Effect of Ethanol-Induced Lipid Interdigitation on the Membrane Solubility of Prodan, Acdan, and Laurdan

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ABSTRACT The effect of ethanol-induced lipid interdigitation on the partition coefficient (K_p) of 6-propionyl-2-(dimethylamino)naphthalene (Prodan) and its two derivatives, 6-acetyl-2-(dimethylamino)naphthalene (Acdan) and 6-lauroyl-2-(dimethylamino)naphthalene (Laurdan), in L-α-dipalmitoylphosphatidylcholine (DPPC) vesicles has been examined by a precipitation method over the ethanol concentration range of 0–1.8 M. At 20°C and in the absence of ethanol, the K_p values for Acdan, Prodan, and Laurdan are 2.0×10^3, 2.8×10^4, and 4.7×10^6, respectively. This result suggests that the K_p of Prodan and its derivatives is not simply a linear function of the polymethylene units. As DPPC undergoes the ethanol-induced phase transition from the noninterdigitated to the fully interdigitated gel state, K_p for Prodan and Acdan decreases by a factor of 5 and 2, respectively, whereas K_p for Laurdan exhibits no detectable changes with ethanol. The differences in K_p are in parallel with the differences in the fluorescence emission spectra of these probes over the ethanol concentration range examined. Previous fluorescence and infrared data indicated that membrane perturbation caused by the probes increases in the order: Laurdan > Prodan > Acdan. Thus, the degree of membrane perturbation also seems to be in parallel with K_p. Among these three probes, Prodan fluorescence reflects most correctly the ethanol-induced lipid interdigitation. In conclusion, the partitioning of small solutes in lipid membranes is significantly reduced by ethanol-induced lipid interdigitation, probably as a result of an increased membrane surface density due to the increased intramolecular lipid acyl chain ordering and a tighter overall intermolecular packing.

INTRODUCTION

Solute partitioning is an important subject in membrane biology (Gennis, 1989; De Young and Dill, 1990); it relates to membrane uptake of drugs, metabolites, and environmental toxics. Previous studies of solute partitioning were focused on noninterdigitated lipid bilayers; little is known about the effect of lipid interdigitation on solute partitioning from the bulk solution to the membrane.

Ethanol-induced lipid interdigitation in L-α-dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles is well documented. DPPC is converted from a noninterdigitated gel state to a fully interdigitated gel state when the ethanol concentration is elevated from 0 to 0.8–1.2 M (Simon and McIntosh, 1984). The formation of the fully interdigitated state depends on lipid acyl chain length (Rowe, 1983), temperature (Nambi et al., 1988), and pressure (Zeng and Chong, 1991) and is hindered by the addition of cholesterol (Komatsu and Rowe, 1991). A temperature/ethanol phase diagram for DPPC has been determined (Nambi et al., 1988; Ohki et al., 1990). Compared with noninterdigitated lipid bilayers, fully interdigitated structures have a shorter membrane thickness (Simon and McIntosh, 1984), a reduced membrane surface charge density, and a higher molecular order in lipid acyl chains (Wu et al., 1982; Simon and McIntosh, 1984; O'Leary and Levin, 1984; Hui and Huang, 1986; Boggs et al., 1989). These structural changes ought to affect membrane properties. It has been shown previously that in DPPC vesicles ethanol-induced lipid interdigitation causes a disappearance of the pretransition (Verio et al., 1987), an increased mobility in the head group (Herold et al., 1987), an increase in proton permeability, and a significant enhancement in membrane aggregation (Zeng and Chong, 1993). However, the effect of ethanol-induced lipid interdigitation on solute partitioning into the membrane has not been studied to any great extent.

In previous studies, we have demonstrated that the fluorescence of 6-propionyl-2-(dimethylamino)naphthalene (Prodan) can be used to monitor the ethanol-induced lipid interdigitation in DPPC multilamellar (Zeng and Chong, 1991) as well as in unilamellar vesicles (Zeng et al., 1993). The emission maximum of Prodan fluorescence shifts to longer wavelengths, and the ratio of the fluorescence intensity at 435 nm to that at 510 nm, F_{435}/F_{510}, decreases abruptly through the ethanol-induced phase transition from the noninterdigitated gel state to the fully interdigitated gel state in DPPC. Using fluorescence anisotropy and lifetime data, we have calculated the percentage of Prodan molecules in the bulk solution relative to DPPC vesicles at various ethanol concentrations (Zeng and Chong, 1991). It was found that, below 1.1 M ethanol, about 80% of Prodan molecules are associated with the membrane. The percentage of Prodan

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Abbreviations used: Acdan, 6-acetyl-2-(dimethylamino)naphthalene; C_t, critical ethanol concentration; DPPC, L-α-dipalmitoylphosphatidylcholine; K_p, partition coefficient for Prodan, Laurdan, or Acdan; Laurdan, 6-lauroyl-2-(dimethylamino)naphthalene; MLVs, multilamellar vesicles; Prodan, 6-propionyl-2-(dimethylamino)naphthalene.

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molecules in the bulk solution increases dramatically at ethanol concentrations higher than 1.1 M. At 1.8 M ethanol about 50% of Prodan molecules are in the bulk solution. Thus, it was concluded that the abrupt spectral change of Prodan fluorescence is due to a decrease in the partitioning of Prodan molecules in the fully interdigitated structure (Zeng and Chong, 1991). However, this determination of partitioning is somewhat indirect, and the approach requires the assumption that the emission peaks in Prodan fluorescence originate from distinct probe locations. Because the fluorescence of Prodan and its derivatives is affected by other events (such as solvent relaxation) than probe location/relocation, it is important to use an independent method to verify our previous results.

In the present study, we used a precipitation method to provide direct evidence that the partitioning of Prodan decreases abruptly when the membrane is converted from the noninterdigitated to the fully interdigitated gel state. In addition, we studied the partitioning of two Prodan derivatives, 6-lauroyl-2-(dimethylamino)naphthalene (Laurdan) and 6-acetyl-2-(dimethylamino)naphthalene (Acdan), in DPPC vesicles at various ethanol concentrations so that the solute structural factors governing the partitioning of naphthalene derivatives in membranes can be elucidated. Laurdan, Prodan, and Acdan differ in the length of the polymethylene chain (structures shown in Fig. 5). Fluorescence and infrared studies show that Laurdan is embedded in lipid bilayers deeper than Prodan (Parasassi et al., 1991; Chong and Wong, 1993). High pressure infrared data further suggest that Laurdan causes more membrane perturbation than Prodan (Chong and Wong, 1993). Thus, using these probes, we can determine whether the differences in the length of the solute hydrocarbon chain and in the probe interactions with the lipid membrane play a role in solute partitioning. Our results indicate that the partitioning of small solute molecules (e.g., Acdan and Prodan) decreases significantly as the membrane becomes fully interdigitated.

MATERIALS AND METHODS

Materials

DPPC was purchased from Avanti Polar Lipids (Alabaster, AL). Prodan and Laurdan were obtained from Molecular Probes (Eugene, OR). Acdan was a gift from Professor Gregorio Weber at the University of Illinois at Champaign-Urbana.

Preparation of liposomes

DPPC(MLVs) were prepared by the method of Bangham et al. (1967). Lipids or lipid/probe mixtures were dried under vacuum overnight. The mixtures were dispersed in a buffer solution containing 100 mM KCl, 10 mM Tris at pH 8.0. Phospholipid concentration was determined as inorganic phosphate by the method of Bartlett (1959). The concentrations of Prodan, Laurdan, and Acdan were determined by using a molar absorption coefficient of 18,400 cm^{-1} M^{-1} at 360 nm in ethanol (Weber and Farris, 1979). In the study of probe partitioning, various lipid concentrations ranging from 0.14 to 5.00 mM were used.

Determination of partition coefficients

The partition coefficient, K, for a solute in the membrane is defined as the ratio of the molality of the solute in the membrane to that in the bulk solution (Massey et al., 1985; Huang and Haugland, 1991). K is described by the equation

$$K = \frac{S_m/L}{S_b/W} = \frac{S_m W}{S_b L}, \quad (1)$$

where S_m and S_b are the moles of solute molecule in the membrane (m) and in the bulk solution (b), respectively, and, L and W are the moles of lipid and water, respectively. $S_m = [S_m]V_m$, $S_b = [S_b]V_b$, $W = 55.6V_b$, and $L = [DPPC]_{av}(V_b + V_m)$, where V_m and V_b are the volumes of the membrane and the bulk solution, respectively. $[DPPC]_{av}$ is the average molar concentration of DPPC in the sample solution.

The partition coefficient K was determined by the procedure outlined in Fig. 1. In brief, a buffer solution containing the solute and a desired amount of ethanol was divided equally into two tubes. DPPC vesicles were added to the sample tube. In the control tube, no DPPC was added. Both the sample and the control were incubated at 20°C for 24 h. After centrifugation in a Sorvall RC 70 ultracentrifuge (Du Pont, Wilmington, DE) at 100,000 × g at 20°C for 30 min, 99.5% of DPPC was found in the pellet, a result similar to that reported by Katz and Diamond (1974). An additional amount of ethanol was then added to the supernatant of the sample and control tubes to adjust the final ethanol concentration to 2.4 M. The purpose of adjusting the final ethanol concentration to 2.4 M is to ensure that fluorescence intensities derived from various samples originally exposed to different levels of ethanol can be compared under the same solvent conditions. The fluorescence intensity of the supernatant resulting from the sample tube, F_b, and the fluorescence intensity of the control, F_c, were measured. F_c reflects the number of moles of

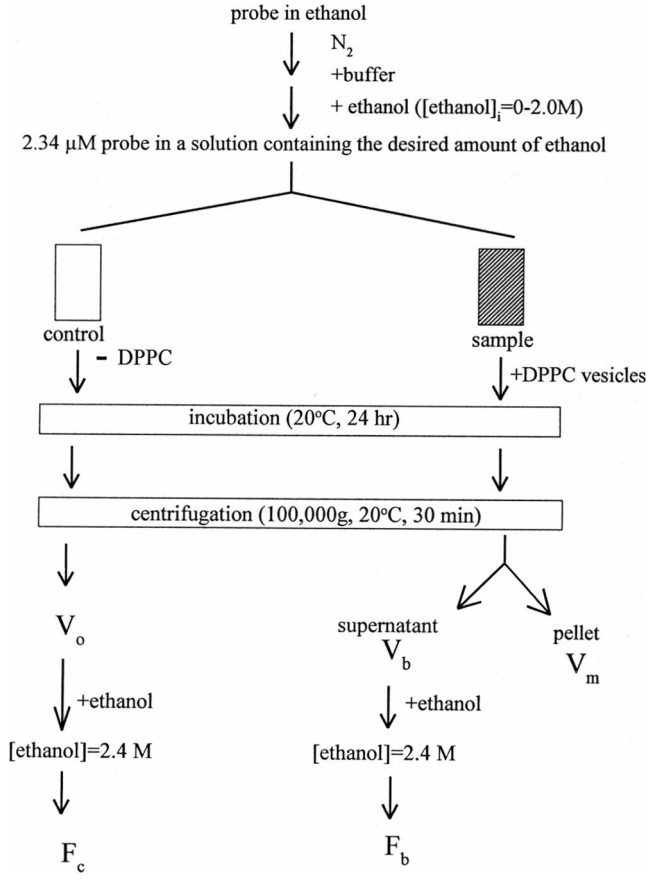

FIGURE 1 A schematic of the experimental procedure for the determination of the partition coefficients of Prodan and its derivatives.

the solute in the control tube, S_c. Note that $S_c = [S_c](V_b + V_m)$ and that $S_m + S_b = S_c$. Now, Eq. 1 can be rewritten as

$$K = \frac{55.6([S_c] - f[S_b])}{[S_b][DPPC]_{av}} \quad (2)$$

and

$$\frac{[S_c]}{[S_b]} = \left\{\frac{K[DPPC]_{av}}{55.6}\right\} + f, \quad (3)$$

where $f = V_b/(V_b + V_m)$. Because the molar concentration of DPPC in the sample tube, $[DPPC]_{av}$, can be determined by the method of Bartlett (1959), K can be calculated if $[S_b]$ and f are known. Here it is assumed that the distribution of the solute between the membrane and the bulk solution is not changed during centrifugation.

The fluorescence intensity of Prodan, Laurdan, and Acdan in the bulk solution increases linearly with probe concentration. Therefore, $[S_c]/[S_b] = F_c/F_b$. It can be assumed that $f = 1$ because $V_m \ll V_b$. Thus, Eq. 3 becomes

$$\frac{F_c}{F_b} = \left\{\frac{K[DPPC]_{av}}{55.6}\right\} + 1. \quad (4)$$

The partition coefficient of the probes, K_p, can be determined from the slope of F_c/F_b vs. $[DPPC]_{av}$.

Fluorescence measurements

Emission spectra were measured at 20°C on a SLM DMX-1000 fluorometer (Urbana, IL). All emission spectra were corrected for instrument response. The excitation wavelength was 359 nm. The bandpass was 4 nm for both excitation and emission monochromators. The total fluorescence intensity was measured from 420 to 620 nm. The residual lipid remaining in the supernatant after centrifugation had virtually no effect on the determination of the total fluorescence intensity.

RESULTS

Partition coefficients for Prodan, Laurdan, and Acdan in DPPC(MLVs) as a function of ethanol concentration

Fig. 2 shows the relationship between F_c/F_b and $[DPPC]_{av}$ for Prodan in DPPC at 20°C in the absence of ethanol.

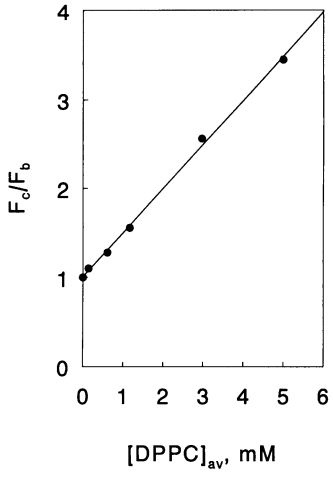

FIGURE 2 Relationship between F_c/F_b for Prodan fluorescence and $[DPPC]_{av}$. Temperature = 20°C for both the fluorescence measurements and the incubation of the lipids with probe.

F_c/F_b increases linearly with increasing DPPC concentration; the slope of the line gives a K_p value of 2.76×10^4. This value, obtained from the noninterdigitated gel phase of DPPC, is significantly lower than the values previously reported for K_p of Prodan in the liquid crystalline state of L-α-dimyristoylphosphatidylcholine (DMPC) and L-α-dioleoylphosphatidylethanolamine (DOPC) (e.g., $3–7 \times 10^5$ in Massy et al. (1985) and in Rottenberg (1992)).

Using the same method, the K_p values for Prodan in DPPC at different ethanol concentrations were determined. These results are shown in Fig. 3. In general, the K_p of Prodan in DPPC decreases with increasing ethanol concentration, a trend similar to that suggested by Rottenberg (1992). Fig. 3 also shows that the K_p undergoes an abrupt decrease at 1.1 M ethanol. This concentration comes close to the critical ethanol concentration, C_r, previously reported for the formation of fully interdigitated DPPC vesicles (Simon and McIntosh, 1984).

The partition coefficients of Laurdan and Acdan in DPPC at various ethanol concentrations were also determined. The K_p values for all three probes in DPPC are summarized in Table 1. The K_p value decreases in the order: Acdan < Prodan ≪ Laurdan. There is a drop in K_p for Acdan at ethanol concentrations between 0.8 and 1.4 M. This concentration region corresponds to the C_r for DPPC, which suggests that the drop in K_p is a result of the ethanol-induced lipid interdigitation. However, this drop is not so dramatic as that observed in the case of Prodan (Fig. 3). In sharp contrast, no abrupt change in K_p is detected at the C_r for Laurdan because the K_p of Laurdan is overwhelmingly in favor of the membrane, and the K_p for Laurdan remains high over the entire range of ethanol concentrations examined.

Relationship between probe partitioning and the emission spectra of probe fluorescence

Fig. 4 *B* (*solid line*) shows that, at 20°C and 0 M ethanol, the fluorescence emission spectrum of Prodan in DPPC multilamellar vesicles exhibits a major peak at 431 nm with a shoulder near 510 nm when $[DPPC] = 1.17$ mM.

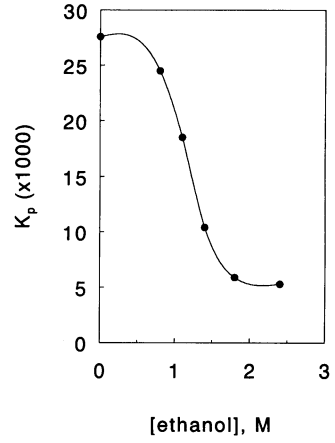

FIGURE 3 Effect of ethanol ($[ethanol]_i$) on the partition coefficient (K_p) of Prodan from the bulk solution to DPPC vesicles at 20°C.

TABLE 1 Effect of ethanol on the partition coefficients of Acdan, Prodan, and Laurdan in DPPC (MLVs) at 20°C

[ethanol] (M)	0	0.8	1.1	1.4	1.8	2.4
			K_p* (10^3)			
Acdan	2.0	2.0	1.5	1.0	1.0	1.0
Prodan	27.6	24.5	18.5	10.4	5.9	5.3
Laurdan	>4700	>4700	>4700	>4700	>4700	>4700

*The errors of K_p were estimated to be ~2%.

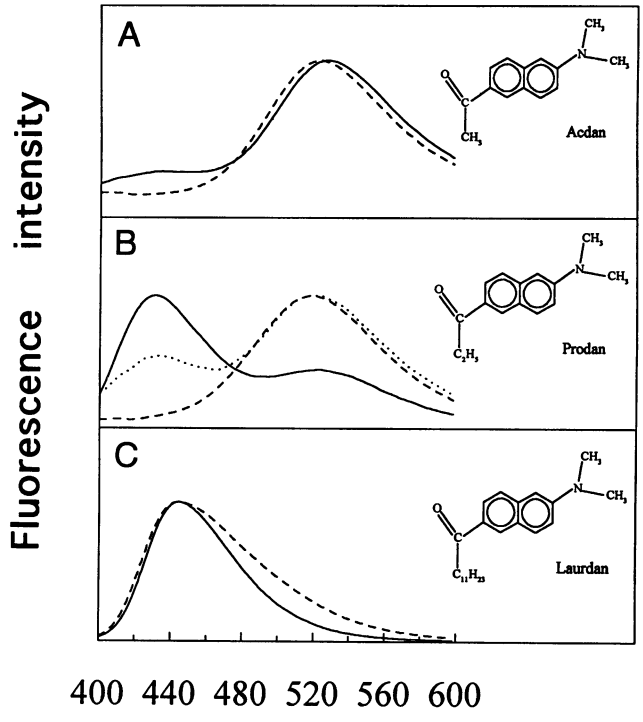

FIGURE 4 Effect of ethanol on the emission spectra of Acdan (*A*), Prodan (*B*), and Laurdan (*C*) fluorescence at 20°C. [*DPPC*] is 1.17 mM for all the samples except for the dotted line, which was obtained from a sample with [*DPPC*] = 0.48 mM. The solid and dotted lines represent samples without ethanol, and the dashed line represents samples containing 1.8 M ethanol.

The emission spectrum of Prodan fluorescence in DPPC vesicles in the presence of 1.8 M ethanol at 20°C displays a dominant peak at 518 nm (*dashed line* in Fig. 4 *B*). Thus, the 518 nm peak can be attributed, at least partially, to Prodan in the bulk solution. As shown earlier, at 1.8 M ethanol and at 20°C, DPPC is fully interdigitated and the partitioning of Prodan is significantly reduced (Figs. 2 and 3).

When [DPPC] is lowered to 0.48 mM (*dotted line* in Fig. 4 *B*), the major peak is shifted to 521 nm. Because the probe amount is fixed in the sample, a decrease in [DPPC] should decrease probe partitioning from the bulk solution to the membrane. This result, again, supports the idea that the fluorescence peak in the 510–518 nm region is associated with Prodan molecules in the bulk solution.

Fig. 4, *A* and *C* show the emission spectra of Acdan and Laurdan, respectively, in DPPC vesicles at 20°C. In the absence of ethanol, the emission maxima of Laurdan and Acdan fluorescence appear at 444 and 527 nm, respectively (*solid lines* in Fig. 4, *A* and *C*). At 1.8 M ethanol, DPPC is turned into the fully interdigitated gel state; yet the emission maximum of Laurdan remains at 444 nm, and the emission maximum of Acdan changes only slightly to 523 nm (*dashed lines* in Fig. 4, *A* and *C*).

Although the emission maxima of Acdan and Laurdan fluorescence show little change with ethanol-induced lipid interdigitation (Fig. 4), the ratio of the fluorescence intensity at 435 nm to that at 510 nm, F_{435}/F_{510}, of Laurdan in DPPC vesicles exhibits an abrupt decrease between 1.0 and 1.8 M ethanol (Fig. 5, *filled diamonds*). A similar distinct drop is seen for Prodan in DPPC (Fig. 5, *filled circles*); however, in the case of Prodan the drop occurs in the region 0.8–1.3 M ethanol. Acdan exhibits a much less dramatic drop in F_{435}/F_{510} at ethanol concentrations between 0.8 and 1.1 M (Fig. 5, *open circles*). Based on the C_r values (0.8–1.2 M) determined by x-ray diffraction (Simon and McIntosh, 1984), it seems that the F_{435}/F_{510} parameter of Prodan and Acdan reports more closely the ethanol-induced lipid interdigitation than that of Laurdan. It is also noticed that the transition in

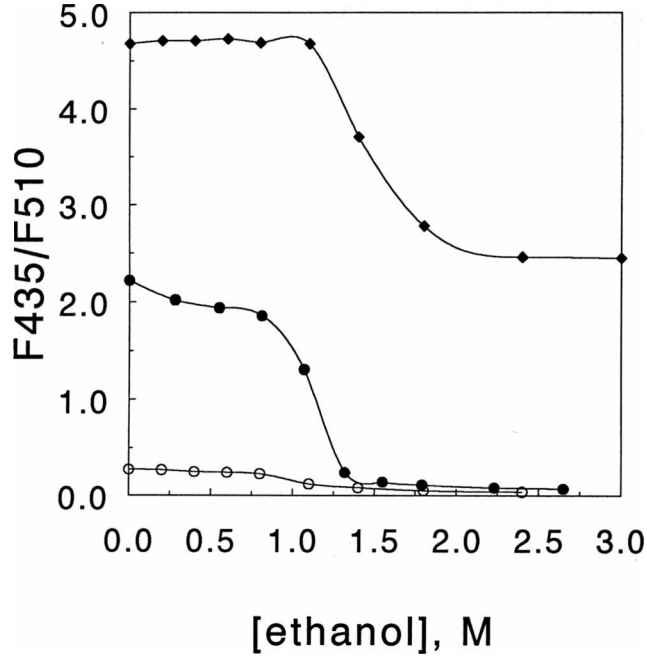

FIGURE 5 Effect of ethanol on F_{435}/F_{510} of Acdan (○), Prodan (●), and Laurdan (◆) fluorescence in DPPC multilamellar vesicles. F_{435}/F_{510} is the ratio of the fluorescence intensity at 435 nm to that at 510 nm. [*DPPC*] = 1.17 mM and the probe-to-lipid ratio is <1/500. Temperature = 20°C.

F_{435}/F_{510} seen in Fig. 5 spans a broad ethanol concentration region for Laurdan (0.8 M) and less for Prodan (0.5 M) and Acdan (0.4 M). This difference suggests that Laurdan causes more perturbation on membranes than do the other two probes, a conclusion in agreement with that previously established from infrared data (Chong and Wong, 1993). In view of the position, the width and the magnitude of the transition in F_{435}/F_{510} as a function of ethanol, it can be concluded that Prodan is the best probe of these three for monitoring ethanol-induced lipid interdigitation.

DISCUSSION

In the absence of ethanol, the partition coefficient of the probes in DPPC increases in the order: Laurdan > Prodan > Acdan. This trend is not surprising because there is much spectroscopic evidence suggesting that the partitioning of Laurdan into membranes is very different from the partitioning of Prodan in membranes. For example, the emission maximum of Prodan fluorescence in DPPC undergoes a dramatic red-shift through the ethanol-induced phase transition from the noninterdigitated gel state to the interdigitated gel state (Zeng and Chong, 1991; also Fig. 4). In contrast, the emission maximum of Laurdan fluorescence remains in the blue region through the phase transition (Fig. 4). This has been interpreted as Prodan undergoes relocation in membranes under physical perturbations, but Laurdan does not (Zeng and Chong, 1991; Chong and Wong, 1993). In addition, the intensity ratio of Prodan fluorescence at 435 nm to that at 510 nm (F_{435}/F_{510}) has been shown to be a useful index for monitoring the phase transition of lipid membranes (Chong, 1988). Merlo and Yager (1990) used a similar parameter (F_{440}/F_{500}) of Laurdan fluorescence to determine the lipid phase transition. It is found that the intensity ratio varies with the Prodan concentration (Chong, 1988), but invariant with the Laurdan concentration (Merlo and Yager, 1990). This further suggests that, in membrane systems, Prodan undergoes relocation through the phase transition, whereas Laurdan is stabilized in the lipid matrix. Moreover, because the pK_a of the amino group in Laurdan and Prodan is about 1.7 (Chong and Wong, 1993), about 5% of Laurdan or Prodan molecules are protonated and positively charged at pH 3. Our infrared data showed that when Laurdan becomes positively charged, Laurdan moves toward the membrane surface, but is still within the membrane (Chong and Wong, 1993). In contrast, at pH 3, the protonated Prodan is situated outside the membrane (Chong et al., 1989). All of these studies already suggest that, compared with Prodan, Laurdan has a much higher partition coefficient in membranes. In this study, we used the precipitation method to demonstrate that this is indeed the case.

The increasing trend, K_p(Laurdan) > K_p(Prodan) > K_p(Acdan), can also be easily understood from the chemical structures of the probes (Fig. 4). The chemical structures of these probes differ only in the hydrocarbon chain attached to the carbonyl group. Laurdan has 10 methylene units and exhibits the highest value in K_p. In contrast, Acdan has no methylene group and has the lowest partition coefficient. It has been known for some years that the membrane:water partition coefficient is increased by a factor of two to three for each additional methylene unit in the linear hydrocarbon solute molecule (Diamond and Katz, 1974) because of the increased van der Waals and hydrophobic interactions (Pope and Dubro, 1986). However, it is interesting to note from Table 1 that the partition coefficients do not increase linearly with the length of the polymethylene chain. The relationship between K_p and the length of the polymethylene side chain in aromatic solute molecules appears to be more complicated than that in linear hydrocarbon solute molecules.

Our previous infrared studies showed that the correlation field-splitting pressure, P_s, of the DMPC methylene scissoring mode is 4.6 kbar for DMPC containing 11 mol% Laurdan at pH 6.8 (Chong and Wong, 1993). This P_s value is much higher than the P_s (3.9 kbar) for DMPC containing the same amount of Prodan (11 mol%) at the same pH (Chong et al., 1989). The pressure-induced correlation field-splitting of the methylene scissoring mode reflects the interchain packing (Boerio and Koenig, 1970; Wong, 1994). The tighter the packing, the lower the P_s is. Thus, the infrared results suggest that Laurdan causes more perturbation to DMPC vesicles than Prodan. This can be understood by considering that the chromophore of Laurdan is embedded deeper in the lipid bilayer than the chromophore of Prodan; the perturbation should mainly arise from the bulky chromophore, rather than from the methylene units of the lauroyl chain. This notion agrees with the conclusion derived from the fluorescence data obtained by Parasassi et al. (1991), who showed that the generalized fluorescence polarization of Laurdan in lipid membranes is independent of the type of the polar headgroup and suggested that Laurdan is embedded in lipid bilayers deeper than Prodan. This conclusion is confirmed in the present study where F_{435}/F_{510} of Laurdan fluorescence in DPPC exhibits a much broader transition than that of Prodan and Acdan (Fig. 5).

Thus, it can be proposed that solute partitioning not only depends on hydrophobicity and van der Waals interactions but also on membrane perturbations and the site of interactions with the lipids. If the probe inserts deeper into the bilayer interior because of strong hydrophobic and van der Waals interactions, membrane perturbation caused by the bulk ring structure of the probe would also be greater. This would create a greater void space in the membrane, which would reduce the membrane surface density, thus resulting in a higher partitioning. Surface density has been proposed to be the main determinant of solute partitioning in lipid bilayers (De Young and Dill, 1988, 1990). When membrane surface density is high because of low temperature, high pressure, or the presence of cholesterol, the partitioning of solutes such as hexane (De Young and Dill, 1990), benzene (De Young and Dill, 1988), chlorpromazine (Luxnat and Galla, 1986), and tetracaine (Auger et al., 1987) in membranes decreases. It is interesting to note that Wimley and White (1993) recently reported that the partitioning of tryptophan side-chain analogs into POPC large unilamellar

vesicles (~100 nm in diameter) is governed by both the hydrophobic and the bilayer effect. They suggested that the partitioning of those solutes leads to changes in lipid bilayer organization that contribute to the "bilayer" effect. Our present study indicates that such a bilayer effect may be related to membrane perturbation.

The partition coefficients of Prodan, Acdan, and ethanol decrease abruptly through the ethanol-induced phase transition from the noninterdigitated to the fully interdigitated gel state of DPPC. These results are in parallel with previous results obtained by the fluorescence lifetime/polarization method (Zeng and Chong, 1991). The abrupt decrease of Prodan partitioning at C_r can be attributed to the changes in membrane structure due to lipid interdigitation. Lipid interdigitation brings about intramolecular acyl chain ordering (Wu et al., 1982; Simon and McIntosh, 1984; O'Leary and Levin, 1984; Hui and Huang, 1986; Boggs et al., 1989) and a tighter acyl chain intermolecular packing (Simon and McIntosh, 1984). These effects would increase membrane surface density. Because membrane surface density is the dominant factor of solute partitioning (Marqusee and Dill, 1986; De Young and Dill, 1988, 1990), solute partitioning decreases when lipids are fully interdigitated.

The fluorescence spectra of Acdan, Prodan, and Laurdan in DPPC vesicles seem to be closely related to their partitioning in the membrane. The fluorescence of Laurdan depends on solvent relaxation and the polarity of the environment (Parasassi et al., 1994). Our present study is confined to the gel phase of DPPC, in which there is virtually no solvent relaxation (Parasassi et al., 1994). Therefore, in this study, the fluorescence of Laurdan reflects only the polarity of the environment. Fig. 4 *C* indicates that although the dominant emission peak remains at 444 nm, the emission intensity in the 510 nm region increases when [*ethanol*] is raised to 1.8 M. This is probably because of a vertical displacement of Laurdan toward a more polar environment in response to the ethanol-induced lipid interdigitation. Another possible explanation for the intensity increase in the 510 nm region is the small decrease in K_p. As such, the decrease must be so small that it cannot be detected by the precipitation method.

The interpretation of Prodan fluorescence in membranes requires the consideration of solvent relaxation (Sommer et al., 1990), environmental polarity (Chong, 1988), and probe location (Chong, 1988; Rottenberg, 1992; Zeng and Chong, 1993). The K_p data listed in Table 1 clearly indicate that Prodan in the bulk solution increases significantly through the ethanol-induced phase transition. This suggests that the increased intensity in the 510–521 nm region (Fig. 4 *B*) originates from Prodan in the bulk solution. However, Prodan in pure water should exhibit an emission maximum at 531 nm (Weber and Farris, 1979). Therefore, the 510–521 nm peak must have two origins, namely, Prodan in the bulk solution and Prodan in a membrane-associated more-polar site, as previously proposed (Chong, 1988; Zeng and Chong, 1991). Note that in the presence of lipid vesicles the bulk solution actually contains a trace amount of monomeric lipid. This explains why the emission maximum of Prodan in pure water is 531 nm (Weber and Farris, 1979), whereas the emission spectra of Prodan in lipid dispersions never exhibit an emission maximum at 531 nm (Chong, 1988; Zeng and Chong, 1991; Rottenberg, 1992), even at conditions that favor Prodan partitioning in the bulk solution.

Like Prodan, the fluorescence of Acdan in lipid vesicles should vary with probe location, the degree of solvent relaxation, and environmental polarity. Using Eq. 1 and $[DPPC]_{av} = 1.17$ mM, it can be calculated that the K_p corresponding to 50% probe molecule in the membrane is 4.75×10^4; K_p for Acdan (Table 1) is much lower than this value. Thus, most Acdan molecules must reside in the bulk solution. This explains why the dominant peak of the Acdan emission spectrum is near 523–527 nm (Fig. 4 *A*). Previous studies showed that Laurdan fluorescence can sense the pressure-induced phase transition in egg yolk phosphatidylcholine (egg-PC) multilamellar vesicles, but Prodan cannot (Chong, 1990). Parasassi et al. (1991) showed that the fluorescence properties of Laurdan in the lipid membrane are independent of the type of polar headgroup. They suggested that Laurdan is embedded in the membrane deeper than Prodan. Using infrared data, Chong and Wong (1993) reached a similar conclusion. Although little has been studied on Acdan in membranes, it may be proposed that the chromophore of Acdan is located at the membrane surface very close to the aqueous phase and the chromophore of Laurdan is embedded deeper in the bilayer whereas the chromophore of Prodan is located in the region between Acdan and Laurdan. This trend is in parallel with the K_p data. The relationship between K_p and the probe location seems to be in qualitative agreement with that previously proposed by Diamond and Katz (1974), who suggested that the partition coefficient of a solute is a function of its position in the bilayer.

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