Effects of Hydrostatic Pressure on the Location of PRODAN in Lipid Bilayers and Cellular Membranes†

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ABSTRACT: The effects of hydrostatic pressure on the location of 6-propionyl-2-(dimethylamino)naphthalene (PRODAN), an environmentally sensitive fluorescent probe, in phosphatidylcholine lipid bilayers and in goldfish brain synaptic membranes have been studied by fluorescence spectroscopy over the pressure range of 0.001–2 kbar. The emission spectrum of PRODAN in all the membrane systems examined exhibits two local maxima: one centers at around 435 nm and the other at about 510 nm. The intensity ratio of these two peaks, F_{435}/F_{510}, increases as pressure increases; in the particular case of dimyristoyl-L-α-phosphatidylcholine multilamellar vesicles [DMPC(MLV)], a dramatic change in F_{435}/F_{510} appears at the lipid phase transition pressure. As pressure varies, an isoemissive point is seen in both egg yolk phosphatidylcholines and goldfish brain synaptic membranes; however, no isoemissive point is observed in DMPC(MLV). The presence of an isoemissive point is attributed to a pressure-induced relocation of PRODAN from the "polar" disposition (the 510-nm peak) to the "less polar" disposition (the 435-nm peak). The absence of an isoemissive point in the case of DMPC(MLV) is probably due to the lack of void space in the lipid matrix, as a result of tight lipid packing. Apparently, the probe relocation takes place in unsaturated systems, and PRODAN favors a more hydrophobic environment under pressure. However, on the basis of the emission spectra, PRODAN seems to remain more or less at the interfacial region over the pressure range examined. In goldfish brain synaptic membranes, the PRODAN polarization increases with pressure, giving dT/dP values of 15–20 °C kbar^{-1} for both dispositions. These values are comparable with those previously reported for lipid-involving processes, suggesting that the two dispositions are indeed lipid-associated. A model has been proposed to describe these two dispositions.

High-pressure biophysics has emerged as an active research area in recent years (Heremans, 1982; Weber & Drickamer, 1983; MacDonald, 1984; Wong, 1984). The effects of hydrostatic pressure on lipid bilayers and cellular membranes have been studied by fluorescence probe techniques as well as other physical techniques [reviewed in MacDonald (1984)]. In previous fluorometric studies, diphenylhexatriene (DPH)[1] (Chong & Weber, 1983; Chong et al., 1983, 1985b; Barkai et al., 1983; Chong & Cossins, 1984; Tamura et al., 1984), perylene (Chong et al., 1985a,b), pyrene (Flamm et al., 1982), dipyrenylpropane (Turley & Offen, 1986), and 12-(9-anthroyloxy)stearate (Chong et al., 1985b) were used to show that hydrostatic pressure either increases the molecular order of the lipids or increases the membrane viscosity, a conclusion which is recently confirmed by the studies of neutron diffraction (Braganza & Worcester, 1986a,b). It has also been demonstrated for membrane-associated (Na,K)-ATPase, using high-pressure fluorescence probe technique, that the changes in membrane order or membrane viscosity are strongly correlated with the changes in enzyme activities (Chong et al., 1985b).

Although much information has been derived from these studies, the interpretation of experimental results seems to be limited by the fact that very little is known about the pressure dependence of the probe location in lipid matrix. As an example, the rotational rate of DPH was found, by differential polarized phase fluorometry, to increase progressively with pressure at high pressure (e.g., 1 kbar) and low temperature (e.g., 4 °C); in contrast, at low pressure and high temperature, an opposite, certainly more reasonable, result was obtained (Chong et al., 1983). The rotational anomaly at high pressure has been interpreted in terms of changes in the rotational mode from "sticking" to "slipping" and in terms of changes in the probe location (Chong et al., 1983, 1985a). Either assertion would be substantiated if the pressure dependence of the probe location could be ascertained.

Employing fluorescent lipid derivatives may circumvent this problem to a considerable extent; however, using free probe has an advantage in shedding light on membrane permeability at high pressures. Chong and Weber (1983) have observed that the fluorescence intensity of DPH in membranes is only slightly changed by pressure. This observation argues against the idea that DPH is being squeezed out of the membrane at elevated pressures, since the low quantum yield of DPH in water would lead to a large drop in the total intensity. Whether at high pressures the probe remains at the same location or migrates into a more hydrophobic environment is still very much a mystery. The latter case would suggest that pressure enhances the partition of hydrophobic molecules into the interior of lipid bilayers, hence facilitating the permeability.

A lipid bilayer possesses a high polarity at the water–lipid interface and a low polarity in the hydrocarbon core. A polarity-sensitive fluorescent probe may prove useful in eluci-

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[1] Abbreviations: (Na,K)-ATPase, sodium plus potassium activated adenosinetriphosphatase; PRODAN, 6-propionyl-2-(dimethylamino)-naphthalene; DMPC, dimyristoyl-L-α-phosphatidylcholine; DPPC, dipalmitoyl-L-α-phosphatidylcholine; egg PC, egg yolk phosphatidylcholine; SUV, small unilamellar vesicle(s); MLV, multilamellar vesicle(s); Tris, tris(hydroxymethyl)aminomethane; DPH, diphenylhexatriene; Laurdan, 6-lauroyl-2-(dimethylamino)naphthalene.

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dating the pressure dependence of probe location in membrane lipids. 6-Propionyl-2-(dimethylamino)naphthalene (PRODAN) is a fluorescent probe particularly suitable for this purpose. Unlike DPH, perylene, or pyrene, PRODAN has a large dipole moment (20 D) at the lowest singlet excited state, giving a 130-nm shift in the emission maximum from 401 nm in cyclohexane to 531 nm in water (Weber & Farris, 1979).

This remarkable spectral property is being utilized in the present study to describe the pressure dependence of the location of PRODAN in membranes. In this paper, I present the results from three membrane systems. They are, in order of simplicity, the following: (1) dimyristoyl-L-α-phosphatidylcholine multilamellar vesicles [DMPC(MLV)] served as an artificial model membrane; (2) sonicated egg yolk phosphatidylcholines (egg PCs) served as a model of natural lipid membranes; (3) synaptosomal membranes isolated from goldfish brain.

Materials and Methods

PRODAN was synthesized by the method of Weber and Farris (1979). Egg PC was a gift from Dr. M. Glasser; the major fatty acids were 16:0 (27%), 18:0 (13%), 18:1 (33%), and 18:2 (18%), as determined by gas–liquid chromatography using the procedures described by Hale et al. (1977). Dimyristoyl-L-α-phosphatidylcholine (DMPC) was purchased from Sigma. Goldfish brain synaptic membrane was prepared as described by Cossins and Prosser (1982). Small unilamellar vesicles (SUV) were made by sonication (Huang, 1969) and were separated from large vesicles by centrifugation at 40 000 rpm for 1 h. Multilamellar vesicles were prepared by the method of Bangham et al. (1967). The concentration of PRODAN in buffer was calculated by using the molar absorption coefficient at 365 nm equal to 14 500 cm^2 mM^{-1} (Weber & Farris, 1979). The concentration of phospholipid was determined as inorganic phosphate following Bartlett (1959).

The membranes were doped with PRODAN by mixing a solution of PRODAN in buffer with the membrane suspensions under rigorous stirring at temperatures either above the phase transition temperatures (for DMPC and egg PC) or at 37 °C (for synaptic membranes).

Emission spectra were obtained by means of the instruments described by Jameson et al. (1976, 1977). The polarization fluorometer and the optical pressure vessel were previously described (Paladini & Weber, 1981). The procedures for the measurements of fluorescence polarization and the emission spectrum under pressure were described by Weber and coworkers (Paladini & Weber, 1981; Chong & Weber, 1983). The polarization values were corrected for both the scattered light from membranes and the pressure-induced birefringency of the windows of the pressure vessel, as described in Paladini and Weber (1981) and in Chong et al. (1983, 1985a).

Results

Emission Spectra of PRODAN in DMPC(MLV). Figure 1A shows the emission spectrum of PRODAN in DMPC(MLV) at three different pressures at 40 °C. At 1 atm, the emission spectrum shows a peak at about 510 nm and a shoulder at around 435 nm. As the pressure is raised to 1.5 kbar, the 510-nm peak decreases, and the intensity at 435 nm increases. The ratio of the fluorescence intensity at 435 nm to that at 510 nm, F_{435}/F_{510}, gives a quantitative measure of the pressure perturbation. The plot of F_{435}/F_{510} vs pressure shows a sharp change somewhere between 0.8 and 1.0 kbar at 40 °C [Figure 2 (△)]. This pressure region is close to the

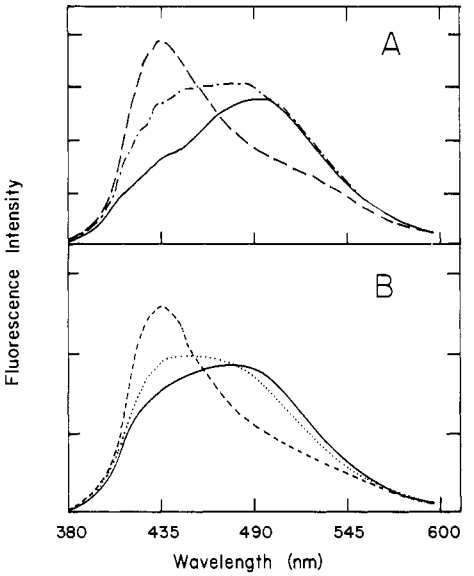

FIGURE 1: (A) Pressure effects on the corrected emission spectra of PRODAN in DMPC(MLV) at 40 °C at 1 atm (—), 0.6 kbar (–·–), and 1.5 kbar (– –). Excitation wavelength = 359 nm. The aqueous phase is 0.1 M KCl/0.01 M Tris at pH 8.0. (B) Same as (A), except that the temperature is at 32 °C and the pressures are 1 atm (—), 0.3 kbar (···), and 1.5 kbar (- - -).

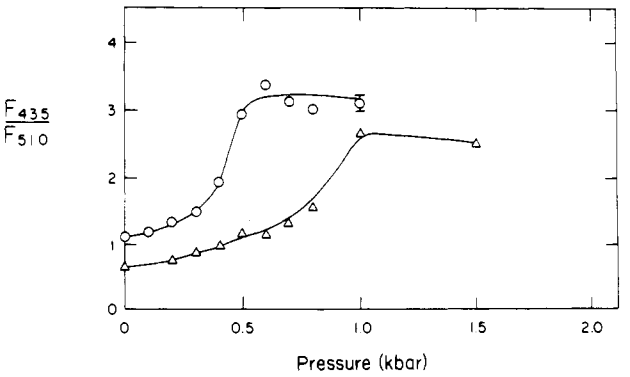

FIGURE 2: F_{435}/F_{510} as a function of pressure for PRODAN in DMPC(MLV). (○) At 32 °C; (△) at 40 °C.

phase transition pressure of DMPC(MLV) determined by DPH fluorescence polarization (0.8 kbar at 42 °C; Chong & Weber, 1983). Apparently, the spectral changes of PRODAN are related to the pressure-induced phase transition of the lipids. In this regard, F_{435}/F_{510} is a useful index of lipid phase transition.

Figure 1B shows the pressure dependence of the emission spectrum of PRODAN in DMPC(MLV) at 32 °C. The plot of F_{435}/F_{510} vs pressure at 32 °C shows a sharp transition at pressures between 0.4 and 0.6 kbar [Figure 2 (○)]. The transition pressure changes from 0.8–1.0 to 0.4–0.6 kbar as the temperature changes from 40 to 32 °C. This shift results in a dT/dP value of about 20 °C $kbar^{-1}$, which is close to the dT/dP values determined by other methods [reviewed in MacDonald (1984)].

To test whether the 510-nm peak originated from free PRODAN in the buffer, the sample was passed through a Sepharose 4B column by which the free PRODAN in buffer would be retarded with respect to PRODAN-containing vesicles. It is found that the emission maxima of the fluorophore-containing vesicles remain unchanged after the column treatment (data not shown). This result shows that the 510-nm peak does not arise from PRODAN in buffer.

Emission Spectra of PRODAN in Egg PC(SUV). The pressure dependence of the emission spectra of PRODAN in

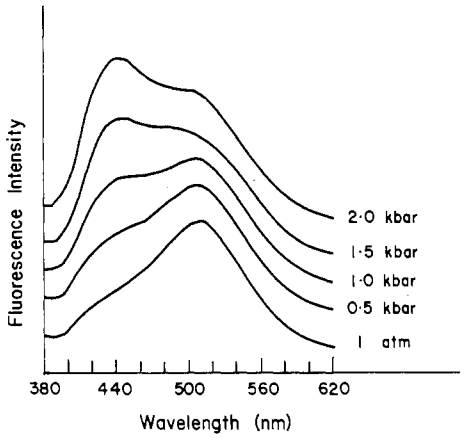

FIGURE 3: Pressure dependence of the emission spectrum of PRODAN in egg PC(SUV) at 26 °C. Excitation wavelength = 370 nm. The aqueous phase is 0.1 M KCl/0.01 M Tris buffer at pH 8.0. PRODAN/vesicle = 30. The fluorescence intensity at 380 nm is set to 0 at each given pressure.

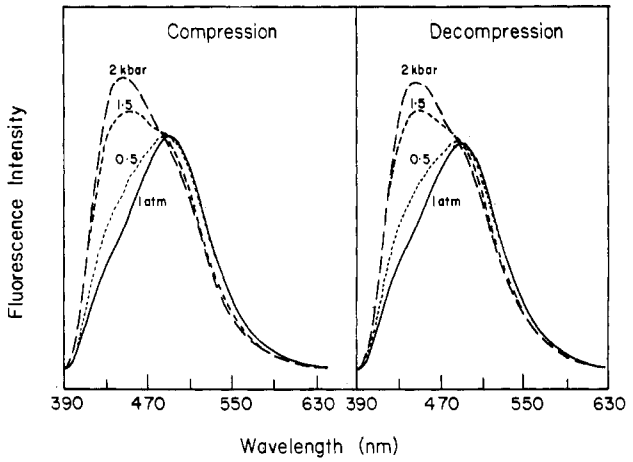

FIGURE 4: Pressure effects on the emission spectrum of PRODAN in egg PC(SUV). PRODAN/vesicle = 3. Other conditions are the same as Figure 3.

egg PC(SUV) at 26 °C is illustrated in Figure 3. The ratio of PRODAN to vesicle is estimated to be about 30, assuming that PC molecules/vesicle = 2000. The emission spectrum varies markedly with pressure. At 1 atm, the maximum of emission is at 510 nm with a noticeable shoulder at 435 nm. As the pressure increases, the intensity at 435 nm increases whereas the 510-nm peak decreases slightly. No apparent shift in the emission maximum is observed under pressure. The plot of F_{435}/F_{510} vs pressure is presented in Figure 5. At each given pressure, F_{435}/F_{510} at 16 °C is higher than that at 26 °C; however, the pressure dependence of F_{435}/F_{510} remains virtually unchanged.

To demonstrate the effect of probe concentration on the pressure dependence of the emission spectrum, a sample of PRODAN/vesicle = 3 is examined (Figure 4). Again, when pressure is applied, the 435-nm peak increases, and the 510-nm peak decreases. An isoemissive point is seen at 490 nm, and the changes in the spectrum are fully reversible after the pressure is released. In fact, when all the spectra in Figure 3 are stacked together, an isoemissive point is also seen for the sample of PRODAN/vesicle = 30. As shown in Figure 5, F_{435}/F_{510} at PRODAN/vesicle = 3 is higher than that at PRODAN/vesicle = 30; however, the general trend of the pressure perturbation is similar in both cases.

Emission Spectra and Polarization of PRODAN in Synaptic Membranes. Figure 6 shows the pressure dependence

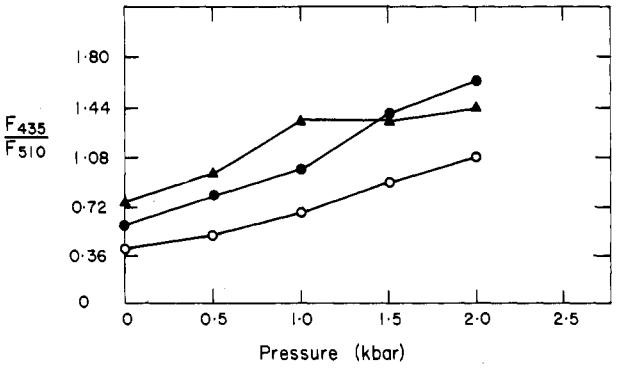

FIGURE 5: F_{435}/F_{510} as a function of pressure for PRODAN in egg PC(SUV). (●) PRODAN/vesicle = 3 at 26 °C; (○) PRODAN/vesicle = 30 at 26 °C; (▲) PRODAN/vesicle = 30 at 16 °C.

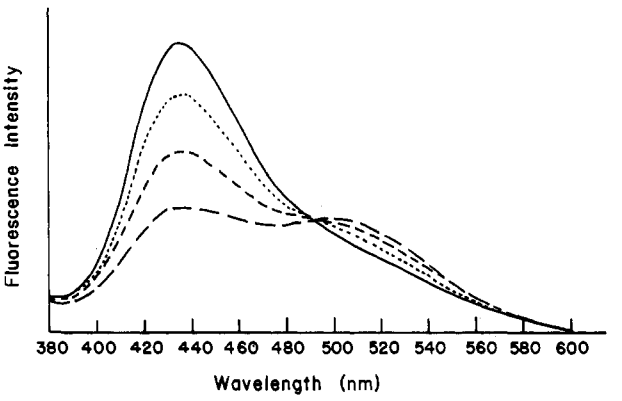

FIGURE 6: Emission spectra of PRODAN in synaptic membranes at various pressures at 32.1 °C. Excitation wavelength = 350 nm. The pressures used are 1.63 kbar (—), 1.09 kbar (···), 0.54 kbar (- - -), and 1 atm (– –).

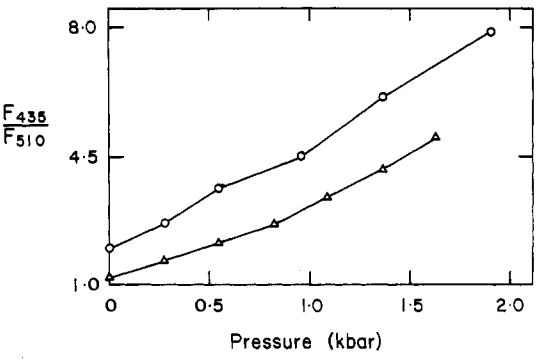

FIGURE 7: F_{435}/F_{510} of PRODAN in synaptic membranes as a function of pressure at 32.6 (△) and 19.8 °C (○).

of the emission spectrum of PRODAN in synaptic membranes at 32.6 °C. The results are similar to those shown in Figures 3 and 4. An isoemissive point is observed at 490 nm. The spectral changes are reversed when pressure is released. The plot of F_{435}/F_{510} vs pressure at two given temperatures, 32.6 °C (△) and 19.8 °C (○), is shown in Figure 7. The plots do not show a phase transition, as expected from our prior results (Chong et al., 1983).

The pressure effects on the fluorescence polarization of PRODAN in synaptic membranes are presented in Figure 8A–C. In Figure 8A, the emission was measured through a Corning 0-51 filter (0% transmittance below 350 nm), which allows for the observation of both the 435-nm peak and the 510-nm peak. The polarization increases as pressure increases, giving a dT/dP value, as estimated by the method previously described (Chong et al., 1983; Chong & Cossins, 1984), of about 16 °C kbar^{-1}. These results are similar to previous

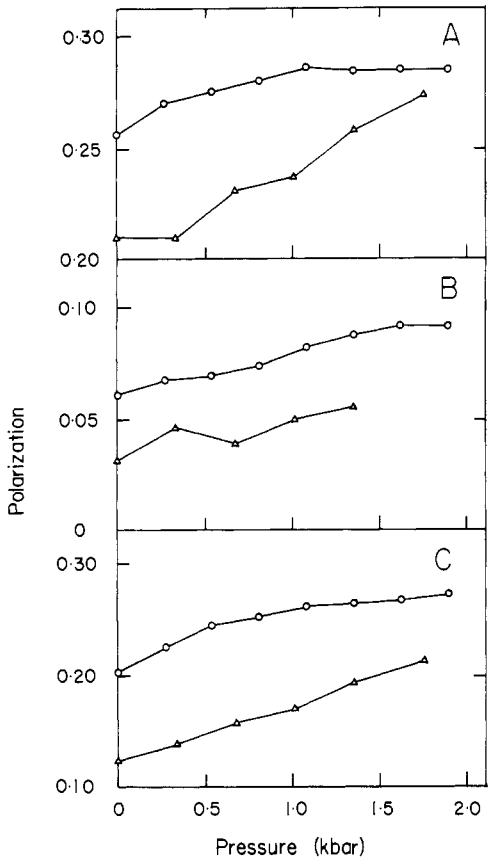

FIGURE 8: Effects of pressure on steady-state polarization of PRODAN in synaptic membranes at 7 °C (○) and 33.7 °C (△). Excitation wavelength = 345 nm. Excitation filter = 7-54. Emission filter: $NaNO_2$ plus Corning filter CS 0-51 (A), 3-68 (B), or 5-58 (C).

observations on DPH in comparable systems (Chong et al., 1983).

The polarization values shown in Figure 8B were measured through a Corning 3-68 filter (0% transmittance below 498 nm) such that only the emission from the 510-nm peak was recorded. Although in this case the polarization values are much lower than those in Figure 8A, the polarization still increases with pressure, and a similar dT/dP value, about 15 °C $kbar^{-1}$, is obtained.

In Figure 8C, the polarization was determined by using a Corning 5-58 filter (0% transmittance below 340 nm and above 482 nm), which allows the observation of the 435-nm peak only. The data give a dT/dP value of about 20 °C $kbar^{-1}$.

Discussion

The energy of the PRODAN fluorescence depends upon its interactions with the neighboring molecules. The possible interactions include hydrogen bonding and solvent dipole–fluorophore monopole interactions (Macgregor & Weber, 1981; Macgregor, 1983). PRODAN possesses a ketone carbonyl group and a tertiary amine, each of which may form hydrogen bonds with the membrane constituents. Mushayakarara et al. (1986a) have reported, using infrared spectroscopy over the pressure range of 1 atm and 35 kbar, that an increase in pressure leads to a strengthening of the hydrogen bonding between water and the ester carbonyl group of 1,2,3-triacetylglycerol. Similar pressure effects on hydrogen bonding strength have been observed in 1,2-dipalmitoylglycerol (Mushayakarara et al., 1986b). Our present study, however, is confined to <2 kbar. This level of pressure should have very little effect on the hydrogen bonding according to Mushayakarara et al. (1986a,b). It is thus unlikely that the remarkable spectral change shown in this study can be attributed to the pressure effect on the hydrogen bonding.

The spectral change, on the other hand, may result from changes in the solvent dipole–fluorophore monopole interactions. During the lifetime of the excited state, the solvent dipole will reorient around the excited fluorophore. When this process is slow, as compared to the fluorescence lifetime, the fluorescence emission emits primarily from the unrelaxed excited state (Franck–Condon excited state). When the solvent reorientation takes place in a very short time, the emission is from the completely relaxed excited state, which has an energy lower than the unrelaxed state. When the reorientation process is comparable to the fluorescence lifetime, the steady-state emission spectrum results from intermediate states between the above two extremes. The rate of solvent reorientation, however, varies with the viscosity of the medium, which can be altered by temperature as well as pressure (Nicol, 1974). Perhaps in our case, pressure decreases the rate of "solvent", which can only be loosely defined as the molecules surrounding the fluorophore, reorientation such that the fluorescence emission emits from a relatively less relaxed excited state, consequently resulting in an increase in F_{435}/F_{510}.

In fact, Parasassi et al. (1986) have clearly demonstrated, using multifrequency phase and modulation fluorometry, that 6-lauroyl-2-(dimethylamino)naphthalene, Laurdan, in dipalmitoylphosphatidylcholine (DPPC) exhibits solvent relaxation and that decreasing temperature causes changes in relaxation, thus altering the steady-state emission spectrum. Laurdan differs from PRODAN only in that Laurdan has a lauric residue instead of a propionic residue. The salient features of the temperature dependence of the Laurdan spectrum in DPPC (Parasassi et al., 1986) resemble those of the pressure dependence of PRODAN in DMPC (Figure 1). This suggests that the remarkable spectral change upon pressure perturbation (Figures 1, 3, 4, and 6) is probably a result of pressure-induced changes in solvent relaxation.

Then, what are the physical origins of the changes in the solvent relaxation? Parasassi et al. (1986) have suggested that the motion of the phospholipid head group may be responsible for the changes in the solvent relaxation. Our present study, however, strongly suggests that, in addition to local lipid motions, a pressure-induced reorientation of PRODAN in membrane may give rise to the changes in the solvent relaxation.

There are two pieces of supporting evidence for this assertion. First, an isoemissive point is observed in both the egg PC (Figure 4) and synaptic membrane case (Figure 6). The presence of an isoemissive point suggests an interexchange of populations between two species (Wehry & Rogers, 1966), in our case, two different dispositions. Second, the spectral change depends upon the ratio of PRODAN/vesicle (Figure 5). An increase of the ratio of PRODAN/vesicle from 3 to 30 is unlikely to alter the average motion of the head group of the phospholipids; the spectral alteration in response to the concentration change (Figures 4 and 5) must arise from the changes in the population between different sites.

The nature of the possible PRODAN sites can be described as follows. The maximum of emission of PRODAN and its derivatives serves as an index of environmental polarity, as previously demonstrated by Weber and Farris (1979) and by Macgregor and Weber (1986). In all the membrane systems examined, two local maxima of PRODAN emission are always observed: one at around 435 nm and the other at 510 nm. Neither of them originates from PRODAN in water, because PRODAN in water would give an emission maximum at 531

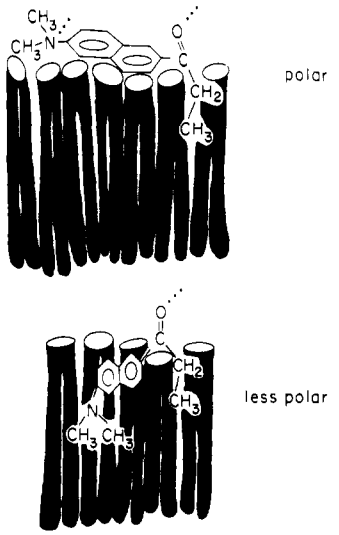

FIGURE 9: Representation of PRODAN at the "polar" and "less polar" dispositions. The dark tails represent the acyl chains of lipids; the open circles represent the lipid head group.

nm (Weber & Farris, 1979) and because the emission spectrum of PRODAN in DMPC(MLV) shows no apparent changes before and after a Sepharose 4B column (see Results). Thus, the 510-nm peak most likely arises from PRODAN residing in the water–lipid interface. This location is now referred to as the "polar" site. The pictorial model of the possible disposition of PRODAN at the polar site is shown in Figure 9. Here the two functional groups of PRODAN contact with neighboring hydrophilic residues, thus reducing the emission from 530 (water) to 510 nm.

On the other hand, if PRODAN were to go all the way into the hydrocarbon core, it would emit at 400–410 nm, according to the polarity studies of PRODAN in organic solvents (Weber & Farris, 1979). Hence, the 435-nm peak must arise from PRODAN at an environment relatively less hydrophobic than the hydrocarbon core. The absorption spectrum of PRODAN in lipid membranes has a maximum at about 350 nm (data not shown). This leads to a Stoke shift of 5583 cm^{-1} for the 435-nm emission peak. By interpolating to the data of Weber and Farris (1979), it is found that the 435-nm emission peak is related to an environment of an orientational polarizability [Δf as defined in Lippert (1957)] of 0.20. This environment is certainly more hydrophobic than that of PRODAN in water [Δf = 0.32 (Weber & Farris, 1979)]. Therefore, it can be suggested that the 435-nm peak originates from the emission of PRODAN at a "less polar" site. A putative less polar disposition of PRODAN in membranes is also presented in Figure 9. In this disposition, the van der Waals interactions and the high polarizability of the ring of PRODAN plus the decreased distance between the hydrocarbon chains and ring compete favorably with the $(CH_3)_2N$–water interaction. Yet, the 435-nm emission still requires some polarity, which is apparently given by the C=O–water interaction.

It is quite possible that there is a dynamic component involved during the pressure perturbation. Suppose that the less polar disposition is the ground state. Then, a change from the less polar to the polar disposition can take place readily at the lower viscosity (e.g., at ambient pressure) but does not change at all or changes very much less at elevated pressures. The fact that dT/dP as measured by spectral changes comes close to that measured by polarization would favor this explanation over any others.

Inability to observe an isoemissive point in the DMPC case is probably due to its tight lipid packing, which either makes the probe relocation difficult or makes the less polar disposition less well-defined. Egg PC contains a number of unsaturated acyl chains (see Materials and Methods), and goldfish brain synaptic membranes consist of a variety of unsaturated lipids and proteins (Cossins, 1977). These features make egg PC and synaptic membranes distinctly different from DMPC in the overall packing. The presence of double bonds and/or proteins would create more dead space (a space not occupied by lipids) through which an interchange of probes between the less polar site and the polar site can readily take place.

It is important to point out that the pressure-induced solvent reorientation does not necessarily produce a continuum of excited-state environments. In the present study, three situations should be considered. (1) If pressure mainly induces changes in the partition of PRODAN between two different, well-defined environments, then an isoemissive point will be seen, and no continuous changes in the emission maximum will be observed. (2) If pressure induces continuous changes in the partition of PRODAN among many different, not well-defined sites, then a continuum appears. (3) If the pressure-induced changes in the viscosity of the medium are continuous and substantial, then a continuum will be observed. The cases of PRODAN in egg PC and in synaptic membranes are close to the first situation whereas PRODAN in DMPC-(MLV) comes close to the second one. Note that the pressure-induced changes of lipid "viscosity" at the polar head group region, where PRODAN presumably resides, are likely to be small and negligible.

Although the increase in intensity at the 435-nm peak by pressure can be interpreted as a result of a relocation of PRODAN from the polar to the less polar disposition, an alternative explanation may account for the pressure effects. It is possible that pressure squeezes water out of the membrane such that the probe environment becomes less polar under pressure. As such, no relocation of PRODAN would be necessary to produce the apparent spectral changes. However, Wong and Mantsch (1987) have recently reported that no hydrogen bonding between the *sn*-1 ester carbonyl group and water is detectable by high-resolution infrared spectroscopy through all the phase transitions of DPPC, while the hydrogen bonding between water and triacetylglycerol can be readily detected (Mushayakarara et al., 1986a,b). These results argue for the idea that, if no membrane defect is present, water actually does not reside in the lipid matrix of diacylphospholipids over a wide range of pressures and temperatures. If this is indeed the case, then the alternative explanation with regard to the water movement can be safely dismissed.

In conclusion, the pressure-induced spectral changes of PRODAN in membranes appear to be a consequence of probe relocation. The physical origin of the differences in the emission spectrum lies in the structural differences among membranes. In the cases of egg PC and synaptic membranes, PRODAN tends to move from the polar disposition to the less polar disposition when pressure is elevated. This is to say PRODAN, although more or less remaining in the head-group region, favors a more hydrophobic environment at high pressures. This conclusion is consistent with the previous thought that probes like DPH are not being squeezed out of membranes under pressure (Chong & Weber, 1983) and that the pressure-induced increase in the surface density of the phospholipid causes solutes to partition more toward mid-bilayer and away from the polar head groups (Marqusee & Dill, 1986). Since a number of hydrophobic molecules such as steroids and drugs being present, either endogenously or exogenously, in biological membranes may undergo similar

relocations under pressure, the finding reported in this paper should be beneficial to future studies of membrane signal transduction, membrane surface phenomenon, and solute permeation at high pressures.

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