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Effects of α-tocopherol on the lipid peroxidation and fluidity of porcine intestinal brush-border membranes

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The effect of α-tocopherol on the lipid fluidity of porcine intestinal brush-border membranes was studied using pyrene as a fluorescent probe. Addition of α-tocopherol to the medium decreased fluorescence intensity and lifetime, but increased the fluorescence polarization of pyrene-labeled membranes. β-, γ-, and δ-Tocopherols gave no appreciable effect on the fluorescence intensity and polarization of the complex. The apparent dissociation constant (3.1 ± 0.12 μM) of the interaction of α-tocopherol with the membranes, estimated from the change in the fluorescence intensity with varying concentrations of α-tocopherol, was in good agreement with the concentration required to cause the half-maximal inhibition of lipid peroxidation of the membranes performed by incubation with 100 μM ascorbic acid and 10 μM Fe^{2+}. Decrease of the slope in the thermal Perrin plot of the polarization of pyrene-labeled membranes by α-tocopherol suggests that the movement of pyrene molecules in the membranes is restricted by binding of the tocopherol. This interpretation was confirmed by an increased harmonic mean of the rotational relaxation time of the dye molecules in the membranes from 10.9 ± 0.16 to 18.5 ± 0.51 μs after addition of 25 μM α-tocopherol to the medium. The perturbation of lipid phase in the membranes induced by α-tocopherol was also suggested from a decreased quenching rate constant of pyrene fluorecence in the membranes for Tl^+. Based on these results, the effect of α-tocopherol on the lipid fluidity of the membranes is discussed.

Introduction

Lipid peroxidation in biological membrane systems is postulated to proceed through a complex process, i.e. it involves a rearrangement and destruction of the double bonds in unsaturated lipids by propagation of lipid free radicals formed during lipid peroxidation [1–3]. The formation of a lipid hydroperoxide in membranes would result in damage of the membrane structure [4–6] and inactivation of membrane-bound enzymes [7–9].

As is well known [10–12], α-tocopherol is a major component of vitamin E and is capable of protecting unsaturated lipids from nonenzymatic autoxidation processes in vivo. This function of α-tocopherol may be assisted by a specific interaction of α-tocopherol with the lipids of biological membranes including hydrophobic binding [13] or hydrogen bonding [14]. Several investigators [15–18] have recently reported that α-tocopherol stabilizes the lipid layers of artificial membranes through the interaction of it with arachidonic acid. From these findings it seems that there is a close relation between the antioxidant activity and the membrane lipid stabilizing effect of α-tocopherol. However, reports on the effect of α-tocopherol on the lipids of biological membranes are few relative to those of artificial membrane systems [19,20].

In the present study, we have analysed the effect of tocopherols, especially that of α-toco-

pherol, on the nature of lipid bilayers of porcine intestinal brush-border membranes with special reference to changes in the fluorescence characteristics of pyrene bound to the membranes.

Materials and Methods

Brush-border membrane vesicles were prepared from porcine small intestine by the calcium-precipitation method as described in our previous paper [21] and suspended in 10 mM Tris-HCl buffer (pH 7.4). Protein concentration was assayed by the method of Lowry et al. [22] using bovine serum albumin as standard.

Pyrene and 2-thiobarbituric acid were purchased from Wako Pure Chemical Co. Pyrene was recrystallized from ethanol once before use and dissolved in ethanol to make a stock solution of 1 mM. Tocopherols (α, β, γ and δ) were obtained from Wako Pure Chemical Co. All other materials were the purest grade obtained from commercial sources.

Lipid peroxidation of the membrane vesicles was performed by incubation of them with 100 μM ascorbic acid and 10 μM $FeSO_4$ in 30 mM Tris-HCl buffer (pH 7.4) at 37°C for 30 min. The amount of thiobarbituric acid-reactive substances formed (as malondialdehyde) during the reaction was determined fluorometrically using 1,1,3,3-tetraethoxypropane as standard [23].

Labeling of membrane vesicles with pyrene was performed as follows. A suspension of the membrane vesicles (1 mg protein/ml) in 10 mM Tris-HCl buffer (pH 7.4) was incubated with 3.3 μM pyrene (dissolved in ethanol) at 0°C for 30 min. The final concentration of ethanol in the reaction mixture was 0.33%. Incubation was terminated by dilution of the mixture with a large volume of the same buffer and centrifugation at $25\,000 \times g$ for 20 min. The pellets were washed twice and resuspended in 10 mM Tris-HCl buffer (pH 7.4).

Fluorescence intensity and polarization measurements of pyrene-labeled membranes were carried out using a Hitachi spectrofluorometer MPF-4 equipped with a rhodamine B quantum counter. The sample compartment was maintained at 25°C by circulating water through the cell holder unless otherwise specified. The excitation and emission wavelengths used were 340 and 392 nm, respectively. The error due to light scattering of the sample at emission could be entirely prevented using a 350 nm cutoff filter.

The degree of fluorescence polarization, P, was expressed as $(I_V - I_H)/(I_V + I_H)$, where I_V and I_H are the intensities of vertically and horizontally polarized emission with excitation of vertically polarized light, respectively. The harmonic mean of the rotational relaxation times of pyrene, ρ_h, in the membranes were determined by the following equation [24] using the slope of a plot of $1/P$ versus T/η and the fluorescence lifetime, τ, measured by separate experiments:

$$\rho_h = \left(\frac{1}{P_0} - \frac{1}{3}\right)\frac{3\tau}{\text{the slope}} \cdot \frac{\eta}{T}$$

where P_0, η and T are the limiting polarization in the absence of rotational motion at $T/\eta = 0$, the solvent viscosity and the absolute temperature, respectively.

The lifetime of pyrene was determined with an Ortec PRA-3000 nanosecond spectrofluorometer (Photochemical Research Associates Inc., Ontario, Canada). Data were deconvoluted as biexponentials using a Digital MINC-11 computer system (Digital Equipment Co., Maynard, MA).

Quenching of the fluorescence with thallium (I) acetate (CH_3COOTI) was determined by titration of pyrene-labeled membranes with very small amounts of 100 mM CH_3COOTl solution in 10 mM Tris-HCl buffer (pH 7.4) at 25°C. The stock solution of the quencher was used within a few days after preparation. The quenching constant (K_Q) was calculated according to the following equation given by Stern and Volmer [25]:

$$(I_0/I) - 1 = K_Q[Q]$$

where I_0 and I denote the fluorescence intensities in the absence and presence of quencher, respectively, and [Q] the concentration of quencher. In other cases of quenching not obeying the simple Stern-Volmer law above, the K_Q value was estimated using the following modified Stern-Volmer equation [26]:

$$I_0/(I_0 - I) = \frac{1}{f_a K_Q} \cdot \frac{1}{[Q]} + \frac{1}{f_a}$$

where f_a is the effective fraction of fluorescence

that is quenchable. The quenching rate constant, k_q, was obtained by dividing the K_Q value by the fluorescence lifetime.

Results

Effect of α-tocopherol on the membrane lipid peroxidation

Fig. 1 shows the relative extent of lipid peroxidation of porcine intestinal brush-border membranes after incubation with 100 μM ascorbic acid and 10 μM Fe^{2+} at 37°C for 30 min with and without various concentrations of α-tocopherol. The amount of thiobarbituric acid-reactive substances formed under these conditions without α-tocopherol, was estimated at 1.02 ± 0.01 (S.D.) nmol/mg protein per 30 min. Addition of α-tocopherol to the incubation medium resulted in suppression of peroxidation at more than 20 μM of α-tocopherol. The α-tocopherol concentration which was required to induce half-maximal inhibition of membrane lipid peroxidation, was about 4.2 μM (inset, Fig. 1).

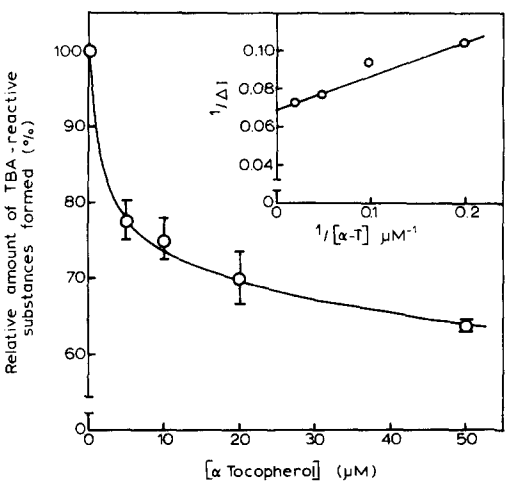

Fig. 1. Concentration dependence of the inhibitory effect of α-tocopherol on the lipid peroxidation of membranes induced by ascorbic acid plus Fe^{2+}. Membrane protein concentration was 0.99 mg/ml. α-Tocopherol concentration was varied from 5 to 50 μM. The amount of thiobarbituric acid (TBA)-reactive substances formed was expressed as relative to that formed in the absence of α-tocopherol. Values are expressed as mean ± S.D. of three membrane preparations. Inset: double-reciprocal plot of ΔI of the TBA complex against the α-tocopherol concentration. ΔI is the difference in fluorescence intensities in the absence and presence of α-tocopherol (α-T).

Alteration of the fluorescence parameters of pyrene-labeled membranes by addition of tocopherols

The difference in potency of four tocopherol congeners (α, β, γ and δ) on the fluorescence intensity of pyrene-labeled membranes was shown in Table I. Among them , α-tocopherol caused the largest decrease of fluorescence intensity, while the others which are known to be very low in antioxidant activity [10], gave less effect on the fluorescence.

The α-tocopherol concentration dependence of fluorescence intensity of pyrene-labeled membranes is presented in Fig. 2. With increasing concentrations of α-tocopherol in the medium, fluorescence intensity of the complex decreased with the minimum value at greater than 20 μM of α-tocopherol. The double-reciprocal plot of the change in fluorescence intensity versus the concentration of α-tocopherol added, revealed a linear relation over the concentration range tested (5–50 μM). The apparent dissociation constant of the complex between α-tocopherol and the membranes was estimated from the plot (inset, Fig. 2) at 3.1 ± 0.12 μM. This concentration of α-tocopherol was in good agreement with that required to induce the half-maximal inhibition of lipid peroxidation of membranes by ascorbic acid and Fe^{2+} (Fig. 1). On the other hand, the apparent dissociation constants of β-, γ- and δ-tocopherols for membranes were estimated at 21.1 ± 1.35, 49.8 ± 9.81 and 64.5 ± 8.10 μM, respectively.

TABLE I

EFFECT OF TOCOPHEROLS ON FLUORESCENCE INTENSITY AND POLARIZATION OF PYRENE-LABELED MEMBRANES IN 10 mM Tris-HCl BUFFER (pH 7.4) AT 25°C

Membrane protein concentration was 0.15 mg/ml. Concentration of each tocopherol was 10 μM. The fluorescence intensity is expressed as relative to that without addition of tocopherol. Values are expressed as mean ± S.D. of three membrane preparations.

Tocopherols	Relative fluorescence intensity (%)	Polarization *P*
No addition	100	0.145 ± 0.001
α-Tocopherol	66.5 ± 1.5	0.170 ± 0.004
β-Tocopherol	86.3 ± 2.8	0.160 ± 0.001
γ-Tocopherol	89.0 ± 1.0	0.157 ± 0.002
δ-Tocopherol	83.0 ± 7.0	0.158 ± 0.001

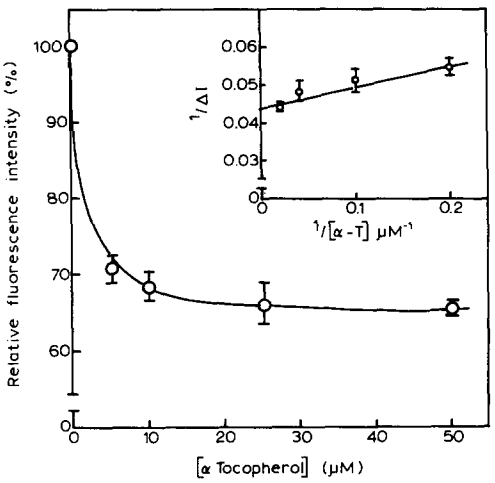

Fig. 2. α-Tocopherol concentration dependence of fluorescence intensity of pyrene-labeled membranes. Membrane protein concentration was 0.041 mg/ml. Other experimental conditions were the same as described in Fig. 1. Values are expressed as mean ± S.D. for three membrane preparations. Inset: double-reciprocal plot of ΔI against the α-tocopherol concentration. ΔI represents the difference in fluorescence intensities in the absence and presence of α-tocopherol (α-T).

In addition, effect on the membrane-bound pyrene fluorescence of the addition of tocopherols was examined with reference to its polarization. As shown in Table I, of the four types of tocopherols tested, α-tocopherol was also the most effective in increasing the fluorescence polarization of pyrene.

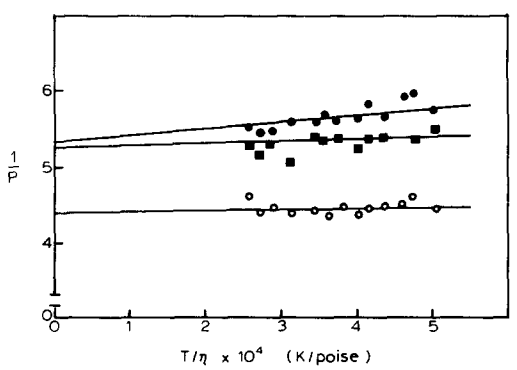

Fig. 3. Temperature dependence of the fluorescence polarization (P) of pyrene-labeled membranes in the presence or absence of α-tocopherol. Membrane protein concentration was 0.102 mg/ml. The temperature was varied progressively from 15 to 45°C. α-Tocopherol: ●, no addition; ■, 25 μM; ○, 50 μM. Other experimental conditions were the same as described in Table I.

Fig. 3 shows the temperature dependence profiles of the polarization of pyrene fluorescence in membranes with and without α-tocopherol. The degree of the polarization of the complex in the absence of α-tocopherol decreased progressively with increasing temperature, and the plot of $1/P$ versus T/η showed a linear relation with a definite slope over the temperature range tested. On the other hand, the slope in the Perrin plot was decreased by addition of α-tocopherol depending on its concentration, suggesting that the temperature-dependent movement of the probe molecules in the membrane lipids is restricted in the presence of antioxidant in the medium [27].

The fluorescence lifetime and rotational relaxation time of pyrene in the membranes

Analysis of the fluorescence lifetime of pyrene in the membranes provides an additional insight into the microenvironment around the probe.

The values of the lifetime of pyrene-labeled membranes in the presence and absence of α-tocopherol are listed in Table II. It should be noted that the fluorescence decay curve of the complex shows a better fit to a two, rather than a single phase exponential decay function, indicating existence of at least two populations of the energy state of the dye. The lifetimes of pyrene in membranes without α-tocopherol, $\tau_1 = 9.63 \pm 1.07$ ns and $\tau_2 = 143.1 \pm 6.00$ ns, were both decreased progressively by the addition of increasing concentrations of α-tocopherol; τ_2 being more effective.

Table II also presents the harmonic means, ρ_h, of the rotational relaxation times of pyrene molecules in the membranes with and without α-tocopherol. An increase of the ρ_h value of pyrene-labeled membranes was clearly dependent on the concentration of α-tocopherol.

Quenching of the fluorescence with CH_3COOTl

The quenching profile of the fluorescence of pyrene alone in the medium and in the presence of CH_3COOTl (1 mM) is shown in Fig. 4. A large quenching (about 30% at the maximal fluorescence) at, concentrations as low as 1 mM of Tl^+, indicates that the Tl^+ ion is very efficient quencher for the fluorescence of pyrene. A typical linear correlation between $(I_0/I) - 1$ and [Q] over a

TABLE II

EFFECT OF α-TOCOPHEROL ON FLUORESCENCE LIMITING POLARIZATION (P_0), FLUORESCENCE LIFETIME AND HARMONIC MEAN OF ROTATIONAL RELAXATION TIMES (ρ_h) OR PYRENE-LABELED MEMBRANES

The fluorescence lifetimes were measured under the following conditions: 10 mM Tris-HCl buffer (pH 7.4); membrane protein concentration, 0.166 mg/ml; 25°C. The limiting polarization (P_0) and harmonic mean of the rotational relaxation times (ρ_h) were determined from the data shown in Fig. 3 and the lifetime values calculated by separate experiments.

α-Tocopherol concn. (μM)	Lifetime (ns) [a]		ρ_h (μs)	P_0 [a]
	τ_1	τ_2		
0	9.63 ± 1.07	143.1 ± 1.07	10.9 ± 0.16	0.187 ± 0.003
10	7.22 ± 0.68	101.7 ± 2.98	–	–
25	6.87 ± 0.72	86.5 ± 2.74	18.5 ± 0.51	0.193 ± 0.003
50	6.76 ± 0.76	74.8 ± 2.70	24.4 ± 1.05	0.230 ± 0.005

[a] Values were expressed as mean ± S.D. of three membrane preparations.

wide range of the quencher concentration (inset, Fig. 4), proves the collisional mechanism of quenching which follows the Stern-Volmer equation [26]. However, the quenching of the fluorescence of pyrene-labeled membranes by Tl^+ did not follow a simple Stern-Volmer law, i.e. the intercept on the ordinate was above zero. This suggests that the probe molecules are arranged within the lipid bilayer of the membranes such that a certain proportion of the molecules are accessible to the quencher [26].

The modified Stern-Volmer plots of the quenching of fluorescence of pyrene-labeled membranes, with and without α-tocopherol are shown in Fig. 5. The plots were linear over a wide concentration range of CH_3COOTl tested, regardless of the presence or absence of α-tocopherol. The

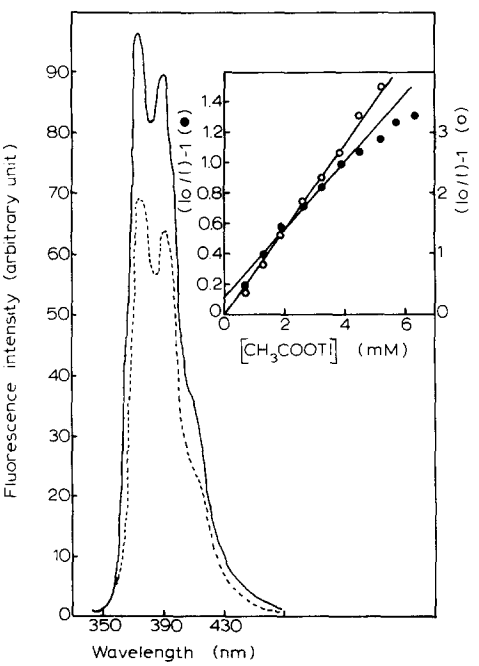

Fig. 4. The fluorescence intensities of pyrene in the presence (- - - - - -) and absence (———) of Tl^+. The concentrations of pyrene and CH_3COOTl were 1 μM and 1 mM, respectively. Inset: plot of $(I_0/I)-1$ against [Q]. Symbols: ○, pyrene alone (1 μM); ●, pyrene-labeled membranes (0.046 mg protein/ml). The CH_3COOTl concentration was varied up to 6.25 mM. Other experimental conditions were the same as described in Table I.

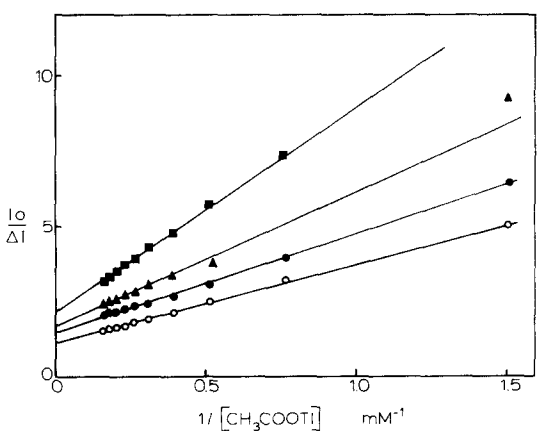

Fig. 5. Modified Stern-Volmer plots of pyrene-labeled membranes in the presence and absence of α-tocopherol. Membrane protein concentration was 0.046 mg/ml. α-Tocopherol: ○, no addition: ●, 10 μM; ▲, 25 μM; ■, 50 μM. ΔI represents the difference in the fluorescence intensities in the absence and presence of quencher. Other experimental conditions were the same as described in Fig. 4.

TABLE III

EFFECT OF α-TOCOPHEROL ON THE QUENCHING PARAMETERS OF PYRENE-LABELED MEMBRANES WITH Tl^+ AT 25°C

The values were calculated from data shown in Fig. 5 and expressed as mean ± S.D. for three membrane preparations.

α-Tocopherol concn. (μM)	K_Q (M^{-1})	k_q ($M^{-1} \cdot s^{-1}$)	f_a
0	612.3 ± 27.9	$5.79 \cdot 10^9$	0.79 ± 0.018
10	483.8 ± 53.2	$4.79 \cdot 10^9$	0.64 ± 0.031
25	537.8 ± 32.5	$6.10 \cdot 10^9$	0.55 ± 0.028
50	351.7 ± 26.5	$4.29 \cdot 10^9$	0.29 ± 0.042

quenching parameters calculated on these data are summarized in Table III.

The quenching constant (K_Q) and the quenching rate constant (k_q) of the system without α-tocopherol were 612.3 ± 27.9 M^{-1} and $5.79 \cdot 10^9$ $M^{-1} \cdot s^{-1}$, respectively. The f_a value obtained from the intercept on the $I_0/\Delta I$ axis is 0.79 ± 0.018, indicating that about 80% of the total pyrene molecules in the membranes can be quenched by Tl^+. This f_a value of the complex decreased by about 60% with the addition of 50 μM α-tocopherol, indicating that a fraction of the total population of fluorescing species accessible to the quencher is markedly decreased in the presence of α-tocopherol [26]. Other quenching parameters such as K_Q and k_q also decreased by the addition of α-tocopherol. These results suggest that the lipid domain of the membranes alters by interaction with α-tocopherol.

Discussion

Addition of tocopherols (α, β, γ and δ) to pyrene-labeled membranes resulted in marked changes in the fluorescence parameters of the complex (Table I and Fig. 3). α-Tocopherol was the most effective of the four tocopherols.

The apparent dissociation constant (3.1 ± 0.12 μM) of the complex between α-tocopherol and the membranes, determined from the decrease in the fluorescence intensity of the complex with increasing concentrations of α-tocopherol, was in good agreement with the concentration of α-tocopherol required to induce the half-maximal inhibition of lipid peroxidation of the membranes with ascorbic acid plus Fe^{2+} (Fig. 1). Based on these results, it is postulated that the fluorescence change of the complex by addition of α-tocopherol reflects the alteration in the environmental properties around the dye molecules due to the antioxidant, and the relation of binding of α-tocopherol with its antioxidant effect.

The fluorescence polarization study is expected to give details about the dynamic nature of the membrane lipids [27–29]. A marked increase in the polarization and a decrease in the slope in the Perrin plot of pyrene-labeled membranes due to addition of α-tocopherol (Table I and Fig. 3) suggest the reduction of the mobility of the dye molecules by the interaction of α-tocopherol with the membrane lipids. In fact, we have demonstrated that the harmonic mean of the rotational relaxation times of pyrene in the membranes markedly increased, depending on α-tocopherol concentration in the medium (Table II).

The discrepancy between the α-tocopherol-induced changes in the fluorescence intensity and the polarization measurements is difficult to understand, because it is generally accepted [30] that the intensity of fluorescence increases as the rotational movement of fluorescent dye molecules is restricted. Although it is difficult at present to characterize this phenomenon, it seems that changes in the organization of the hydrocarbon region in the membranes induced by interaction with α-tocopherol may affect the aligment of the pyrene molecules in configurations which produces decrease in the fluorescencse intensity (Table I and Fig. 2) and lifetime (Table II).

Perturbation of lipid domain in the membranes by α-tocopherol was also suggested by the quenching experiment with CH_3COOTl (Table III). As is well known [25], the quenching rate constant, k_q, is dependent on diffusion-controlled processes, so that a decrease of the k_q value suggests, in general, a decreased accessibility of the quencher for fluorophore. However, it has been reported by several investigators [26,31,32] that quenching of a fluorophore attached to macromolecules by an ionic quencher is influenced by not only the accessibility of the quencher but also other factors such as electrostatic forces and configuration of the binding site. Since membrane lipids are known to bind cations, the fluorescence dependence on the

concentration of Tl^+ may be also influenced by the binding. Recently, Ando and Asai [33] have demonstrated the existence of electrostatic interaction between Tl^+ and the phosphate groups of $1,N^6$-ethanoadenosine oligophosphates. Therefore it seems that the effect of binding of Tl^+ ions to negatively charged groups on the membrane surface is also partly contributed to the α-tocopherol-induced changes in the quenching parameters observed in the present study. In any way, α-tocopherol binding resulted in marked decreases in the quenching parameters (Table III), suggests a reduction of sensitivity of pyrene fluorescence in the membranes for Tl^+ due to alteration in the nature of lipid domain in the membranes.

On the basis of these results, we concluded that the interaction of membrane lipids with α-tocopherol induces perturbation of the lipid organization. Especially, the increases of the fluorescence polarization (Table I) and the harmonic mean of the rotational relaxation times of pyrene molecules (Table II) strongly suggest that α-tocopherol induces restriction of movement of the dye molecules in the membranes due to intensified molecular rigidity around the region where the dye molecules are located, i.e. decrease of membrane fluidity [27,28].

Recently, several investigators have proposed the contribution of the hydroxy group on the chromanol ring [14,34] and the polyisoprenoid side-chain [13,15,35] to the antioxidant activity of α-tocopherol, but the specific mechanism of the interaction between them still remaines obscure. Therefore, although the nature of the interaction between α-tocopherol and the membrane lipids is not clear at present, the results described above indicate that the physical state of the lipid phase in biological membranes should provide one of the important clues elucidating the antioxidant activity of α-tocopherol.

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