Preferential Interactions of Fluorescent Probe Prodan with Cholesterol

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ABSTRACT The fluorescent probe Prodan has been widely used as a probe of model and biological membranes. Its fluorescent maxima in phospholipid bilayers vary as a function of phase state, with maxima at 485 for the liquid crystal L_α, 435 nm for the gel L'_β, and 507 nm for the interdigitated gel $L_\beta I$ phase, with excitation at 359 nm. These spectral changes have been used for the detection of phase changes among these phases. In the present study, the fluorescent properties and partition coefficients of Prodan in model membranes of phosphatidylcholines and phosphatidylethanols have been studied as a function of lipid phase state and cholesterol content. It is shown that the Prodan spectrum in the presence of cholesterol no longer reflects the known phase state of the lipid; in each phase state, the presence of cholesterol leads to a spectrum with the maximum at 435 nm, characteristic of the noninterdigitated gel phase. The partition coefficient of Prodan into these lipids also varies with the phase state, giving values of 0.35×10^4 in the interdigitated gel, 1.8×10^4 in the noninterdigitated gel, and 7.6×10^4 in the liquid crystal phase. In the presence of cholesterol these partition coefficients are increased to 13×10^4 for the liquid crystal and the gel phase, and 5.1×10^4 in the presence of 100 mg/ml ethanol. These results suggest that Prodan has preferential interactions with cholesterol, and is thus not a randomly distributed fluorescent reporter probe in membranes containing cholesterol. These results suggest that Prodan should be used only with great caution in complex lipid mixtures, particularly biological membranes.

INTRODUCTION

Since its introduction by Weber and Farris (Weber and Farris, 1979; Macgregor and Weber, 1986), the hydrophobic probe Prodan has been widely used to determine the physical properties of phospholipid membranes. It has been shown that in the gel phase the emission spectrum of Prodan has a maximum at ~435 nm, with excitation at 359 nm, which is interpreted as Prodan in hydrophobic surroundings, while in the liquid-crystalline phase the emission maximum shifts to the region around 485–490 nm, suggesting a more hydrophilic environment. Prodan fluorescence has been shown to respond to the formation of interdigitated lipids in the presence of ethanol (Zeng and Chong, 1991, 1995; Zeng et al., 1993; Bondar and Rowe, 1996). The emission maximum of Prodan fluorescence is shifted dramatically to longer wavelength, i.e., 507 nm, by the ethanol-induced phase transition from the noninterdigitated to the fully interdigitated gel phase. Prodan fluorescence has also been used to study lipid behavior in cell membranes, for example goldfish brain synaptic membranes, liver microsomes, inner membranes of mitochondria, and red blood cells (Rottenberg, 1992). Microsomes and inner mitochondrial membranes, which contain little or no cholesterol, exhibit a Prodan fluorescence spectrum similar to phospholipids in the liquid-crystalline state. The Prodan spectra in synaptic membranes and red blood cell plasma membranes, which contain cholesterol and sphingolipids, were more similar to phospholipids in the gel phase.

Prodan has a weak affinity for most proteins, and it has not been used effectively to probe hydrophobic binding sites of proteins (Mazumdar et al., 1992). Thus, Prodan binds BSA with an affinity of $1 \times 10^5\ M^{-1}$ and tubulin with an affinity of $5 \times 10^4\ M^{-1}$, and these affinities are lower, for example, than for ANS (Weber and Farris, 1979; Mazumdar et al., 1992). At the same time it has been shown that Prodan can bind to spectrin with high affinity (Chakrabarti, 1996). The fluorescence of Prodan exhibits a wavelength shift from 520 nm in water to 433 nm in the presence of dimeric spectrin.

In the present investigation we have studied the effect of increasing cholesterol concentration on Prodan fluorescence in lipid bilayers under various phase conditions. We have also measured the partition coefficients of Prodan into lipids under these conditions. Our results show that cholesterol increases the partition coefficient of Prodan and produces a Prodan spectrum that is characteristic of the cholesterol environment rather than being characteristic of the average membrane environment, suggesting that Prodan has preferential interactions with cholesterol. These results suggest that Prodan should be used with great caution in studying complex lipid mixtures, particularly biological membranes.

Received for publication 30 July 1998 and in final form 29 October 1998.

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Abbreviations used: Prodan, 6-propionyl-2(*N′N*-dimethyl) aminonaphthalene; ANS, anilinonaphthalene-sulfonic acid; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPeth, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanol (sodium salt); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPeth, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol (sodium salt); FME, 4′-dimethylaminoflavonol; MLV, multilamellar vesicles; PC, phosphatidylcholine; Peth, phosphatidylethanol; PG, phosphatidylglycerol.

0006-3495/99/02/956/07 $2.00

MATERIALS AND METHODS

Chemicals

DMPeth and DPPC were obtained from Avanti, Birmingham, AL; the ethanol was purchased from PhaRmco, Linfield, PA; and Prodan was obtained from Molecular Probes (Eugene, OR). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO).

Sample preparation

The MLVs were prepared using Bangham's method (Bangham et al., 1967). Chloroform stock solutions of lipids and ethanol solution of Prodan were dried under a gentle stream of nitrogen gas and then kept overnight on a vacuum pump to remove all solvent residuals. Distilled water or 50 mM Tris-HCl buffer was added to the dry film and the suspensions were hydrated at a temperature above the temperature of the phase transition for at least 2 h with occasional vortexing. Lipid concentrations were determined by Bartlett's method (Bartlett, 1959).

Fluorescence measurements

The fluorescence experiments were performed using the SLM 8300 spectrofluorometer. The temperature of samples was monitored with an Instrulab Model 700 digital thermometer, with the thermistor placed in a parallel reference cuvette. The sample was magnetically stirred during the measurements. The lipid:probe ratio used was 500:1 and the concentration of lipids was 1.1–1.2 mM. The excitation wavelength was 359 nm, and the fluorescence spectra were measured from 400 to 610 nm, and were not corrected for instrument response. The excitation and emission slits were 4 nm and 16 nm, respectively. A control sample prepared without a fluoroprobe had <1% scattering signal for all fluorescence experiments.

Determination of partition coefficients

The partition coefficients for Prodan into lipids were measured according to the methods described by Huang and Haugland (1991). In this method, Prodan is located in the fluorescence cuvette and its fluorescence at 435 nm measured as a function of added lipid.

DMPC MLVs were used for the determination of the partition coefficient of Prodan into the various phases of lipid bilayers. The partitioning of Prodan into the gel phase was measured at 10°C, and into the liquid-crystalline phase at 30°C. In the case of the interdigitated gel phase, DMPC in the presence of 2.5 M ethanol was added to the cuvette containing Prodan in the presence of the same concentration of ethanol.

The partition coefficient, K_p, of the Prodan is defined as

$$K_p = (C_b/L)/(C_f/W) \quad (1)$$

where C_b and C_f are the molar concentrations of the membrane-bound and free probe, respectively, and L and W are the concentration of lipid and water (Huang and Haugland, 1991; Harris, 1971). Since free Prodan in water at 435 nm is essentially nonfluorescent (Hutterer et al., 1996), the fluorescence (F) should be proportional to the concentration of membrane-bound probe, i.e.,

$$F = kC_b \quad (2)$$

where k is a proportionality constant. Since the total probe concentration $C = C_b + C_f$, Eq. 1 can be rearranged to

$$F = F_0L/(W/K_p + L) \quad (3)$$

where $F_0 = kC$ is the maximum fluorescence resulting from total probe incorporation into membrane. Assuming the concentration of water to be 55.6 M, Eq. 3 may be rewritten as

$$F = F_0L/(55.6/K_p + L) \quad (4)$$

or

$$1/F = [55.6/(K_pF_0)](1/L) + 1/F_0 \quad (5)$$

K_p can then be calculated from the plot of $1/F$ vs. $1/L$:

$$K_p = -55.6(x\text{-intercept}) \quad (6)$$

RESULTS

Prodan fluorescence

Prodan fluorescence has been widely used to characterize lipid phase behavior. Fig. 1 shows the fluorescence spectra of Prodan for DPPC at 23.5°C in the presence of increasing cholesterol concentration. Prodan in pure DPPC in the gel phase has its main fluorescence maximum at 435 nm and a weak shoulder at ~530 nm (Fig. 1 *B*). Increasing the concentration of cholesterol significantly increases the fluorescence intensity at 435 nm. At the same time, as low as 5 mol % of cholesterol reduces the shoulder at 530 nm; this suggests that increasing cholesterol concentration causes decreasing polarity in the probe environment. The increasing intensity at 435 nm can be attributed to increased partitioning (see below) as well as possible probe relocation.

The phase transition of DPPC from the gel to liquid-crystalline phase dramatically changes the shape of the fluorescence spectrum. Fig. 2 shows the spectra of Prodan in DPPC at 50°C with a series of cholesterol concentrations. The fluorescence maximum of Prodan in pure liquid-crystalline DPPC shifts to 480 nm, compared to the gel phase maximum at 435 nm, indicating that the probe is located in a more polar region compared to the gel phase. In the

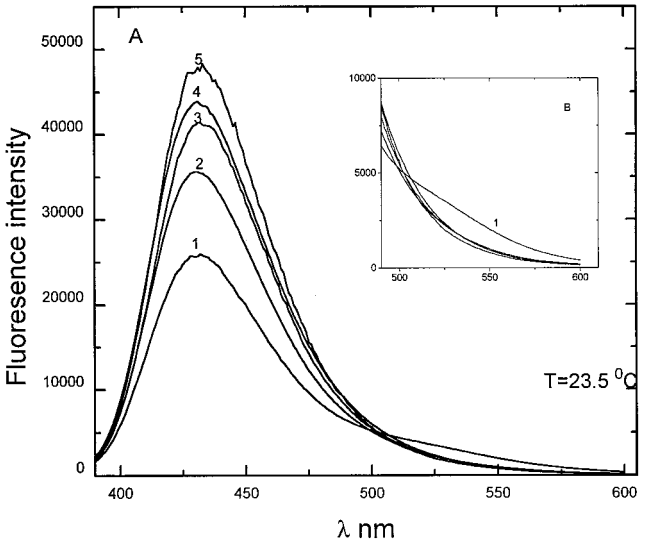

FIGURE 1 (*A*) Prodan fluorescence spectra in DPPC in the presence and absence of cholesterol at 23.5°C. Excitation at 359 nm. *1*, 0% cholesterol; *2*, 5 mol % cholesterol; *3*, 10 mol % cholesterol; *4*, 40 mol % cholesterol; *5*, 50 mol % cholesterol. (*B*) Expansion of region 500–600 nm.

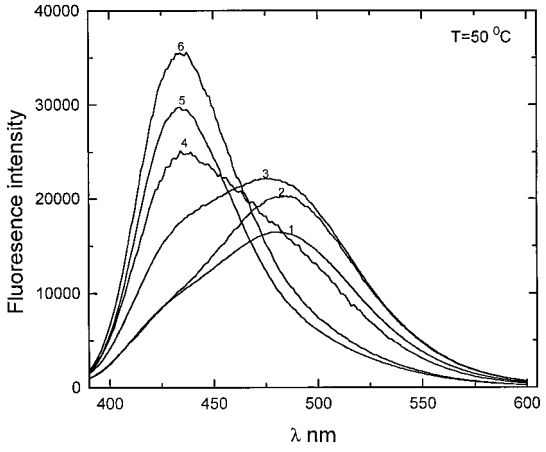

FIGURE 2 Prodan fluorescence spectra in DPPC in the presence and absence of cholesterol at 50.5°C. Excitation at 359 nm. *1*, 0% cholesterol; *2*, 5 mol % cholesterol; *3*, 10 mol % cholesterol; *4*, 20 mol % cholesterol; *5*, 40 mol % cholesterol; *6*, 50 mol % cholesterol.

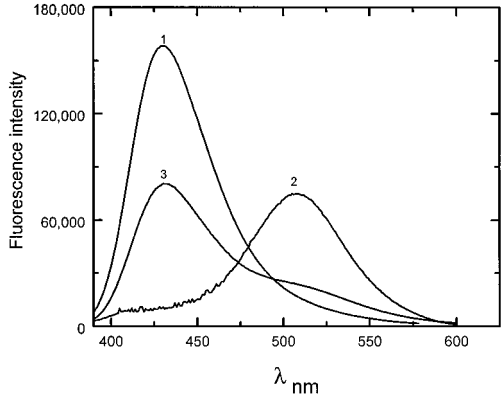

FIGURE 3 Prodan fluorescence in DPPC in the presence of ethanol and cholesterol in water at 22°C. Excitation at 359 nm. *1*, DPPC-0 mol % cholesterol; *2*, DPPC in the presence of 100 mg/ml ethanol; *3*, DPPC-5 mol % cholesterol in the presence of 100 mg/ml ethanol.

presence of 5 mol % cholesterol the fluorescence intensity increases without changing the spectral shape. Increasing the cholesterol concentration to 10 mol % leads to a broadening of the fluorescence peak with a shift of the maximum to 470 nm. Beginning from 10 mol % cholesterol a shoulder appears at 430 nm. Increasing the cholesterol concentration up to 50 mol % shifts the fluorescence maximum to 430 nm with increasing fluorescence intensity, indicating that the probe has relocated to a less polar environment. The spectral shape for 40 mol % cholesterol and above is similar to that seen in the gel phase (Fig. 1).

Prodan fluorescence spectra in DPPC in the interdigitated phase in the presence and absence of cholesterol were studied. The interdigitated gel phase ($L_\beta I$), in which the acyl chains from opposing monolayers interpenetrate, is among the most recently described stable lipid phase states, and has been well demonstrated for the saturated symmetrical and asymmetrical PC (McDaniel et al., 1983; McIntosh et al., 1983, 1984; Rowe, 1985; Wilkinson et al., 1987; reviewed in Slater and Huang, 1988). It has been demonstrated previously that increasing concentrations of cholesterol abolish the interdigitated phase in DPPC, but ethanol can partially restore the interdigitation of DPPC in the presence of 5–20 mol % of cholesterol (Komatsu and Rowe, 1991; Bondar and Rowe, 1998). Fig. 3 shows the Prodan spectra of DPPC with 5 mol % cholesterol in the presence and absence of ethanol, and the spectrum of pure DPPC with ethanol. For pure DPPC in the presence of ethanol Prodan gave a fluorescence maximum at 507 nm, which is characteristic of interdigitation of the gel phase (*spectrum 2*). The DPPC with 5 mol % cholesterol in the absence of ethanol shows a Prodan spectrum typical of the normal L'_β gel phase. In the presence of ethanol the spectrum in DPPC with 5 mol % cholesterol remained characteristic of the noninterdigitated gel phase. This is in contrast to previous studies showing that under these conditions DPPC containing 5 mol % cholesterol is ~50% in the fully interdigitated phase (Komatsu and Rowe, 1991; Bondar and Rowe, 1998).

Prodan fluorescence has been used to detect the interdigitated phase in Peths (Bondar and Rowe, 1996) as well as PCs (Zeng and Chong, 1991, 1995; Zeng et al., 1993; Chong, 1988; Chong et al., 1989). It was shown previously in our laboratory that Peth can form the interdigitated gel phase in the presence of Tris (Bondar and Rowe, 1996). Prodan fluorescence data correlated with DSC in the demonstration of interdigitation for DMPeth and DPPeth (Bondar and Rowe, 1996). Fig. 4 *A* shows the Prodan spectra in DPPeth in 50 mM Tris-HCl and in a mixture of DPPeth with 5 mol % cholesterol in the presence and absence of ethanol. The main fluorescence maximum at 507 nm for pure DPPeth (*spectrum 1*) indicates that the Prodan environment is more polar and the partitioning of the probe into the lipid bilayer is restricted. As was shown earlier, this spectrum of Prodan corresponds to the interdigitated gel phase of DPPeth in Tris-HCl (Bondar and Rowe, 1996). The combination of 5 mol % cholesterol with DPPeth significantly changes the shape of the fluorescence spectrum (*spectrum 2*). The fluorescence maximum at 507 nm disappears and the intensity of the fluorescence at 435 nm increases. This Prodan spectrum is similar to that for DPPC in the normal L'_β phase (Zeng and Chong, 1995). Adding 100 mg/ml ethanol to the DPPeth-cholesterol mixture does not significantly change the shape of the fluorescence spectrum (*spectrum 3*), giving only a weak shoulder in the region of 507 nm. Fig. 4 *B* shows the series of Prodan spectra for mixtures of DPPeth with 20 mol % cholesterol in the presence of increasing concentrations of ethanol. As seen here, the fluorescence spectra do not change with ethanol addition. The data shown in Fig. 3, *A* and *B* suggest that no interdigitation occurs in the presence of any amount of cholesterol, even with the addition of up to 100 mg/ml ethanol. These results are not consistent with the DSC results indicating that DMPeth and DPPeth containing up to 20 mol % cholesterol are at least partially interdigitated in the presence of ethanol (Bondar and Rowe, 1998).

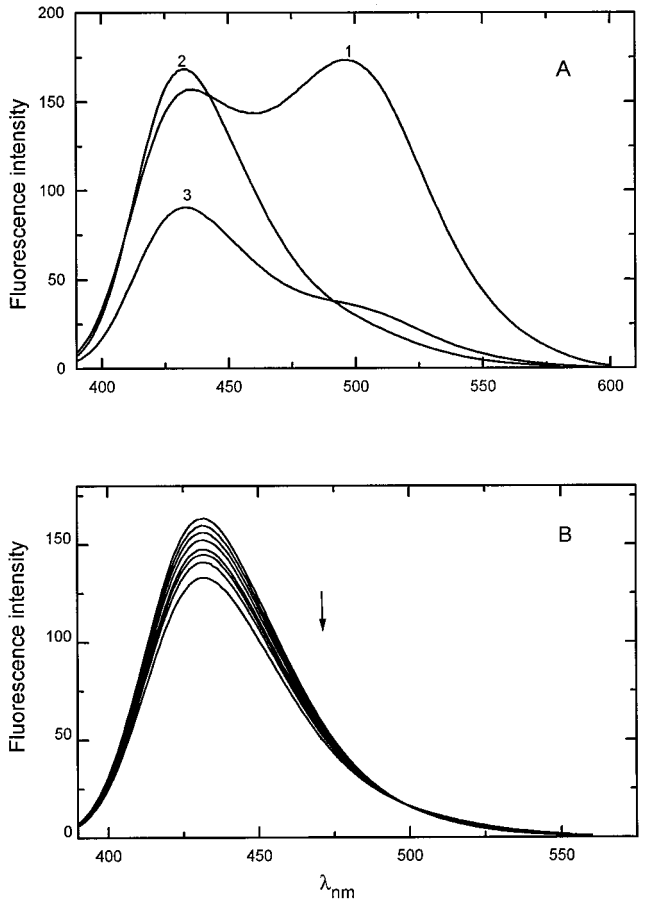

FIGURE 4 Prodan fluorescence spectra of DPPeth in the presence of cholesterol in 50 mM Tris-HCl at 23.5°C. Excitation at 359 nm. (*A*) effect of cholesterol on the emission spectra of Prodan fluorescence in DPPeth: *1*, 0 mol % cholesterol; *2*, 5 mol % cholesterol; *3*, 5 mol % cholesterol in the presence of 100 mg/ml ethanol. (*B*) Effect of ethanol on the emission spectra of Prodan fluorescence in DPPeth-20 mol % cholesterol in 50 mM Tris-HCl at 22°C. The arrow shows the increasing concentration of ethanol from 0 (*top spectrum*) to 100 mg/ml ethanol (*bottom spectrum*).

Partition coefficient for Prodan

The present data suggest that Prodan is not uniformly distributed in the lipid in the presence of cholesterol. To further understand the distribution of Prodan in lipid bilayers in the absence and presence of cholesterol, we have determined the partition coefficient of Prodan in lipid bilayers as a function of cholesterol concentration and phase state. According to Eq. 4 and as shown in Fig. 5 *A*, the Prodan fluorescence enhancement by titration with DMPC liposomes exhibits a saturating tendency. Fig. 5, *B* and *C* show, as examples, the corresponding double reciprocal $1/F$ vs. $1/L$ plots for pure DMPC (*line 1*), in the presence of 10 mol % cholesterol (*line 2*), and 20 mol % cholesterol (*line 3*). $1/F$ increases linearly with increasing concentration of lipid, and the *x*-intercepts of these lines give the values for calculation of the Prodan partition coefficient, K_p. Partition coefficients of Prodan into DMPC in various phase states in the presence of increasing concentrations of cholesterol are shown in Table 1. In the case of pure DMPC in the gel phase the K_p of Prodan was 1.84×10^4. This value of K_p for DMPC is close to the value previously reported for K_p of Prodan into DPPC in the gel phase by Zeng and Chong (1995). The K_p of Prodan into the gel phase increased with increasing cholesterol concentration, giving a value of 13.2×10^4 at 40 mol % cholesterol. This suggests that Prodan preferentially locates in the vicinity of cholesterol within the bilayer.

The transition of DMPC from the gel phase to the liquid-crystalline phase increases the partitioning of Prodan into the lipid bilayers. The Prodan K_p in DMPC in the liquid crystalline phase was 7.6×10^4. The addition of cholesterol increases the K_p of Prodan in the liquid-crystalline phase. Increasing cholesterol concentration abolishes the gel-to-liquid-crystalline phase transition of DMPC, and the values of K_p at the liquid crystal temperature were the same as at the gel phase temperature in the presence of 40 mol % cholesterol. In the presence of 40 mol % cholesterol Prodan exhibited the characteristic gel phase spectrum even at 50°C (Fig. 2).

In the presence of 2.5 M ethanol DMPC forms the interdigitated gel phase and the partitioning of Prodan into the lipid decreases significantly. The K_p of Prodan for the interdigitated gel phase was only 0.35×10^4. As little as 5 mol % of cholesterol significantly increased K_p, supporting our suggestion that the probe is preferentially located in the cholesterol-rich regions. Again, increasing cholesterol leads to an increasing partition coefficient for Prodan. At 40 mol % cholesterol the interdigitated phase is abolished, so it might be expected that the partition coefficient would be the same as for the gel phase lipid at the same temperature. The lower value found suggests that the presence of 2.5 M ethanol probably reduces the partitioning because the solubility of Prodan in the aqueous phase is enhanced.

DISCUSSION

Prodan has found widespread use in membrane studies because of its large spectral shifts associated with phospholipid phase transitions. Prodan possesses a ketone carbonyl, –C = O, and dimethyammonium $(CH_3)_2$ N– groups, each of which may form hydrogen bonds with water or with membrane components. In addition, it is known that the dimethylammonium group plays an important role in the interaction of probes with the lipid headgroups (Hsieh and Wu, 1995; Sprinz et al., 1986). These structural properties of Prodan explain its behavior in lipid bilayers. Some previous investigations of Prodan fluorescence in lipid bilayers have been made by Chong and co-workers (Zeng and Chong, 1991, 1995; Zeng et al., 1993; Chong, 1988; Chong et al., 1989). They showed that in the gel phase the fluorescence emission maximum is around 435 nm, which was interpreted as a low polarity environment in the region of the lipid acyl chains. In the liquid-crystalline phase the fluorescence emission maximum is shifted to the region of 485 nm, suggesting a hydrophilic high polarity location near the

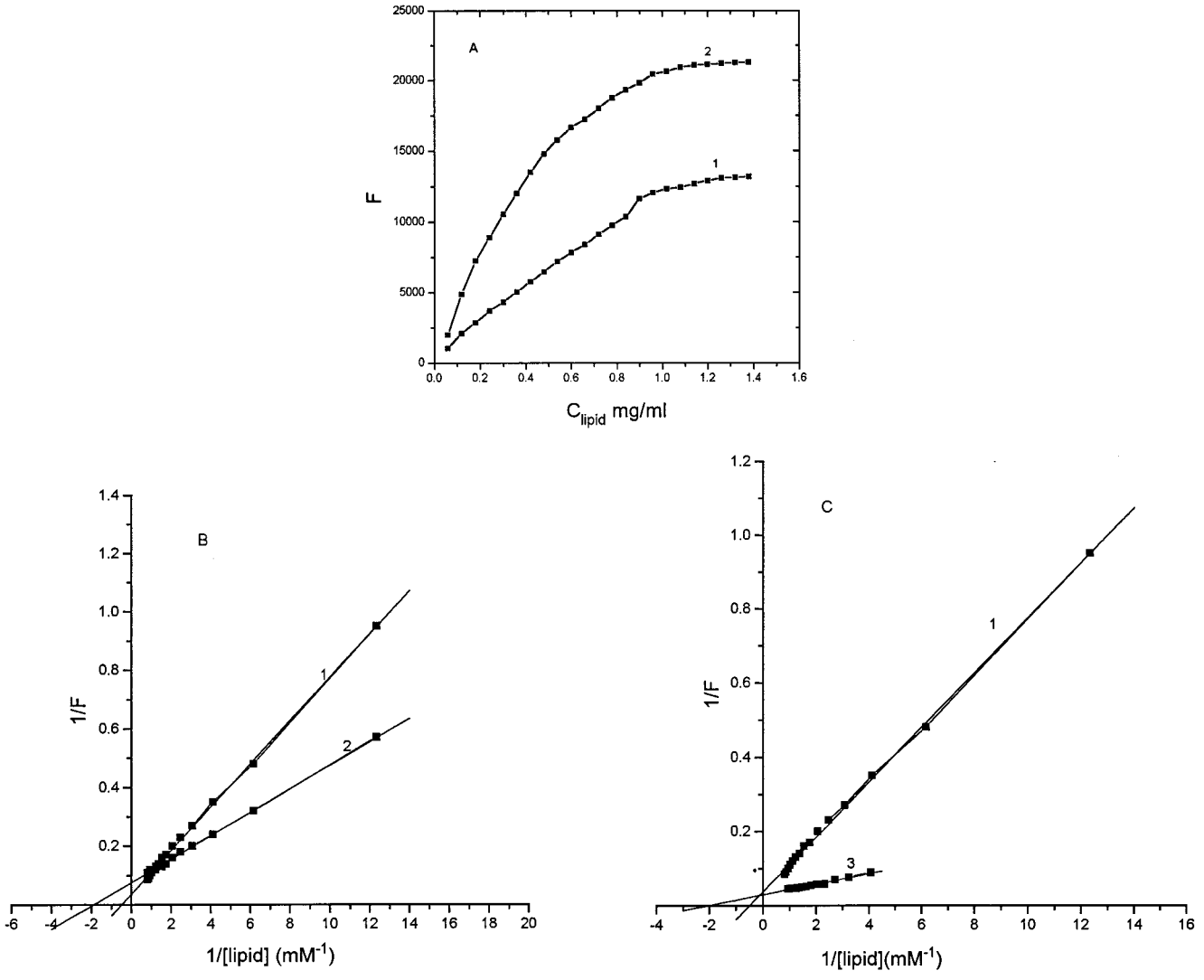

FIGURE 5 (*A*) Effect of increasing concentration of lipid on the fluorescence intensity of Prodan in DPPC. Excitation at 359 nm. *1*, pure DPPC; *2*, DPPC in the presence of 10 mol % cholesterol. (*B* and *C*) Reciprocal plots of fluorescence intensity versus concentration of lipid. *1*, pure DPPC; *2*, DPPC in the presence of 5 mol % cholesterol; *3*, DPPC in the presence of 20 mol % cholesterol.

phospholipid headgroup. Formation of the interdigitated gel phase shifted the main fluorescence maximum to the region of 510 nm, suggesting an additional increase in the polarity of the Prodan environment (Zeng and Chong, 1991, 1995; Zeng et al., 1993; Bondar and Rowe, 1998). This suggests that in the interdigitated gel phase the Prodan has been squeezed out of the acyl chain region to the interfacial region, or into the aqueous phase.

Our results have correlated the partition coefficient of Prodan into the gel or liquid-crystalline phases with the spectrofluorescence characteristics of the probe. The formation of the liquid crystalline phase increases the partitioning

TABLE 1 Partition coefficients of Prodan in DMPC MLVs

	K_p (10^4)				
Cholesterol	0	5 mol %	10 mol %	20 mol %	40 mol %*
Gel Phase (T = 10°C)	1.8	5.5	5.8	9.2	13.2
Liquid-Crystalline Phase (T = 25°C)	7.6	9.6	7.8	11.1	13.0
Interdigitated Gel Phase[#] (T = 10°C)	0.35	3.6	2.8	6.7[§]	5.1[§]

*Forty mol % of cholesterol abolishes the phase transition.
[#]These samples contain 2.5 M ethanol.
[§]The interdigitated phase is abolished above 20 mol % cholesterol.

of Prodan into DMPC bilayers compared to the gel phase, even while the fluorescence maximum shifts to 485 nm, indicating a more polar environment for the Prodan in the liquid-crystalline relative to the gel phase. This could occur either by relocation of the probe nearer to the interfacial region or by a greater penetration of solvent water into the acyl chain region in this phase due to the greater disorder and decreased packing density.

The partition coefficients of Prodan are significantly decreased for the interdigitated gel phase, suggesting that the tighter packing of the acyl chains in this phase compared to the noninterdigitated gel phase has "squeezed" the Prodan out. This is consistent with the large shift of the fluorescent maximum to 510 nm, suggesting that the Prodan is located on the lipid surface or even in the aqueous phase. These results are similar to those obtained by Zeng and Chong (1995). It is this large fluorescent maximum difference between the interdigitated gel phase and the noninterdigitated gel phase which has made Prodan a useful probe for studying the interdigitated phase.

The addition of cholesterol to the lipid bilayers dramatically changes the behavior of Prodan. In the presence of cholesterol in the gel phase the surface density of lipid bilayers increases (De Young and Dill, 1988, 1990; Luxnat and Galla, 1986), which might be expected to decrease Prodan partitioning. However, in contrast, as low as 5 mol % of cholesterol in the gel phase increases the partitioning of Prodan into the PC bilayers, with increasing fluorescence intensity at 435 nm. Raising the concentration of cholesterol up to 20 mol % leads to an increase in fluorescence intensity without a change in the position of the fluorescence maximum. This increase of intensity of the emission spectrum reflects the increased partitioning resulting from the presence of cholesterol.

In the liquid-crystalline phase, at 50°C for DPPC and at 35°C for DMPC, Prodan exhibited its maximum fluorescence at 485 nm, indicating decreased hydrophobicity in its environment. At this temperature the introduction of cholesterol leads to a shift in the maximum fluorescence of Prodan to 435 nm and an increase in Prodan partitioning. This spectrum is similar to that of Prodan in the gel phase. These results suggest the probe has preferential interactions with cholesterol and locates in a cholesterol-rich region of the bilayer, which has a polarity similar to the gel phase.

Previously, Prodan fluorescence has been successfully used to study the formation of interdigitated lipid bilayers. Fig. 4 shows the clear difference in its spectrum between the fully interdigitated and the noninterdigitated gels for DPPC. We have also used Prodan in our study of the phase behavior of DMPeth and DPPeth to investigate the effects of Tris-HCl and other solvents on its tendency to become interdigitated (Bondar and Rowe, 1996). In the present study, however, Prodan did not give results consistent with the other methods used in investigating the effect of cholesterol on the interdigitation of DPPeth and DMPeth. As shown in Fig. 4 for DPPC containing cholesterol, whose behavior is well documented, the Prodan spectrum was characteristic of the noninterdigitated gel phase for all conditions, including those where it is well established that the lipid is at least partially interdigitated. This is consistent with the Prodan locating near the cholesterol in noninterdigitated regions of the bilayer.

The mechanism of the spectral changes in Prodan that leads to the large change during the transition from gel to interdigitated gel shown in Fig. 4 for DPPC has been discussed by ourselves and others (Zeng and Chong, 1991, 1995; Zeng et al., 1993; Bondar and Rowe, 1996; Chong, 1988; Chong et al., 1989; Komatsu and Rowe, 1991; Bondar and Rowe, 1998). The behavior of Prodan with regard to these two lipid phases has been described as being caused by changes in partitioning of the probe between the aqueous solvent and the lipid bilayer (Zeng and Chong, 1995). The spectrum for the gel phase represents Prodan in the lipid phase, whereas the spectrum for the interdigitated phase is similar to that of Prodan in water, indicating that the Prodan is either in the solvent phase or in the aqueous interfacial region. Our measurement of the partition coefficient supports this interpretation that the partitioning of Prodan into the interdigitated phase is low because of the tighter acyl chain packing in this phase. The smaller bilayer thickness could also be a factor. The present study has shown that Prodan continues to exhibit the characteristic noninterdigitated spectrum in the presence of cholesterol, even when a majority of the membrane lipids are interdigitated. This again suggests that Prodan interacts preferentially with cholesterol, and remains in the lipid phase in the cholesterol-rich regions, even in membranes containing only 5 mol % cholesterol.

Similar results have been observed for FME, a compound structurally similar to Prodan, in DPPC-cholesterol and DPPeth-cholesterol membranes (Bondar et al., 1998). This probe is also effective for monitoring formation of the interdigitated gel phase in pure lipid bilayers. In the presence of increasing concentration of cholesterol the FME spectra were indicative of the noninterdigitated gel phase even where it is well known that lipid is at least partially interdigitated (Bondar et al., 1998).

We have shown that Prodan fluorescence in the phospholipid membranes increases in the presence of cholesterol, with the fluorescence maximum shifting to a characteristic wavelength of 435 nm. A similar result has been shown by Massey et al. (1985) for Prodan fluorescence in high-density lipoprotein (HDL) model membranes in the presence of apoA with increasing concentrations of cholesterol. They also showed that the emission maximum of Prodan in apoA/DMPC vesicles with 18 mol % of cholesterol was 433 nm both in the gel and the liquid-crystalline phases, whereas in the absence of cholesterol the maximum fluorescence occurred at 435 nm in the gel phase and at 493 nm in the liquid-crystalline phase. However, this effect of cholesterol on Prodan fluorescence was attributed to changes in hydration rather than to increased Prodan partitioning or redistribution due to preferential cholesterol-Prodan interactions. Similarly, alterations in the wavelength maxima of Prodan

fluorescence observed in biological membranes in the presence of cholesterol from a wavelength characteristic of the liquid crystal phase to one characteristic of the gel phase were also interpreted in terms of changes in hydration and structure of the membrane as a whole, without consideration of the possibility of nonrandom distribution of the Prodan probe molecules (Rottenberg, 1992).

The results presented here raise serious questions about the use of Prodan to probe the membrane environment in complex systems such as biological membranes. In addition to the nonrandom behavior of Prodan noted here, it was previously shown that a spectrum similar to the interdigitated spectrum of Fig. 3 *A* occurs in the inverted hexagonal phase of DEPE (Bondar and Rowe, 1996). These observations have important consequences for the use of Prodan as a probe of the membrane environment in membranes that contain mixtures of lipids. In the present study it appears that Prodan preferentially interacts with cholesterol; it is possible that there are also other lipids having positive or negative preferences for Prodan. This study indicates that Prodan should be used with extreme caution in probing the environments of cellular membranes, with their great heterogeneity. For studying model membranes of defined composition it can be useful, but it is necessary to perform the appropriate controls for each condition and type of lipid.

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