Probing the Interactions of Alcohols with Biological Membranes with the Fluorescent Probe Prodan†

Hagai Rottenberg

Department of Pathology, Hahnemann University, Broad and Vine, Philadelphia, Pennsylvania 19102

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ABSTRACT: Prodan [6-propionyl-2-(dimethylamine)naphthalene] is a hydrophobic fluorescent probe which is extremely sensitive to both the polarity and the hydrogen-bond donating capacity of the solvent. In binary mixtures of solvents, the hydrogen-bond donating effect on Prodan fluorescence saturates at relatively low concentrations of protic solvent while the polarity effect is proportional to the mixture's dielectric constant. The fluorescence emission maximum is approximately a linear function of the dielectric constant in both protic and aprotic solvents, and this allows estimation of the dielectric constant in both environments. In phospholipid bilayers and biological membranes, Prodan exhibits two distinct emission peaks: blue (430–445 nm) and green (470–505 nm). Temperature determines the relative intensity of the two peaks, but their wavelengths depend on the type of membrane and appear to reflect a specific membrane environment. In phospholipid vesicles, alcohols reduce the fluorescence intensity of the blue peak and produce a red-shift in the emission maximum of the green peak. Taking the partition coefficients of the alcohols into account, short-chain alcohols are much more effective than longer-chain alcohols in red-shifting the emission maximum of the green peak. Alcohols have similar effects on Prodan fluorescence in liver microsomal and mitochondrial membranes, synaptosomal membranes, and red blood cell plasma membranes. However, in liver organelle membranes the red-shift of the green peak is the dominant effect while in plasma membranes the quenching of the fluorescence of the blue peak is dominant. These effects are observed at low (pharmacological) ethanol concentrations and provide a unique tool for probing the interactions of ethanol with biological membranes. The effect of alcohols on the relative intensity of the emission of the blue and green peaks could be interpreted as resulting either from enhanced solvent dipolar relaxation or from transfer of dye from the membrane core to the surface. The effect of alcohols on the position of the green emission peak is most likely the result of an increased polarity of the environment.

Ethanol differs from many psychoactive drugs in that its effects do not depend on binding to specific receptors. Although several channels, receptors, and membrane enzymes are affected by relatively low concentrations of ethanol, there is no evidence that ethanol interacts with specific binding sites on these proteins (Deitrich et al, 1989). It appears that the effects of ethanol depend on its partitioning into the lipid bilayer. However, the interactions of ethanol with lipid bilayers and biological membranes have not been characterized in as great detail as those of longer-chain alcohols and other drugs. This is because the hydrophilic nature of ethanol does not allow a sufficient amount of membrane-bound ethanol to be detected by most methods. Ethanol effects on membranes are usually investigated with the aid of fatty acid spin probes, which detect its effect on the membrane order parameter (Goldstein, 1984), or with fluorescent probes, which detect its effect on membrane order and viscosity (Wood & Schroeder, 1988). Direct binding of ethanol was estimated from radiolabeled ethanol distribution (Rottenberg et al; 1981) and from ^{2}H-NMR spectra of [^{2}H]ethanol (Kreishman et al., 1985). The effect of ethanol on the phase transitions of phospholipid bilayers was also used to probe ethanol interaction with phospholipids (Rowe, 1983; Rowe et al., 1987). However, the information obtained by these methods is very limited and largely contradictory. For example, partition coefficients estimated from binding of [^{2}H]ethanol (Kreishman et al., 1985) are 100-fold larger than those estimated from the phase-transition shifts (Rowe, 1983). Even though it is expected that ethanol does not distribute equally in all regions of the membrane (Brasseur et al., 1985), there is little evidence for the exact location(s) of ethanol in biological membranes.

The hydrophobic fluorescent probe Prodan, like several other aminoacylnaphthalenes, is extremely sensitive to solvent polarity (Weber & Farris, 1979) and exhibits complex fluorescence responses when bound to phospholipid membranes (Massey et al., 1985; Chong, 1988; Zeng & Chong, 1991). In the gel phase, the fluorescence emission maximum is around 435 nm, which was interpreted as suggesting a hydrophobic, low dielectric environment, while in the liquid-crystalline phase the fluorescence emission maximum is red-shifted (around 485 nm), suggesting a hydrophilic, high-dielectric environment (Massey et al., 1985). However, even in this state a distinct blue shoulder exists, and the fluorescence intensity can be shifted from the green peak to the blue peak by hydrostatic pressure (Chong, 1988). Prodan fluorescence was also shown to respond to the formation of interdigitated lipids in DPPC liposomes when induced by a high concentration of ethanol (Zeng & Chong, 1991). In this study the use of Prodan as a probe for the interactions of alcohols with biological membranes was further explored. It is shown that Prodan fluorescence can be used to probe the partition of alcohols (and other solvents) into biological membranes. The information obtained from such measurements could enhance our understanding of the action of ethanol on biological membranes.

MATERIALS AND METHODS

Rat liver mitochondria and inner membranes of mitochondria (Hashimoto & Rottenberg, 1983), microsomes (Ponapa

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et al., 1982), red blood cell plasma membranes (Kelly-Murphy et al., 1984), and rat brain synaptosomes (Rottenberg et al., 1981) were prepared as described previously. Egg lecithin and dioleoylphosphatidylcholine (DOPC) multilamelar liposomes were prepared by evaporating chloroform solutions of lipids in a wide flask to form a thin layer and then shaking gently with the suspension buffer (10 mM Tris-HCl, 100 mM KCl, pH 7.5). Prodan (Molecular Probes) was dissolved either in dimethylformamide (DMF) or acetone. In most experiments an aliquot of Prodan stock solution was dried in a flask by a stream of nitrogen and then dissolved in the proper solvent or medium. In a few experiments a concentrated DMF solution (5 mM) was diluted to 0.5–1 μM directly into the membrane suspension (the DMF concentration never exceeded 0.02%). There was no difference in the fluorescence properties of suspensions containing a very low concentration of DMF. All the spectra with pure solvents, which required accurate determination of peak positions, were performed on a SLM 8000 spectrofluorometer employing correction programs. Most of the experiments with membranes were carried out on a Spex spectrofluorometer without correction. Several experiments (e.g., with DOPC vesicles) were conducted on both instruments. Because the emission spectra in natural membranes are very broad and are located in a wavelength region in which the photomultiplier response is independent of wavelength (430–510 nm), there was no significant differences between the emission spectra collected on these instruments. All spectra were collected at 1-nm intervals with each point integrated from 1 to 3 s to obtain smooth curves. The indicated maxima are the wavelength in which the maximum peak intensity was observed. In cases where identical maximal intensity was obtained over a range of the spectrum, the median of the peak values is given as the maximum wavelength. All solvents were of the highest grade available commercially (Aldrich). Aprotic solvents were used only from freshly-opened bottles to minimize water contamination. Lipids were obtained from Sigma.

RESULTS

Effect of Solvents on Prodan Fluorescence. Weber and Farris (1979) have determined the emission and absorption maxima of Prodan in a number of polar and nonpolar solvents. These measurements were extended to a number of additional solvents, particularly alkanols. Based on these measurements, we can divide these solvents into five distinct groups. The typical spectra obtained with these solvents groups are shown in Figure 1. Figure 1A shows the absorption spectra of Prodan in five solvents: hexane (curve 1), which was typical for paraffins (e.g., cyclohexane, heptane); benzene (curve 2), which was typical for aromatic nonpolar solvents (e.g., toluene); acetone (curve 3), which was typical for polar aprotic solvents (e.g., chlorobenzene, acetonitrile); propanol (curve 4), which was typical for alcohols; and water (curve 5). In paraffins the absorption peak at 340 nm was sharp with a small shoulder at 370 nm; in aromatic solvents the absorption peak was broader and red-shifted (347 nm), and the shoulder at 370 nm was much more pronounced. The absorption spectra in polar nonprotic solvents were very similar to those in aromatic solvents. In alcohols the absorption spectra were much broader and were further red-shifted (360 nm). Finally, in water the absorption spectrum was broader and the extinction coefficient was only about one-half the value in other solvents. Typical excitation and emission spectra are shown in Figure 1B. In a paraffin the quantum efficiency was very low compared to other solvents, the excitation peak was at short wavelength (344 nm), and the emission was also at short wavelength (400 nm). In aromatic nonpolar and in aprotic polar solvents Prodan had very similar excitation spectra (347–352 nm), but the emission spectra in polar solvents were red-shifted and broader than those in nonpolar solvents. In alcohols excitation peaks were at longer wavelengths (370–380 nm) and the emission spectra were red-shifted further and were broader. Finally, in water, the excitation and emission spectra were very flat with a low quantum efficiency but with the largest apparent separation between the excitation maximum and the emission maximum. These differences in the spectra of Prodan in different types of solvents suggest that, in addition to the effect of solvent polarity, specific effects of solvent–dye interactions contribute to the fluorescence characteristics. Prodan is an amphiphilic solute and is only sparingly soluble in paraffins and water. The unusual absorption and fluorescence properties in both types of solvents suggest that dimerization or higher aggregates occur in these solvents.

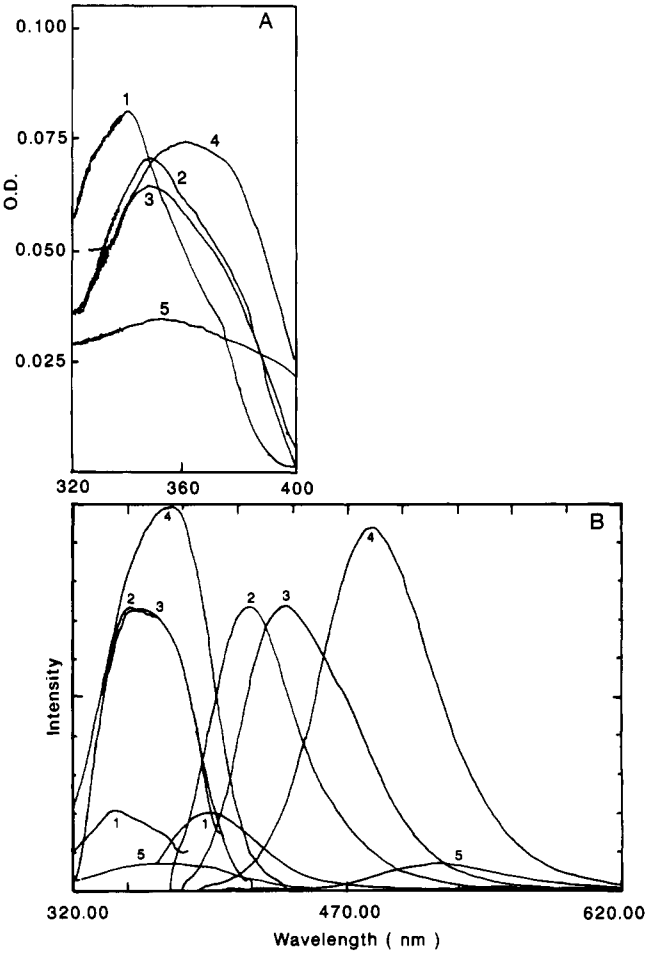

FIGURE 1: Absorption, excitation, and emission spectra of Prodan in different types of solvents. Solvents are hexane (1), benzene (2), acetone (3), propanol (4), water (5). (Panel A) Absorption spectra of 5 μM solution. (Panel B) Excitation and emission spectra of 0.5 μM solution. Spectra of hexane, $F \times 10$; other spectra were taken at the same sensitivity. Emission spectra were taken with excitation at the maximum of each solvent, and excitation spectra were taken with emission at the maximum of each solvent.

Figure 2 shows an empirical plot of the fluorescence emission maximum of Prodan in 24 different solvents against the solvent dielectric constant. Most of the data points fall on two curves. Nonprotic solvents fit one linear curve ($r = 0.87$) and *n*-alcohols fit another linear curve ($r = 0.998$). Secondary alcohols, diols, and even glycerol also fit the *n*-alcohols curve reasonably well. The only significant deviations are observed with water and paraffins. The two curves have similar slopes, 0.87 nm/*D* for

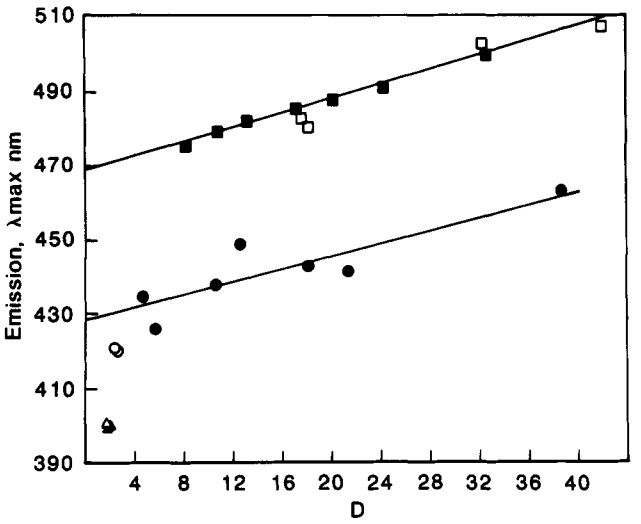

FIGURE 2: Effect of the solvents dielectric constant (D) on the fluorescence emission maximum of Prodan. Paraffins (hexane, cyclohexane, heptane) (△), aromatic (benzene, toluene) (○), aprotic polar (chloroform, chlorobenzene, pyridine, cyclohexanone, acetone, acetonitrile) (●), n-alcohols (decanol, octanol, hexanol, butanol, propanol, ethanol, methanol) (■), secondary and polyalcohols (2-butanol, 2-propanol, 1,2-propanediol, glycerol) (□).

aprotic solvents and 0.95 nm/D for protic solvents, and are separated by about 40 nm.

The effect of alcohols on Prodan fluorescence is thus attributed to two distinct parameters: hydrogen bonding and the dielectric constant. Since hydrogen bonding is essentially a chemical reaction with a typical equilibrium constant, it can be expected that relatively low concentrations of the solvent will saturate this effect (Rapp et al., 1971). This can be seen in Figure 3, which shows the shift in the position of the emission maximum of Prodan in binary solvent mixtures as a function of the mole fraction of the titrating solvent. In a mixture of two solvents, aprotic and protic, with approximately the same dielectric constant, increasing concentrations of the protic solvent caused a red-shift in the emission spectrum which was entirely due to hydrogen bonding and occurred at very low solvent concentrations. Thus, in a titration of the aprotic solvent acetone (D = 21.4) with the protic solvent propanol (D = 20.1), the entire red-shift (47 nm) was due to hydrogen bonding. 50% of the shift occurred at 0.05 mol fraction of protic solvent. Similarly in a titration of the aprotic solvent 1,2-dichloroethane (D = 10.65) with the protic solvent octanol (D = 10.8), the entire shift (39 nm) was due to hydrogen bonding. 50% of the shift occurred at 0.03 mol fraction in this case. In contrast, in titrations of one alcohol with another the entire spectra shift was due to modulation of the dielectric constant and was proportional to the mole fraction. Thus when Prodan in decanol (D = 8.1) was titrated with ethanol (D = 24.3), the entire spectral shift was linear with the mole fraction and the slope (0.93 nm/D) was the same as that obtained in Figure 2B. Titrations of the aprotic solvent of moderate dielectric constant acetone (D = 21.4) with aromatic nonpolar solvent (benzene) or with more polar aprotic solvent (acetonitrile) also yielded linear titrations with similar slopes. On the other hand, when Prodan in acetone was titrated with cyclohexane, the initial slope was the same as that obtained with benzene, but at very high cyclohexane concentration a sharp decrease of the emission maxima occurred, most probably due to the formation of Prodan aggregates. Titration of Prodan in acetone with methanol produced a more complex shift, which at low concentrations was dominated by the effect of hydrogen bonding, while at high concentrations it was

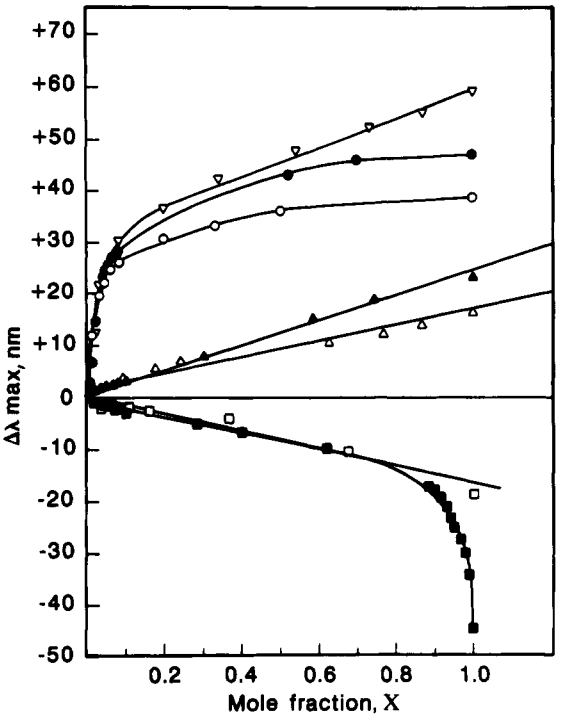

FIGURE 3: Titrations of the emission maxima of Prodan in binary mixtures of solvents: Prodan concentration was 0.5 μM. The abscissa shows the mole fraction of the titrating solvent, and the ordinate shows the corresponding shift in the emission maxima. Prodan in acetone titrated with propanol (●), Prodan in acetone titrated with cyclohexane (■); Prodan in decanol titrated with ethanol (△), Prodan in 1,2-dichloroethane titrated with octanol (○), Prodan in acetone titrated with benzene (□), Prodan in acetone titrated with acetonitrile (▲). Prodan in acetone titrated with methanol (▽).

dominated by the increase of the dielectric constant. This more complex curve can be simulated by assuming a 50% formation of hydrogen-bonded Prodan at 0.05× and an increment due to the dielectric constant with a slope of 0.9 nm/D. The large difference in the concentration dependence of the hydrogen-bonding effect and the dielectric effect can be utilized to discriminate between the two effects in an unknown environment.

Prodan Fluorescence in Biological Membranes. Previous studies established that, in phospholipid vesicles, two distinct emission peaks are observed. In the gel phase the blue peak (~435 nm) was dominant while at the liquid-crystalline phase the green peak (~485 nm) was dominant (Massey et al., 1985). A shift of the intensity from the green to the blue peak in the liquid-crystalline state could also be induced by the application of high pressure (Chong, 1988). Figure 4A shows the emission spectra (25 °C) of Prodan in phospholipid membranes (DOPC) (a), rat liver microsomes (b), inner membranes of rat liver mitochondria (c), rat synaptosomal membranes (d), and plasma membranes of rat red blood cells (e). It is observed that these diverse membranes, at room temperature, exhibit the whole spectrum of fluorescence intensity distribution between the blue and green peaks. Moreover, the maxima of the green peaks are different with each membrane. Microsomes and inner mitochondrial membranes which contain little or no cholesterol and exist in the liquid-crystalline state are more similar to DOPC liposomes in the liquid-crystalline state. Synaptic membranes and RBC plasma membranes which contain cholesterol and sphingolipids and do not show a clear phase transition are more similar to phospholipid in the gel phase where the blue peak is dominant. Even though the five membrane preparations shown in Figure 4A do not exhibit major phase transition at the range 5–50

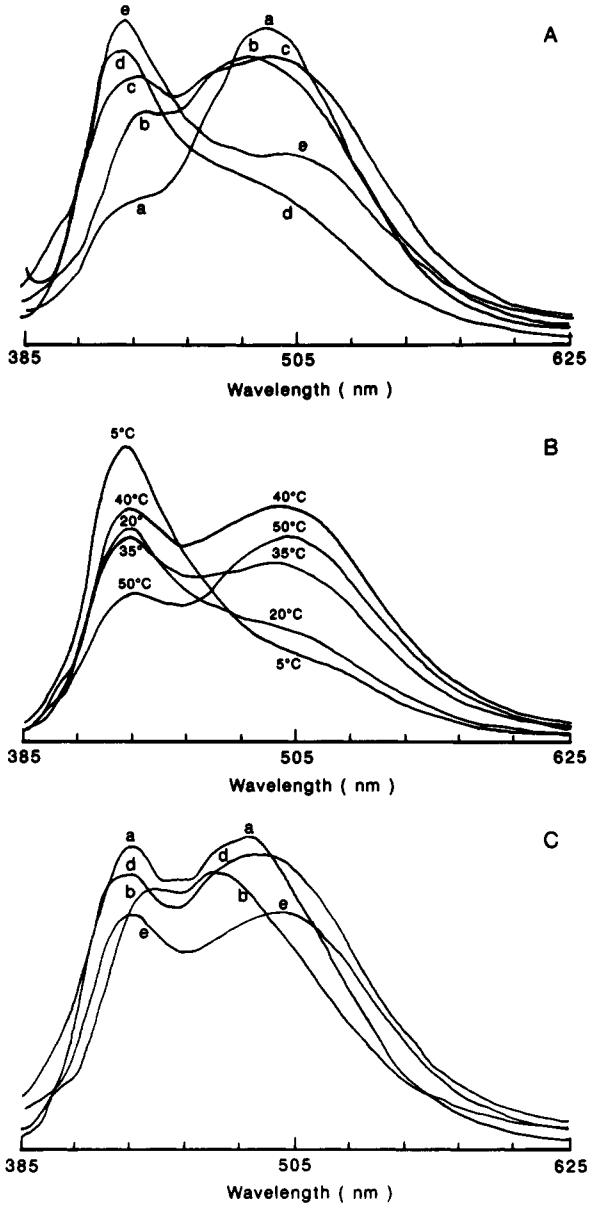

FIGURE 4: Prodan fluorescence in biological membranes. (Panel A) Emission spectra of Prodan, at room temperature (25 °C), in different membranes: a, DOPC; b, liver microsomes; c, mitochondrial inner membranes; d, synaptosomal membranes; e, RBC ghosts. Prodan concentration 0.5 μM; lipid concentration approximately 1 mg/mL; medium, 100 mM KCl and 50 mM Tris-HCl, pH 7.0; excitation, 350 nm. (Panel B) Emission spectra of Prodan in RBC ghosts as function of temperature. (Panel C) Emission spectra of Prodan in different biological membranes and different temperatues at approximately equal distribution of fluorescence between peaks: a, PC at 10 °C; b, microsomes at 5 °C; d, synaptosomes at 50 °C; e, RBC ghosts at 40 °C.

°C, the spectra could be shifted by appropriate change in temperature from one form to another. This is demonstrated in Figure 4B, which shows the emission spectrum of Prodan in plasma membranes from red blood cell in the temperatures range 5–50 °C. At 5 °C the blue peak was dominant while the green peak was hardly apparent; at 40 °C the two peaks were of equal intensity while at 50 °C the green peak was dominant, much like the spectrum of Prodan in phospholipid vesicles at the liquid-crystalline phase. However as Figure 4B demonstrates, these shifts in the fluorescence distribution were not associated with shifts in the wavelength of the maximum which appeared to be independent of temperature but characteristic for each membrane. This is best demonstrated in Figure 4C which shows the spectra of Prodan from

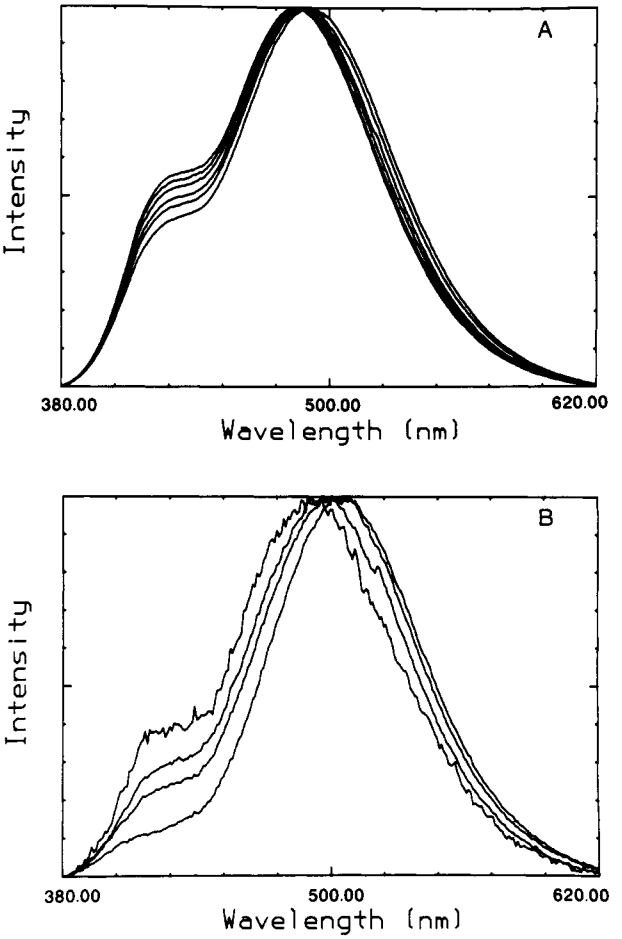

FIGURE 5: Effect of ethanol on the emission spectrum of Prodan in multilameler vesicles of egg lecithin. Phospholipid suspension (0.5 mg/mL), incubated with 1 μM Prodan and titrated with increasing concentration of ethanol from left to right 0, 2, 4, 6, 8, and 10% (v:v). (Panel A) Normalized spectra. (Panel B) Normalized difference spectra (ethanol – control).

these membranes in different temperatures selected to yield approximately equal intensity in the blue and green peaks. The wavelength of the green peak was different in each membrane. In microsomes the blue peak was red-shifted compared to DOPC while the green peak was blue-shifted and the peaks nearly overlapped. In synaptosomal membranes the green peak was red-shifted, thus producing a larger separation between peaks than DOPC. Finally in red blood cells the green peak was even further shifted compared to synaptosomal membrane, producing a very large separation (72 nm) between the peaks. This was in contrast with the very small separation (24 nm) observed in microsomal membranes. The independence of the peak position of temperature and the large variations between membranes are important for the interpretation of the fluorescence spectrum of Prodan in biological membranes (see Discussion).

Effect of Alkanols on Fluorescence of Prodan in Phospholipid Bilayers. Figure 5A shows the effects of increasing concentrations of ethanol on the normalized emission of Prodan embedded in multilameler liposomes prepared from egg lecithin. In the absence of alcohols, the dominant-corrected emission maximum was at 484 nm and a prominent shoulder was observed at 435 nm. The addition of ethanol enhanced the fluorescence of Prodan in these vesicles; the green peak was red-shifted and was enhanced much more than the blue peak.

Because the spectra are normalized to the maximum emission of the green peak, there is an apparent quenching

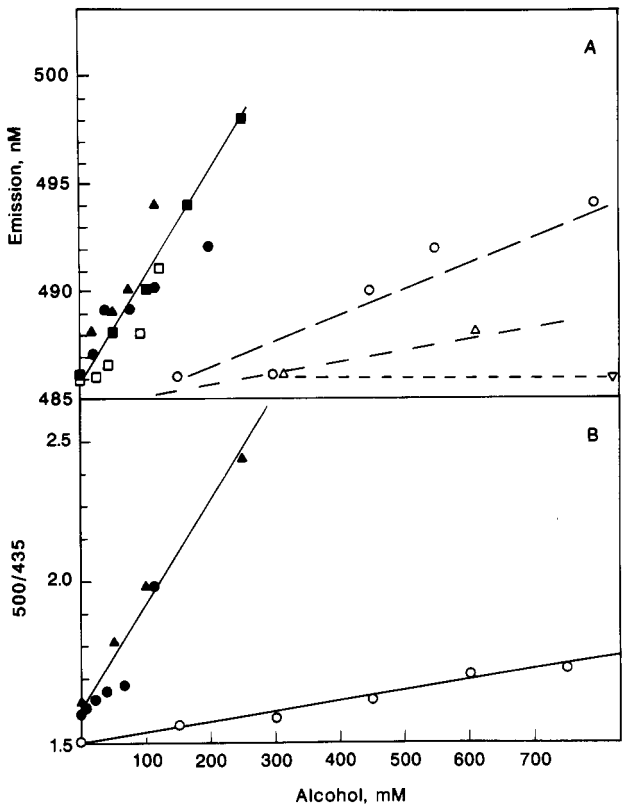

FIGURE 6: Effect of alcohols on the emission maximum of Prodan and the green/blue intensity ratio in lipid bilayers. The Prodan-labeled vesicles were titrated with different alcohols in experiments similar to those shown in Figure 5. The results are plotted against the membrane alcohol concentration, which is calculated from the buffer alcohol concentrations using the following partition coefficients: methanol (▲), 0.045; ethanol (●), 0.14; propanol (■), 0.45; butanol (□), 1.5; hexanol (○), 13.0; octanol (△), 152; decanol (▽), 1222 (Seeman, 1972). (Panel A) Fluorescence emission maxima. (Panel B) Green/blue intensity ratio.

of the blue peak. Figure 5B shows the normalized difference spectra (alcohol–control) of these titrations. Plotted in this manner, the ethanol-induced shift of intensity from the blue peak to the green peak is separated from the spectral shift of the green peak. The shift observed in these curves, from 484 nm in control to 505 nm in 10% ethanol, average 2 nm for each 1% ethanol.

Similar shifts in the emission maximum of Prodan were observed in titration with several alkanols. The concentrations of alcohols which were needed to produce shifts comparable in magnitude to those observed with ethanol decreased with increased chain length. This suggests that the effects depend on the membrane concentration of the alcohols since the partition coefficients of long-chain alcohols are much larger than those of short-chain alcohols. However, when the values of the partition coefficients of alkanols were used to calculate the membrane concentrations of the alkanols and the shifts were plotted against the calculated membrane concentrations (Figure 6A), it is evident that short-chain alcohols were actually much more effective than long-chain alcohols in shifting the emission maxima of Prodan. The effect of alcohols on Prodan fluorescence distribution between the two membrane peaks is demonstrated in Figure 6B, which shows the ratio of intensities at the two peaks as a function of the calculated alcohols membrane concentration. In this effect too, short-chain alcohols are more effective than long-chain alcohols.

Effect of Alcohols on Prodan Fluorescence in Biological Membranes. Figure 7 shows the effect of alcohols on Prodan

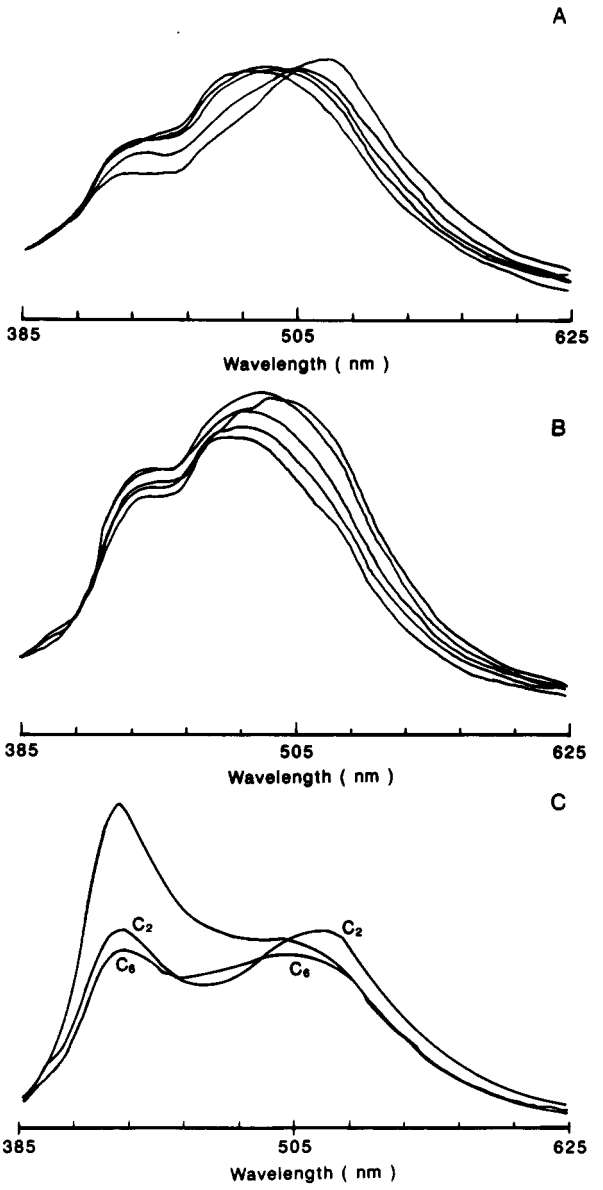

FIGURE 7: Effect of alcohols on Prodan fluorescence in biological membranes. Conditions are the same as in Figure 4A. (Panel A) Effect of ethanol on microsomes. (Panel B) Effect in mitochondria: ethanol 0, 114 mM, 278 mM, 807 mM, and 1.54 M. (Panel C) Effect of ethanol (C2, 1.1 M) and hexanol (C6, 100 mM) on plasma membranes from red blood cells.

fluorescence emission spectra in different biological membranes. Increasing concentrations of ethanol quenched the blue peak fluorescence and red-shifted the green peak both in microsomes (Figure 7A) and mitochondria (Figure 7B). In general the effects were similar to those observed with phospholipid vesicles. The red-shift was particularly large in liver mitochondrial membranes. The effect of ethanol and hexanol on Prodan fluorescence in plasma membrane from red blood cell was similar (Figure 7C). However, in plasma membrane the dominant effect was the quenching of the fluorescence of the blue peak where as the shift of the fluorescence maximum of the green peak was not as pronounced. Figure 7C also shows that at concentrations of these alcohols that produced approximately the same extent of quenching of the blue peak fluorescence, ethanol produced a larger shift of the emission maximum of the green peak than hexanol.

The effects of alcohols on the emission maximum of the green peaks and the intensity ratio of the green/blue peaks

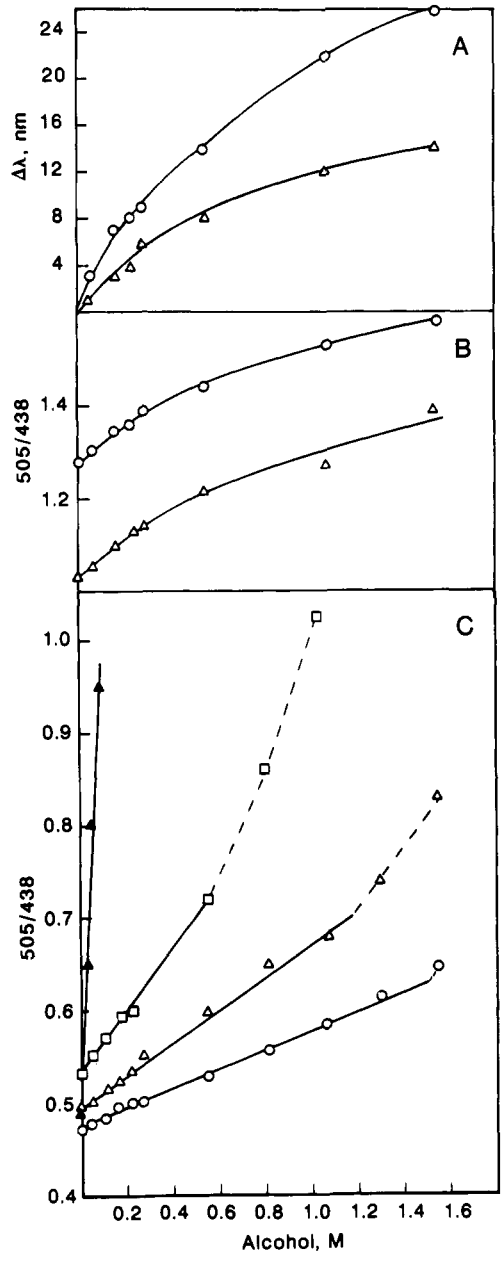

FIGURE 8: Effect of ethanol on Prodan emission maximum (panel A) and fluorescence intensity distribution (panel B) in liver microsomes (Δ) and mitochondria (O). Conditions are the same as in Figure 7. (Panel C) Effect of alkanols and membrane concentrations on fluorescence intensity distribution in synaptosomal membranes. Hexanol (0.8 nmol of Prodan/mg of protein) (Δ), ethanol (0.8 nmol of Prodan/mg of protein) (Δ), ethanol (4 nmol of Prodan/mg of protein) (□), ethanol (0.4 nmol of Proden/mg of protein) (O). Condition are as in Figure 4A, except for the synaptosomal membrane concentration.

in several biological membranes are summarized in Figure 8. Figure 8A shows the effect of increasing ethanol concentrations on the emission maximum of the green peak in microsomes and mitochondria. Figure 8B shows the effect of ethanol on the ratio of intensity of the two peaks in microsomes and mitochondria. These effects were not specific to ethanol and could be induced by other short-chain alcohols. The effect of hexanol and ethanol on the ratio of the intensity of the two peaks in synaptosomal membranes is shown in Figure 8C.

To better understand the effect of alcohols on Prodan fluorescence in biological membranes, we have attempted to determine the partition coefficient of Prodan into biological membranes in the presence and absence of ethanol. It is

Table I: Effect of Ethanol on Apparent Partition Coefficients of Prodan in Various Membranes[a]

membrane	blue peak + ethanol		green peak + ethanol		green/blue	green/blue (+ ethanol)
DOPC	3.69	2.22	7.81	7.35	2.12	3.31
microsomes	4.19	0.33	37.7	24.1	9.00	73.0
mitochondria	5.96	0.972	7.87	25.3	1.32	26.0
RBC						
25 °C	12.48	9.25	24.6	65.6	1.97	7.09
50 °C	4.26		10.1		2.37	
synaptosomes	5.30	1.69	11.2	10.9	2.11	6.45

[a] Apparent partition coefficients were estimated from the fluorescence intensity of each peak as a function of dye/membrane ratios. Conditions are the same as in Figure 4A, except for the membrane concentration that was varied from 50 μg of lipid/mL to 3 mg of lipid/mL. The partition coefficient was calculated by linear regression analysis of the plot of $1/I$ versus H_2O/lipid according to the following equation: $1/I = (1/K_p)(1/I_{max})(H_2O\ (mol)/lipid\ (mol) + 1/I_{max})$ (Sklar, 1980). K_p shown is multiplied by 10^{-5}. Ethanol when added was 0.78 M.

possible to estimate partition coefficients from the dependence of the fluorescence intensity on the dye/membrane ratio (Sklar, 1980). This analysis is fairly straightforward in regard to the intensity of the blue peak. However, the fluorescence maxima of the green peak are sufficiently close to that of Prodan in water that the two peaks are merged, and it is not always possible, at least in high dye/membrane ratios, to separate the fluorescence of the bound and free dye. Moreover, the fluorescence of the green peak appears to saturate so that the binding site may not be adequately described by a partition coefficient. Thus, the partition coefficients determined by this method for the green peak are less accurate than those determined for the blue peak. Moreover, if these two peaks represent independent binding sites (see Discussion) with independent partition coefficients, the determination of partition coefficients by this method is not strictly valid. Nevertheless, comparison of the results obtained by this method is revealing (Table I). In general, the partition coefficients calculated for the green peak in the various membranes were higher than the calculated partition coefficients of the blue peak. In liposomes, RBC plasma membrane, synaptosomes, and mitochondria, the partition coefficient of the green peak was approximately twice that of the blue peak, but in microsomes the ratio appeared to be much higher (9.0). Ethanol appeared to decrease significantly the partition coefficient of the blue peak in all membranes, while the effect on K_p of the green peak varied from slight reduction (microsomes), to no effect (DOPC, synaptosomes), to a large increase (mitochondria, red blood cells). Nevertheless in each case the K_p ratio (green/blue) was increased significantly by ethanol. The extent of increase of the partition coefficient ratio was proportional to the effect of ethanol on the intensity ratio in these various membranes. Thus, it appears that the changes observed in the intensity ratio of the peaks were not the result of selective quenching but resulted from the ethanol-induced changes in the partition coefficients of Prodan. The alcohol effect clearly differed from the temperature effect on Prodan fluorescence; temperature affected the apparent partition coefficients of the green and red peak in RBC to the same extent (Table I) while the fluorescence ratio was greatly increased (Figure 4B).

If alcohol reduces the partition coefficient of Prodan between the membrane and the medium, it is possible that the increased intensity of the green peak is partially due to the fluorescence of the free Prodan. However, because of the lower quantum efficiency of Prodan in water, the contribution of this species

is only observed at high ethanol concentration and very high dye/membrane ratios. This is demonstrated in Figure 8C, which shows the effect of ethanol on the fluorescence intensity ratio at various dye/membrane ratios. At high membrane/dye ratios the slope of the curves increased at high-ethanol concentrations. Under these conditions free Prodan contributed to the fluorescence intensity of the green peak [see also Zeng and Chong (1991)]. This conclusion was verified by measuring the fluorescence of the supernatant after precipitation of the synaptosomes by centrifugation. At low dye/membrane ratios, the supernatant fluorescence was negligible compared to the suspension fluorescence, but at high ethanol concentrations and high dye/membrane ratio the supernatant fluorescence was significant. Moreover, hexanol, which also increases the green/blue intensity ratio, did not increase the fluorescence contribution of the free dye (Figure 8C).

DISCUSSION

Effect of Solvents on Fluorescence of Prodan. Weber and Farris (1979) have previously examined the fluorescence of Prodan in different solvents. Although Prodan was designed to be a polarity-sensing probe, it was clear that other solvent properties contributed to the fluorescence characteristics. Most conspicuously was the enhancement of the red-shift in the emission peak in protic solvents, far exceeding the increments expected from the calculated orientational polarizabilities in accordance with Lippert theory (Lippert, 1957). The effects of solvents on Prodan fluorescence, as summarized in Figure 1 suggest that the tested solvents could be divided into five different groups. The spectra of Prodan in very hydrophobic solvents (paraffins) and very hydrophilic solvents (water) suggest that the limited solubility of Prodan in these solvents induces dye–dye interactions that result in low quantum yield and spectral shifts. Most other tested solvents fall into two groups: protic polar and aprotic solvents. Within each of these two groups the fluorescence emission depends on the polarity of the solvent in a predicted manner (a Lippert plot of these results also shows two separate linear curves for protic and aprotic solvents, not shown). The data suggest that the difference between polar protic and polar aprotic solvents is due to hydrogen-bond donation by protic solvents. This is apparent both from the difference in the absorption spectrum (Figure 1A), the excitation and emission spectrum (Figure 1B), and, most convincingly, by the magnitude of the spectral shifts induced by titrations of aprotic solvents with protic solvents (Figure 3). The strong dependence of the emission spectra on the protic solvent concentration in binary mixtures of solvents of identical dielectric constant indicates that this spectral shift is the result of specific solvent–dye interactions (Rapp et al., 1971). The fact that the same concentration dependence is observed with propanol (in acetone) and octanol (in dichloroethane) suggests that it is the interactions of Prodan with the hydroxyl groups of the protic solvent that lead to the spectral shifts. From these titration curves, the difference between the free energy of hydrogen bonding in alkanols and the alkanol Prodan hydrogen bond is estimated as follows: $\Delta G^\circ = -RT \ln ([\text{Prodan}-\text{propanol}]/\{[\text{Prodan}][(\text{propanol})_2]\}) = -1.38 \log (1/0.409) = 0.54$.

The strong correlation between the dielectric constant and the emission maxima in both protic and aprotic solvents (Figure 2) should provide useful information about the environment in which Prodan is embedded both in regard to the dielectric constant and the hydrogen-bonding state of Prodan.

In very viscous solvents (e.g., glycerol) Prodan fluorescence exhibited two emission maxima (not shown). The green peak fits the curve for protic polar solvents (Figure 2) and appears to represent the relaxed spectrum; the blue peak corresponds to emission from protic solvents of low dielectric and appears to represent the nonrelaxed spectrum as predicted from the two-state model (Lakowicz, 1983). When we compared the emission spectra at low and high temperatures, we observed that the main effect of increased relaxation rate is to increase the fraction of emission from the relaxed species as predicted by the two-state model.

Prodan Fluorescence in Phospholipids Bilayers and Biological Membranes. Previous studies with Prodan have demonstrated the existence of two emission peaks in suspensions of lipoproteins (Massey, 1985) and in suspensions of phospholipid bilayer vesicles (Massey, 1985; Chong, 1988). As discussed above, one possible explanation for the appearance of these spectra is the existence of a nonrelaxed state in viscous environment. This has been demonstrated previously with the fluorescent probe Patman (Lakowicz et al., 1984). Based on the temperature dependence of the emission maximum of Prodan and the bandwidth, Weber and Farris (1979) have suggested that the blue peak that appears in albumin suspension is largely due to the nonrelaxed state of the albumin–Prodan complex. The two-state relaxation model for the fluorescence of Lordan (which is similar to Prodan) in phospholipid membranes was tested by Parasassi et al. (1986) by time-resolved fluorescence, and the results gave partial support for this interpretation. However, Massey et al. (1985) interpreted the different spectra obtained from Prodan in lipoproteins and lipid vesicles at different temperatures as representing different solvent environments. It was concluded that the major factor that determines the emission maximum is accessibility of water to bound Prodan. Thus the blue peak, which is dominant at the gel phase, was attributed to the hydrophobic environment of the dye at this state, while the green peak, which is dominant at the liquid-crystalline state, was attributed to water–dye interactions. It was also shown that cholesterol affects the emission spectrum of lipoprotein-bound Prodan (Massey et al., 1985). The coexistence of the two peaks in phospholipid vesicles at intermediate temperature was attributed to the coexistence of the two phases in the transition state. It is clear, however, from Chong's (1988) work and ours that the two peaks coexist even in the liquid-crystalline state. Chong (1988) interpreted this as evidence of two binding sites of Prodan, one (blue) deeper in the membrane core and the other (green) at the membrane surface. It was demonstrated that the fluorescence intensity can be shifted from the green peak to the blue peak by the application of hydrostatic pressure, which was considered as evidence favoring this interpretation (Chong, 1988). In principle it should be possible to decide between these two contrasting interpretations of Prodan fluorescence in phospholipid vesicles by time-resolved fluorescence measurements. However, in biological membranes, which are composed of many different types of both polar and nonpolar lipids and proteins, it is very likely that different environments exist around the dye, which will most likely result in multiple fluorescence lifetimes. Indeed, the excitation spectra of Prodan in membranes are very broad and also show at least two distinct peaks, suggesting that even in the ground state at least two different species coexist. However, the emission spectrum did not show a strong dependence on the excitation wavelength (results not shown). Thus, on the basis of the available data, it is not possible to decide whether the blue peak arises from a distinct nonpolar binding site or from an unrelaxed polar environment.

Nevertheless, the position of the green peak is different in different membranes and is not affected by temperature and, thus, appears to reflect different environments. The differences between the various membranes can be related to the very different surface composition of these membranes. The microsomal membranes are mostly pure phospholipid membranes, and Prodan spectra from these membranes are the most similar to those obtained from phospholipid vesicles. Since the green peak cannot be located on the curve for aprotic solvents, we may conclude that the membrane-bound Prodan corresponding to the green peak is fully hydrogen-bonded and the corresponding dielectric constant from Figure 2 is 12. This value is the dielectric constant expected at the ester bond region of glycerophospholipids (H. Rottenberg, manuscript in preparation). Notice that even though the Prodan acyl group appears to be hydrogen-bonded with water in this site, the dielectric constant is not significantly affected because one molecule of water is not sufficient to significantly change the polarity of the environment of the membrane-bound Prodan. In mitochondria, which are similar in composition and fluidity to microsomes, there is a considerable red-shift in the maximum of the green peak. While the reason for this shift cannot be ascertained, it is possible that in these membranes, which are much richer in proteins than microsomes and appear to have higher dielectric constant than phospholipids membranes (Dilger et al., 1979), the surface dielectric constant is higher (25 according to the curve for polar protic solvent). Finally, the green peak in plasma membranes is further red-shifted up to 505 nm, in RBC membranes. Plasma membranes contain large amounts of cholesterol and sphingolipids. These lipids possess very polar groups (hydroxyls and amides), which are located at the membrane surface and can greatly increase the surface dielectric constant (the value obtained from Figure 2 is 38). Alternatively, the difference in the location of the green maxima in these membranes may relate to differences in the degree of hydration of the surface-bound dye in these membranes.

Effect of Alkanols on Prodan Fluorescence in Phospholipids and Biological Membranes. As our study shows, two distinct effects of alkanols on the fluorescence of membrane-bound Prodan can be observed. There is a shift in the fluorescence intensity from the blue to the green peak and there is a shift in the emission maximum of the green peak. The data indicate that these two effects are independent of each other. In some membranes the first effect was strong while the second was weak, while in other membranes the opposite occurred. Moreover, there was a difference in the relative potency of alkanols in regard to the two effects. Thus a concentration of hexanol, which produced a greater quenching of the blue peak than ethanol, produced a much smaller shift in the green peak emission maximum. Moreover, the changes in peak intensity can be mimicked by raising the temperature, but temperature had no effect on the position of either emission peak. It is therefore apparent that two different mechanisms are responsible for the two effects. Assuming the two sites model, the alcohol effect on the intensity ratio of the two peaks can be interpreted as transfer of dye from the hydrophobic core to the surface (Chong, 1988). Alternatively, assuming the two-state relaxation model, the alcohol effect on the intensity ratio can be interpreted as resulting from increased dipolar relaxation.

Regardless of the correct explanation of the effect of alcohols on the intensity ratio of the two peaks, the effect of alcohols on the fluorescence emission maximum of the green peak appears to be a totally independent effect. The most straightforward interpretation is that alcohols, by partitioning into the membrane surface, increase the surface dielectric constant and hence the shift. This is a direct effect which results either from the presence of the alcohol hydroxyl groups at the surface or from alcohol-induced accessibility of water to membrane-bound Prodan. Since alcohols increase Prodan solubility in water, it is possible that the apparent maxima shift is also due to fluorescence of water-soluble Prodan. This is indeed observed under conditions of high alcohol concentrations and high dye/lipid ratio [see Figure 8 and Zeng and Chong (1991)]. However, under most conditions, when the dye/membrane ratio is kept low and the alcohol concentrations are also low, the contribution of free dye to the suspension fluorescence is negligible.

The difference in the potency of the various alkanols in producing the two effects is of great interest. Although, the potency of alkanols in both effects increases with increasing chain length, the increments are much less than expected from the membrane buffer partition coefficients and are larger for the effect of alkanols on the intensity ratio than on the emission maxima. If the alkanols-induced shift of the green emission is an effect on the surface-bound Prodan, then these data suggest that short-chain alkanols bind preferentially to the membrane surface. This conclusion is compatible with both empirical observations (Pope & Dubrow, 1986) and theoretical considerations (Brasseur et al., 1985).

What can be learned regarding the interactions between ethanol and biological membranes from its effect on Prodan fluorescence? First, the magnitude of the effect should be proportional to the partition of ethanol into the membrane surface. It is believed that the partition coefficient of ethanol varies greatly between membranes and also in certain pathological conditions and that the determination of alcohol partition is of great interest (Rottenberg, 1987). It is interesting that the effect of ethanol on the emission maximum of Prodan is greatest in mitochondria, which have also yielded the highest partition coefficient of ethanol by direct measurement of ethanol binding (Rottenberg, 1987). Because of the very low partition coefficient of ethanol, it is not possible to estimate the absolute partition coefficient from the effect of ethanol on Prodan fluorescence by a simple titration with increasing membrane concentration. However, the relative partition of ethanol can be estimated from the relative effect of ethanol on Prodan fluorescence in different membrane preparations. For instance, the shift of the emission maximum of the green peak in mitochondria induced by 1.0 M ethanol is 21 nm, the highest found for all tested membranes. Direct determination of partition coefficients of radiolabeled ethanol has yielded a value of 3.6 (Rottenberg, 1987). In microsomes the shift is about half as much (11 nm), and it can be predicted that the partition coefficient of ethanol in this membrane is about half as much as that of mitochondria. In synaptic membranes the shift is even smaller: about 5 nm at 1.0 M ethanol (results not shown), suggesting a partition coefficient which is about one-fourth of that of mitochondria. This is approximately the same difference between mitochondria and synaptosomes as found by direct determination of ethanol partition (Rottenberg, 1987).

The apparent higher potency of short-chain alcohols (C_1–C_4) in their effect on the emission maxima of the green peak suggests that these alcohols bind preferentially to the membrane surface. It is interesting to note that there are several reports in which there are specific effects of short-chain alcohols on membrane processes, and these may be related to

specific effects on the membrane surface. We have recently found that alcohols stimulate Na–Ca exchange in brain mitochondria and that this effect is specific to short-chain alcohols (Rottenberg & Marbach, 1992). It is possible that the specific stimulations by short-chain alcohols of ion transport in biological membranes (Orme et al., 1988) are related to the specific effects of short-chain alcohols on the dielectric constants of the membrane surface.

Recently, it was found that in dipalmitoylphosphatidylcholine (DPPC) liposomes very high concentrations of ethanol red-shifted and increased the intensity of the green peak of Prodan. This observation, which is similar to the results reported here, was interpreted as alcohol-induced formation of an interdigitated lipid phase (Zeng & Chong, 1991). The effects of low concentrations of ethanol in biological membranes and lecithin liposomes as reported here are not related to lipid interdigitation, which does not occur in these membranes.

In summary, Prodan is a sensitive probe for the effects of ethanol (and other short-chain alcohols) on biological membranes. Of particular interest is the apparent modulation of the membrane surface dielectric constant by short-chain alcohols, which may be correlated with the effects of alcohol on Prodan fluorescence.

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