\beta\)-Car for \(1\textsuperscript{1}O\textsubscript{2}\) scavenging in EtOH and liposomes in the RB system. The original value for \(\alpha\)-Toc in liposomes was higher than that in EtOH solution, though its mobility was lowered in liposomes. Since \(\alpha\)-Toc and \(\beta\)-Car are assumed to be present in membranes, the \(k_s\) values were revised, taking into account their concentrations in liposomes (Table 2). The revised \(k_s\) values for \(\alpha\)-Toc and \(\beta\)-Car in liposomes were 26 and 634 times lower, respectively, than those in EtOH solution (Table 3). The \(k_s\) values for \(\beta\)-Car were 4.2 times and 1.5 times higher than those for \(\alpha\)-Toc in EtOH and liposomes, respectively (Table 4). The mobility of \(\beta\)-Car would be suppressed more than that of \(\alpha\)-Toc in membranes because the lateral diffusion coefficient of \(\beta\)-Car in 1-palmitoyl-2-oleoyl PC liposomes was calculated to be
5.2×10\(^{-7}\) cm\(^2\) s\(^{-1}\) (32), which is 10 times lower than that of \(\alpha\)-Toc (4.8×10\(^{-6}\) cm\(^2\) s\(^{-1}\)) in EYPC liposomes (10), resulting in greater decreases in the \(k_s\) values than those of \(\alpha\)-Toc in liposomes than in EtOH solution. This is supported by the report that carotenoid molecules are anchored across the hydrophobic bilayer, bringing their two head groups in contact with opposite polar sides of the membranes, but the \(\alpha\)-Toc molecule is located in the monolayer of the bilayer membranes (16). The \(k_s\) value for astaxanthin (Astax) was 1.9 times of that for \(\beta\)-Car in EtOH, but 0.88 times of that in liposomes (Table 4). Zeaxanthin, which has two OH groups at the head ring structure, has been reported to strongly interact with the head group of membrane phospholipids by its OH groups (33, 34), but \(\beta\)-Car, which has no OH groups, does not. Because Astax also has an OH group, the mobility of Astax would be suppressed more than that of \(\beta\)-Car in liposomes, resulting in a greater decrease in the \(k_s\) value of Astax than that of \(\beta\)-Car in membranes.

### 3.5. Effect of \(^1\)O\(_2\) generation site in membrane on rate constants of \(\alpha\)-Toc for its scavenging

To investigate the effect of the \(^1\)O\(_2\) generation site in membranes on its scavenging by \(\alpha\)-Toc, the \(k_s\) values were measured in the water-soluble MB system and the water-insoluble PDA system (Table 5) (14–16). The Apparent and Revised 1 values in membranes were 6 times higher in the MB system than in the PDA system, although little difference was observed between them in EtOH solution. The difference between the revised \(k_s\) value (Revised 1) of \(\alpha\)-Toc in membranes in the MB system and the PDA system was supposed to be caused by the differences in the local concentrations of the OH group, depending on its membrane localization due to the biphasic structure of \(\alpha\)-Toc, and the membranous characteristics such as the dielectric constant and fluidity around the OH groups of \(\alpha\)-Toc. The higher concentration of OH groups in the region close to the membrane surface where \(^1\)O\(_2\) is generated in the MB system may result in higher values, and the lower concentration of OH groups in the hydrophobic membrane region where \(^1\)O\(_2\) is generated and diffused in the PDA system may result in lower values; the higher dielectric constant at the membrane surface is partially responsible for higher values in the MB system than in the PDA system because the \(k_s\) value of \(\alpha\)-Toc is higher in solvents with higher dielectric constants.

On the other hand, the revised values for \(\beta\)-Car were almost the same in both photosensitizing systems (data not shown). The quenching site of \(\beta\)-Car, probably the center of the conjugated polyene chain may be localized in the hydrophobic region where the concentration of \(^1\)O\(_2\) is also high, irrespective of its generation site, because \(^1\)O\(_2\) is more soluble in the hydrophobic region than in the polar region, and thus the \(k_s\) values of \(\beta\)-Car were similar irrespective of the photosensitizer.

### 3.6. \(^1\)O\(_2\) scavenging rate constants of \(\alpha\)-Toc at membrane sites where its OH groups were distributed

The \(k_s\) values for \(\alpha\)-Toc in liposomes were further revised in consideration of the site-specific \(^1\)O\(_2\) scavenging reaction of \(\alpha\)-Toc, which depends on the localization of the OH groups of \(\alpha\)-Toc and the generation sites of \(^1\)O\(_2\) in the membranes (14–16). Equation (3) was used to revise the \(k_s\) value of \(\alpha\)-Toc in liposomes assuming the following two points; the local concentrations of the OH groups of \(\alpha\)-Toc in DMPC liposomes are about 0% in the polar zone (PZ), 50–60% in the hydrogen belt (HB), and 40–50% in the hydrophobic core (HC) of the membranes, and the lengths and dielectric constants (\(\varepsilon\)) of these membrane regions of DMPC liposomes are about 10 Å, \(\varepsilon=40\) (PZ), 4 Å, \(\varepsilon=25\) (HB), and 20 Å, \(\varepsilon=\sim2\) (HC) (Fig. 15), and \(^1\)O\(_2\) generated by MB is only scavenged at the membrane surface where \(^1\)O\(_2\) is generated in the MB system, resulting in a greater decrease in the \(k_s\) value of \(\alpha\)-Toc than that of \(\beta\)-Car in membranes.

| EtOH | Liposomes | \(k_s\) (\(M^{-1}\) s\(^{-1}\)) |
|------|-----------|-----------------------------|
| MB-system | | |
| Apparent | Revised 1 | Revised 2 |
| EtOH | 3.1×10\(^8\) | 3.5×10\(^8\) | 4.5×10\(^7\) | 3.1×10\(^6\) |
| PDA-system | 2.1×10\(^8\) | 0.66×10\(^8\) | 0.25×10\(^7\) | 3.25×10\(^6\) |

### Table 4. Ratios of \(k_s\) values (\(\beta\)-Car/\(\alpha\)-Toc, Astax/\(\beta\)-Car) in EtOH solution and DMPC liposomes in RB system (6, 13–17).

|          | \(\beta\)/\(\alpha\) | Astax/\(\beta\) |
|----------|---------------------|----------------|
| EtOH     | 41.9                | 1.8            |
| Liposomes| 1.5                 | 0.88           |

### Table 5. \(k_s\) values of \(\alpha\)-Toc for scavenging \(^1\)O\(_2\) generated with MB and PDA in EtOH solution and DMPC liposomes (6, 13–17).

The concentrations of reactants (MB system) were 25–50 \(\mu\)M \(\alpha\)-Toc, 5 mM DMPC liposomes with 0.5 mM DCP, 0.5 mM DTPA, and 50 \(\mu\)M MB in 10 mM HEPES buffer (\(\text{pH } 7.0\)). Photolab irradiation was carried out at 37°C for 8 min.
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...enged by the OH groups localized in the HB of membranes, whereas that generated by PDA is scavenged mainly by the OH groups in the HC of membranes because of the slight diffusion of 1O2 generated in the HC into the HB due to its higher solubility in solvents with higher hydrophobicities.

\[ \frac{S_0}{S_{\text{α-Toc}}} = 1 + (\text{ks/kd}) \text{ [OH-group of α-Toc]} \]  

(3)

The revised ks values for α-Toc in liposomes were recalculated as $3.10 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$ and $3.25 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$ in the MB and PDA systems, respectively (Revised 2 in Table 5). This result indicates that the 1O2 scavenging ability of OH groups in the HB zone is almost the same as that in the HC zone. The site specific 1O2 scavenging ability of an OH group in membranes was supposed to depend on the mobility of OH-group and the membrane polarity around it. Because the order parameter of 5-NS in EYPC membranes was reported to be 0.587 (35) which is 6 times higher than that of 16-NS (36), the degree of packing of phosphatidylcholine molecules is higher in the region close to the membrane surface (HB) than in the inner membrane region (HC). The motion freedom of the 5α-methyl group on the chromanol moiety of α-Toc is more restricted than that of the methyl groups on the isoprenoid side chain in the HC zone because the values of spin-spin relaxation time measured by 19F NMR of 19F-labeled methyl groups of α-Toc in EYPC liposomes were 0.016 s for 5α-CF3 on the chromanol and 0.040, 0.060, and 0.107 s for 4′α-, 8′α-, and 12′α-CF3 on the isoprenoid side chain, respectively (24). The OH group on the chromanol moiety may be packed in the HB zone and its mobility is restricted possibly by hydrogen bonding with ester carbonyl moieties of membrane phosphatidylcholine (23, 24). On the other hand, the polarity (dielectric constant) around the OH groups which reflects their reactivity with 1O2. Because the ks value of α-Toc is higher in a solvent with a higher dielectric constant (14, 23), the polarity dependent reactivity of OH groups would be higher in the HB with a higher dielectric constant than that in the HC with a lower dielectric constant. Accordingly, the OH groups of α-Toc in the HB are supposed to have higher reactivity but lower mobility than those in the HC, resulting in unexpectedly similar ks values (Revised 2 in Table 5) in membranes in the MB system and PDA system. The ks value of α-Toc is 2.9 $\times$ 10^8 M^{-1} s^{-1} in EtOH, the dielectric constant of which is similar to that of the HB, is 3 times higher than that in cyclohexane (0.9 $\times$ 10^8 M^{-1} s^{-1}), the dielectric constant of which is similar to that of the HC (Table 6), indicating that the 1O2 scavenging ability of an OH group, depending on the dielectric constant, is 3 times higher in the HB than in the HC. Thus, the 1O2 scavenging ability of an OH group, depending on the mobility of the OH group, is calculated to be 3 times lower in the HB than in the HC because of the similar ks values obtained in the HB and HC in membranes.

In considering the antioxidant potencies of α-Toc in membranes, we experimentally confirmed the impor-

![Fig. 15. Localization of percentage of OH group of α-Toc in DMPC liposomes (6, 15). The lengths (Å) and dielectric constants (ε) in the polar zone (PZ), hydrogen belt (HB), and hydrophobic core (HC) of the membranes are from the report by Fragata and Bellemare (23). Percentage localizations of the OH groups of α-Toc were deduced from the results in Refs. 8, 20, 22). Order parameters in HB and HC were from Refs. 35, 36).](image)

| Solvents   | ε   | ks (10^8 M^{-1} s^{-1}) |
|------------|-----|------------------------|
| MeOH       | 32.6| 5.3–6.7                |
| EtOH       | 25.5| 2.1–3.6                |
| Cyclohexane| 2.02| 0.9                    |

Dielectric constants in the HB and HC of PC membranes are 25 and ~2, respectively.
tance of three factors: the concentration and mobility of the $\alpha$-Toc molecule in membranes, especially those of its active moiety at the membrane regions where it functions, and the dielectric constant in membrane regions. These factors are useful to evaluate drug functions at the membrane, and my explanation of the antioxidant mechanism of vitamin E in membranes would contribute the investigation of the functions of membranous drugs.

4. Conclusions

(1) The initiation mechanism of lipid peroxidation in PC liposomes under biological conditions: Membrane lipid peroxidation was not directly induced by $O_2^-$, $H_2O_2$, or $\cdot OH$ itself. The initiation of $O_2^-$-dependent peroxidation of PC liposomes required the presence of preformed PC-OOH and a transition metal catalyst such as $Fe^{3+}$-chelate. As the mechanism I propose that an alkoxy radical moiety of PC-OH, which is derived from degraded PC-OOH by the reaction with reduced $Fe^{3+}$-NTA by $O_2^-$, penetrates from the membrane surface into the inner hydrophobic region and triggers the initiation of lipid peroxidation by abstraction of a hydrogen from the unsaturated bond moieties of fatty acids of PC.

(2) The location of $\alpha$-Toc in liposome membranes and its dynamics in inhibiting lipid peroxidation and being recycled by AsA: The intrinsic fluorescence of $\alpha$-Toc was little quenched by acrylamide, a water-soluble fluorescence quencher with a very low capacity to penetrate into the phospholipid membranes, suggesting little exposure of the OH group of $\alpha$-Toc at the membrane surface. The oxidation of $\alpha$-Toc by aqueous $Fe^{3+}$ was 500 times slower in negatively charged liposomes than in negatively charged SDS micelles, indicating that probably less than 0.2 mol% of the OH groups of $\alpha$-Toc was exposed at the membrane surface. The dynamic quenching abilities of n-NS labeled at different depths of membranes on the intrinsic fluorescence of $\alpha$-Toc were in the order 5-NS > 7-NS > 12-NS > 16-NS, indicating that the OH groups of $\alpha$-Toc are located mainly at a position corresponding to 5-methylene carbon within the surface, with not a few in the hydrophobic region. AsA in the bulk water phase completely suppressed the consumption of $\alpha$-Toc during lipid peroxidation induced by a hydrophobic radical precursor AMVN in negatively charged liposomes, although the ESR spectra of 5-NS and 16-NS labeled in negatively and neutrally charged liposomes were not changed by the addition of negatively charged AsA (AsA could not penetrate into these membranes), indicating that the OH-groups of $\alpha$-Toc at the inner region of the membranes oxidized by lipid radicals floats up to the surface, where they are regenerated to $\alpha$-Toc by AsA.

(3) The rate constant ($ks$) of $\alpha$-Toc for deactivating $^3O_2$, which was generated by photoradiation at the membrane surface, in the hydrophobic inner region, and in bulk water: Three factors experimentally confirmed to be important for consideration of the $ks$ value of $\alpha$-Toc in membranes were (1) the concentration of $\alpha$-Toc was higher in membranes than in EtOH solution; the local concentration of the active moiety (OH group) in membranes, which was 0% at the membrane surface polar zone (PZ), 50–60% in the inner hydrogen belt (HB), and 40–50% in the hydrophobic core (HC), (2) the mobility of $\alpha$-Toc was higher in EtOH solution than in membranes, and higher in the liquid crystalline state than in the gel state of membrane phospholipids; the mobility of $\alpha$-Toc, which was higher than that of $\beta$-carotene because $\alpha$-Toc locates at one half of the bilayer membrane, but $\beta$-carotene locates across the bilayer: the local mobility of the OH group of $\alpha$-Toc was higher in the HC region than in the HB region, and (3) the dielectric constant (micropolarity) was higher in the HB region than in the HC region (the reactivity of the OH group of $\alpha$-Toc and $^3O_2$ was high in the solvent with high dielectric constant).

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