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Interactions between Pressure and Ethanol on the Formation of Interdigitated DPPC Liposomes: A Study with Prodan Fluorescence†

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ABSTRACT: Steady-state fluorescence of 6-propionyl-2-(dimethylamino)naphthalene (Prodan) has been employed to study the interacting effects between ethanol and pressure on the formation of the fully interdigitated dipalmitoylphosphatidylcholine (DPPC). At 1 atm and 20 °C, a dramatic change in the emission spectrum of Prodan fluorescence is observed at about 1.1–1.3 M ethanol. The emission maximum shifts to longer wavelengths, and the intensity ratio of Prodan fluorescence at 435 nm to that at 510 nm, F_{435}/F_{510}, decreases abruptly with increasing ethanol content. The spectral changes are correlated to the ethanol-induced phase transition of DPPC from the noninterdigitated gel state to the fully interdigitated gel state [Rowe, E. S. (1983) *Biochemistry 22*, 3299–3305; Simon, S. A., & McIntosh, T. J. (1984) *Biochim. Biophys. Acta 773*, 169–172]. The spectral changes are attributed to the probe relocation from a less polar environment to a more polar environment due to lipid interdigitation. This relocation is either due to the bulky terminal methyl group of the lipids or due to the partition of Prodan into the bulk solution or both. The present study demonstrates that Prodan is a useful probe in monitoring the formation of the ethanol-induced fully interdigitated DPPC gel phase. Pressure is found to produce spectral changes similar to those induced by ethanol when the ethanol content amounts to 0.8–1.1 M. At lower (e.g., <0.4 M) and higher ethanol (e.g., >2.4 M) concentrations, pressure is unable to induce such spectral changes. The critical ethanol concentrations for the formation of the fully interdigitated DPPC gel phase (Cr) have been determined. Cr decreases with increasing pressure in a nonlinear manner, and the Cr's at 50 °C are less than those at 20 °C. These results suggest that high pressure and high temperature assist ethanol in forming the fully interdigitated gel phase in DPPC. The results imply that ethanol "toxicity" as a result of lipid interdigitation can be enhanced under pressure.

Ethanol produces pronounced effects on membranes. The main phase transition temperature (T_m)[1] of dipalmitoylphosphatidylcholine (DPPC) is reduced at low concentrations (<0.87 M) of ethanol but increased at high concentrations (>1.10 M) (Rowe, 1983, 1985). Above a critical ethanol concentration (0.98 M), the main phase transition of DPPC shows marked hysteresis (Rowe, 1985), and the pretransition of DPPC is completely abolished (Veiro et al., 1987). These ethanol-induced effects have been attributed to a transition from the bilayer phase to a gel phase in which the lipid acyl chains from opposing leaflets are fully interdigitated (designated as $L_{\beta}I$) (Simon & McIntosh, 1984). Using X-ray

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[1] Abbreviations: Cr, critical ethanol concentration; DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPHPC, 1-palmitoyl-2-[3-(1,3,5-hexatrienyl)propanoyl]phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; Laurdan, 6-lauroyl-2-(dimethylamino)-naphthalene; L_{α}, liquid-crystalline phase; $L_{\beta'}$, tilted bilayer gel phase; $L_{\beta}I$, interdigitated gel phase; MLV, multilamellar vesicles; $P_{\beta'}$, rippled gel phase; Prodan, 6-propionyl-2-(dimethylamino)naphthalene; POPOP, *p*-bis[2-(5-phenyloxazolyl)]benzene; T_m, main phase transition temperature.

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diffraction, Simon and McIntosh (1984) determined that the $L_\beta I$ phase appears between 0.8 and 1.2 M ethanol. The induction of lipid interdigitation by ethanol is temperature dependent, with higher temperatures favoring interdigitation (Nambi et al., 1988). A temperature/ethanol phase diagram for DPPC has been determined (Nambi et al., 1988; Ohki et al., 1990). In addition to structural changes, short-chain alcohols such as ethanol also produce varying degrees of anesthetic effects. The potency of anesthesia of alcohols has been linked to the ability of alcohols to lower the T_m of phosphatidylcholines (Trudell et al., 1973; Macdonald, 1978; Kamaya et al., 1979; Kita et al., 1981).

Such alcohol-induced structural and pharmacological effects in membranes should vary with pressure since pressure is known to induce lipid interdigitation (Braganza & Worcester, 1986) and to antagonize anesthesia. At the present time, the effect of pressure on alcohol-induced lipid interdigitation is poorly documented. Also, the current knowledge of the antagonism between pressure and alcohol-induced anesthesia has been confined to low alcohol concentrations. It is not at all clear what would be the interacting effects between pressure and alcohols at high alcohol concentrations, where lipid interdigitation occurs.

Fluorescence probe techniques have proven to be useful in detecting the formation of interdigitated lipid phases. Nambi et al. (1988) observed that the fluorescence intensity of 1,6-diphenyl-1,3,5-hexatriene (DPH) is decreased during the ethanol-induced phase transition from the noninterdigitated to the fully interdigitated phases. Using the fluorescence polarization of 1-palmitoyl-2-[3-(1,3,5-hexatrienyl)-propanoyl]phosphatidylcholine (DPHPC), Veiro et al. (1987) observed the 1-propanol-induced interdigitation in DPPC. Kao et al. (1990) demonstrated that fluorescence dynamic parameters of DPH as well as its intensity can be used to monitor the liquid-crystalline to gel phase transition between partially and mixed-interdigitated C(18):C(10) phosphatidylcholine.

Unlike DPH and DPHPC, 6-propionyl-2-(dimethylamino)naphthalene (Prodan) is an environmentally sensitive fluorescent probe (Weber & Farris, 1979). At low probe-to-lipid ratios (<0.4 mol %), Prodan is believed to reside in the lipid–water interfacial zone (Chong, 1988). At higher probe-to-lipid ratios (e.g., 4% by weight) and at lower water contents, it penetrates into the hydrocarbon region (Chong et al., 1989). The emission spectrum of Prodan has been shown to change markedly through the phase transition between the liquid-crystalline and gel state of phospholipid (Chong, 1988). The ratio of the fluorescence intensity at 435 nm to that at 510 nm, F_{435}/F_{510}, exhibits an abrupt increase from the liquid-crystalline state (L_α) to the gel state of dimyristoylphosphatidylcholine (DMPC) (Chong 1988). F_{435}/F_{510} was also found to be sensitive to pressure and temperature in egg yolk phosphatidylcholine vesicles as well as in the synaptic membrane isolated from goldfish brain (Chong, 1988). Prodan and its derivatives have been utilized in a number of other membrane studies (Lakowicz et al., 1984; Massey et al., 1985; Parasassi et al., 1986, 1990; Chong, 1990; Sommer et al., 1990). As an environmentally sensitive probe, Prodan should be useful in studying the ethanol-induced phase transition of DPPC from the noninterdigitated to the fully interdigitated gel phase ($L_\beta I$), since the polarity near the lipid–water interfacial region changes greatly through the phase transition. In the present study, we have employed the steady-state fluorescence of Prodan to examine the overlapping influence of ethanol and pressure on the formation of the fully interdigitated DPPC at two temperatures: 20 °C (below T_m) and 50 °C (above T_m). A preliminary report of this work has appeared elsewhere (Zeng & Chong, 1991).

Materials and Methods

Sample Preparation. DPPC was purchased from Avanti Polar Lipids (Alabaster, AL). Prodan was obtained from Molecular Probes (Eugene, OR). DPPC and Prodan dissolved in chloroform were dried under vacuum overnight and then suspended in a buffer solution containing 100 mM KCl and 10 mM Tris at pH 8.0 to form multilamellar vesicles (MLV). A solution of Tris buffer with $K^+/Tris^+ < 30$ has been reported to cause acyl chain interdigitation in phsophatidylglycerol (Wilkinson et al., 1987). However, the Tris buffer used in the present study gives $K^+/Tris^+ = 1000$, under which conditions interdigitation does not occur (Wilkinson et al., 1987).

Fluorescence Measurements. Emission spectra were measured isothermally on a SLM DMX-1000 fluorometer (Urbana, IL). For high-pressure measurements, about 1.2 mL of lipid dispersions were placed in an SLM high-pressure optical cell. The quartz cuvette used for pressure measurements was made according to the design of Paladini and Weber (1981). The band-pass is 4 nm for both excitation and emission monochromators. The probe-to-lipid ratio is about 1:500. The emission spectra were corrected for instrument response. Measurements of fluorescence anisotropy and lifetime were made with an ISS Greg 200 fluorometer (Champaign, IL). Blank readings from ethanol/DPPC dispersions without probes were subtracted from the sample anisotropy readings. The excitation wavelength is 359 nm for all fluorescence measurements. Lifetime values were determined relative to a *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP) solution (1.35 ns; in ethanol) at a modulation frequency of 100 MHz.

Results

Interacting Effects of Pressure and Ethanol on the Emission Spectra of Prodan Fluorescence in DPPC Vesicles at 20 °C

Spectra at 1 atm. In order to test whether Prodan fluorescence can be used to monitor the ethanol-induced phase transition from the noninterdigitated gel to the fully interdigitated gel state in DPPC, we have measured the emission spectra of Prodan in DPPC (MLV) as a function of ethanol concentration at 1 atm and at 20 °C (Figure 1A). At this temperature and pressure, pure DPPC in an aqueous medium is in the tilted noninterdigitated bilayer gel phase known as the $L_{\beta'}$ state.

The emission spectrum of Prodan in DPPC (MLV) in the absence of ethanol exhibits (Figure 1A) an emission maximum at 431 nm and a shoulder at about 520 nm. This spectrum is similar to the emission spectrum of Prodan in the noninterdigitated gel state of DMPC (MLV), as previously reported by Chong (1988). The wavelength of the emission maximum shifts to 434 nm at 1.07 M. At 1.32 M ethanol, the dominating peak is at 515 nm with a shoulder at about 435 nm. At higher ethanol concentrations, there is a further red-shift in the emission maximum: 518 nm at 1.55 M ethanol and 522 nm at 2.23 M. The data in Figure 1A clearly indicate a dramatic change in the emission spectrum of Prodan fluorescence in the neighborhood of 1.07–1.32 M ethanol. These concentrations are close to the values previously reported for the formation of $L_\beta I$ (Rowe, 1983, 1985; Simon & McIntosh, 1984; Veiro et al., 1987). It appears that the steady-state fluorescence of Prodan varies with the ethanol-induced phase change in DPPC.

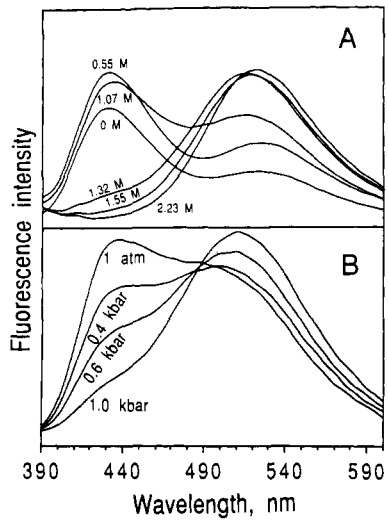

FIGURE 1: (A) Effects of ethanol on the emission spectra of Prodan fluorescence in DPPC at 20 °C and 1 atm. The sample size was 1.8 mL of 1.17 mM lipid. (B) Effects of pressure on the emission spectra of Prodan fluorescence in DPPC at 20 °C and at 0.8 M ethanol.

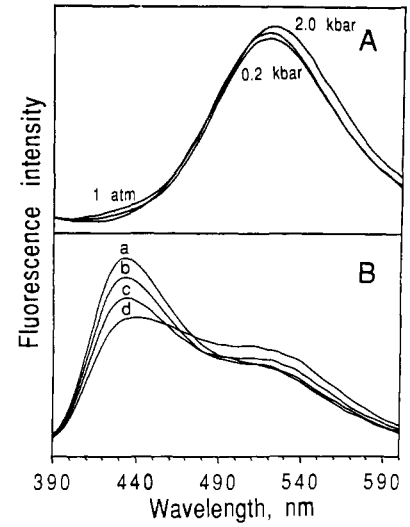

FIGURE 2: (A) Effects of pressure on the emission spectra of Prodan fluorescence in DPPC at 20 °C and at 1.8 M ethanol. (B) Fluorescence emission spectra of Prodan in DPPC at 20 °C at (a) 1 atm, (b) 0.2 kbar, (c) 0.6 kbar, and (d) 2.0 kbar, in the absence of ethanol.

Spectra at Elevated Pressures. In order to study the interacting effects of pressure and ethanol on the formation of $L_\beta I$ in DPPC, we have conducted isothermal measurements of Prodan emission spectra in DPPC as a function of pressure at various ethanol concentrations. Figure 1B shows the effects of pressure on the emission spectra of Prodan in DPPC at 20 °C and at 0.8 M ethanol. Under these conditions, DPPC at ambient pressure is in the $L_{\beta'}$ gel state [see Figure 11 of Nambi et al. (1988)] and the emission spectrum exhibits a peak at 434 nm with a shoulder near 510 nm (Figure 1B). As the pressure is increased, the intensity at 434 nm decreases with an accompanying increase in intensity at 510 nm. At 1.0 kbar, the 510-nm peak is dominating. The emission spectrum at 1.0 kbar (Figure 1B) is similar to the emission spectrum at 1.32 M ethanol shown in Figure 1A, suggesting that pressure assists ethanol in forming the $L_\beta I$ phase in DPPC. In a previous study (Chong, 1988) of noninterdigitated systems, pressure was found to increase the Prodan fluorescence in the blue region, a result just opposite to the case involving ethanol and the $L_\beta I$ phase as shown here in Figure 1B.

Figure 2A shows the effect of pressure on the emission spectrum of Prodan in DPPC in the presence of 1.8 M ethanol and at 20 °C. Under these conditions and at 1 atm, DPPC is already in the $L_\beta I$ state, according to the phase diagram shown in Nambi et al. (1988). It is therefore anticipated that elevated pressures will not further change the emission spectrum. As shown in Figure 2A, this is almost the case.

Figure 2B shows the effects of pressure on the emission spectra of Prodan in DPPC (MLV) at 20 °C in the absence of ethanol. The emission maximum undergoes small changes in the pressure range examined (433 nm at 1 atm vs 440 nm at 2 kbar). The pressure-induced $L_\beta I$ phase of DPPC has been previously detected by neutron diffraction methods to occur at about 1.6 kbar at 50 °C but not at 20 °C (Braganza & Worcester, 1986; Winter & Pilgrim, 1989). Thus, the low pressure sensitivity of the emission spectra shown in Figure 2B can be taken to indicate that, at 20 °C, pressure alone (up to 2 kbar) cannot induce the $L_\beta I$ phase of DPPC.

Figure 3A summarizes the effects of pressure on the wavelength of the emission maximum of Prodan fluorescence in DPPC at various ethanol concentrations at 20 °C. It is interesting to note that pressure induces an abrupt increase in the emission maximum at intermediate ethanol concentrations (e.g., 0.6–0.8 M) but not at low (<0.4 M) nor at high (e.g., >1.1 M) concentrations. On the basis of the results shown in Figure 3A, it can be suggested that, at intermediate ethanol concentrations, pressure assists ethanol in the formation of $L_\beta I$.

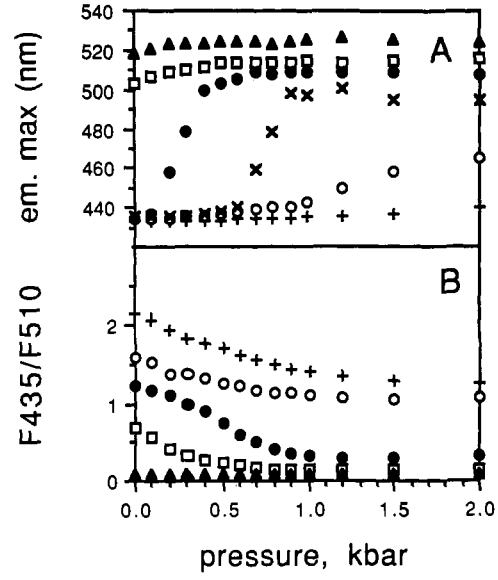

FIGURE 3: Pressure dependence of emission maximum (A) and F_{435}/F_{510} (B) on Prodan fluorescence in DPPC at 20 °C and at various ethanol concentrations. Shown are data for 0 M (+), 0.4 M (○), 0.6 M (×), 0.8 M (●), 1.1 M (□), and 2.4 M (▲) ethanol. The typical standard deviation of F_{435}/F_{510} is 0.05.

However, during the pressure-induced phase transition, the emission spectra of Prodan may consist of two peaks of similar intensities, making the selection of the emission maxima difficult and less meaningful. The interacting effects of pressure and ethanol on the formation of interdigitated DPPC can be better described in terms of the parameter F_{435}/F_{510}. F_{435}/F_{510} has been shown to be a useful index monitoring lipid phase transition (Chong, 1988). The pressure dependence of F_{435}/F_{510} at varying amounts of ethanol at 20 °C is illustrated in Figure 3B. As shown in Figure 3B, F_{435}/F_{510} at 2.4 M ethanol is low (0.07–0.10) and virtually invariant with pressure. This result is not surprising since at this ethanol concentration

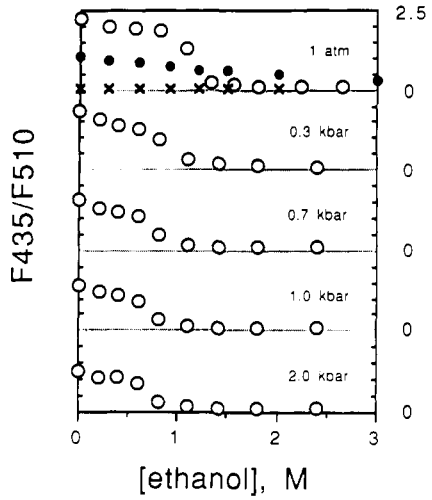

FIGURE 4: Effects of ethanol on F_{435}/F_{510} of Prodan fluorescence in DPPC (○) at various pressures. Shown are the data obtained with Prodan in Tris buffer (×) and in DPPE (●). The temperature was 20 °C. The sample size for DPPE measurement is the same as that for DPPC.

DPPC is already in the interdigitated gel state at ambient pressure (Rowe, 1983) and should remain in the interdigitated state at elevated pressures. The low F_{435}/F_{510} value of 0.07–0.10 is thus indicative of the $L_{\beta}I$ phase. At 1.1 M ethanol, F_{435}/F_{510} decreases steadily with pressure and levels off at 0.15, indicating that pressure provides additional driving force to bring about the $L_{\beta}I$ state. A similar curve is seen for the case of 0.8 M ethanol. In contrast, at 0 and 0.4 M ethanol, pressure lowers F_{435}/F_{510}, but the ratio never reaches the level below 1.0. This implies that certain amounts of ethanol are required in order to observe a positive interacting effect between pressure and ethanol on the formation of interdigitated DPPC gel phase.

Determination of the Critical Ethanol Concentration for the Formation of Interdigitated DPPC. The effects of ethanol on F_{435}/F_{510} of Prodan fluorescence in DPPC at 20 °C and at various pressures are illustrated in Figure 4. In all the curves presented in Figure 4, there is a general trend: F_{435}/F_{510} first decreases linearly with ethanol content. Then, there follows a region where F_{435}/F_{510} decreases abruptly. Finally, F_{435}/F_{510} reaches a leveling-off value of about 0.07–0.10. The leveling-off region can be easily fitted into a line. Using the fitted straight lines at both low and high concentrations, one can determine the beginning and ending points of the nonlinear region from which a midpoint can be estimated. For example, the midpoint at 1 atm is estimated to be 1.16 M ethanol.

The midpoint ethanol concentrations determined at 1 atm come close to the values for the break in T_m (Rowe, 1983), the removal of the pretransition (Veiro et al., 1987), the marked hysteresis (Rowe, 1985), and the appearance of interdigitated phase (Simon & McIntosh, 1984). For these reasons, the midpoint ethanol concentrations determined from Figure 4 are believed to reflect the ethanol-induced phase transition of DPPC from the noninterdigitated gel phase to the interdigitated gel phase. The midpoint values are now referred to as the critical ethanol concentrations (Cr) for the formation of the $L_{\beta}I$ phase. Figure 5 shows that Cr at 20 °C (●) decreases with pressure in a nonlinear manner. A break point is obvious at 0.9 kbar. Below 0.9 kbar, the decreasing rate is 0.44 M/kbar. Above 0.9 kbar, the rate is 0.02 M/kbar.

The effect of ethanol on Prodan fluorescence in Tris buffer in the absence of lipid is shown in Figure 4 (x). F_{435}/F_{510} remains virtually unchanged with ethanol in the ethanol concentration range examined. This suggests that the abrupt change in F_{435}/F_{510} observed in the lipid systems is not a result of an ethanol effect on Prodan fluorescence.

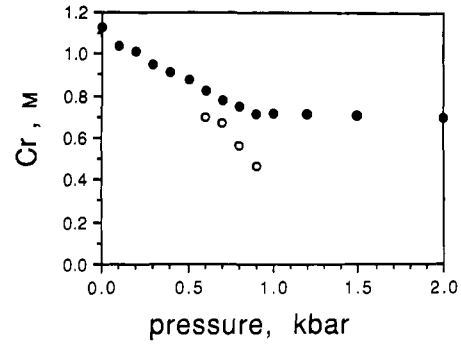

FIGURE 5: Effects of pressure on the critical ethanol concentration (Cr) for the formation of interdigitated DPPC at 20 °C (●) and at 50 °C (○).

In order to ensure that the abrupt change in F_{435}/F_{510} is the result of lipid interdigitation, a control experiment using dipalmitoylphosphatidylethanolamine (DPPE) was carried out. According to Rowe (1985), ethanol cannot induce an interdigitated structure in DPPE, therefore, no abrupt changes in F_{435}/F_{510} are expected in the presence of ethanol. Our data (Figure 4, ●) showed that this is indeed the case. This control experiment strongly suggested that the abrupt change in F_{435}/F_{510} observed with DPPC is related to lipid interdigitation. It is important to mention that addition of ethanol to DPPE results in an abrupt change in the emission maximum of Prodan fluorescence (a red-shift; data not shown) but not in F_{435}/F_{510}. We would like to stress again that using the emission maximum alone may lead to a wrong conclusion. F_{435}/F_{510} should be used to reflect the lipid phase transition. The decrease in F_{435}/F_{510} (or a red-shift in the emission spectrum) is probably due to the partition of probe into the buffer/ethanol bulk solution (discussed later).

Interacting Effects of Pressure and Ethanol on the Formation of Interdigitated DPPC Gel Phase at 48–50 °C

Emission Spectra at 1 atm. In the absence of ethanol, the emission spectrum of Prodan fluorescence in DPPC at 48 °C and 1 atm gives a maximal intensity at about 488 nm (curve a in Figure 6A). This result is in good agreement with that for DMPC reported previously (Chong, 1988). In the presence of ethanol, the emission spectrum shifts only slightly toward longer wavelengths, and there is no dramatic change in the wavelength of emission maximum. The phase diagram shown by Nambi et al. (1988) indicates that at 48 °C ethanol cannot induce the $L_{\beta}I$ phase in DPPC. For this reason, the spectra shown in Figure 6A can be taken to indicate that DPPC remains in the L_{α} state over the ethanol concentrations examined.

Interacting Effects of Pressure and Ethanol. Figure 6B shows the emission spectrum of Prodan fluorescence in DPPC in the absence of ethanol at 50 °C and at various pressures. As shown in Figure 6B, pressure initially shifts the emission peak to the blue and then to the red. The blue-shift can be attributed to the pressure-induced phase transition from L_{α} to the noninterdigitated gel state of DPPC. The red-shift is probably due to the transition from the noninterdigitated gel phase to the $L_{\beta}I$ phase. The results obtained from the sample containing 0.4 M ethanol (data not shown) are similar to those shown in Figure 6B. In sharp contrast, at 1.8 M ethanol, no blue-shift in the emission maximum is observed (data not

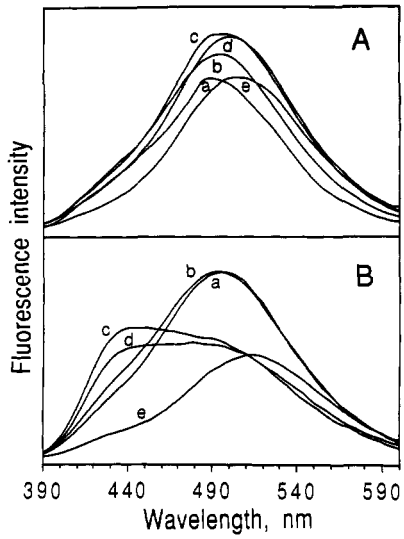

FIGURE 6: (A) Effects of ethanol on the emission spectra of Prodan fluorescence in DPPC at 1 atm and at 48 °C. (a) 0 M ethanol, (b) 0.55 M, (c) 1.07 M, (d) 1.55 M, and (e) 2.23 M. (B) Effects of pressure on the emission spectra of Prodan fluorescence in DPPC at 0 M ethanol, 50 °C and at (a) 0.001 kbar, (b) 0.2 kbar, (c) 0.4 kbar, (d) 1.0 kbar, and (e) 2.0 kbar.

shown); instead, a red-shift from 509 to 524 nm is seen from 0.6 to 1.0 kbar. At 1 atm and at 50 °C, DPPC in the presence of 1.8 M ethanol is in the L_α state (Nambi et al., 1988), which gives an emission maximum of Prodan fluorescence at 508 nm. The red-shift from 508 to 524 nm may be attributed to the pressure-induced phase transition from L_α to $L_\beta I$. According to the phase diagram constructed by Nambi et al. (1988), it is possible to convert DPPC from L_α to $L_\beta I$ by lowering the temperature from 50 to 40 °C. Note that an increase in pressure is equivalent to a decrease in temperature in terms of maintaining a constant membrane order in the acyl chain and that the pressure-to-temperature equivalence for DPPC is about 24 °C/kbar (Chong & Weber, 1983).

In summary, at 50 °C, below a threshold ethanol concentration, pressure first induces a phase change in DPPC from L_α to one of the noninterdigitated gel phases and then from the noninterdigitated gel phase to $L_\beta I$. Above the threshold concentration, pressure probably induces the phase transition from L_α to $L_\beta I$ without the intermediate noninterdigitated gel state. It needs to be mentioned that at the present time we do not know the nature of the pressure-induced noninterdigitated gel phase observed at 50 °C. Using Raman spectroscopy, Wong and Mantsch (1985) have detected five pressure-induced gel phases in DPPC. Their study, however, was conducted in the absence of ethanol.

Figure 7A shows the pressure dependence of F_{435}/F_{510} at different ethanol concentrations at 50 °C. At 0 M ethanol, F_{435}/F_{510} increases with pressure until 0.47 kbar. This initial increase can be attributed to the pressure-induced L_α-to-gel phase transition. After reaching the maximal value, F_{435}/F_{510} decreases with pressure, in a manner similar to the curves shown in Figure 3B. This decrease is believed to reflect the phase transition from the gel phase to $L_\beta I$. The midpoint of the initial increase in F_{435}/F_{510} can be termed the phase transition pressure, $P_{1/2}$, which is 0.36 kbar in the case of 0 M ethanol. This value is close to the value obtained by Chong and Weber (1983). Using DPH fluorescence, Chong and Weber (1983) have determined $P_{1/2}$ for DPPC (MLV) at 42 °C to be 0.02–0.06 and determined a dT/dP value of 24.3 °C/kbar. On the basis of their data, one can calculate a $P_{1/2}$ value for DPPC (MLV) to be 0.39 °C/kbar at 50 °C.

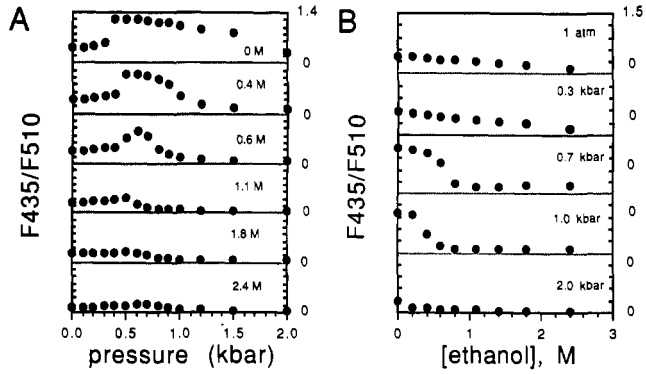

FIGURE 7: (A) Pressure dependence of F_{435}/F_{510} at different ethanol concentrations and at 50 °C. (B) Effects of ethanol on F_{435}/F_{510} of Prodan fluorescence in DPPC at 50 °C and at various pressures.

At 0.4 and 0.6 M ethanol, the curves (Figure 7A) are similar to that at 0 M. Above 1.1 M ethanol, the shape of the curve changes to a rather flat line.

In order to determine the effects of pressure on the critical ethanol concentration of interdigitation at 50 °C, the data shown in Figure 7A were replotted in Figure 7B. It can be seen from Figure 7B that an abrupt change in F_{435}/F_{510} is not observed at pressures below 0.3 kbar. At 0.7 kbar, ethanol induces an abrupt change in F_{435}/F_{510}, giving a sigmoidal curve. The sigmoidal curve migrates to the left as the pressure is raised and eventually disappears at 2 kbar. As discussed before, the abrupt change in F_{435}/F_{510} is indicative of the ethanol-induced phase transition of DPPC. Hence, it can be suggested from Figure 7B that ethanol alone cannot induce the $L_\beta I$ phase at 50 °C and that pressures exceeding $P_{1/2}$ are required in order to assist ethanol in forming the $L_\beta I$ phase in DPPC. The midpoints of the sigmoidal curves were determined and designated as the critical ethanol concentrations for the formation of $L_\beta I$ at 50 °C. The results are presented in Figure 5 (○). It is found that the critical concentration at 50 °C is less than the corresponding value at 20 °C (● in Figure 5). This implies that high temperatures favor the ethanol-induced interdigitation in DPPC under pressure. This implication is consistent with that made previously by Nambi et al. (1988). Here we have extended their assertion to include the situations at high pressures (up to 2.5 kbar).

Discussion

The results presented in this paper clearly demonstrate that Prodan is a useful probe for monitoring the ethanol-induced phase transition of DPPC from the noninterdigitated gel to the fully interdigitated gel state. The F_{435}/F_{510} is shown to be sensitive to such a phase transition. As the lipid transforms from a noninterdigitated bilayer structure to a fully interdigitated "monolayer" structure, as depicted in Figure 8, the polarity near the water–lipid interface undergoes dramatic changes. In the fully interdigitated structure, the polar head group of DPPC and the terminal methyl group in the acyl chain are alternating in the membrane surface. In sharp contrast, the membrane surface in a noninterdigitated structure is completely made of the polar head group of the lipid. Since Prodan is an environmentally sensitive probe (Weber & Farris, 1979) residing in the water–lipid interfacial region at low probe-to-lipid ratios [e.g., 1:500, Chong (1988)], it is not surprising that Prodan can sense the ethanol-induced phase transition in DPPC.

It is, however, surprising that F_{435}/F_{510} drops through the phase transition and that the emission maximum shifts to the red. Weber and Farris (1979) demonstrated that the emission

Table I: Steady-State Anisotropy, Lifetime, and the Amount of Prodan in the Bulk Solution at 1 atm and 20 °C

[ethanol] (M)	Schott KV 389		Schott KV 389 + BG-12		Schott KV 520		Schott KV = 520 extrapolated to [lipid] = ∞		Schott KV 520 (bulk solution)		% Prodan in the bulk solution
	A_t	τ_t (ns)	A_{lp}	τ_{lp} (ns)	A_{mp}	τ_{mp} (ns)	A_m	τ_m (ns)	A_{bs}	τ_{bs} (ns)	
0	0.125 ± 0.007	3.86 ± 0.03	0.153 ± 0.006	4.91 ± 0.10	0.043 ± 0.001	1.87 ± 0.04	0.048	2.99	0.029 ± 0.000	1.54 ± 0.00	19
0.8	0.119 ± 0.003	3.49 ± 0.05	0.160 ± 0.005	4.76 ± 0.06	0.043 ± 0.002	2.00 ± 0.05	0.047	3.04	0.034 ± 0.000	1.64 ± 0.01	25
1.1	0.093 ± 0.002	3.27 ± 0.06	0.124 ± 0.003	3.08 ± 0.08	0.062 ± 0.002	2.19 ± 0.05	0.071	2.95	0.032 ± 0.003	1.71 ± 0.04	20
1.4	0.067 ± 0.002	2.49 ± 0.05	0.095 ± 0.009	1.89 ± 0.10	0.060 ± 0.001	2.24 ± 0.05	0.069	2.86	0.033 ± 0.001	1.71 ± 0.00	28
1.8	0.052 ± 0.007	2.28 ± 0.03	NA	NA	0.051 ± 0.003	2.12 ± 0.03	0.064	2.70	0.031 ± 0.001	1.75 ± 0.01	50
2.4	0.038 ± 0.003	2.11 ± 0.02	NA	NA	0.040 ± 0.002	2.11 ± 0.02	0.047	2.51	0.033 ± 0.001	1.84 ± 0.00	58

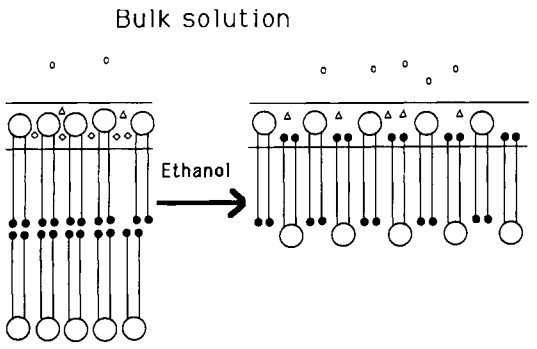

FIGURE 8: Schematic diagram showing the polarity change in the water–lipid interfacial region from the noninterdigitated gel to the interdigitated gel state. The dark circles represent the terminal methyl groups. The symbols ◇, △, and **0** represent the centers of the chromophores in Prodan molecules; ◇ is Prodan in the membrane-associated, less polar environment; △ is Prodan in the membrane-associated, more polar environment; and **0** is Prodan in the bulk solution.

spectrum of Prodan gives a 130-nm shift in the emission maximum from 401 nm in cyclohexane to 531 nm in water. Suppose that Prodan remains at the same location when the matrix lipid undergoes a phase transition from the noninterdigitated gel to the interdigitated gel state. Then Prodan in the interdigitated gel state should experience a less polar environment due to the alternating methyl/head group arrangement, hence giving a blue-shift in the emission maximum through the phase transition. This is apparently not the case. One would also expect that F_{435}/F_{510} increases when pressure and ethanol assert a positive interacting effect with regard to the formation of $L_\beta I$, since such an increase is seen in DMPC, egg yolk phosphatidylcholine, and the synaptic membrane isolated from goldfish brain under pressure (Chong, 1988). This is obviously not the case either.

The red-shift in the emission spectrum and the decrease in F_{435}/F_{510} cannot be interpreted as the result of the higher affinity of ethanol to the $L_\beta I$ phase. Nambi et al. (1988) suggested that the affinity of ethanol in DPPC is increased in the order $L_{\beta'} < P_{\beta'} < L_\alpha < L_\beta I$. Assuming that the location of Prodan in the membrane remains unchanged and that the ethanol concentration around Prodan increases as the lipid is converted from the noninterdigitated gel to the interdigitated gel state, one would expect a red-shift in the emission maximum and a reduction in F_{435}/F_{510} through the phase transition, due to the increase in local ethanol content. However, the increased ethanol concentration in $L_\beta I$ cannot exceed 100%, and Prodan in 100% ethanol should give an emission maximum at 496 nm (Weber & Farris, 1979). This implies that the emission maximum shifts at most to 496 nm. Thus, the observation of an emission maximum in the $L_\beta I$ phase at about 505–515 nm cannot be interpreted by the increase in ethanol content in membranes.

The surprising results can be attributed to the probe relocation from a less polar environment (◇) to a more polar environment (△ or **0**), as depicted in Figure 8. We propose that the relocation of Prodan is caused either by the bulky terminal methyl groups in the lipid acyl chain or the partition of Prodan into the bulk solution or both. In the fully interdigitated state, the methyl groups are located near the membrane surface. The effective volume of the terminal methyl group is about twice that of a methylene unit in the hydrocarbon chain (Reiss-Husson & Luzzati, 1964; Flory, 1969). In order to avoid the steric hindrance imposed by the bulky terminal methyl group, Prodan may move into a more polar environment in the membrane surface (△) or into the bulk solution (**0**) (Figure 8). In a more polar environment, the emission maximum of course shifts to the red, and the F_{435}/F_{510} ratio becomes smaller. Note that the relocation of Prodan has been previously suggested to occur in membranes under pressure (Chong, 1988). At the present time, we cannot give a detailed molecular description of the "more polar" environment in the membrane surface. Certainly, the model of the "more polar" disposition of Prodan proposed by Chong (1988) is one of the possibilities.

In order to determine the amount of Prodan in the bulk solution, we have measured steady-state anisotropy of Prodan fluorescence at 1 atm. The A_t, A_{lp}, and A_{mp} shown in Table I are the anisotropy of Prodan fluorescence in DPPC measured through a Schott KV 389 cut-on, KV 389 cut-on plus a BG-12 band-pass, and Schott KV 520 cut-on filter, respectively. The A_{lp} is the anisotropy of Prodan fluorescence from the 389–430-nm region, which can be considered as the anisotropy of Prodan from the "less polar" site (◇ in Figure 8). The A_{mp} is the anisotropy from the 510–530-nm region, which can be assigned as the anisotropy of Prodan from the "more polar" disposition. As expected, A_{mp} is lower than A_{lp}. The τ_s (Table I) are the corresponding modulation lifetimes. A_{bs} and τ_{bs} are the anisotropy and lifetime of Prodan in Tris buffer/ethanol mixtures in the absence of lipids measured by using the Schott KV 520 filter. The A_{bs} values are significantly lower than those of A_{mp}. A_m and τ_m are the anisotropy and lifetime of A_{mp} and τ_{mp} when the DPPC concentration is very high. A_m and τ_m were estimated from the y-intercept of a plot of A_{mp} and τ_{mp} vs 1/[DPPC]. Assume that A_t is contributed from two populations (A_{mp} and A_{lp}) and that A_{mp} is contributed from A_m and A_{bs}. Then, $A_t = f_1A_{lp} + f_2A_{mp}$ and $A_{mp} = f_3A_m + f_4A_{bs}$ where f_s are the fractional intensity of each component, $f_1 + f_2 = 1$, and $f_3 + f_4 = 1$. To this end, one can estimate the percentage of Prodan molecules in the bulk solution using the anisotropy and lifetime data listed in Table I. The amount of free Prodan is calculated on the basis of the relationship $f \propto nQ \propto n\tau$, where Q is the quantum yield and n the number of Prodan molecules. The percentage of Prodan molecules in the bulk solution is shown in Table I. Below 1.1 M ethanol, most Prodan molecules (~80%) remain associated with the membrane rather than going into the bulk solution. However, free Prodan increases dramatically at ethanol concentrations higher than 1.1 M. It is necessary to point out that partition

of Prodan into the bulk solution should vary with the water-to-lipid ratio. The conclusion derived from the present fluorescence data may not be comparable to that derived from the infrared study (Chong et al., 1989), for which a very low water-to-lipid ratio (about 30–50% by weight) was used.

Pressure will in principle alter the partition of Prodan between the bulk solution and the membrane site. Previous infrared results show that Prodan stays in the membrane at pressures as high as 25 kbar (Chong et al., 1989). Those results, although obtained in the absence of ethanol, argue for higher pressures favoring Prodan in the membrane site, rather than the bulk ethanol/water solution. Suppose that pressure pushes all the Prodan molecules back into the membrane. This would affect the steady-state fluorescence by only 12% (fractional intensity) at 1.1 M ethanol. At ethanol concentrations higher than 1.4 M, more Prodan may be pushed from the bulk solution back to the membrane site at high pressures. However, this should have little effect on the determination of Cr since, at 1.4 M or higher ethanol concentrations, F_{435}/F_{510} is in the leveling-off region (Figure 4). Note that Cr can be determined without the F_{435}/F_{510} values at ethanol concentrations higher than 1.4 M. This assertion is affirmed by a recent study (Zeng and Chong, unpublished results) which shows that the pressure dependence of Cr determined by Laurdan [6-lauroyl-2-(dimethylamino)naphthalene] is very similar to that determined by Prodan. Laurdan is much more stabilized in membranes, as compared to Prodan (Chong, 1990).

It can be concluded that the abrupt change in F_{435}/F_{510} obtained in DPPC can be attributed to lipid interdigitation, which results in the relocation of Prodan. This relocation is either due to the bulky terminal methyl group of the lipid or due to the partition of Prodan into the bulk solution or both. This conclusion is further strengthened by the DPPE result. No abrupt change in F_{435}/F_{510} is seen in the case of DPPE, a lipid not interdigitated in ethanol (Rowe, 1985) (Figure 4, ●).

Ethanol is known to cause anesthesia and lipid interdigitation. Is lipid interdigitation related to the effect of anesthesia? The question is answered indirectly by our pressure study. Pressure is known to antagonize ethanol-induced anesthesia. Pressure reversal of anesthesia may be caused by the increase in membrane order and/or the displacement of anesthetics from the membrane (Kaneshina et al., 1983; Auger et al., 1987). In this study, we have demonstrated that pressure facilitates, rather than antagonizes, the effects of ethanol on the formation of interdigitated gel phase of DPPC. Thus the antagonism between pressure and ethanol-induced anesthesia must not originate from lipid interdigitation. It can be further implicated that lipid interdigitation is unlikely to be the main cause of the ethanol-induced anesthesia.

High doses of ethanol cause lipid interdigitation, which ought to affect membrane functions since the membrane thickness and the overall packing are changed by interdigitation (Simon & McIntosh, 1984; Ohki et al., 1990). If so, ethanol-induced lipid interdigitation may be one of the factors contributed to ethanol toxicity. In this regard, ethanol toxicity would be enhanced under pressure since pressure assists ethanol in forming interdigitated gel phase (Figure 5). The enhancement rate of toxicity due to interdigitation in DPPC at 20 °C is estimated to be 41% per kbar. This rate will be even higher at elevated temperatures according to the data shown in Figure 5.

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