Dipolar Solvent Relaxation on a Nanosecond Time Scale in Ether Phospholipid Membranes As Determined by Multifrequency Phase and Modulation Fluorometry†

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ABSTRACT: The present study reports on the observation of dipolar solvent relaxation in phospholipid membranes using multifrequency phase and modulation fluorometry. We measured the time-resolved emission spectra of 6-propionyl-2-(dimethylamino)naphthalene (PRODAN) in artificial bilayer membranes of chemically defined acyl-, alkyl-, and alkenyl-substituted phospholipids at 15 °C. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 3-*O*-hexadecyl-2-oleoyl-*sn*-glycero-1-phosphocholine, or 1-*O*-hexadec-1′-enyl-2-oleoyl-*sn*-glycero-3-phosphocholine (plasmalogen) were used as matrix lipids. The chemical structures of these lipids differ only with respect to the type of linkage (carboxyl ester, ether, or enol ether bond) between glycerol and the hydrophobic chain linked to the primary hydroxyl of glycerol. At 15 °C, all the lipids are in the liquid crystalline state. PRODAN probably localizes at the hydrophobic–hydrophilic interface of the phospholipid bilayer [Chong, P. L. (1988) *Biochemistry 27*, 399–404]. We found faster solvent relaxation of PRODAN in membranes composed of the ether lipid compared to that in the ester lipid membranes. On the other hand, the fluorescence anisotropies of the label were very similar, showing that the motion of the label itself is similar in ether and carboxyl ester lipids. We conclude that the spectral differences observed for PRODAN in ether and ester lipids could be due to different dipolar relaxation of the immediate surroundings of the label, i.e., reorientation of lipid dipoles in the glycerol region and of water molecules residing therein.

Glycerophospholipids containing glycerol (enol) ether bonds are widely abundant in different types of cells. Most animal cell membranes contain significant proportions of 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerophospholipids (plasmalogens). Although increasing evidence has already been accumulated that alkenylacyl (enol ether) and diacyl phospholipids behave differently as constituents of artifical and biological membranes (Hermetter, 1988), the biological function of plasmalogens is still unknown. Therefore, it is desirable to gain additional information that could help elucidate a possible role of plasmalogens in cellular membranes.

It was the purpose of the present study to derive information on the molecular dynamics of the surface of membranes composed of ether or ester derivatives of choline glycerophospholipids. The investigated alkenylacyl-, alkylacyl-, and diacylglycerophosphocholines differ only with regard to the chemical bonding between glycerol and the hydrophobic chains linked to the primary hydroxyl of glycerol. Thus, differences observed in the molecular dynamics in the hydrophobic–hydrophilic interface of the corresponding bilayers could be attributed to the differences in the chemical structure of the constituent phospholipids.

The fluorescence label 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN)[1] is supposed to reside in the hydrophobic–hydrophilic membrane interface (Chong, 1988). Its fluorescence properties are remarkably sensitive to solvent polarity. The emission maximum of PRODAN is strongly shifted depending on the nature of the surrounding medium, e.g., from 401 nm in cyclohexane to 531 nm in water (Weber & Farris, 1979).

"Solvent effects" in phospholipid membranes and also in proteins have already been demonstrated, using various fluorescent labels such as some PRODAN analogues (LAURDAN, PATMAN) or TNS (De Toma et al., 1976; Lakowicz et al., 1983, 1984a,b; Parasassi et al., 1986). In such systems solvent relaxation occurs on a nanosecond time scale and therefore time-resolved fluorescence emission spectra are observed for the respective labeled bilayers. The observed spectral relaxation phenomena were attributed to time-dependent reorientation of the molecular domains (e.g., of phospholipids or proteins and water) surrounding the fluorescent compound.

We used multifrequency phase and modulation fluorometry (Gratton et al., 1984; Gratton & Limkemann, 1984; Lakowicz & Maliwal, 1985) to determine time-dependent emission spectra of PRODAN in ether and ester lipid membranes. The data showed that "solvent relaxation" in ether lipid bilayers is faster than in diacyl lipid membranes. Taking into account that the fluorophore is localized in the region that is different for (enol) ether and carboxyl ester phospholipid bilayers, the spectral data indicate a more polar and flexible interface of the former systems.

Experimental Procedures

Materials. The following phospholipids (Figure 1) were prepared: Isomerically pure 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was synthesized as described (Hermetter et al., 1989); 3-*O*-hexadecyl-2-oleoyl-*sn*-glycero-1-phosphocholine (AOPC; Hermetter & Paltauf, 1983) and

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[1] Abbreviations: PRODAN, 6-propionyl-2-(dimethylamino)-naphthalene; HOPC, 1-*O*-hexadec-1′-enyl-2-oleoyl-*sn*-glycero-3-phosphocholine; AOPC, 3-*O*-hexadecyl-2-oleoyl-*sn*-glycero-1-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Tris, tris(hydroxymethyl)aminomethane; POPOP, *p*-bis(5-phenyloxazol-2-yl)benzene.

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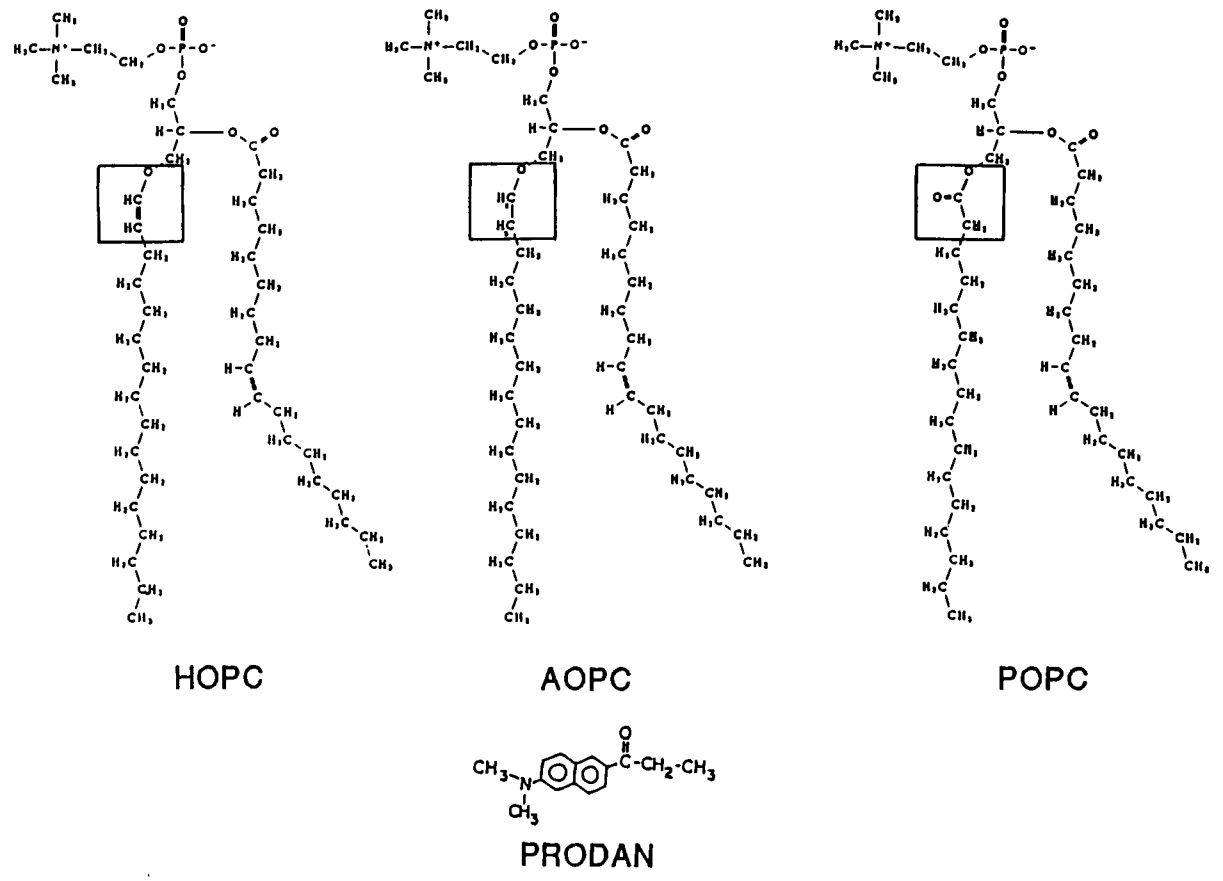

FIGURE 1: Chemical structures of glycerophosphocholine lipids and the fluorescence label. HOPC = 1-*O*-hexadec-1′-enyl-2-oleoyl-*sn*-glycero-3-phosphocholine; AOPC = 3-*O*-hexadecyl-2-oleoyl-*sn*-glycero-1-phosphocholine; POPC = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PRODAN = 6-propionyl-2-(dimethylamino)naphthalene.

1-*O*-hexadec-1′-enyl-2-oleoyl-*sn*-glycero-3-phosphocholine (HOPC, plasmalogen) were prepared as described (Hermetter & Paltauf, 1982). Phospholipids were quantitated according to Bartlett (1959). The fluorescence label 6-propionyl-2-(dimethylamino)naphthalene (PRODAN, Figure 1) was obtained from Molecular Probes, Eugene, OR; standard laboratory chemicals were purchased from Merck, Darmstadt, FRG.

Vesicle Preparations. Unilamellar vesicles composed of HOPC, AOPC, or POPC were prepared by the ethanol injection method (Batzri & Korn, 1973; Kremer et al., 1977). A chloroform–methanol (2/1, v/v) solution containing 6 μmol of phospholipid and 60 nmol of the fluorescence marker PRODAN was brought to dryness under a stream of nitrogen and residual solvent was removed under vacuum. Lipids were dissolved in 200 μL of ethanol and the resulting solution was injected with a Hamilton syringe into 2 mL of 10 mM Tris-HCl buffer (pH 7.4) at 37 °C under stirring. The vesicle suspensions had optical densities of less than 0.3 at 360 nm. The molar ratio of matrix phospholipid to the label was 100:1.

The vesicle suspensions were then dialyzed against 200 mL of Tris-HCl (pH 7.4) for about 20 h at 4 °C under protection from light. The preparations were stored not longer than three days at 4 °C.

Fluorescence Measurements. Lifetime measurements were performed by using a GREG 200 (I.S.S., La Spezia, Italy) variable-frequency cross-correlation phase and modulation fluorometer with a frequency range from 1 to 200 MHz (Gratton & Limkemann, 1984). The measurements were carried out at 15 °C. The sample temperature in the cuvette was maintained by an external thermostatic bath.

A He–Cd laser (Liconix 4207 NB) was used as an excitation light source at 325 nm. The incident light was modulated by a Pockel's cell. POPOP in ethanol (Lakowicz et al., 1981) served as a lifetime reference (τ = 1.35 ± 0.2 ns).

Phase shifts (Φ) and demodulations (m) were determined by the cross-correlation method (Spencer & Weber, 1969) at modulation frequencies of 10, 20, 40, 80, and 160 MHz for different wavelengths across the emission spectrum. Data measured at each modulation frequency were means out of at least 10 measurements.

The steady-state fluorescence emission spectra from 400 to 600 nm were recorded by using the same instrument with the modulation device off. The slit of the emission monochromator was 4 nm.

Anisotropy Measurements. Anisotropy measurements were carried out by using a Shimadzu RF-540 spectrofluorometer. Fixed excitation and emission wavelengths were 360 nm (5-nm slit) and 490 nm (10-nm slit). The anisotropies were measured at 15 °C. The results are means out of two experiments. Fluorescence anisotropies r were determined according to

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

$$G = I_{HV}/I_{HH} \quad (2)$$

I_{VV} and I_{VH} are the fluorescence intensities parallel and normal relative to the vertically polarized excitation light. I_{HV} and I_{HH} are the fluorescence intensities determined with the emission polarizer oriented vertically and horizontally when the excitation polarizer was set in the horizontal position.

Evaluation of Time-Resolved Parameters. Apparent fluorescence lifetimes were determined from the measured phase angles and demodulations, using a least squares program

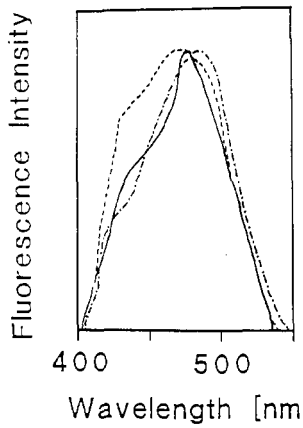

FIGURE 2: Normalized steady-state spectra of PRODAN in unilamellar phospholipid vesicles consisting of HOPC (---), AOPC (—), or POPC (---).

from I.S.S. minimizing χ^2_R as described (Jameson & Gratton, 1983).

The experimental data were analyzed by assuming a sum of lifetime exponentials with experimental errors of $\sigma(\Phi) = 0.4°$ and $\sigma(m) = 0.008$.

From the resulting apparent lifetimes and the respective fractional intensities together with the steady-state fluorescence intensities at different emission wavelengths, the time-dependent parameters such as fluorescence intensity, center of gravity, and full width at half-maximum were determined (Lakowicz, 1983; Lakowicz et al., 1984a).

Results

Steady-State Emission Spectra. The normalized steady-state spectra of PRODAN in unilamellar vesicles made of HOPC, AOPC, or POPC are shown in Figure 2.

The emission maximum of PRODAN in the plasmalogen matrix (490 nm) is shifted to longer wavelengths compared with the alkylacyl phospholipid (482 nm) and is lowest for the diacyl-substituted choline phospholipid (475 nm). In addition, the steady-state spectrum of PRODAN in POPC is somewhat broader compared with the PRODAN spectra in ether lipids.

Furthermore, a shoulder appears in all three spectra at about 430–440 nm. Chong suggests, on the basis of hydrostatic pressure effects on the localization of PRODAN in lipid bilayers, that the label probably binds to two different sites in the membrane, the shoulder being referred to as the so-called "less polar site" of the fluorophore in the lipid–water interface of the membrane (Chong, 1988).

Lifetime Measurements. From the phase-shift and demodulation values measured at different frequencies, the apparent phase $[\tau(\Phi)]$ and modulation $[\tau(m)]$ lifetimes were calculated (Lakowicz, 1983). If excited-state reactions such as dipolar solvent relaxation occur and the spectral relaxation rate is comparable to the fluorescence lifetime of the label, $\tau(\Phi)$ becomes larger than $\tau(m)$ at increasing frequencies on the long-wavelength side. This effect is called "inversion" (Lakowicz et al., 1980). It is caused by emission originating mainly from the relaxed excited state of the probe, while on the blue edge of the spectrum emission is dominated by fluorophore molecules in the unrelaxed excited state.

We actually observed such an effect with PRODAN in vesicles of the different phospholipid subclasses HOPC, AOPC, and POPC. At 480 nm and higher wavelengths larger values for $\tau(\Phi)$ compared with $\tau(m)$ were obtained for these systems. The frequency dependence of apparent phase and modulation lifetimes at 450 and 500 nm is shown in Figure 3. The values of $\tau(\Phi)$ at 160 MHz are not shown at 500 nm; the respective

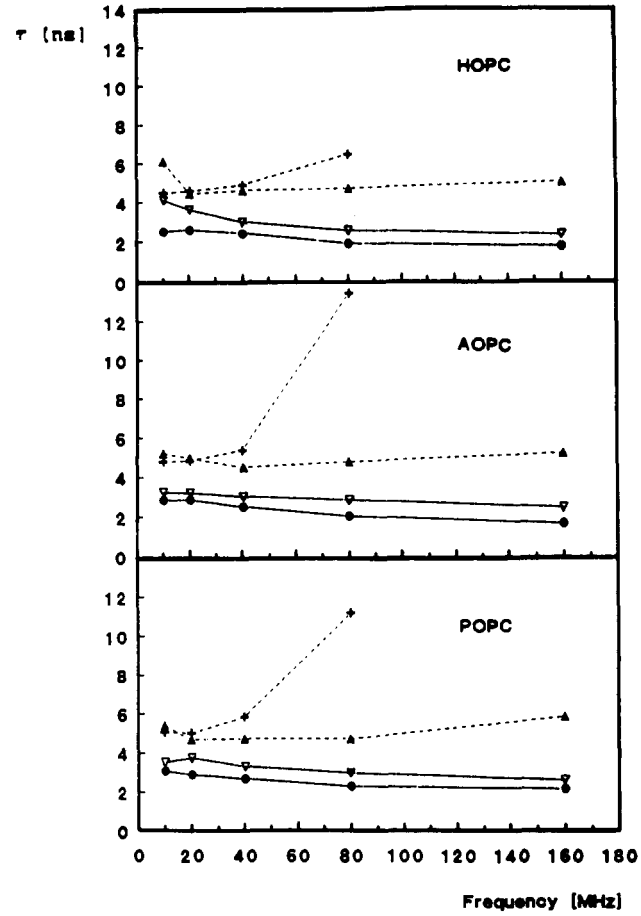

FIGURE 3: Apparent phase $[\tau(\Phi)]$ and modulation lifetimes $[\tau(m)]$ determined at various frequencies for PRODAN-labeled phospholipid vesicles at 450 nm $[\tau(\Phi) = \bullet; \tau(m) = \nabla]$ and 500 nm $[\tau(\Phi) = +; \tau(m) = \blacktriangle]$.

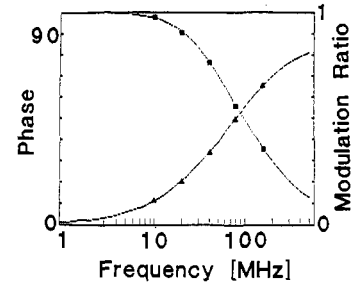

FIGURE 4: Frequency-dependent phase (▲) and modulation values (■) for PRODAN-labeled POPC vesicles at 450 nm fitted to a double-exponential decay.

phase angles exceeded 90°, thus leading to negative apparent phase lifetimes (Lakowicz et al., 1984a). The latter effect is also in agreement with a nanosecond relaxation process and provides evidence that the longer wavelength emission is at least to some extent due to an excited-state process (Lakowicz, 1983).

If the measured phase shift and demodulation data were fitted to a double-exponential decay (for PRODAN-labeled POPC vesicles, see Figure 4), the values of χ^2_R indicating the goodness of a fit were significantly lower as compared to values fitted to a monoexponential decay. On the other hand the χ^2_R could not be further decreased when a third lifetime component was added. Nevertheless, more complex decays cannot be excluded, taking into account that the χ^2_R values are significantly larger than unity. Thus, we might have additional fractional components that cannot be resolved from the observed decay.

Table I: Two-Component Fits of Phase-Shift and Demodulation Data for Different Emission Wavelengths[a]

lipid	λ (nm)	τ_1 (ns)	f_1	α_1	τ_2 (ns)	f_2	α_2	χ^2_R
HOPC	450	1.39 ± 0.11	0.52 ± 0.07	0.77	4.27 ± 0.45	0.48	0.23	2.50
	470	3.09 ± 0.26	0.87 ± 0.13	0.95	9.51 ± 7.30	0.13	0.05	7.92
	490	0.62 ± 0.24	−0.05 ± 0.01	−0.46	4.25 ± 0.07	1.05	1.46	1.12
	500	0.91 ± 0.30	−0.09 ± 0.03	−0.60	4.33 ± 0.15	1.09	1.60	3.74
	510	1.52 ± 0.27	−0.30 ± 0.12	−1.47	3.88 ± 0.22	1.30	2.47	4.31
	520	2.08 ± 1.44	−0.27 ± 0.50	−0.80	4.29 ± 0.65	1.27	1.80	4.61
AOPC	450	1.08 ± 0.11	0.31 ± 0.05	0.61	3.74 ± 0.21	0.69	0.39	1.79
	460	2.69 ± 0.24	0.77 ± 0.14	0.91	8.16 ± 3.57	0.23	0.09	6.34
	470	3.31 ± 0.32	0.84 ± 0.17	0.93	8.48 ± 5.11	0.16	0.07	3.88
	480	0.75 ± 0.44	−0.05 ± 0.03	−0.33	4.14 ± 0.12	1.05	1.33	2.84
	490	0.38 ± 0.26	−0.05 ± 0.02	−1.26	4.30 ± 0.16	1.05	2.26	6.77
	500	0.97 ± 0.95	−0.20 ± 0.16	−2.39	4.16 ± 0.40	1.20	3.39	9.72
	510	2.37 ± 0.82	−0.54 ± 0.79	−1.45	3.97 ± 0.60	1.54	2.45	6.26
	520	1.40 ± 1.18	−0.23 ± 0.27	−1.46	4.38 ± 0.57	1.23	2.46	8.50
POPC	430	1.29 ± 0.09	0.56 ± 0.06	0.80	4.09 ± 0.42	0.44	0.20	2.05
	440	0.72 ± 0.16	0.17 ± 0.04	0.45	2.95 ± 0.13	0.83	0.55	1.78
	450	1.74 ± 0.09	0.52 ± 0.05	0.74	4.69 ± 0.32	0.48	0.26	0.75
	460	1.76 ± 0.29	0.26 ± 0.10	0.47	4.27 ± 0.33	0.74	0.53	1.76
	470	3.13 ± 0.65	0.64 ± 0.52	0.76	5.40 ± 2.40	0.36	0.24	2.33
	480	0.94 ± 0.29	−0.05 ± 0.02	−0.26	4.10 ± 0.08	1.05	1.26	0.94
	490	1.41 ± 0.30	−0.16 ± 0.07	−0.68	4.15 ± 0.17	1.16	1.68	2.86
	500	1.92 ± 0.35	−0.39 ± 0.21	−1.45	4.05 ± 0.29	1.39	2.45	4.01
	510	1.63 ± 0.19	−0.29 ± 0.08	−1.44	4.23 ± 0.16	1.29	2.44	2.22
	520	1.30 ± 0.22	−0.20 ± 0.06	−1.38	4.50 ± 0.19	1.20	2.38	4.73

[a] τ_1 = apparent fluorescence lifetime; f_1 = fractional intensity; α_1 = preexponential factor; χ^2_R = reduced χ^2 with assumed errors for phase angles $\sigma(\Phi)$ and demodulations $\sigma(m)$ of 0.4° and 0.008, respectively.

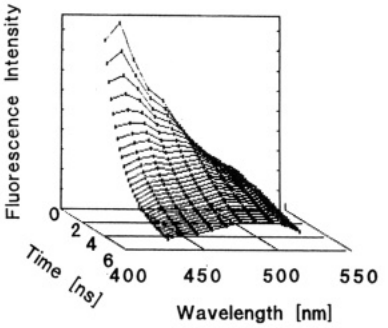

FIGURE 5: Time- and wavelength-dependent emission spectra of PRODAN-labeled POPC vesicles.

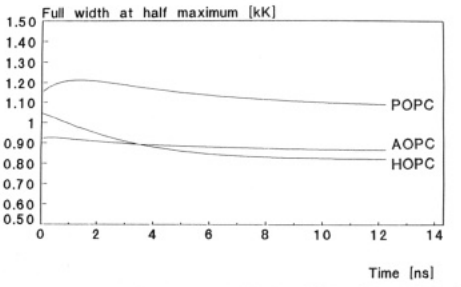

FIGURE 6: Time-resolved spectral widths of PRODAN in different matrix lipids (1 kK = 1000 cm^{-1}).

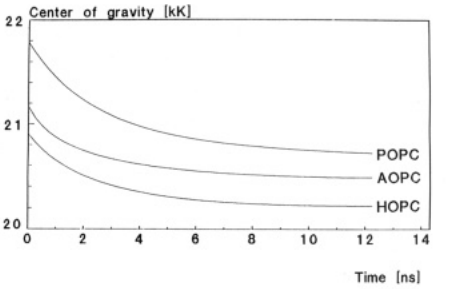

FIGURE 7: Time-resolved centers of gravity of PRODAN in different matrix lipids (1 kK = 1000 cm^{-1}).

This fitting procedure was carried out for the phase and modulation data obtained at all chosen wavelengths of the PRODAN spectra in the different phospholipid vesicles. The results of the double-exponential decay analyses of PRODAN emission in ether and carboxyl ester phospholipid vesicles are summarized in Table I.

The preexponential factors and the fractional intensities become negative for the higher wavelengths. This is further evidence that PRODAN exhibits dipolar solvent relaxation leading to the observed "multiexponential decay", which is in contrast to the emission of a mixture of directly excited fluorophores displaying ground-state heterogeneity (Gratton et al., 1984; Lakowicz et al., 1984a). Thus, the apparent lifetimes and fractional intensities as determined for PRODAN in vesicles do not have any physical meaning.

Time-Resolved Emission Spectra. The results of the double-exponential analyses shown in Table I were used to calculate the time-resolved emission spectra characterized by fluorescence intensity, full width at half-maximum, and center of gravity (Lakowicz et al., 1984a).

Figure 5 shows a three-dimensional plot of the time-resolved emission spectra of PRODAN in POPC. Two observations can be made: First, the emission maxima shift to longer wavelengths as expected for the emission from a relaxed excited state of lower energy, and, second, the relative fluorescence intensity decreases at the red edge of the spectrum. This is explained by the fact that the population in an excited state decreases with time.

In Figure 6 the time dependence of the spectral widths is illustrated for PRODAN in vesicles of HOPC, AOPC, or POPC. The half-widths determined for AOPC and HOPC are decreasing moderately as a function of time. The half-widths of the POPC spectra show a maximum at short times (approximately 1 ns) followed by a decrease at longer decay times. Such an effect is not detectable with HOPC or AOPC, probably due to the fast relaxation time of PRODAN in this system (see below).

The time-dependent red shift of the spectra is demonstrated by Figure 7, showing the centers of gravity as a function of time. First of all, PRODAN emission in POPC is observed at higher energies immediately after excitation (unrelaxed

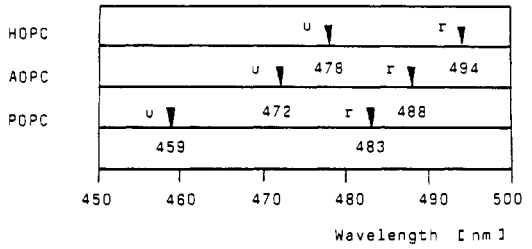

FIGURE 8: Relative positions of the emission maxima determined for the unrelaxed (u) and relaxed (r) excited states of PRODAN in vesicles of HOPC, AOPC, or POPC.

Table II: Apparent Relaxation Times and Standard Deviations of PRODAN in Different Phospholipid Vesicles According to the Continuous Model for Dipolar Solvent Relaxation

lipid	apparent relaxation times of PRODAN (ns)
HOPC	2.1 ± 0.1
AOPC	2.2 ± 0.2
POPC	3.0 ± 0.2

excited state) as well as at very long times after excitation (emission from the relaxed excited state). In addition, the total shifts from the unrelaxed to the relaxed excited state are smaller (approximately 700 cm^{-1}) for both ether lipids compared with the carboxyl ester derivative (1100 cm^{-1}, Figure 7). The larger shift observed for POPC means that a larger amount of energy is released in the relaxation process, mainly caused by a rather high energy level for the unrelaxed state of POPC (Figure 8). The respective value determined for PRODAN is in close agreement with the wavenumber shift of 1000 cm^{-1} observed for LAURDAN relaxation in fluid DPPC bilayers (Parasassi et al., 1986); the latter label differs from PRODAN only by a larger (lauroyl) acyl chain. The centers of gravity that are directly proportional to the transition energy were transformed into wavelengths (Figure 8). The obtained values, although not exactly representing the emission maxima since the spectra are not symmetrical, are compared for the ether and ester phospholipids in Figure 8. Spectra of POPC are blue-shifted relative to those of AOPC and HOPC, the latter showing emission of the relaxed and unrelaxed excited state at the longest wavelengths, respectively.

We determined apparent relaxation times, using the continuous model of Bakhshiev for the analysis of our results (Mazurenko & Bakhshiev, 1970). For 10 values of the center of gravity at different times (from Figure 7) we calculated the relaxation times according to

$$\bar{\nu}_m(t) = \bar{\nu}_\infty + (\bar{\nu}_0 - \bar{\nu}_\infty)e^{-t/\tau_s} \quad (3)$$

where $\nu_m(t)$ is the center of gravity of the emission spectrum; ν_0 and ν_∞ represent the emission maxima (in reciprocal centimeters) of the initially excited and the relaxed states, respectively; τ_s is the relaxation time.

Means out of 10 τ_s values calculated for different times t were determined for the different lipids and the results (apparent relaxation times) are shown in Table II. The relatively small standard deviations indicate that our data can be well fitted to the continuous model of solvent relaxation. The relaxation times of the ether lipids HOPC and AOPC cannot be distinguished on the basis of a statistical two-tailed t-test, whereas the mean apparent relaxation time calculated for POPC is significantly higher ($p < 0.001$).

Besides the mentioned fact that our time-dependent fluorescence data (centers of gravity) fit the continuous model, there is even more important evidence for a continuous relaxation process: the time-resolved emission spectra do not exhibit isoemissive points (Wehry & Rogers, 1964). Thus, continuous loss of energy might be taking place in the excited state, rather than the exchange between populations of two well-defined emitting species.

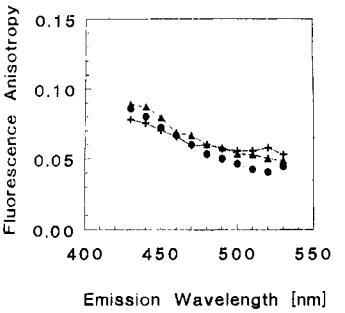

FIGURE 9: Fluorescence anisotropies of PRODAN in vesicles of HOPC (+), AOPC (▲), or POPC (●) determined at different emission wavelengths. Excitation wavelength = 360 nm. (Experimental errors were ±0.001.)

On the other hand, there is only one observation that is not in agreement with the assumed continuous model: the intermediate increase in the spectral half-widths, at least for POPC. Therefore, we cannot exclude a number of different continuous relaxation processes occurring simultaneously in the same time domain. Such effects could partly be explained by the existence of slightly different binding sites or orientations of PRODAN in the phospholipid bilayer, thus leading to different interactions with the "solvent dipoles".

However, we wish to emphasize that both models are mathematical expressions and none of them will necessarily be able to fully describe the evidently more complex processes in a complex lipid aggregate. It was suggested (Lakowicz, 1983) that time-dependent spectra of fluorophores in complex environments can be more adequately described by the very general Bakhshiev model, since transformation of the discontinuous model by including an increasing number of individual steps finally leads to the formulation of the continuous model.

Anisotropy Measurements. In order to obtain information on PRODAN mobility in bilayers of HOPC, AOPC, or POPC, we determined the fluorescence anisotropy of the solvent-sensitive label over the entire emission range. Thus, we obtained the anisotropy values at the spectral extremes corresponding to the predominantly unrelaxed and relaxed excited states and, in addition, the wavelength-dependent changes in anisotropy due to solvent effects in a given bilayer. According to Figure 9, the fluorescence anisotropies and, thus, the mobilities of PRODAN in the choline phospholipid membranes are not very different. The label anisotropy changes slightly less in HOPC membranes as compared to in AOPC and POPC bilayers on going across the emission spectrum. Nevertheless, all the anisotropy values are rather similar for the lipids under investigation.

Similar anisotropies were also found for diphenylhexatriene-labeled phospholipid probes in artificial membranes of choline plasmalogen compared with those in diacyl glycerophosphocholines, indicating similar "fluidity" of plasmalogen membranes. On the other hand, parinaroyl phospholipids exhibited somewhat higher fluorescence anisotropies in alkenylacyl than in diacyl phospholipid bilayers (Hermetter et al., unpublished results).

Discussion

PRODAN is a solvent-sensitive fluorophore that binds to phospholipid bilayer membranes in the gel or fluid state. It is supposed to be localized within the hydrophobic–hydrophilic

membrane interface (Chong, 1988). According to results obtained by IR spectroscopy, almost all of the PRODAN molecules are embedded in a fluid phospholipid bilayer at low probe to lipid ratios (4%, w/w) under atmospheric pressure (Chong, 1988). Partition of the label into the bilayer is reported to be favored in the presence of unsaturated lipids due to their lower packing density. In our study, we exclusively investigated unsaturated glycerophospholipids well above their phase transition temperature. In addition, we performed our experiments at even lower label to lipid ratios (0.3%, w/w, corresponding to 1/100 mol/mol) compared with the work cited above.

The notion that PRODAN preferably localizes in the lipid phase of aqueous vesicle suspensions containing ether or ester phospholipids is supported by our experiments, too. The quantum yield of PRODAN in water is very low (Weber & Farris, 1979). In the presence of phospholipid (HOPC, AOPC, or POPC), the fluorescence intensity of PRODAN (label to lipid ratio, 1/100 mol/mol) increases 25-fold at the emission maximum (data not shown). The fluorophore binds very tightly to the lipid bilayers as it cannot be removed by extensive dialysis (for more than 20 h). Before and after dialysis, the labeled vesicle suspensions showed the same fluorescence intensities.

2-(Dimethylamino)-6-acylnaphthalenes undergo excited-state relaxation processes on a nanosecond time scale. After excitation of PRODAN, its dipole moment increases in magnitude and the dipole orientation changes (Weber & Farris, 1979). The surrounding "solvent" dipoles reorient around the fluorophore, leading to a relaxed excited state of lower energy. This effect causes a shift of the emission spectrum from the blue to the red edge, according to the transition from the unrelaxed to the relaxed excited state of the label, respectively. PRODAN is a useful membrane probe as it does not possess any electrostatic charge and, therefore, preferential interactions with the charged phospholipid polar head groups are not very likely.

We determined the steady-state and time-resolved emission spectra of this label in unilamellar vesicles of alkenyloleoyl-GPC, alkyloleoyl-GPC, or palmitoyloleoyl-GPC in the liquid-crystalline state. The respective chemically defined phospholipids differ with respect to the chemical linkage between the hydrophobic side chain and the primary hydroxyl of the glycerol backbone, localized in the hydrophobic–hydrophilic interface of vesicle membranes made of either phospholipid. The chemical differences between the single phospholipid molecules lead to significantly altered dipolar properties of ether lipid membranes according to their dipolar surface potentials determined in lipid monolayers (Smaby et al., 1983). Surface potentials of ether lipids are much lower compared with those of diacyl glycerophospholipids. Thus, it could be expected that a fluorescent probe bound to the membrane interface region would also respond to the resultant physicochemical differences.

We observed time-resolved emission spectra in bilayers of alkenylacyl-, alkylacyl-, or diacylglycerophospholipids in the fluid state. The dipolar relaxation in such systems may be considered to be a process including reorientation of lipid and/or water dipoles around the electronically excited state of the label. Thus, dipolar solvent relaxation of PRODAN in phospholipid membranes is a rather complex process. Most of our data support a relaxation mechanism following the continuous model according to Bakhshiev (Mazurenko & Bakhshiev, 1970) implying the emission from intermediate excited states (in contrast to a two-state model assuming emission from two distinct excited states). The time-dependent spectra do not exhibit an isoemissive point and the corresponding time-dependent centers of gravity are well fitted to this model for excited-state reactions. Therefore, we used the continuous model to determine apparent relaxation times of PRODAN in vesicles of the different lipid model compounds in order to obtain information on the interfacial properties of the respective bilayers.

Apparent relaxation times were found to be significantly shorter in vesicles of alkenyl and alkyl ether lipids compared with those of the diacyl analogue ($p < 0.001$), suggesting a more flexible molecular environment of the label in bilayers of the former lipids. According to the slower relaxation time determined for PRODAN in POPC, this system shows a broader steady-state emission spectrum as compared to the label in ether lipid membranes (Figure 2). The larger spectral width is due to the contribution of a larger number of emitting species to the overall spectrum.

Faster relaxation of PRODAN in HOPC and AOPC is in agreement with ^2H NMR experiments showing that the flexibility of the α-methylene segment is much higher in the *sn*-2 chain of choline plasmalogen compared with that of phosphatidylcholine (Malthaner et al., 1987).

Furthermore, the presence of water and its influence on solvent relaxation determined for PRODAN in phospholipid bilayers may also depend on the type of linkage between glycerol and the hydrophobic chains, although the contribution of water molecules to the overall membrane surface dipole moment has remained unknown so far. Continuous fluorescence lifetime distributions of *sn*-2-(diphenylhexatrienyl)-propionyl phospholipids suggest a less steep dielectric (water) gradient across the hydrophobic–hydrophilic interface of plasmalogen bilayers (Hermetter et al., 1988). Thus, plasmalogen bilayers may be subject to more effective water penetration into the glycerol region, leading to a more polar label environment.

Such effects would also be in agreement with the steady-state emission spectra (Figure 2) and the emission spectra of the relaxed and unrelaxed excited state of PRODAN being shifted to larger wavelengths in ether lipid compared with diacyl phospholipid bilayers (Figure 8). Inspection of Figure 8 shows, in addition, that the emission spectra of PRODAN in the alkenyl ether lipid (HOPC) are even more red-shifted than in the alkyl ether compound (AOPC). This spectral phenomenon might be due to the presence of the polar enol ether double bond in HOPC penetrating more deeply into the membrane interior (Figure 1) and, thus, creating a more polar environment around the fluorophore. Rather similar emission maxima were found for PRODAN in bilayer vesicles of di-*O*-alkylglycerophosphocholines and diacyl analogues (Massey et al., 1985; Jonas et al., 1987). However, from the lowering of the quantum yields of PRODAN it was concluded that water penetrates more effectively into diether phospholipid membranes (Massey et al., 1985). Evidently, the presence of (enol) ether bonds in a phospholipid seems to have a significant impact on the molecular dynamics and possibly the water penetration into the lipid glycerol region.

The altered physical membrane properties of ether lipid bilayers come into play if the respective membranes interact with other—biologically important—water-soluble components such as proteins. For example, phospholipid transfer proteins, catalyzing the intermembrane transport of phospholipid molecules, act superficially on lipid membranes. Experiments with cytosolic phospholipid transfer proteins specific for phosphatidylinositol and phosphatidylcholine (from yeast) and

for phosphatidylcholine only (from beef liver) showed significantly enhanced transfer rates for alkenylacyl- and alkylacylglycerophosphocholines compared with those for the diacyl analogue. This result was interpreted in terms of facilitated interaction of the protein with the membrane surface (Szolderits et al., 1990).

The lipid mobilities in the hydrophobic region of alkenylacyl- and diacylglycerophosphocholine bilayers as determined from the fluorescence anisotropies of fluorescent [(diphenylhexatrienyl)propionyl and parinaroyl] phospholipids are very similar (Hermetter et al., unpublished results). Thus, motional properties in the membrane interior are not influenced significantly by the (physico)chemical properties of the glycerol region of a bilayer.

In addition, the fluorescence anisotropies of PRODAN localized in the hydrophobic–hydrophilic membrane interface are also similar in alkenyl-, alkyl-, or acyl-substituted choline phospholipids (Figure 9). Accordingly, the motion of PRODAN itself in the respective membranes is probably similar too. Therefore, we may conclude that the time-resolved emission spectra obtained with this label in membranes of the different phospholipid subclasses monitor differences in the reorientation of the lipid and/or water dipoles in the hydrophobic–hydrophilic membrane interface rather than differences in the dynamics of the label.

From neutron diffraction studies it can be concluded that the hydrophobic–hydrophilic membrane interface should be localized in the glycerol region of diacylglycerophospholipids, as the water does not penetrate beyond the fatty acid ester groups (Franks & Lieb, 1979, 1981). So far we have no direct information about the penetration of water into ether lipid bilayers. Investigation of bilayers of alkenylacyl or alkylacyl glycerophosphocholines by such a technique could provide more direct evidence whether water molecules penetrate more deeply into an ether lipid membrane, thus contributing to the observed relaxation processes.

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