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Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence

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ABSTRACT The sensitivity of Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) excitation and emission spectra to the physical state of the membrane arises from dipolar relaxation processes in the membrane region surrounding the Laurdan molecule. Experiments performed using phospholipid vesicles composed of phospholipids with different polar head groups show that this part of the molecule is not responsible for the observed effects. Also, pH titration in the range from pH 4 to 10 shows that the spectral variations are independent of the charge of the polar head. A two-state model of dipolar relaxation is used to qualitatively explain the behavior of Laurdan. It is concluded that the presence of water molecules in the phospholipid matrix are responsible for the spectral properties of Laurdan in the gel phase. In the liquid crystalline phase there is a relaxation process that we attribute to water molecules that can reorientate during the few nanoseconds of the excited state lifetime. The quantitation of lipid phases is obtained using generalized polarization which, after proper choice of excitation and emission wavelengths, satisfies a simple addition rule.

INTRODUCTION

Modifications of the composition and physical properties of the phospholipid matrix in biological membranes accompany several physiological processes (1–4). The variation of phospholipid structure and phase state is believed to affect membrane function and the partition and activity of membrane-associated proteins (5). The large compositional heterogeneity of phospholipids in natural membranes can give rise to heterogeneous physical properties in the physiological temperature range, namely the coexistence of the gel and the liquid crystalline phases (6, 7).

Different spectroscopic techniques have been used to characterize membrane structure and dynamics (8–10). There are several advantages that suggest the use of fluorescence spectroscopy, among which are the high sensitivity of the technique; the perturbing effect of the probe on membrane structure is virtually nil; the response of fluorescence parameters to the physical properties of the environment; the intrinsic time scale of the fluorescence phenomenon, typically in the range of nanoseconds which is considered the time scale of most

membrane processes; finally, the possibility of resolving spectroscopic properties arising from sample heterogeneity.

The observation and quantitation of coexisting gel and liquid crystalline phases in phospholipid bilayers have been attempted by using different fluorescent membrane probes. The decay of the popular probe 1,6-diphenyl-1,3,5-hexatriene (DPH)¹ shows little spectroscopic variation in the two phospholipid phases. Moreover, the measured DPH decay in phospholipid vesicles can be equally described by the linear superposition of the properties of the two phases or by a continuous variation of the fluorescence properties along the phase transition (11). For example, the lifetime value of DPH in dipalmitoyl-phosphatidylcholine (DPPC) gel phase vesicles is ~10.5 ns, in liquid crystalline phase vesicles it is ~7.5 ns, and at the transition midpoint it is ~9 ns. The lifetime value at the transition midpoint can arise either from the superposition of two components with equal contribution or from a unique species characterized by a lifetime value of 9 ns. Present instrumentation is incapable of distinguishing between these two possibilities, due to the relatively small changes in the lifetime values between the two phases. Neither can the larger variation of the DPH steady-state anisotropy be used to resolve and quantitate the fraction of the different phases. Following the accepted model for DPH orientational distribution in membranes, the value of the steady-state anisotropy depends on several factors. The order parameter, r/r_0 , depends on the phase state,

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¹Abbreviations used in this paper: DLPC: dilauroyl-phosphatidylcholine; DMPA: dimyristoyl-phosphatidic acid; DMPC: dimyristoyl-phosphatidylcholine; DMPE: dimyristoyl-phosphatidylethanolamine; DPPC: dipalmitoyl-phosphatidylcholine; DPH: 1,6-diphenyl-1,3,5-hexatriene; DPPG: dipalmitoyl-phosphatidylglycerol; DPPS: dipalmitoyl-phosphatidylserine; GP: generalized polarization; Laurdan: 6-dodecanoyl-2-dimethylaminonaphthalene; POPOP: 2,2'-p-phenylenebis-(5-phenyl)oxazole.

whereas the rotational rate of the molecule depends not on the phase state, but on the temperature (12–13). The lifetime value is dependent both on the phospholipid phase state and on the temperature. In principle, only by measurement of the anisotropy decay and of the lifetime can the fraction of each phase be resolved. However, in a mixture of two coexisting phases, the number of variable parameters is large and the relative fraction can be obtained only by a global analysis of measurements at several temperatures or quencher concentrations to reduce the lifetime value without affecting the rotational motion (13–14). This complexity prevents the use of DPH to quantitate coexisting phospholipid phases in most biological samples.

Large spectroscopic differences between the two phases have been observed using parinaric acid fluorescence (15–16). Nevertheless, the resolution of the two spectroscopic properties requires quite delicate time-resolved techniques and the interpretation of the measured decays is complicated by their intrinsic heterogeneity (17).

Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) is a fluorescent membrane probe that has the advantage of displaying spectral sensitivity to the phospholipid phase state. Its steady-state excitation and emission spectra can be resolved into two spectral components that correspond to the two phases (18). Laurdan has been reported to be located at the hydrophilic-hydrophobic interface of the bilayer (19–20) with the lauric acid tail anchored in the phospholipid acyl chains region. The typical time-dependent red spectral shift, observed in the liquid crystalline phase and during the phospholipid phase transition, can be attributed to dipolar relaxation phenomena, originating from the sensitivity of the probe to the polarity of its environment (21). The time evolution of the Laurdan emission spectrum has been measured to detect the coexistence of different phospholipid phases in mixed vesicles and to determine the interconversion rate between the phases, which occurs in ~30–40 ns in a mixture of (dilauroyl-phosphatidylcholine) DLPC: DPPC (1:1) at 20°C (18).

In a previous study an approach to quantitate both the relative amount of gel and liquid crystalline phases and the fluctuation between the phases has been proposed (18). In that article we suggested that the quantitation of the phases can be obtained using steady-state generalized polarization (*GP*), while phase fluctuations were measured using time-resolved techniques.

We now present a steady-state generalized polarization study of Laurdan in multilamellar vesicles composed of phospholipids with various polar head residues at different pH values. On the basis of our results, we describe a method that uses the generalized polarization to quantitate the two phases in synthetic membranes.

MATERIALS AND METHODS

DLPC, dimyristoyl-phosphatidylcholine (DMPC), dimyristoyl-phosphatidic acid (DMPA), DPPC, and dipalmitoyl-phosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Dipalmitoyl-phosphatidylserine (DPPS) was from Sigma Chemical Co. (St. Louis, MO). Laurdan was purchased from Molecular Probes, Inc. (Eugene, OR). Solvents were spectroscopic grade. Multilamellar phospholipid vesicles were prepared by mixing the appropriate amounts of stock solutions of phospholipid in chloroform and Laurdan in chloroform in a glass tube, then evaporating the solvent by nitrogen flux. The dried samples were resuspended in phosphate buffered saline solution (pH 7.4). The samples for the pH and absorption measurements were then heated above the phase transition temperature of the phospholipids and vortexed. The sample for the isosbestic point determination was instead sonicated in an ultrasonic cleaner (model 2200; Branson, Shelton, CT) for 30 min at a temperature greater than the phase transition temperature. Then the sample was frozen and allowed to thaw at room temperature. All samples were prepared in red light and used immediately after preparation.

For the experiments performed as a function of pH, the final concentrations of the probe and phospholipid were 0.3 μ M and 0.2 mM, respectively (probe:phospholipid = 1.5:1,000). The pH was adjusted in the prepared samples. For the experiment to determine an isosbestic point, the concentrations of the probe and phospholipid were 2 μ M and 1.36 mM, respectively (probe:phospholipid = 1.5:1,000). For the absorption measurements, the concentrations of the probe and phospholipid were 8 μ M and 0.4 mM, respectively (probe:phospholipid = 1:50). For the experiments performed with Laurdan in solvents, the absorbance of the samples never exceeded 0.1 at the wavelength of excitation.

Steady-state excitation and emission spectra were measured on a phase fluorometer (model GREG 200; ISS Inc., Champaign, IL) equipped with a Xenon-arc lamp, using 8 or 16 nm bandwidth and using the ISS Inc. software. The *GP* values were acquired using the ISSPC software (ISS Inc.). Some of the spectra were acquired on a photon counting spectrofluorometer (model GREG PC; ISS Inc.). Emission spectra are uncorrected for instrument response. The temperature was controlled by a circulating bath and the actual temperature was measured in the sample cuvette.

Absorption spectra were acquired using a DW 2,000 spectrophotometer (SLM/AMINCO®, Champaign, IL).

Laurdan lifetimes in phospholipid vesicles were measured using a multifrequency phase and modulation fluorometer, described by Gratton and Limkeman (22). The excitation was the 325 nm line of a He-Cd laser (model 4240N; Liconix, Sunnyvale, CA) and the emission was observed through a KV370 cutoff filter (Schott Optical Glass Co., Duryea, PA). Phase and modulation data were acquired at 10 frequencies of modulation, in the range from 2 to 150 MHz. A solution of 2,2'-p-phenylenebis(5-phenyl)oxazole (POPOP) in ethanol was used as a reference ($\tau = 1.35$ ns). Data acquisition and analysis were performed using software from ISS Inc.

RESULTS

Fluorescence excitation and emission spectra are shown in Fig. 1 for Laurdan in DPPC multilamellar vesicles at 20°C (gel phase) and 60°C (liquid crystalline phase). The spectra are pH independent from pH 4 to 10 when compared at the same temperatures. The spectra in

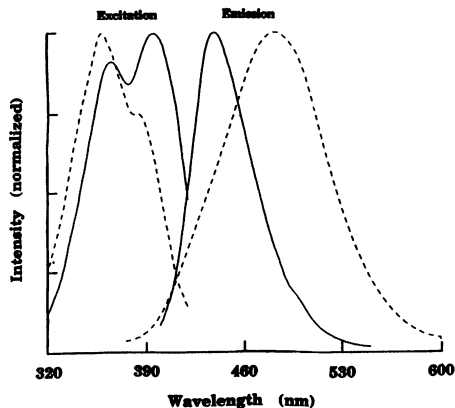


FIGURE 1 Normalized excitation and emission spectra of Laurdan in DPPC multilamellar vesicles at 20°C (—) and at 60°C (- - -). The excitation spectra were measured at an emission wavelength of 440 nm, and the emission spectra were measured at an excitation wavelength of 340 nm.

Fig. 1 show that in the liquid crystalline phase Laurdan emission is red shifted (maximum at 490 nm) with respect to the emission observed in the gel phase (maximum at 440 nm). During the phospholipid phase transition the spectra show a continuous shift to longer wavelengths with no isosbestic point (Fig. 2), in contrast with results reported by Merlo et al. (23). The Laurdan excitation spectrum in the liquid crystalline phase shows a decrease in intensity at longer wavelengths with respect to the excitation spectrum observed in the gel phase. These special characteristics of Laurdan excitation and emission spectra have been previously reported (18) and have been used to study phase coexistence and interconversion in mixed phase phospholipid vesicles.

Excitation and emission spectra were also measured

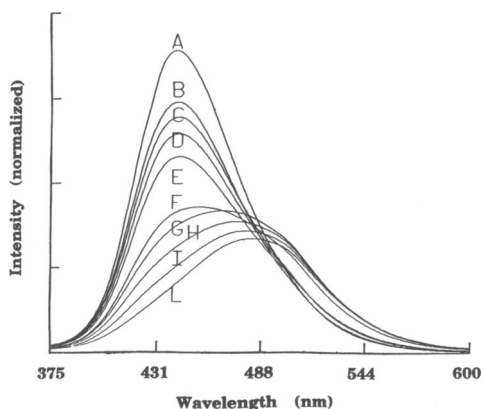


FIGURE 2 Fluorescence emission spectra for Laurdan in multilamellar DPPC vesicles at different temperatures, measured at excitation wavelength 350 nm. (A), 30°C; (B), 35°C; (C), 37°C; (D), 38°C; (E), 39°C; (F), 41°C; (G), 42°C; (H), 45°C; (I), 50°C; (L), 60°C.

for Laurdan in samples of mixed phospholipid composition: DPPS:DPPC (2:1) (molar ratio), DPPG:DPPC (2:1), and DMPA:DMPC (2:1). At physiological pH, DMPA, DPPG, and DPPS have a charge of -1 , and DPPC has no net charge. The pK 's for these phospholipids are: DPPG, $pK_{PO_4^-} = 2.9$; DPPS, $pK_{PO_4^-} < 1$, $pK_{COO^-} = 5.5$, $pK_{NH_3^+} = 11.5$; DMPA, $pK_{PO_4^-} = 4.0$, $pK_{PO_4^{2-}} = 9.5$; and, DPPC, $pK_{PO_4^-} < 1$ (24). The spectra of these samples are identical to those obtained in the pure choline vesicles, both in the gel and in the liquid crystalline phases, showing that different polar heads do not affect the characteristic Laurdan excitation and emission in phospholipids. In a previous study, spectra were reported for Laurdan in DMPC:DMPE (dimyristoyl-phosphatidylethanolamine) (1:1) which also shows that the PE head group does not affect the Laurdan spectra (25). In Fig. 3 the excitation and emission spectra for Laurdan in vesicles composed of DPPS:DPPC (2:1) at 20°C and at pH 7.4 are superimposed to the spectra of pure DPPC vesicles.

The generalized polarization (18) is given by:

$$GP = \frac{I_B - I_R}{I_B + I_R}, \quad (1)$$

where I_B and I_R are the emission intensities at the blue and red edges of the emission spectrum, respectively. We have measured the GP for phospholipid vesicles at emission wavelengths of 440 and 490 nm (or 500 nm), which correspond to the emission maxima of Laurdan in the gel and in the liquid crystalline phases, respectively. In Fig. 4 the GP for Laurdan in phospholipid vesicles of mixed composition (that is, different polar head groups) at different pH's is plotted as a function of temperature.

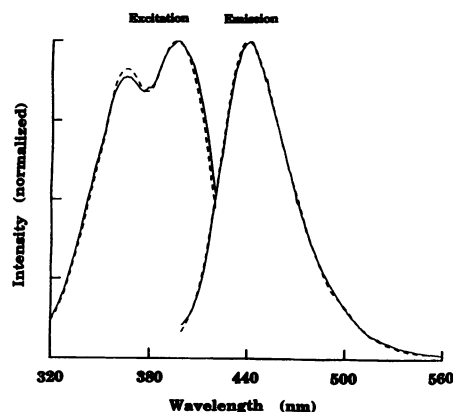


FIGURE 3 Normalized excitation and emission spectra of Laurdan in multilamellar vesicles composed of DPPS:DPPC (2:1) (—) and (- - -) at 20°C, pH 7.4. The excitation spectra were measured at an emission wavelength of 440 nm, and the emission spectra were measured at an excitation wavelength of 340 nm.

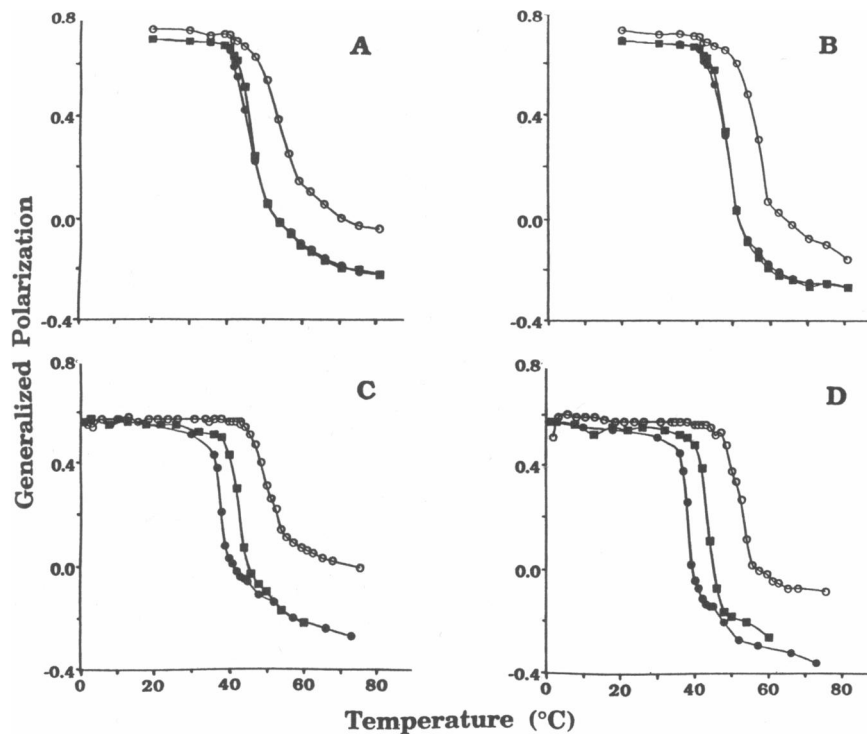


FIGURE 4 Generalized polarization values as a function of temperature for Laurdan in multilamellar vesicles of DPPS:DPPC (2:1) (*A* and *B*) and of DPPG:DPPC (2:1) (*C* and *D*) using excitation at 340 and 410 nm. For DPPS:DPPC (2:1), pH values were 4.1 (○), 7.4 (●), and 10.4 (■). For DPPG:DPPC (2:1), pH values were 3.0 (○), 7.4 (●), and 10.2 (■). The fluorescence intensity was measured at emission wavelengths of 440 and 500 nm for DPPS:DPPC (2:1), and at 440 and 490 nm for DPPG:DPPC (2:1).

Fig. 4, *A* and *B*, correspond to DPPS:DPPC (2:1) vesicles, for which the excitation wavelength is 340 nm (*A*) or 410 nm (*B*), and the emission intensity is measured at 440 and 500 nm. Fig. 4, *C* and *D*, correspond to DPPG:DPPC (2:1) vesicles, for which the excitation wavelength is 340 nm (*C*) or 410 nm (*D*), and the emission intensity is measured at 440 and 490 nm.

The GP is strongly affected by the phase state of the membrane, and as a function of temperature, shows an abrupt change at the phospholipid phase transition. The temperature shift of the phase transition region is in good agreement with the previously reported temperature shift of the phase transition as a function of pH for different polar heads (26).

At low pH, an increase in the GP is observed for both DPPS:DPPC (2:1) and DPPG:DPPC (2:1) samples at temperatures above the phospholipid phase transition, as well as for vesicles composed of DPPC and DMPA:DMPC (2:1) (not shown). This effect occurs at ~pH 4 for vesicles containing negatively charged polar heads, and at ~pH 3 for vesicles composed of neutral polar heads.

In Fig. 5 the GP as a function of pH is plotted for

Laurdan in DPPC vesicles at 60°C, exciting at 340 and 410 nm. pH has no effect on GP in the pH range 4–10, but increases below pH 4.

The GP for Laurdan in phospholipid vesicles composed of different polar head groups at pH 7.4 as a

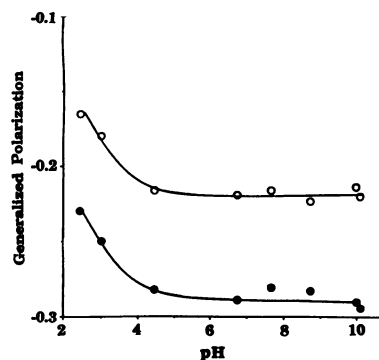


FIGURE 5 Generalized polarization values for Laurdan in DPPC multilamellar vesicles at 60°C vs. pH. Excitation at 340 nm (●) and at 410 nm (○).

function of temperature is plotted in Fig. 6. The *GP* vs. temperature shows different transition temperatures, depending on the polar head composition of the vesicles, in good agreement with data obtained using different techniques (11, 26). The phase transition temperatures (T_m) for these phospholipids are: DPPS, $T_m = 52^\circ\text{C}$; DMPA, $T_m = 50^\circ\text{C}$; DPPG, $T_m = 41.5^\circ\text{C}$; DPPC, $T_m = 41.5^\circ\text{C}$; DMPC, $T_m = 23^\circ\text{C}$; and DLPC, $T_m = -1^\circ\text{C}$ (24). When the *GP* value is calculated using fixed emission wavelengths, 440 and 490 nm, or 440 and 500 nm, a similar *GP* value is obtained for all samples in the pure gel or in the pure liquid crystalline phase, ~ 0.6 for the gel phase and -0.2 for the liquid crystalline phase. Of

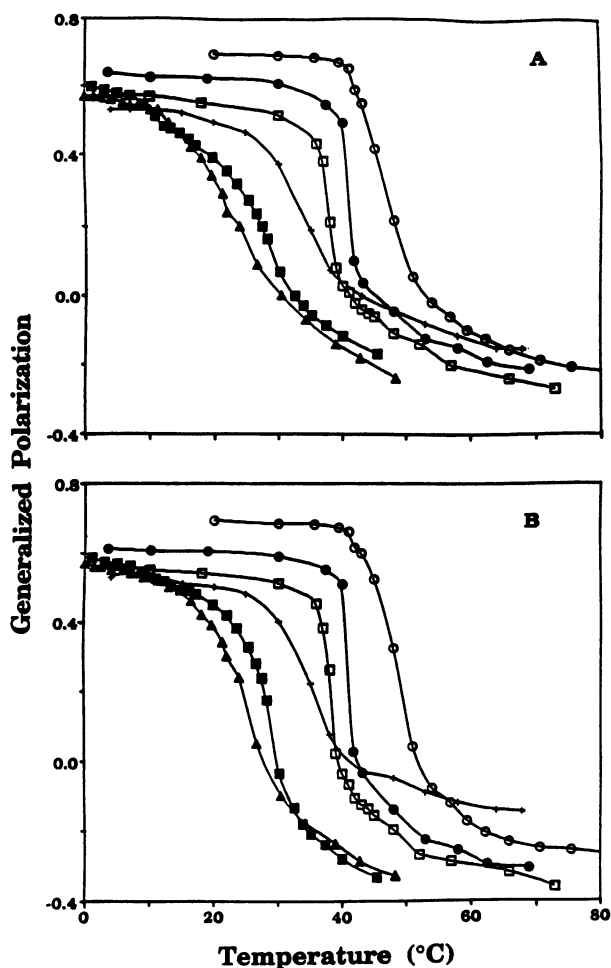


FIGURE 6 Generalized polarization values for Laurdan in phospholipid vesicles composed of different polar head groups at pH 7.4 as a function of temperature: DPPC (●); DPPS:DPPC (2:1) (○); DPPG:DPPC (2:1) (□); DMPA:DMPC (2:1) (+); DPPG:DLPC (1:1) (■); DPPC:DLPC (1:1) (▲). The *GP* was calculated using excitation wavelengths of 340 nm (A) and 410 nm (B), and measuring the fluorescence intensity at emission wavelengths of 440 and 500 nm for DPPS:DPPC (2:1), and at 440 and 490 nm for all other samples.

course, if other wavelength combinations are used, the *GP* value is different.

The spectral dispersion of the *GP* can be used to determine the coexistence of different species. A dependence of the *GP* on the excitation wavelength (measured at fixed emission wavelengths, in our case 440 and 490 nm) can signify that more than one phase is present because the excitation spectrum of Laurdan is different in the gel and liquid crystalline phases. In Fig. 7, A and B, we see that the *GP* value is independent of the excitation wavelength for the pure gel phase (low temperature). In the pure liquid crystalline phase (high temperature) the *GP* is higher exciting in the wavelength region ~ 320 nm then gradually decreases to a minimum in the wavelength region ~ 410 nm. During the phospholipid phase transition the *GP* shows a gradual increase proceeding toward longer excitation wavelengths. In Fig. 7, C and D, we see that the *GP* value is independent of the emission wavelength at low temperatures (gel phase) whereas at high temperatures it gradually increases toward longer emission wavelengths. During the phospholipid phase transition the *GP* decreases at longer emission wavelengths. These results indicate that in the mixed phases and in the liquid crystalline phase there are different molecular environments for the Laurdan molecule.

To investigate the physical origin of the *GP* change with excitation and emission wavelengths, we performed experiments in solvents of different polarity. The Laurdan excitation and emission spectra in solvents are red shifted with increasing solvent polarity (Fig. 8). For the solvent experiments we calculated the *GP* values using emission wavelengths of 387 and 503 nm, which correspond to the maximum emission of Laurdan in dodecane and in methanol, respectively. The *GP* values as a function of the solvent dielectric constant are shown in Fig. 9. The limiting *GP* values have been obtained in methanol (-1.0 at 20°C) and in dodecane ($+1.0$ at 20°C).

In Fig. 10 absorption spectra of Laurdan in DLPC and DPPC vesicles at 20°C are reported. The band at ~ 390 nm is much more intense in the DPPC sample. This result indicates that in the gel phase (DPPC at 20°C) the band with a maximum at 390 nm is enhanced in the more rigid environment.

DISCUSSION

We have shown that for Laurdan in phospholipid vesicles, the *GP* has two well defined values, corresponding to the gel phase and to the liquid crystalline phase.

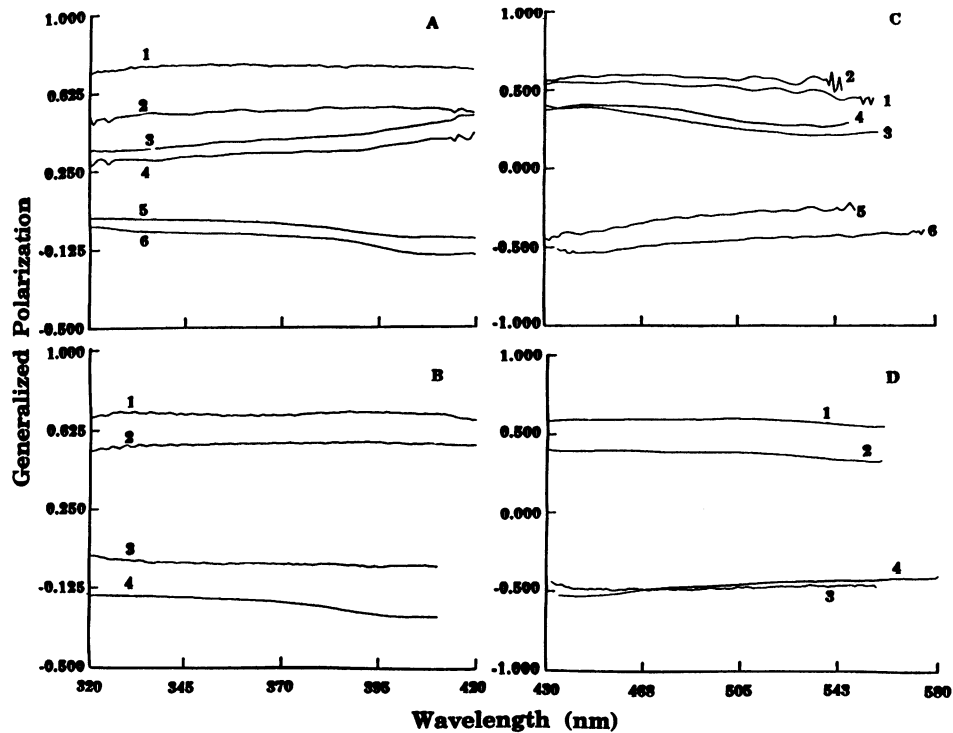


FIGURE 7 Excitation and emission generalized polarization values for Laurdan in vesicles composed of different polar head groups and at different pH's. Excitation GP of: (A) DPPS:DPPC (2:1) at pH = 4.1, 20°C (1), 54°C (3) and 75°C (5); DMPA:DMPC (2:1) at pH = 4.7, 11°C (2), 48°C (4), and 75°C (6). (B) DPPS:DPPC (2:1) at pH = 10.4, 20°C (1), and 75°C (4); DMPA:DMPC (2:1) at pH = 9.3, 7°C (2), and 68°C (3). Emission GP of: (C) DPPS:DPPC (2:1) at pH = 4.1, 20°C (1), 54°C (3), and 75°C (6); DMPA:DMPC (2:1) at pH = 4.7, 11°C (2), 43°C (4), and 75°C (5); (D) DPPS:DPPC (2:1) at pH = 10.4, 20°C (1), and 75°C (4); DMPA:DMPC (2:1) at pH = 9.3, 7°C (2), and 68°C (3). The excitation GP spectra were measured using emission wavelengths of 440 and 500 nm for DPPS:DPPC (2:1) samples and at 440 and 490 nm for DMPA:DMPC (2:1) samples. The emission GP spectra were measured using excitation wavelength of 340 and 410 nm for both samples.

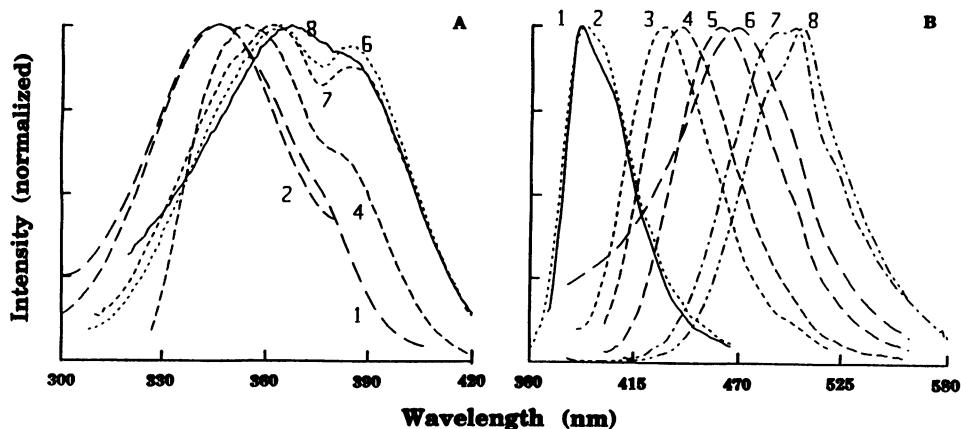


FIGURE 8 Excitation (A) and emission (B) spectra for Laurdan in various solvents at 20°C, measured at the excitation or emission wavelength maximum for each solvent. Dodecane (1), cyclohexane (2), tetrahydrofuran (3), acetone (4), dimethylsulfoxide (5), cyclohexanol (6), ethanol (7), and methanol (8).

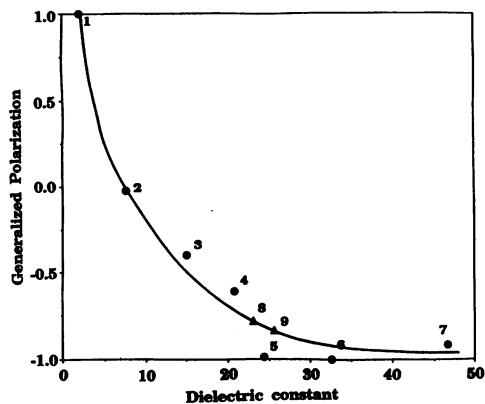


FIGURE 9 Generalized polarization values for Laurdan in solvents and in phospholipids as a function of dielectric constant. Dodecane and cyclohexane (1), tetrahydrofuran (2), cyclohexanol (3), acetone (4), ethanol (5), methanol (6), dimethylsulfoxide (7), DLPC at 20°C (8), and DPPC at 20°C (9). The *GP* was measured at emission wavelengths of 387 and 503 nm for all solvent and phospholipid samples.

By exciting the samples at 340 nm and measuring the fluorescence intensity at 440 and 490 nm, *GP* values of 0.6 and -0.2 were determined for the gel phase and liquid crystalline phase, respectively. Choosing different excitation and emission wavelengths would simply result in different numerical values for the *GP*, as illustrated by the excitation and emission *GP* spectra in Fig. 7. Once the wavelengths for excitation and emission are chosen, the *GP* does not change with polar head group or pH (in the range 4–10); it changes only with phase state. In pure isotropic solvents, it can be more convenient to choose the two emission wavelengths that correspond to the maximum emission of the bluest (dodecane) and of the reddest (methanol) Laurdan spectra (Fig. 9).

One characteristic feature of *GP* in phospholipids is its dependence on temperature and excitation wavelength. This behavior can be explained by dipolar relaxation processes (27).

Since Laurdan is a fluorescent probe sensitive to the "polarity" of the environment, the question arises as to the physical origin of the spectral shifts observed between the two phospholipid phases. The model of dipolar relaxation assumes that the spectral shift occurs after excitation and is due to the interaction between the probe excited state dipole and the surrounding solvent dipole. The red spectral shift of the fluorescence emission originates from the energy required for such an interaction. The total energy released in the relaxation process depends both on the dipole moment and on the dielectric constant of the solvent (28, 29). The relaxation

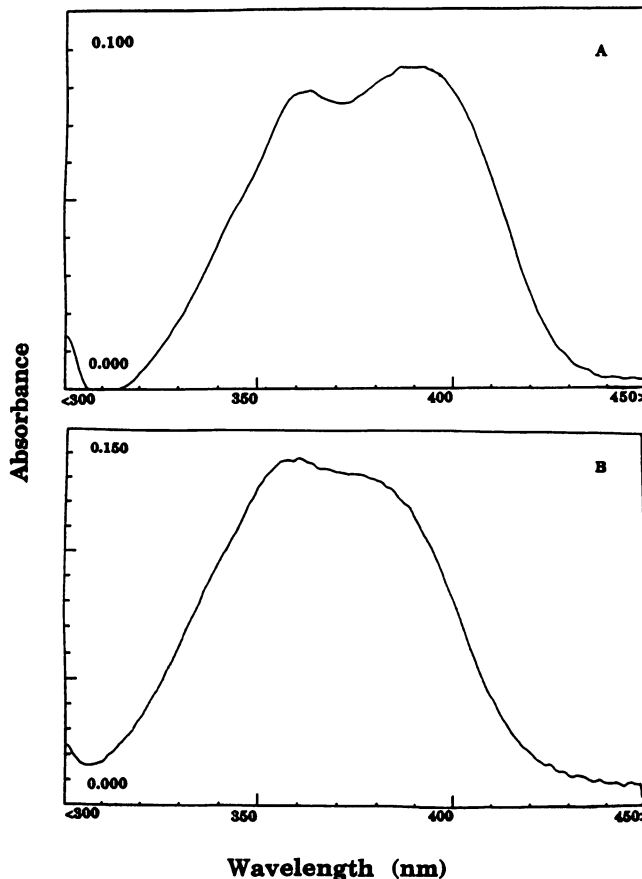


FIGURE 10 Absorption spectra for Laurdan in phospholipid vesicles at 20°C: (A) DPPC (gel phase); (B) DLPC (liquid crystalline phase).

process also requires some time to occur and is temperature and viscosity dependent. If the excited state lifetime is very long compared to the solvent dipolar relaxation time, the solvent relaxation can complete before emission and the spectral shift only reflects the dielectric property of the solvent. On the other hand, if the fluorescence lifetime is very short compared to the solvent relaxation time, only the Stoke's shift of the particular solvent will determine the position of the emission spectra, because there is no reorientation of the solvent dipoles. In general, combination of the above effects can arise and must be discussed in some detail. We will develop our discussion using a simple model, assuming that after excitation only two states are possible, the unrelaxed and the relaxed states (27). Of course this model has several limitations but is useful as a first approximation to qualitatively describe several characteristic features of dipolar relaxation. In this model, the general expression of the steady-state *GP* has been

derived (18) and is reported here:

$$\frac{B - R}{GP} - (B + R) = \frac{2\left(b_0 + \frac{k_{ba}}{k_a}\right)}{a_0 \frac{k_b}{k_a} - b_0 + \frac{k_{ab}}{k_a} - \frac{k_{ba}}{k_a}}, \quad (2)$$

where GP is the generalized polarization (Eq. 1), B and R represent the fractional emission of the unrelaxed state in the blue and red edges of the spectrum, a_0 and b_0 are the relative absorption of the unrelaxed and relaxed state at the wavelength of excitation, k_a and k_b are the intrinsic decay rates of the unrelaxed and relaxed state, and k_{ba} and k_{ab} are the forward and the backward relaxation rates. We can reasonably assume that $k_a = k_b$ and that $k_{ab} = 0$, i.e., the decay rate is independent of the relaxation process and that the back reaction rate is slow. Then Eq. 2 reduces to:

$$\frac{B - R}{GP} - (B + R) = \frac{2\left(b_0 + \frac{k_{ba}}{k_a}\right)}{a_0 - b_0 - \frac{k_{ba}}{k_a}}. \quad (3)$$

It is the ratio k_{ba}/k_a that determines the modalities of the relaxation process and the value of the GP . By inspection of Eq. 3, we can distinguish three limiting cases: (a) $k_{ba} \gg k_a$: fast relaxation or long lifetime value. The GP value depends only on the spectral emission properties of the unrelaxed state at the wavelength of excitation, i.e., on B and R , and not on the relaxation properties of the solvent. (b) $k_{ba} \ll k_a$: slow relaxation or short lifetime value. The GP value depends also on the relative absorption of both the unrelaxed and relaxed state at the wavelength of excitation. If no relaxed state is excited ($b_0 = 0$), then there should be no dependence of the GP value on the excitation wavelength because $a_0 = 1$. (c) $k_{ba} = k_a$: the GP value also depends, in a complicated fashion, on the dynamic properties of the solvent.

On the basis of the above considerations, we can discuss some of our results. In the gel phase, the dynamics of the dipolar moieties surrounding the Laurdan molecule are likely to be slow compared to the Laurdan fluorescence decay rate, and condition b should be satisfied. In the gel phase, no relaxed state is excited and the GP value should be independent of the excitation wavelength. Instead, when the relaxed and unrelaxed state coexist, i.e., in the phospholipid liquid crystalline phase, the GP should depend on the excitation wavelength. In the reported GP excitation spectra (Fig. 7) a variation is observed during the phospholipid phase transition and in the liquid crystalline phase only in the region between 350 and 410 nm, where the relaxed

state can also be excited. Similarly, the emission GP spectrum is wavelength dependent for the liquid crystalline phase and also in the temperature range where the gel and the liquid crystalline phase coexist. According to our previous considerations, using the simple two-state model, we can qualitatively explain the difference in the GP values in the liquid crystalline phase obtained using excitation at 340 nm (higher GP values) and at 410 nm (lower GP values) (Fig. 5) as arising from the relaxation process.

In the above discussion we have considered the relaxation process in single phase phospholipids, not to be confused with the existence of two phospholipid phases, nor with the possible interconversion between them. The dipolar relaxation in a given phase physically corresponds to the reorientation of the surrounding dipoles around the Laurdan excited-state dipole. Our results show that this process is relatively slow in the gel phase (condition b is satisfied) whereas it is faster, and comparable to the excited state lifetime, in the liquid crystalline phase (condition c is satisfied). We have determined that the dipolar relaxation process is independent of the type of polar head group and of pH in the range 4–10. We propose that this process is caused by the presence of water molecules in the bilayer, in the region of Laurdan location. This hypothesis is consistent with several other observations based on fluorescence and on NMR techniques (30, 31). Changes in the “static” dielectric constant between the two phospholipid phases, due to increased water penetration, are not sufficient to explain our results. We observe a relaxation process in the liquid crystalline phase with a relaxation time in the nanosecond time scale that we attribute to water molecules with restricted mobility with respect to bulk water molecules. Bulk water has an orientational relaxation time below one picosecond (32) and cannot explain the spectral shift that we observe.

The problem of phase interconversion at the transition temperature and the extra relaxation associated with this process has been previously discussed (18). To quantitate this more complicated process, time-resolved spectral information is needed.

To proceed with the quantitation of coexisting phospholipid phases, we need information on the quantum yield and the absorption and partition coefficients of Laurdan in the two phospholipid phases. The absorption spectrum of Laurdan is definitely different in the two phases (Fig. 10). In particular, the absorption at 390 nm is higher in the gel with respect to the liquid crystalline phase. However, the absorption spectrum at 340 nm is nearly identical in the two phases and no photoselection should occur using excitation at 340 nm. The partition coefficient between the two phases can be different. Some preliminary results (subject to a manuscript in

preparation, Parasassi et al.) show that the differences are relatively small and can be explained as due to the variation of the interconversion rate between the phospholipid phases in systems in which there is phase coexistence.

There are further comments in the spectroscopic properties of Laurdan in the two phospholipid phases. The absorption spectra of Laurdan in phospholipid vesicles show two distinct peaks at ~ 370 and 390 nm (Fig. 10). In the excitation spectra (Fig. 1) this spectral feature is even more evident. Surprisingly, the part of the spectrum which predominates in the gel phase, which is assumed to be "less polar" with respect to the liquid crystalline phase, is the red part, with a larger contribution in the region around 390 nm. This is in contrast to the spectral shifts reported in Fig. 8A where the most polar solvents shift the excitation spectrum toward the red. The splitting of the excitation spectrum into two bands is not unique to phospholipids because in solvents we also observe the enhancement of the intensity at higher wavelengths. However, in solvents there is a direct correlation between "polarity" and spectral position. Furthermore, there is a correlation between the appearance of the band at 390 nm and the enhancement of the quantum yield. The more pronounced is this feature, the larger is the quantum yield and, of course, the longer is the lifetime value. For example, very short decay times are observed in cyclohexane (~ 0.2 ns) whereas a longer lifetime value is observed in methanol and ethanol (~ 3.5 ns). Correspondingly, we have found that the average lifetime of Laurdan in the gel phase is 6.9 ns whereas in the liquid crystalline phase it is 4.3 ns. It appears that in some solvents there is a stabilization in the ground state of a dipole transition at lower energy, from which most of the fluorescence arises. The excitation *GP* is relatively flat in the spectral region between 320 to 420 nm, excluding that this spectral feature is due to the coexistence of the L_α and L_β transitions. In the Laurdan molecule the emission comes from a state in which there is a charge separation at the carbonyl and the amino residue (28, 29). The stabilization of this transition gives a very large dipole moment and a relatively large quantum yield. Polar solvents, especially alcohols, seem to stabilize this transition. In phospholipids this stabilization can arise from the difference in water penetration and mobility between the two phases. Again, this effect cannot be due to interactions with the polar phospholipid residues, since the effect is not dependent on different polar heads nor on pH in the range 4–10.

With respect to the failure of observing an isoemissive point in our experiment, we must consider that there are two different physical processes in our membrane system. One process corresponds to the reorientation of

the "solvent" dipole around the excited state dipole of Laurdan. This multistep process causes a continuous spectral shift and is temperature dependent. A different process is the gel to liquid crystalline phase transition, which is of course temperature dependent. Because Laurdan has two different spectra in the two phases, this two-state process should give rise to an isoemissive point. However, the change in temperature also causes a spectral shift in the pure liquid crystalline phase due to the relaxation process. The combination of these two processes prevents observation of an isoemissive point. This is not a general rule and under different circumstances in which there is only one process, an isoemissive point can be observed (19).

In experiments by Chong (19), two different locations of Prodan at the polar head region are proposed to explain the different spectral characteristics of the probe in the gel and liquid crystalline phases. Our pH experiments and the use of different polar heads seem to rule out the probe relocation mechanism because the final state is independent of pH in the range 4–10 and of the solvation and type of polar head. Furthermore, Laurdan is located deeper in the membrane due to the anchoring effect of the lauroyl tail (20). The conclusions of Chong from the Prodan experiments are not necessarily applicable to Laurdan.

In conclusion, the simple two-state model can qualitatively explain most of the observed features of Laurdan *GP*. The penetration of water molecules and their interaction with the excited state dipole are of relevance in understanding the different spectroscopic properties of Laurdan observed in the two phases. In the liquid crystalline phase the dynamics of water molecules surrounding Laurdan must occur in the nanosecond time-scale, providing evidence that these water molecules have different dynamic properties than the bulk water. However, there are some open problems in regard to the interpretation of the excitation and absorption spectra of Laurdan in the region between 320 and 420 nm. Two different bands are clearly identifiable in the spectra. The larger contribution of the gel phase to the red part of the spectrum is unusual. Incomplete understanding of the details of the photophysics of Laurdan does not preclude its use for the quantitative determination of the coexisting gel and liquid crystalline phases in a mixed bilayer.

We have shown that the *GP* value of Laurdan in different phospholipid vesicles has a well defined value for the gel phase ($GP = 0.6$) and for the liquid crystalline phase ($GP = -0.2$) once excitation and emission conditions are chosen. Given the addition rule for generalized polarization (18), the determination of the relative fraction of the gel and liquid crystalline phases can be achieved by a steady-state measurement at two

emission wavelengths. This is in contrast with the ratio-metric methods used with the popular calcium or pH-sensitive probes. The ratiometric methods provide a concentration independent measurement, but the ratio of the intensities at two different wavelengths does not satisfy the simple addition rule of the GP. The addition rule for the GP provides a method for the determination of the concentration of two different species, without an ad hoc calibration curve. The GP is a relatively simple quantity to measure in comparison to the classical polarization, requiring only two intensity measurements, and can also be applied in a simple manner to flow cytometry and to fluorescence microscopy.

The addition rule can be expressed in the following way

$$GP = \frac{xGP_g S_g + (1-x)GP_l S_l}{xS_g + (1-x)S_l}, \quad (4)$$

where GP_g and GP_l are the GP of the pure gel and pure liquid crystalline phases, respectively. S_g is the sum of the intensities $I_B + I_R$ for the gel phase, and S_l is the same but for the liquid crystalline phase. x is the relative fraction of gel phase. If we use emission wavelengths such as $S_g = S_l$, then Eq. 4 will take the simple form

$$GP = xGP_g + (1-x)GP_l. \quad (5)$$

This form is particularly attractive and it is satisfied using emission wavelengths of 440 and 490 nm and can be used to directly quantitate lipid phases.

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