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Effect of the Membrane Boundary Potential on the Phase Transition of Dipalmitoylphosphatidylcholine Bilayer Liposomes†

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Effect of the membrane boundary potential on the thermotropic fluid-solid phase transition of the uniformly sized single lamellar liposomes of dipalmitoylphosphatidylcholine, DPPC, has been investigated by measuring the fluorescence intensity of N,N'-distearyloxacarbocyanine, DSOCC, embedded in the liposomes. The change in the electric field at the polar head group region of the lipid bilayers was induced by the addition of sodium tetraphenylborate, Na^+TPB^- and/or tetraphenylphosphonium chloride, TPP^+Cl^-, in the liposome dispersions and it was confirmed to be established by the measurements of the electrochromic absorption change of DSOCC. An addition of Na^+TPB^- at the concentration of 0.1 mM resulted in the drop of the phase transition temperature of the lipid bilayers for about 7°C, while TPP^+Cl^- gave a detectable but very small effect on the phase transition temperature. The relationships between the phase transition temperature and the amount of TPB^- absorbed in the bilayers and the electrochromic absorption change were obtained to discuss the effect of the electric field on the phase transition.

INTRODUCTION

Fluidity of biological membranes has been known to affect the intra- and intermolecular structure and functional activity of membrane bound proteins.[1,2,3] Since an abrupt change in the fluidity of the biological membranes is

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usually associated with the fluid to solid phase transition with regard to the acyl chains of lipid molecules, in certain cases, the change in some physicochemical conditions of the surroundings of the membranes results in the alternation of the structure and properties of the biological membranes through the phase transition of the lipids.[4,5,6] From this point of view, many works have been carried out to examine the external variables which may give a significant effect on the phase transition of the lipid bilayers. The pressure dependence of the phase transition was investigated for DPPC liposome dispersions in water by Srinivasan *et al.* and the transition temperature was found to increase about 2.5°C at 100 bars.[7] The effect of the additives on the phase transition was examined by Jain *et al.* with more than ninety lipid-soluble compounds such as uncouplers, alkanols, fatty acids, detergents, organic solvents, ionophores, inorganic ions.[8] The type of the effect was found to depend on the nature and concentration of the additives. Electrostatic effects at charged membranes have been studied to find that the phase transition can be regulated electrostatically, e.g., by varying the external pH and ionic strength.[9] Jähnig *et al.* found that with increasing surface charge in the solid phase, the bilayer expands laterally at the expense of the thickness, whereas the hydrocarbon chain packing stays constant and the internal chain ordering does not change. From these results, they suggested that a tilt of the chains is caused by the surface charges on the polar head and results in the shift of the phase transition temperature.[10] In neutral lipid bilayers, however, the electrostatic effect on the phase transition has never be shown experimentally. In this paper, we intend to show experimental evidences which suggest that the change in the electric field perpendicular to the membrane surfaces at the polar head region may result in the alternation of the phase transition temperature of single lamellar DPPC liposomes. We expect that the addition of the salt composed of a lipophilic ion and a hydrophilic ion such as Na^+TPB^- and TPP^+Cl^- in the liposome dispersions induces the charge separation at the membrane solution interfaces by the absorption of the lipophilic ions on the membrane surfaces. This charge separation produces not only the electrostatic potential difference between the bulk aqueous phase and the plane of the absorbed ions, but also the electric field within the membrane phase by the partial penetration of the absorbed lipophilic ions into interior of the membrane phase. In the presence of the electric field, thus induced, there will be an additional energy terms, U_f, due to the field-dipole interaction, which may lead to the alternation of the phase transition temperature.

EXPERIMENTS

MATERIALS

l-α-dipalmitoylphosphatidylcholine, DPPC, purchased from Sigma Chemical Co. was purified by thin layer chromatography on silica gel (Merck 60 F254).

N,N''distearyloxacarbocyanine iodide, DSOCC, in a crystalized form was purchased from the Japan Research Institute for Photosensitizing Co. Ltd. and was used without further purification. Sodium tetraphenylborate, Na^+TPB^-, tetraphenylphosphonium chloride, TPP^+Cl^-, tris-HCl and NaCl of analytical reagent grade from Wako Pure Chemicals Ltd., were used without further purification. Sepharose 2B and Sephadex G-50 molecular sieves were purchased from Pharmacia Fine Chemicals.

PHASE TRANSITION MEASUREMENTS

The uniformly sized single lamellar liposomes of DPPC containing 0.4 weight percent DSOCC were prepared in a buffered solution (1 mM tris-HCl, 0.1 M NaCl) at pH 8.00 by the alcohol method[11] followed by molecular sieve chromatography on a Separose 2B gel, according to the early work of this series.[12] The molecular weight of the liposomes was determined as 1.4×10^7 daltons from the partition coefficient of 0.32 on the Sepharose 2B gel by using the previously obtained equation.[12] The thermotropic phase transition behavior of the liposomes in the presence and absence of TPB^- and/or TPP^+ was examined by measuring the fluorescence intensity of DSOCC embedded in the liposome bilayers, according to the method previously reported.[13,14] Fluorescence measurements were performed on a Hitachi Model MPF-4 spectrofluorometer with a temperature controlled cell holder. The temperature profile of the fluorescence intensity was obtained for cooling and heating scans between 50 to 20°C at the rate of 0.5°C/min using a circulating water bath with temperature programmer (Neslab. Instruments INC.RTE-8). The temperature was monitored with a copper-constantan thermocouple dipped in the sample cell.

Analyses of TPB^- and TPP^+ contents absorbed in the liposome bilayer

DPPC liposome dispersions were prepared in the buffered solution (1 mM tris-HCl at pH 8.00) with various concentrations of NaCl by the sonication method[15] at 45°C and their multilamellar fractions were removed by the gel filtration. DPPC concentrations in the liposome dispersions were determined by phosphate assey, according to the Fiske-Subbarow method.[16] After the given amounts of TPB^- or TPP^+ solution and the liposome dispersions were added into the definite volume of the buffered solution and incubated at 45°C for 1 hour, the liposomes were removed from the solution by the ultrafiltration (Amicon Diaflo ultrafiltration membranes, XM100A) at the same temperature and the concentration of TPB^- or TPP^+ in the filtrate was determined spectrophotometrically at 240 nm. All experiments were repeated on two separate preparations at least.

Measurements of electrochromism of DSOCC in the liposome bilayers

A generation of electric field by the addition of Na^+TPB^- or TPP^+Cl^- was confirmed by measuring the electrochromic absorption change of DSOCC with a Hitachi 320 spectrophotometer at 4.00 ± 0.05°C. For this purpose, egg phosphatidylcholine, egg PC was used instead of DPPC, because egg PC liposomes are more stable than DPPC liposomes. The absorption change induced by the addition of TPB^- was measured as a function of TPB^- concentration.

RESULTS AND DISCUSSION

Relationship between the phase transition temperature and the amount of the lipophilic ions absorbed in the liposome bilayers

In the early works,[13,14] we demonstrated that the fluorescence quantum yield, ϕ, of DSOCC in solution satisfies the following equation

$$\ln[\phi/\{1 - \phi(1 + k_1/k_f)\}] = \ln(k_f/k_2) + E/RT \qquad (1)$$

where k_f is the rate constant of the fluorescence process and k_1 and $k_2 \exp(-E/RT)$ are those of the temperature independent and dependent nonradiative processes, respectively. A test of the applicability of Eq. 1 with the data obtained in methanol and in DPPC and dimyristoylphosphatidylcholine, DMPC, single lamellar liposomes is presented in Figure 1 with the approximation of $k_f \gg k_1$. The results show good linear relations between $\ln[\phi/(1 - \phi)]$ and $1/T$ not only in methanol but also in the DMPC liposomes over the temperature range studied, while for the DPPC liposomes, the plots are clearly devided into three parts. We already showed that in the case of the DPPC liposomes, the low and high temperature parts in which the plots show the straight lines correspond to the monophase regions, namely the solid and fluid phases, respectively and the intermediate region does to the state in which both phases coexist.[14] Assuming the partition coefficients of the dye in the both phases of the lipid bilayers to be equivalent, the fluid phase fraction of the lipids, θ, is calculated by the equation:

$$\theta = \frac{\phi_f(T) - \phi_s(T)}{\phi_{fl}(T) - \phi_s(T)} \qquad (2)$$

Here $\phi_f(T)$ is the observed fluorescence quantum yield at temperature T in the region of the phase transition and $\phi_s(T)$ and $\phi_{fl}(T)$ are those in the solid and fluid phases, respectively. The values of $\phi_s(T)$ and $\phi_{fl}(T)$ in the region of the phase transition were estimated by extraporating the straight lines given in the low and high temperature parts into the phase transition region. Figure 2 illustrates the temperature profiles of θ thus obtained as a function of Na^+TPB^- concentration. The results indicate a drop of the midpoint of the phase transition, T_m, for several degrees (from 41 to 34°C) as Na^+TPB^- concentration increases from 10 μM to 0.5 mM. It was also found that the magnitude of T_m

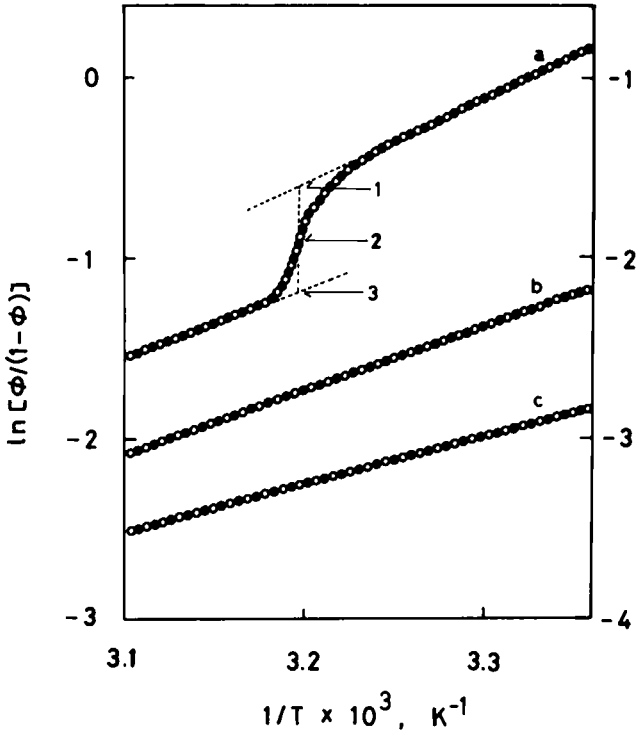

FIGURE 1 Plots of $\ln [\phi/(1 - \phi)]$ vs. $1/T$. (a) in DPPC, (b) in DMPC, (c) in methanol. Arrows 1, 2 and 3 indicate the values of $\ln [\phi/(1 - \phi)]$ from which the values of ϕ corresponding to the solid, intermediate and fluid states were determined, respectively. ●; cooling scan, ○; heating scan.

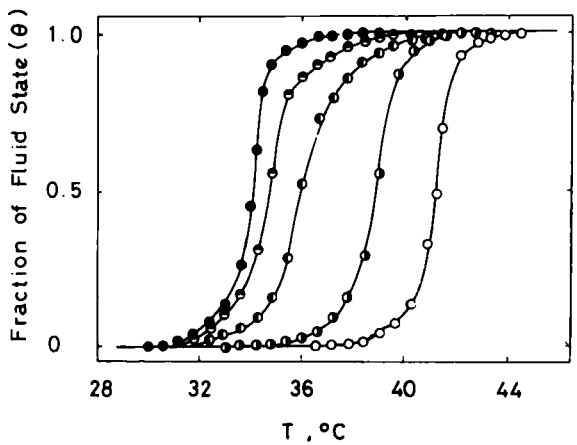

FIGURE 2 Temperature profiles of θ obtained as a function of Na^+TPB^- concentration. Na^+TPB^- concentration: ○; 0 M, ◑; 10 μM, ◐; 50 μM, ◒; 0.1 mM, ●; 0.5 mM.

shift depended on the ionic strength as well as the Na^+TPB^- concentration, as increased with increasing the ionic strength. While, the addition of TPP^+Cl^- gave little effect on T_m even when its concentration and the ionic strength were considerably high (T_m = 40.0°C at $[TPP^+Cl^-]$ = 0.5 mM and [NaCl] = 0.1 M). Thus, the presence of the lipophilic anion has a pronounced effect on the phase transition of the DPPC liposomes but the corresponding cation has no significant effect, although the hydrophobicities of these ions are considered to be equivalent. These results may be interpreted in terms of the preferential absorption of DPPC bilayers for TPB^- to TPP^+. Figure 3-A shows the plots of

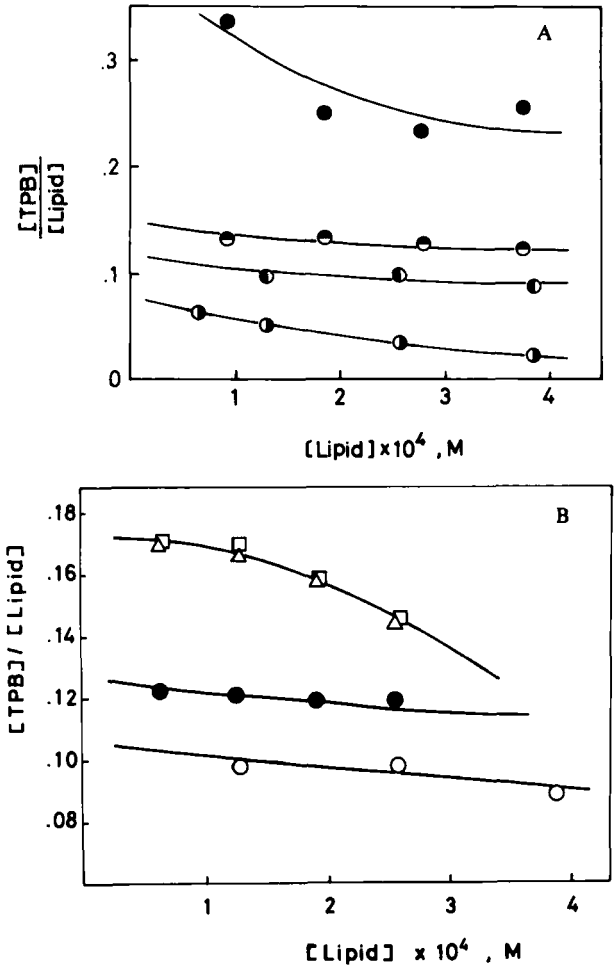

FIGURE 3 Plots of the amount of the absorbed TPB^- relative to DPPC molecule vs. DPPC concentration. (A) The effect of Na^+TPB^- concentration at a given ionic strength (1 mM NaCl, 1 mM tris-HCl). Na^+TPB^- concentration: ◑; 10 μM, ◐; 50 μM, ◒; 0.1 mM, ●; 0.5 mM. (B) The effect of the ionic strength at a given concentration of Na^+TPB^- (50 μM). NaCl concentration: ○; 1 mM, ●; 10 mM, △; 30 mM, □; 50 mM. The tris-HCl concentration was fixed at 1 mM.

the number of the absorbed TPB^- relative to DPPC molecule against the lipid concentration varing the added Na^+TPB^- concentration at a given ionic strength (1 mM NaCl, 1 mM tris-Cl). Figure 3-B is the corresponding plots varing the ionic strength at a given Na^+TPB^- concentration (50 μM). The results show that a considerable amount of TPB^- is absorbed on the liposome bilayers, as increases with increasing the ionic strength as well as the Na^+TPB^- concentration. In constrast to this, the absorption of TPP^+ on the DPPC bilayers was not detected by the present method. These facts are interpreted in terms of the arrangement of the dipole moments of the lipid head groups in the bilayer. In the case of phosphatidylcholine bilayers, the external surfaces adjacent to the aqueous phases comprise the positively charged choline groups which may prohibit the cations from binding on the membrane surfaces.

The relationships between the change in T_m, ΔT_m, and the amount of the absorbed TPB^- relative to the lipid molecule is shown in Figure 4. The results clearly demonstrate that ΔT_m monotonically increases with the amount of the absorbed TPB^- relative to the lipid, irrespective of the concentrations of Na^+TPB^- and the lipids and the ionic strength themselves.

The effect of coexistance of TPB^- and TPP^+ in the dispersions on the phase transition was also examined as follows. At first, T_m of the DPPC liposomes was lowered at 37°C by the addition of Na^+TPB^- at 50 μM and then TPP^+Cl^- was added to the dispersion at various final concentrations. After incubated at

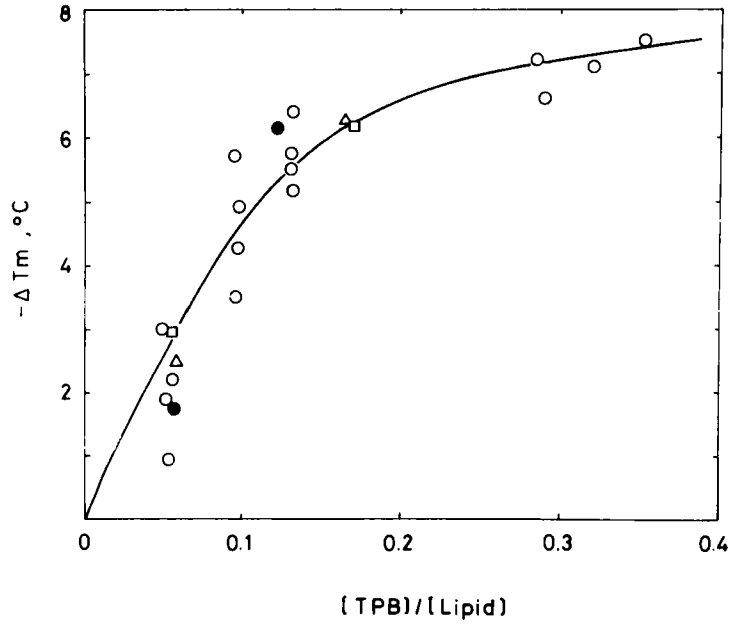

FIGURE 4 Plots of ΔT_m against the amount of absorbed TPB^- relative to DPPC molecule at various concentrations of Na^+TPB^- and NaCl. NaCl concentration: ○; 1 mM, ●; 10 mM, △; 30 mM, □; 50 mM. The tris-HCl concentration was fixed at 1 mM.

TABLE I

Effect of TPP^+ concentration on T_m in the presence of TPB^- ions.

TPP^+ concentration(M)	T_m(°C)
0	37.0
5.0×10^{-6}	37.0
1.0×10^{-5}	37.1
2.5×10^{-5}	37.8
3.5×10^{-5}	38.0
4.0×10^{-5}	39.0
5.0×10^{-5}	40.7

TPB^- concentration: 5.0×10^{-5} M.

45°C for 30 min., T_m of each sample was measured. As shown in Table 1, T_m increased with TPP^+Cl^- concentration up to 40.7°C at 50 μM which coincides with the value of T_m in the absence of TPB^- and TPP^+. These results may rule out the possibility that the T_m drop by the addition of Na^+TPB^- is due to a simple impurity effect of TPB^- and they may be interpreted as the electrostatic field induced by the absorption of TPB^- in the membrane phase was compensated by the charge neutralization due to the absorption of TPP^+. Although other possibilities still remain, the evidences shown here seem to suggest that the T_m shift induced by TPB^- is attributed to the change in the electrostatic field in the membrane phase at the vicinity of the surfaces.

Relationship between ΔT_m and the electrochromic absorption change of DSOCC in the bilayers

Since DSOCC has a lipid-like structure with two long acyl chains, the hydrophilic chromophore of the dye is considered to locate at the polar head group region of the bilayers, laying the methene bond axis parallel to the membrane surface. In this case, the change in the spectrum of the dye is expected to be associated with the change in the electric field perpendicular to the membrane surface as shown by Witt *et al.* with using various kinds of amphiphilic dyes.[17,18,19] In Figure 5, the difference spectra of DSOCC embedded in the DPPC liposomes in the presence of Na^+TPB^- against in the absence of Na^+TPB as a function of the Na^+TPB^- concentration and compared with the first derivative of the absorption spectrum in the absence of Na^+TPB^-. The shapes of the difference spectra are essentially similar to that of the first derivative independent of the amount of added Na^+TPB^-, suggesting that these absorption changes are due to the shift of the absorption band by the electric field. In Figure 6, the values of ΔT_m induced by TPB^- under various conditions

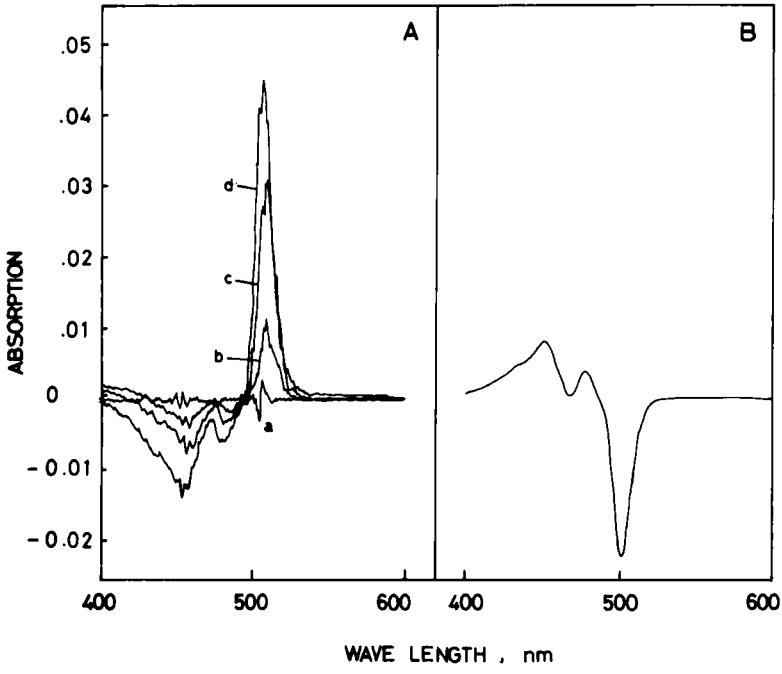

FIGURE 5 Comparison of the difference spectrum with the first derivative of absorption spectrum of DSOCC in the liposome bilayers. (A) The difference spectra in the presence of Na^+TPB^- against in the absence of Na^+TPB^-. a, b, c and d indicate the concentrations of Na^+TPB^- in the dispersions to be 0 μM, 10 μM, 25 μM and 50 μM, respectively. NaCl concentration was fixed at 1 mM. (B) The first derivative of absorption spectrum of DSOCC in the absence of Na^+TPB^-.

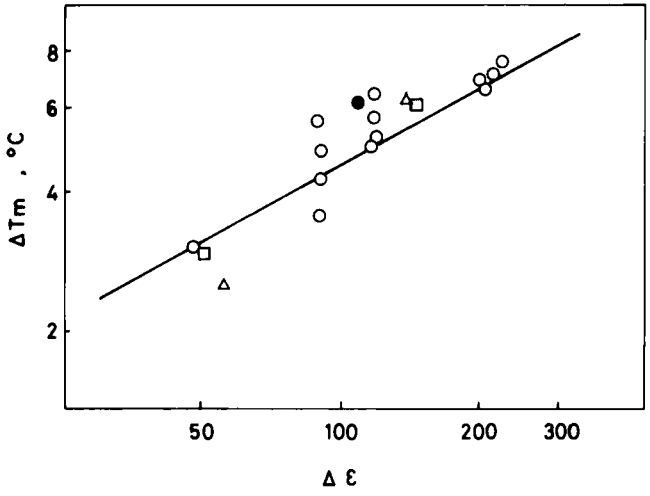

FIGURE 6 Plots of ΔT_m vs. $\Delta\epsilon$. Notations are same in Figure 4.

are plotted against the change in the molar extinction coefficient, $\Delta\epsilon$, at 504 nm obtained at the same conditions both in logarithmic scale. The results show a linear relationship between them with a slope of ca. $\frac{1}{2}$, irrespective of the concentrations of Na^+TPB^- and the lipids and the ionic strength. In general, the electrochromic band shift, $\Delta\nu$, is expressed by the equation:

$$h\Delta\nu = -(\mu_e - \mu_g)F - (\tfrac{1}{2})(\alpha_e - \alpha_g)F^2 \tag{3}$$

where μ_g and α_g are the permanent dipole moment and polarizability of the dye in the ground state and μ_e and α_e are the corresponding values in the excited state and F is the electric field. A shift of the absorption band by $\Delta\nu$ yields a change of the molar extinction coefficient at a fixed frequency of

$$\Delta\epsilon = (\partial\epsilon/\partial\nu)_F(-\Delta\nu) + (\tfrac{1}{2})(\partial^2\epsilon/\partial\nu^2)F(-\Delta\nu)^2 + \cdots \tag{4}$$

Inserting Eq. 3, one obtains approximately

$$\Delta\epsilon = [(\mu_e - \mu_g)/h]F(\partial\epsilon/\partial\nu)_F + (\tfrac{1}{2})[(\alpha_e - \alpha_g)/h]F^2(\partial\epsilon/\partial\nu)_F + (\tfrac{1}{2})[\mu_e - \mu_g)^2/h^2]F^2(\partial^2\epsilon/\partial\nu^2)_F \tag{5}$$

If the chromophore of the dye has a permanent dipole moment difference with a fixed preferential orientation relative to the field, the first term in Eq. 5 is predominant at field changes in the order of $F = 10^5$ V/cm, otherwise the second term may mainly contribute to $\Delta\epsilon$. Unfortunately we have no experimental evidence showing which term is predominant in the present system. Since the first term depends linearly on the field strength, if it is the predominant term, the results shown in Figure 6 suggest that ΔT_m is proportional to the electric field strength, but if the second term is predominant, it should be concluded that ΔT_m is proportional to square of the field strength. We are now working on this point, so the experimental evidences and detailed discussion concerning the field dependency of T_m will be presented in the succeeding paper of this series.

Tentative models on the electric field dependency of the phase transition temperature in a neutral lipid bilayer

If there is a difference in the orientation of the dipole axis of the head group between below and above T_m, we can expect that the change in the electric field yields an additional energy term due to the field-dipole interaction to the free energy change associated with the transition. It has been well established that at least above T_m, the dipole axis of DPPC is almost parallel to the surface of the bilayer by using a variety of methods, such as x-ray diffraction,[20] neutron diffraction[21] and ^1H, ^2H and ^{31}P nmr.[22,23] Recently Büldt *et al.* examined the average orientation of the head group dipole of DPPC multilamellar dispersion at 28°C($L_{\beta'}$ phase) and 50°C(L_β phase) by neutron diffraction with selectively deuterated lipids at four different positions in the head group region and

presented the results indicating the angle between the dipole and the bilayer surface to be less than 20° at both temperatures.[24] Taking these results into account, we tentatively propose a simple model to interpret the field dependence of T_m. We assume that the zwitterionic dipole head group is nearly parallel to the surface of the membrane below T_m as well as above T_m, but their absolute values are slightly different from each other, presumably the tilt angle against the normal of the membrane surface in the solid phase might be smaller than the corresponding value in the fluid phase, reflecting the stiffness of the hydrocarbon chain region in the solid phase. Using electrostatics, one has $U_f = \mu \cdot F$ per dipole, where U_f is the additional energy due to the field-dipole interaction and μ is the dipole moment of the head group of lipid. Thus, $U_f = -|\mu| F \cos\theta$, where θ is the angle between the field direction and the dipole axis. If the angle might change during the solid to fluid transition from θ_s to θ_f, the resulting change in T_m would be as follows;

$$\Delta T_m = -(|\mu| \cdot F/\Delta S_m)(\cos\theta_f - \cos\theta_s)$$

where ΔS_m is melting entropy. Consequently, if $\theta_f > \theta_s$, T_m is expected to decrease proportionally with increasing the field strength in the DPPC-Na^+TPB^- system.

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