Antioxidative Activities of Tocotrienols on Phospholipid Liposomes

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Antioxidative activity of tocotrienol (Toc3) was studied in the oxidation of dilinoleoylphosphatidylcholine (DLPC) liposomes. The objective was to measure the differences in the antioxidative activities between α-Toc3 and α-tocopherol (α-Toc), and between γ-Toc3 and γ-Toc. When each antioxidant was added to the already prepared DLPC liposome solution, the antioxidative activity of Toc3 was larger than that of Toc. However, when incorporated into the liposomal membrane, the antioxidative activities of Toc3 and Toc were the same and were intermediate between those of the added Toc3 and Toc.

When added to the liposome solution, the consumption of Toc3 during the induction period was larger than that of Toc. When incorporated into the liposomal membrane, the consumptions of Toc3 and Toc were the same and were intermediate between those of the added Toc3 and Toc.

These results suggest that the reactions of Toc3 and Toc with phospholipid peroxide within the membrane are inhibited to a different degree depending on the dosing manner of Toc3 and Toc. Namely, the degree of inhibition decreases in the following order: Toc(added)> Toc(incorporated)= Toc3(incorporated)> Toc3(added).

KEY WORDS: Addition to the liposome solution, antioxidative activity, incorporation into the liposomal membrane, phospholipid liposomes, tocotrienol and tocopherol.

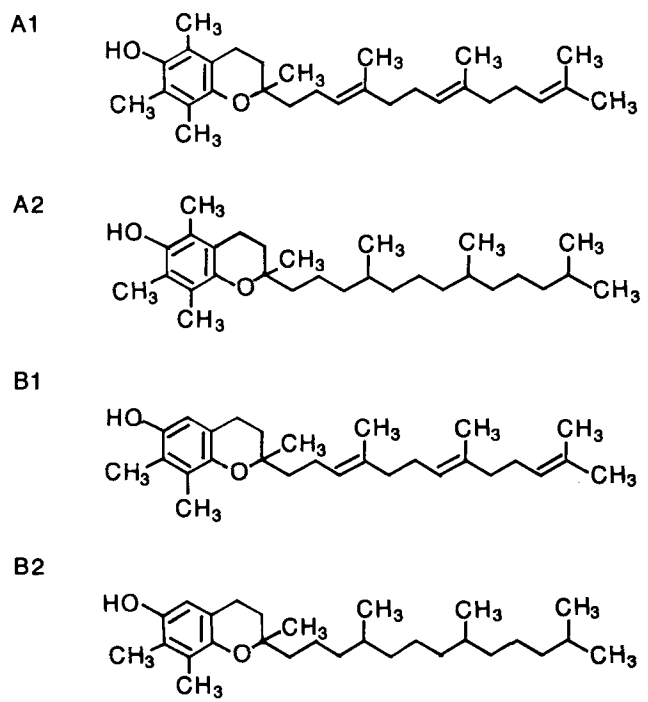

FIG. 1. Chemical structures of α-Toc3(A1), α-Toc(A2), γ1Toc3(B1), and γ-Toc(B2).

Tocotrienol (Toc3), a vitamin E homologue, has a different chemical structure from the corresponding tocopherol (Toc) in the unsaturated long side-chain (Fig. 1). Although the presence of Toc3 was reported in vegetable oils (1-3) such as palm oil, rice bran oil, wheat germ oil, barley oil, coconut oil, corn oil, and rubberseed oil, few studies have been reported on the physiological activity of Toc3 (4-7).

Recently, Kato *et al.* (8) and Komiyama *et al.* (9) found that some transplantable murine tumors inoculated intraperitoneally (i.p.) into mice were cured by i.p.-injected Toc3. It was noticed that Toc3 inhibited the adriamycin-induced oxidation of murine liver microsomes more efficiently than did Toc. Few studies have been reported on the antioxidative activity of Toc3 in a heterogeneous system (10,11). Tatsuta (11) reported that α-Toc3 was more efficient in protecting red blood cells from hemolysis than was α-Toc *in vitro*. However, these results were opposite to those observed *in vivo*. Yamaoka and Komiyama (12) preliminarily observed that α-Toc3 had more antioxidative activity than did α-Toc when added as an ethanol solution to a phospholipid liposome solution. On the contrary, α-Toc3 and α-Toc had equal antioxidative activities when incorporated into the liposomal membrane. These results were not, however, discussed in detail (12).

In this paper, we further investigated the antioxidative activity of Toc3 by using α-Toc3 and γ-Toc3. We used the sets of concentrations of the oxidation substrate and the oxidation initiator. Dilinoleoylphosphatidylcholine (DLPC) and 2,2′-azobis(2-amidino-propane)-dihydrochloride (AAPH) were employed as the substrate and the initiator, respectively.

The residual amount of Toc3 and Toc after the induction period was measured. The antioxidative activity in the binary mixture of liposome solution also was measured.

MATERIALS AND METHODS

Materials. The Toc and pentamethylchromanol (PMC) of more than 98.5% purity were obtained from Eizai Co., Ltd. (Tokyo, Japan). Carboxytetramethylchromanol (CTMC) of 97% purity was purchased from Aldrich Chemical Co. (Milwaukee, WI). The Toc3 was extracted from rice bran oil scum and was purified by the method described earlier (13). Purity of Toc3 was checked by both a normal and a reverse phase high-performance liquid chromatography (HPLC). The HPLC pump was model LC-5A (Shimadzu Corporation, Kyoto, Japan). The column and the mobile phase for the reverse phase HPLC were Zorbax ODS column (Shimadzu Corporation, 4.6 × 250 mm) and the mixture of methanol and water (95:5, v/v), respectively. The column and the mobile phase for the normal phase HPLC were Zorbax Sil column (Shimadzu Corporation, 4.6 × 250 mm) and the mixture of hexane, tetrahydrofuran and methanol (972.5:25:2.5, v/v/v), respectively. An ultraviolet (UV) spectrophotometer (model SPD-2A, Shimadzu Corporation) was used as

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the detector at 298 nm in both HPLC procedures. Analysis from each phase showed a single peak, and the purity was confirmed with a mass spectrometer (model 5995, Hewlett-Packard Co., Palo Alto, CA) by using a direct inlet method.

DLPC and dimyristoylphosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids, Inc. (Arabaster, AL) and were stored at −20°C in a chloroform solution before use. AAPH was purchased from Wako Pure Chemicals, Inc. (Osaka, Japan). Other chemicals were of guaranteed grade from Nakalai Tesque, Inc. (Kyoto, Japan).

Preparation of liposome solution. The DLPC liposomes were prepared in the following manner. An aliquot of DLPC in chloroform kept at −20°C in the freezer was transferred into an amber-colored round-bottomed flask. The chloroform was evaporated at room temperature under reduced pressure to obtain a thin film. After introducing nitrogen to the flask, deionized and distilled water was added. The solution was shaken for 1 min with a mixer (model S-5F, Taiyo Kagaku Kogyo Co., Tokyo, Japan) and sonicated for 2 min with a bath-type sonicator (Bransonic 1200, Branson Ultrasonic Co., CT) to obtain multilamellar liposomes at room temperature. Molar concentrations of DLPC in water were 0.3 mM and 1.93 mM.

The DMPC liposomes were prepared in the same manner as mentioned above except for the sonication time and temperature. The DMPC aqueous solution was kept at 30°C and was sonicated for 4 min.

The terms "incorporation" and "addition" were used in this report as follows: for incorporation of the antioxidant, the antioxidant was mixed with DLPC or DMPC chloroform solution before liposome formation, and was incorporated into the liposomal membrane. For addition of the antioxidant, 0.01M ethanol solution of the antioxidant was added to the liposome solution after liposome formation.

Measurement of antioxidative activity. Antioxidative activity was measured by analyzing the dissolved oxygen as described previously (12). The apparatus for the dissolved oxygen analysis consisted of a branched testing bottle which had a stopcock at the branch, an oxygen sensor (model 39557, Beckman Instruments Inc., Fullerton, CA), a thermostat bath and a magnetic stirrer. The DLPC liposome solution was transferred to the testing bottle and the oxygen sensor was attached to the bottle at the neck with care so as not to leave air bubbles around the sensor in the bottle. The bottle with the sensor was kept at 37°C with stirring for approximately 10 min to saturate the solution with air at that temperature. Oxidation of the liposomes was then initiated with the addition of AAPH aqueous solution through the branch. The oxidation of the liposomes was monitored by measuring the consumption of the dissolved oxygen with the oxygen sensor. The stopcock at the branch was closed during the measurement. The measurement of the antioxidative activity was not replicated but was carried out randomly at various concentrations of the antioxidant.

Analysis of antioxidant with HPLC. A Zorbax ODS column (4.6 × 250 mm) and Asahipak GS-310H column (Asahi Chemical Industry, Kawasaki, Japan, 7.6 × 250 mm) were employed for Toc3 and Toc analyses and for CTMC analysis, respectively. The mobile phase was a mixture of methanol and water (95:5, v/v). An aliquot of the liposome solution (50 μL) was taken out from the testing bottle through the branch after opening the stopcock, and directly injected onto the HPLC column. Samples were detected by the spectrofluorophotometer (model RF-500, Shimadzu Corporation). The excitation wave length and the emission wave length of the detector were 298 nm and 325 nm, respectively. The measurement was duplicated. The mean values were shown in Table 1. The spreads in the induction periods and in the remaining percentages in Table 1 were <±120 s and <±5%, respectively.

Antioxidative activity in the binary mixture of liposomes. Each antioxidant was added to the DMPC liposome solution or was incorporated into the DMPC liposomal membrane. The DMPC liposome solution was then mixed with the DLPC liposome solution, which did not contain the antioxidant. The mixture of the two kinds of liposome solutions also was kept standing for 10 min before initiation of oxidation by AAPH. The oxidation of the DLPC liposomes was monitored by the oxygen sensor in the same manner as mentioned above.

RESULTS

Antioxidative activity of Toc3. Figures 2 through 4 show plots of the concentration of each antioxidant vs the induction period. Concentrations of DLPC and AAPH in Figures 2A, 3A, and 4A were 0.3 mM and 0.15 mM,

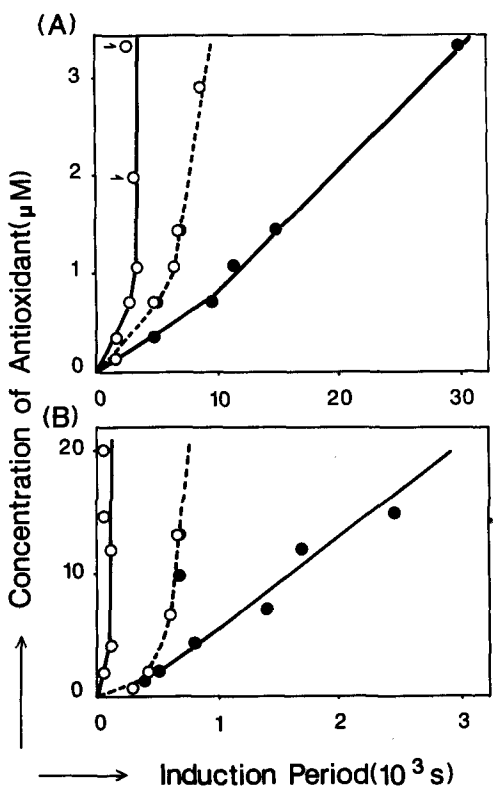

FIG. 2. Plots of the concentration of antioxidant vs the induction period. Each antioxidant was added to (—) or incorporated into (– – – –) the liposomes. ○: α-Toc, ●: α-Toc3, [DLPC]: (A) 0.30 mM, (B) 1.93 mM, [AAPH]: (A) 0.15 mM, (B) 10.0 mM. In Figure 2A, data were cited from previous work (12) except for two points shown with arrows.

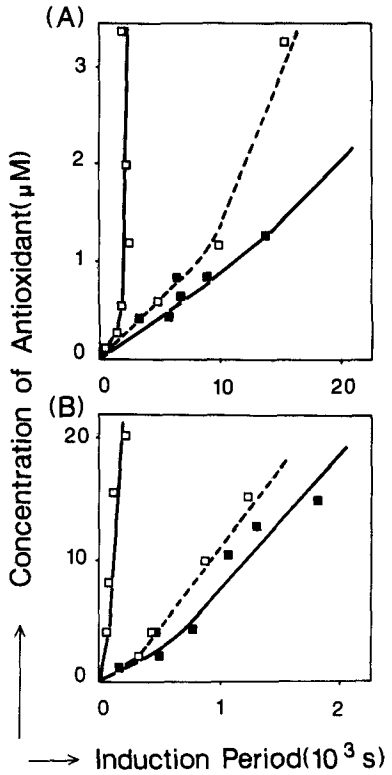

FIG. 3. Plots of the concentration of antioxidant vs the induction period. Each antioxidant was added to (——) or incorporated into (– – – –) the liposomes. □: γ-Toc, ■: γ-Toc3, [DLPC]: (A) 0.30 mM, (B) 1.93 mM, [AAPH]: (A) 0.15 mM, (B) 10.0 mM.

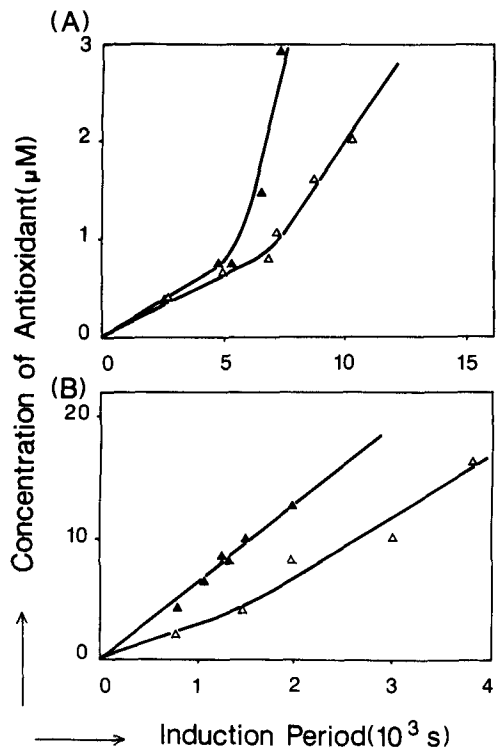

FIG. 4. Plots of the concentration of antioxidant vs the induction period. Each antioxidant was added to the liposomes. △: PMC, ▲: CTMC, [DLPC]: (A) 0.30 mM, (B) 1.93 mM, [AAPH]: (A) 0.15 mM, (B) 10.0 mM.

respectively. In Figures 2B, 3B, and 4B, their concentrations were 1.93 mM and 10.0 mM, respectively. The data of α-Toc3 and α-Toc in Figure 2A (except for two points shown with arrows) and the data of CTMC in Figure 4 were cited from the previous work of Yamaoka and Yomiyama (12) for comparison.

Figure 2 showed that α-Toc3 had a higher antioxidative activity than did α-Toc, when each antioxidant was added to DLPC liposome solution as an ethanol solution. When either α-Toc3 or α-Toc was incorporated into the liposomal membrane, their antioxidative activities were the same and were intermediate between those of α-Toc3 and α-Toc which has been added to the liposome solution. By extending the experimental conditions, the authors observed an inflection also for the added α-Toc3 and α-Toc, which was not found previously (12).

Figure 3 showed that γ-Toc3 had a higher antioxidative activity than did γ-Toc, when each antioxidant was added to DLPC liposome solution. When either γ-Toc3 or γ-Toc was incorporated into the liposomal membrane, their antioxidative activities were the same and were intermediate between those of γ-Toc3 and γ-Toc which has been added to the liposome solution.

The antioxidative activities of the incorporated Toc3 and Toc decreased in the following order: γ-Toc3>α-Toc3 and γ-Toc>α-Toc. When added to the already formed liposomes, however, the opposite order was observed: γ-Toc3≤α-Toc3, and γ-Toc≤α-Toc. A comparison of the antioxidative activities among the antioxidants having the 5,7,8-trimethyl chromanol nucleus such as α-Toc, α-Toc3, PMC, and CTMC (in Figs. 2 and 4) revealed that the antioxidative activity decreased in the following order when added to the liposome solution: (A) α-Toc3>PMC>CTMC >α-Toc, and (B) PMC>α-Toc3>CTMC>α-Toc, where concentrations of DLPC and AAPH were (A) 0.30 mM and 0.15 mM, and (B) 1.93 mM and 10.0 mM, respectively.

Consumption of Toc3 during the induction period. Table 1 shows the remaining percentage of the antioxidants after the induction period. Concentrations of DLPC and AAPH were 1.93 mM and 10.0 mM, respectively. It is shown in Table 1 that the antioxidants, except CTMC, were not completely consumed during the induction period. A comparison of Toc3 with Toc at the same concentration showed that the larger the induction period, the larger the consumption. Namely, both the induction period and the consumption decreased in the following order: Toc3(added)>Toc3(incorporated)=Toc (incorporated) >Toc(added).

Transfer from DMPC liposomes to DLPC liposomes. Table 2 shows the antioxidative activity in the binary mixture of DMPC liposomes and DLPC liposomes. Because DLPC liposomes did not contain any antioxidant at the beginning, and because the oxidation of the DLPC liposomes was inhibited, the antioxidant incorporated into or added to the DMPC liposomes was transferred from the DMPC to the DLPC liposomes. When transferred, the antioxidants were released from the DMPC liposomes and were added to the DLPC liposomes. Because the concentration of DLPC was much higher than that of DMPC, the desorption from the DMPC liposomes was the rate-limiting step of the transfer (14). Therefore, by comparison of the induction periods shown in Table 2 and in Figures 2 through 4, it can be estimated how readily the antioxidants are released from the DMPC liposomes.

TABLE 1

The Residual Antioxidant Found After the Induction Period; Concentrations of DLPC and AAPH—1.93 mM and 10.0 mM, Respectively

Antioxidant (μM)[a]	Induction period[b] (s)	Remaining percentage[b] (%)[c]
α-Toc[d] (13.5)	180	77
α-Toc3[d] (13.5)	2,400	18
γ-Toc[d] (13.5)	210	95
γ-Toc3[d] (14.9)	1,800	19
α-Toc[e] (13.2)	720	30
α-Toc3[e] (13.3)	990	26
α-Toc3[e] (2.7)	300	10
γ-Toc[e] (13.2)	1,050	45
γ-Toc3[e] (12.5)	1,230	42
CTMC[d] (13.3)	1,890	0

[a] Initial concentration added to or incorporated into the DLPC liposomes.

[b] Values are the average of duplicate runs.

[c] The percentages of the antioxidant remaining after the induction period.

[d] Added to the DLPC liposomes.

[e] Incorporated into the DLPC liposomal membrane.

TABLE 2

Antioxidative Activity in the Binary Mixture of DMPC Liposomes and DLPC Liposomes

Antioxidant (μM)[a]	[DMPC] (mM)	[DLPC] (mM)	[AAPH] (mM)	Induction period[b] (s)
α-Toc3[c] (0.82)	0.028	0.30	0.15	3,630
α-Toc3[c] (4.73)	0.161	1.91	1.12	3,660
CTMC[c] (0.81)	0.028	0.30	0.15	4,140
CTMC[c] (4.70)	0.161	1.91	1.12	3,960
α-Toc[c] (0.81)	0.028	0.30	0.15	1,380
γ-Toc[c] (0.81)	0.028	0.30	0.15	2,130
α-Toc3[d] (1.58)	0.161	1.91	1.12	135
α-Toc3[d] (4.73)	0.161	1.91	1.12	240
PMC[d] (4.71)	0.161	1.91	1.12	3,432

[a] Initial concentration added to or incorporated into the DMPC liposomes.

[b] The antioxidants were released from the DMPC liposomes and were added to the DLPC liposomes to inhibit the oxidation of the DLPC. By comparison of the induction periods shown in Table 2 and in Figures 2 through 4, it can be estimated how readily the antioxidants are released from the DMPC liposomes.

[c] Added to the DMPC liposomes.

[d] Incorporated into the DMPC liposomes.

Thus, the incorporated PMC appeared to be more readily released from the DMPC to the DLPC liposomes when compared with the incorporated α-Toc3. The induction period of the incorporated PMC was more than ten times as long as that of the incorporated α-Toc3 (see Table 2), and the difference in the antioxidative activity between the added PMC and the added α-Toc3 was not as large (see Figs. 2 and 4). Fukuzawa *et al.* (15) and Urano *et al.* (16) also described that PMC was readily released compared with that of α-Toc. Likewise, it was noticed that the added CTMC was more readily released from the DMPC to the DLPC liposomes than were the added α-Toc and α-Toc3 (see Figs 2, 4 and Table 2).

DISCUSSION

Comparison of Toc3 with Toc. Figures 2 and 3 show that both α-Toc3 and γ-Toc3 had the same antioxidative activity as that of their corresponding Toc when the antioxidant was incorporated into DLPC liposomal membrane. However, when the antioxidant was added to the liposome solution, the antioxidative activity of Toc3 was greater and that of Toc was less when compared with the incorporated Toc3 and Toc. The decrease in the efficiency of antioxidative activity of α-Toc when added to the liposome solution agrees with the data of Leibowitz and Johnson (17). However, no explanation was given as to why Toc's antioxidative activity depended on its dosing manner such as addition or incorporation. To settle this question, first we studied the consumption of the antioxidant during the induction period.

Judging from the remaining percentage of antioxidant after the induction period, the consumption and the antioxidative activity of Toc3 were larger than those of the corresponding Toc at the same initial concentration when added to the liposome solution. When incorporated into the liposomal membrane, both the consumption and the antioxidative activity of Toc3 and Toc were intermediate between those of the added Toc3 and Toc. This means that the antioxidative activities of Toc3 and Toc were related to the consumptions during the induction period.

Because Toc is a chain-breaking antioxidant (18), the differences in the consumptions of Toc3 and Toc can be explained by some inhibition in their reaction with phospholipid peroxide within the liposomal membrane. This interpretation is based on the assumption that Toc3 and its corresponding Toc have approximately the same k_{inh} value in a homogeneous organic solution, where the k_{inh} value is the rate constant for antioxidant's reaction with peroxyl radicals. However, there is no data of the k_{inh} value of Toc3 in a homogeneous organic solution (19,20). Burton *et al.* (20) estimated the linear correlation between the k_{inh} value with the σ_I constant of the 2-position's substituent of the chromanol nucleus, where the value of σ_I constant was the inductive effect of the substituent. No data of the σ_I constant for the long side-chain of both Toc and Toc3 is available (21). However, the difference in the σ_I constant of butyl and 3′-butenyl substituents was small (21), so it is likely that the σ_I constant and the k_{inh} value for Toc3 and Toc also are small.

Differences in the reactions of Toc3 and Toc with phospholipid peroxide within the membrane, when added to the liposome solution, may be related to the changes of the physicochemical property of the liposomal membrane. The membrane fluidity decreased not only by the incorporation of α-Toc into the liposomal membrane (15,22) but also by the addition of Toc3 and Toc to the liposome solution (23). In the latter report, the decrease in the membrane fluidity and the change in the membrane potential of the liposomes were affected in the following order: α-Toc>α-Toc3 and γ-Toc> γ-Toc3, respectively. However, there are other possible interpretations for Toc's poor consumption such as distribution of Toc and Toc3 to the inner bilayers of multilamellar vesicles or

non-monomeric dispersion of Toc in the liposomal membrane (24). Mehlhorn *et al.* (25) recently reported that a significant residual fraction of α-Toc remained after all the electron spin resonance (ESR) signal of tocopheroxyl had disappeared, and speculated that the decomposition products of α-Toc would act as antioxidants and would prevent net α-Toc consumption.

Comparison of α-Toc3 with γ-Toc3, and α-Toc with γ-Toc. The antioxidative activities of the incorporated Toc3 and Toc decreased in the following order: γ-Toc3> α-Toc3 and γ-Toc>α-Toc. This order agreed with that observed in the homogeneous system (26). When added, however, the opposite order was observed: γ-Toc3≤-Toc3, and γ-Toc≤α-Toc. It was reported in the homogeneous system that α-Toc and γ-Toc have different k_{inh} values and stoichiometric factors of the reaction between Toc and radicals (19). Therefore, it seemed that the differences in the antioxidative activities between α-Toc3 and γ-Toc3, or α-Toc and γ-Toc, could not be explained by their consumptions during the induction period. But, as it is clear that some portions of the antioxidant remain even after the induction period, we estimate that the differences in the antioxidative activities between α-Toc3 and γ-Toc3, or α-Toc and γ-Toc, can be explained by the different inhibition of their reaction with the phospholipid peroxide within the membrane.

Antioxidative activities of PMC and CTMC. Among antioxidants having the 5,7,8-trimethyl chromanol nucleus, the order of the antioxidative activities was (A) α-Toc3>PMC>CTMC>α-Toc ([AAPH]=0.3 mM) and (B) PMC>α-Toc3>α-Toc ([AAPH]=10 mM). It is not clear why the order of the antioxidative activities of PMC and α-Toc3 varied depending on the concentration of AAPH, although PMC may scavenge AAPH peroxy radicals as Niki *et al.* (27) described. However, by considering the factors such as the inhibition of the reaction with peroxyl radicals within the membrane and the k_{inh} value, the reactivity with peroxyl radicals in a homogeneous solution, other orders of the antioxidative activities among α-Toc3, α-Toc, PMC, and CTMC can be explained. The explanation will hereinafter be described.

By comparison of the induction periods shown in Table 2 and in Figures 2 through 4, the desorption of the antioxidants can be compared as described in the results of the text. Because the hydrophilic antioxidants were readily released (14), PMC and CTMC were more hydrophilic than α-Toc and α-Toc3. It was also reported (23) that PMC and CTMC were less hydrophobic and were less effective to the physicochemical property of the membrane. By analogy of the preceding discussion that the changes of the physicochemical property of the membrane were related to the inhibition of the reaction with peroxyl radicals, it was estimated that both PMC and CTMC interact with the membrane to a lesser extent and are less inhibited in the reaction within the membrane. Therefore, the difference in the antioxidative activities of PMC and CTMC may be explained by the difference in the k_{inh} values, because the k_{inh} values of PMC and CTMC were reported as 380×10^{-4} and 110×10^{-4} ($M^{-1}s^{-1}$), respectively (20). On the other hand, because α-Toc was strongly inhibited in the reaction with peroxyl radicals within the membrane compared with CTMC, and because the k_{inh} value of α-Toc was $320 \times 10^{-4}(M^{-1}s^{-1})$ (20), the difference in the antioxidative activities of α-Toc and CTMC may be explained by the difference in the inhibition rather than the difference in the k_{inH} values. The difference in the antioxidative activities of α-Toc3 and CTMC may be explained by the difference in the k_{inh} values, because α-Toc3 is not inhibited in the reaction within the membrane compared with α-Toc.

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