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Influence of Cholesterol on Phospholipid Bilayers Phase Domains as Detected by Laurdan Fluorescence

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ABSTRACT Coexisting gel and liquid-crystalline phospholipid phase domains can be observed in synthetic phospholipid vesicles during the transition from one phase to the other and, in vesicles of mixed phospholipids, at intermediate temperatures between the transitions of the different phospholipids. The presence of cholesterol perturbs the dynamic properties of both phases to such an extent as to prevent the detection of coexisting phases. 6-Lauroyl-2-dimethylaminonaphthalene (Laurdan) fluorescence offers the unique advantage of well resolvable spectral parameters in the two phospholipid phases that can be used for the detection and quantitation of coexisting gel and liquid-crystalline domains. From Laurdan fluorescence excitation and emission spectra, the generalized polarization spectra and values were calculated. By the generalized polarization phospholipid phase domain coexistence can be detected, and each phase can be quantitated. In the same phospholipid vesicles where without cholesterol domain coexistence can be detected, above 15 mol % and, remarkably, at physiological cholesterol concentrations, ≥30 mol %, no separate Laurdan fluorescence signals characteristic of distinct domains can be observed. Consequences of our results on the possible size and dynamics of phospholipid phase domains and their biological relevance are discussed.

INTRODUCTION

Together with phospholipids, cholesterol is ubiquitous in animal cell membranes and is one of the major modifiers of the phospholipid membrane structure and dynamics. In most biological membranes cholesterol concentration is relatively high, generally ≥30 mol %. Phospholipids in biological membranes have a complex composition, including differences in length and in unsaturation of their acyl residues and in the type of polar heads. These chemical differences cause different dynamic properties and, in synthetic phospholipid membranes, they were shown to favor the appearance of segregated coexisting domains having different phase states and dynamical properties, close to the pure gel and liquid-crystalline phases (Shimshick and McConnel, 1973; Parasassi et al., 1984; Parasassi et al., 1993). The gel phase domain is composed of the phospholipid with higher transition temperature (T_m) in the presence of the phospholipid with lower T_m at concentration ≤30 mol %. The liquid-crystalline phase domain has a complementary composition. Thus, the properties of the two phase domains are close to the pure phases, modified by the presence of the other phospholipid, but still resolvable (Parasassi et al., 1993). Important questions remain about the influence of cholesterol on the dynamic properties and on the coexistence of phospholipid phases. Cholesterol effect on phospholipid bilayers has been studied by a variety of spectroscopic techniques, including nuclear magnetic resonance (NMR) (Vist and Davis, 1990; Sankaram and Thompson, 1990a), electron spin resonance (ESR) (Subczynski et al., 1990; Sankaram and Thompson, 1990b), calorimetry (Vist and Davis, 1990; Tampé et al., 1991; Keough et al., 1989), dilatometry (Melchior et al., 1980), small angle neutron scattering (Mortensen et al., 1988), and fluorescence (van Langen et al., 1989; Schroeder et al., 1991; Ben-Yasar and Barenholz, 1989; Nemecz and Shroeder, 1988). Abrupt variations of several structural and dynamical parameters have been found at critical cholesterol concentrations. These variations have been interpreted as arising from specific complexes between lipids and cholesterol in the molar ratios 3:1, 2:1, and 1:1. Ipsen et al. (1987) proposed an alternative and more satisfactory interpretation. Their model does not require specific complexes between phospholipids and cholesterol. The important ingredient of this model is the different interaction free energy between all possible pair combinations of chain-ordered, chain-disordered, and cholesterol. Phase diagrams of cholesterol in dimyristoylphosphatidylcholine (DMPC) (Tampé et al., 1991), in deuterated dipalmitoylphosphatidylcholine (DPPC) vesicles (Vist and Davis, 1990), and partial phase diagrams in DPPC and sphingomyelin (Sankaram and Thompson, 1990b) have been constructed, based on NMR, ESR, differential scanning calorimetry, fluorescence, and theoretical studies as shown by Ipsen et al. (1987). As a first approximation, at concentrations above 6 mol %, cholesterol has a disordering effect on the gel phase and an ordering effect on the liquid-crystalline phase (Vist and Davis, 1990). Specifically, in the gel phase, lateral diffusion and axial rotation are increased by cholesterol, while in the liquid-crystalline phase axial rotational motion is decreased (Sankaram and Thompson, 1990a, Rubenstein et al., 1979). Above a cholesterol concentration of 30 mol %, in the liquid-crystalline phase, lateral diffusion is decreased (Ipsen et al., 1987; Mouritsen, 1991). From the phase diagrams, apart

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from the pure gel and liquid-crystalline phases observed in the absence of cholesterol, four main regions have been classified by Vist and Davis (1990): (*a*) at low temperatures and above 6 mol % cholesterol, the coexistence of gel and liquid-ordered phases can be observed; (*b*) at high temperatures and at low cholesterol concentrations, the liquid-disordered phase is observed; (*c*) at high temperatures and at high cholesterol concentrations, the liquid-ordered phase is observed; (*d*) at high temperatures and intermediate cholesterol concentrations, the coexistence of liquid-ordered and liquid-disordered phases occurs. Following the above classification, the coexistence of gel and liquid-crystalline phases can only be observed at cholesterol concentrations below 6 mol % in a narrow temperature interval, about 1°C wide (Vist and Davis, 1990; Tampé et al., 1991) corresponding to the transition temperature of the phospholipid.

The major feature derived from the above results is that because of the combined effects on the lateral diffusion and on the axial rotational motion of phospholipids, and because of the appearance of the new liquid-disordered and liquid-ordered phases, the transition of phospholipids from the gel to the liquid-crystalline phase progressively disappears by increasing cholesterol concentration. As a result, no calorimetric transition can be detected in phospholipids at cholesterol concentration >17 mol % (Vist and Davis, 1990; Keough et al, 1989). At cholesterol concentrations above 6 mol %, the phospholipid pretransition is suppressed (Vist and Davis, 1990).

The regions in which two phases coexist have been carefully analyzed by a number of techniques. The basic idea is that in the region of phase coexistence, two separate signals indicating two sets of different spectroscopic properties should be detected. In their NMR study, Vist and Davis (1990) proved that it is possible to resolve the NMR spectra of DPPC at various cholesterol concentrations using the linear superposition of two components, one characteristic of the gel phase with low cholesterol concentration and one characteristic of the liquid-crystalline phase formed at high cholesterol concentration. However, this decomposition can be performed only at temperatures below the major phase transition of DPPC but not at high temperatures. The reason for this failure is attributed to a rapid mixing (in the NMR characteristic time scale) of the signals from the two phases at high temperatures. A similar conclusion was reached by Sankaram and Thompson (1990b) using ESR in various phospholipids and sphingomyelin. Also in this case there is not direct observation of two separate signals characteristic of two distinct phases. An average signal is instead observed. The failure to observe two separate spectroscopic properties was again attributed to rapid mixing—rapid as compared to the ESR characteristic time scale. This characteristic rate is on the order of 5×10^7 s^{-1}.

In principle, fluorescence spectroscopy can explore a much faster rate, in the range of 10^8-10^9 s^{-1}. Therefore, fluorescence spectroscopy offers the opportunity of verifying the rapid mixing hypothesis as the reason for the failure of the observation of distinct spectroscopic properties where the coexistence of different dynamical properties is expected. However, the difficulty of applying fluorescence spectroscopy to this problem resides in finding a fluorescent probe that displays characteristic distinct properties in the two phases. In the present study we used the spectral sensitivity of the fluorescent probe 2-dimethylamino-6-lauroylnaphthalene (Laurdan) to investigate the influence of cholesterol on the structure and dynamics of the two phases of phospholipid bilayers, with the specific purpose of further exploring the rapid mixing hypothesis.

LAURDAN FLUORESCENCE PROPERTIES

In this section we review the fluorescence properties of Laurdan that are relevant to the present study of domain coexistence and dynamics. We have shown that the fluorescence properties of Laurdan are extremely sensitive to the polarity of the environment of its fluorescent moiety (Parasassi et al., 1991). Laurdan emission spectrum is bluer in apolar solvents. In addition to the relatively small emission shift due to changes in polarity, in phospholipid vesicles there is a 50-nm emission red spectral shift of Laurdan fluorescence when passing from the gel to the liquid-crystalline phase (Parasassi et al., 1990). This large spectral shift is due to dipolar relaxation processes that occur in the liquid-crystalline phase. Since the amount of spectral shift depends on the rate of dipolar relaxation, a small modification of the dynamical properties of the membrane can cause very large effects on Laurdan emission properties. Also, Laurdan excitation spectrum is different in the two phospholipid phases (Parasassi et al., 1990). The excitation maximum in gel phase phospholipids is at about 390 nm, while in the liquid-crystalline phase the excitation maximum is at about 360 nm. Because of this difference in excitation, a partial photoselection of Laurdan molecules surrounded by the gel phase with respect to those in the liquid-crystalline environment is possible. For analogy with the concept of photoselection that can be performed using polarized excitation, spectroscopic properties of Laurdan have been described (Parasassi et al., 1990 and 1991) by the generalized polarization (GP) defined as

$$GP = (I_g - I_l)/(I_g + I_l) \quad (1)$$

where I_g and I_l are the fluorescence intensities at the maximum emission in phospholipids in the gel and in the liquid-crystalline phase, respectively (excitation GP). In addition, the GP value can be obtained using the fluorescence intensities at the maximum excitation in phospholipids in the gel and in the liquid-crystalline phase (emission GP).

A further peculiar feature of Laurdan spectroscopy concerns the red band of the excitation spectrum, centered at about 390 nm. This second excitation band is absent in apolar solvents, appears in polar solvent capable of hydrogen bonding, and is quite intense in phospholipid vesicles (Parasassi et al., 1991). In phospholipids, the fluorescent moiety of Laurdan is located at the level of the glycerol backbone, where a few partially immobilized water molecules may be

present. Thus, the red excitation band seems to originate from the stabilization of Laurdan ground state by a local polar environment. Moreover, this band is populated by Laurdan molecules with a favorable orientation of surrounding dipoles, already relaxed, but is associated to gel phase, unrelaxed phospholipids. By selectively exciting this red band, the resulting shape of Laurdan emission depends on the balance between the amount of relaxation, i.e., the phospholipid phase state, and the local polarity of the Laurdan environment.

The GP value offers several advantages for the measurement and analysis of Laurdan fluorescence data. In phospholipids of different polar heads and of various acyl residues, and at pH values varying from 4 to 10, the GP has a unique characteristic value for the gel and for the liquid-crystalline phase (Parasassi et al., 1991). The excitation GP value in the gel phase is high, of about 0.6, while in the liquid-crystalline phase is low and negative, of about −0.3. Moreover, the property of additivity of the GP can be used to quantitate coexisting phases (Parasassi et al., 1991). Laurdan GP value can be obtained both from emission and from excitation spectra, providing independent information on the dipolar relaxation process and on the polarity of the environment. The particular wavelength behavior of the GP value gives information on the coexistence of different phases and on their interconversion (Parasassi et al., 1993).

When Laurdan is used in cell membranes, where cholesterol and a variety of lipids are present, a blue shift of its emission and excitation spectra is observed (Parasassi et al., 1992). A similar blue shift was observed in DMPC vesicles after the addition of 30 mol % cholesterol. In cell membranes Laurdan GP value is relatively high, and in DMPC vesicles the GP value is higher compared with the value obtained in the absence of cholesterol at all temperatures (Parasassi et al., 1992).

With the purpose of 1) verifying the existence and kinetics of the mixing between separate domains in phospholipid vesicles and 2) investigating the origin of the blue spectral shift and of the relatively high GP value observed in cell membranes, we explored the influence of cholesterol on Laurdan spectroscopic properties. The study was performed in various phospholipid vesicles with cholesterol concentrations up to 60 mol %.

MATERIALS AND METHODS

Laurdan-labeled liposomes

Multilamellar phospholipid vesicles were prepared using conventional methods. The appropriate amounts of phospholipids in chloroform solution (dilauroyl-, dimyristoyl-, and dipalmitoyl-phosphatidylcholine; DLPC, DMPC and DPPC, respectively) (Avanti Polar Lipids, Inc., Alabaster, AL), cholesterol (Sigma Chemical Co., St. Louis, MO), and Laurdan (Molecular Probes Inc., Eugene, OR) were mixed. The solvent was evaporated by nitrogen flow. The dried samples were resuspended in phosphate-buffered saline solution (ICN Biomedicals, Irvine, CA), heated to 70°C, and vortexed. All samples were prepared in red light and used immediately after preparation. The final lipids and probe concentrations were 0.3 mM and 0.3 μM, respectively. Chloroform was spectroscopic grade.

Fluorescence measurements

Laurdan excitation and emission spectra in the lipid vesicles were obtained using a GREG 200 fluorometer, equipped with photon-counting electronics (PX01, ISS Inc., Champaign, IL) and the accompanying software. The fluorometer uses a xenon arc lamp as the light source. Monochromator bandpass was 4 nm. The spectra were only corrected for lamp intensity variations. For the DLPC-DPPC samples, the corresponding blank spectra were acquired and subtracted from the fluorescence spectra. The emission GP spectra were constructed by calculating the GP value for each emission wavelength as follows:

$$GP = (I_{410} - I_{340})/(I_{410} + I_{340}) \quad (2)$$

where I_{410} and I_{340} are the intensities at each emission wavelength, from 425 nm to 550 nm, obtained using fixed excitation wavelength of 410 nm and 340 nm, respectively. The excitation GP spectra were constructed in a similar way from the excitation spectra using:

$$GP = (I_{440} - I_{490})/(I_{440} + I_{490}) \quad (3)$$

where I_{440} and I_{490} are the intensities at each excitation wavelength, from 320 nm to 420 nm, obtained using fixed emission wavelength of 440 nm and 490 nm, respectively. For the calculation of GP, fixed excitation and emission wavelengths were chosen as previously discussed (Parasassi et al., 1990). In particular, in the case of the calculation of emission GP, excitation wavelengths of 340 nm and 410 nm do not correspond to the maximum excitation of the liquid-crystalline and of the gel phase, respectively. They were chosen as convenient extremes of the excitation spectrum to improve the photoselection of the two Laurdan populations. Temperature was controlled to ± 0.1°C by a water circulating bath.

Oxygen-quenching experiments were performed using a photon-counting fluorometer, model GREG PC (ISS Inc.) equipped with a xenon arc lamp and an oxygen pressure cell described in Lakowicz and Weber (1973) holding a 2 × 2 cm cuvette and thermostated at 20°C by a circulating water bath. During measurements, samples were continuously stirred. The measurements in the absence of oxygen were performed in N_2 atmosphere. After the application of each value of oxygen pressure, samples were allowed to equilibrate for 45 min in the dark with continuous stirring.

The three-dimensional plots reported in Figs. 9–11 were created using the software Axum from TriMetrix Inc. The underlying grid was obtained from the same data of the plots, using the three-dimensional spline surface option of the Axum program.

RESULTS

We have performed four different kinds of experiments on phospholipid vesicles labeled with Laurdan with the aim of studying the coexistence of domains in the presence of cholesterol: 1) effect of different cholesterol concentration in phospholipid vesicles on Laurdan excitation and emission spectra to identify the effect of polarity changes; 2) temperature dependence of Laurdan GP value in phospholipid vesicles and in the presence of different cholesterol concentrations to assess the effect of cholesterol on Laurdan dipolar relaxation; 3) wavelength dependence of Laurdan excitation and emission GP spectra in phospholipid vesicles with various cholesterol concentrations to detect the coexistence of phase domains; 4) a surface for each of the phospholipids used, built with the purpose of separating the influence of cholesterol on Laurdan dipolar relaxation from the polarity changes.

Laurdan excitation and emission spectra

Laurdan excitation and emission spectra were measured in DLPC, DMPC, and in an equimolar DLPC-DPPC mixture,

from 1°C to 65°C, at cholesterol concentrations from 0 to 60 mol %. As representative examples, Laurdan excitation and emission spectra obtained in multilamellar vesicles composed of DMPC or of an equimolar mixture of DLPC and DPPC with no cholesterol and with 30 mol % cholesterol at 1°C are reported in Fig. 1, *A* and *C*, respectively. Both excitation and emission spectra are blue shifted in the presence of cholesterol. At 65°C the emission blue shift in the presence of cholesterol is quite relevant (Fig. 1, *B* and *D*), while the excitation spectrum shows a red shift accompanied by the increase in intensity of the red band of excitation at about 390 nm.

Temperature dependence of Laurdan GP

The excitation GP values obtained in DMPC vesicles with cholesterol concentrations from 5 to 30 mol % using an excitation wavelength of 340 nm and emission wavelengths of 440 and 490 nm versus temperature are reported in Fig. 2 *A*. In Fig. 2 *B* the emission GP values obtained in the same samples using an emission wavelength of 440 nm and excitation wavelengths of 410 and 340 nm are reported versus temperature. The temperature interval of the phase transition is progressively broader and shifted at higher temperature by increasing the cholesterol concentration in DMPC vesicles as detected by both the excitation and emission GP values. In the temperature range of DMPC liquid-crystalline phase, the increase of cholesterol concentration produces an increase of both excitation and emission GP values to the GP values of the gel phase, so that at higher cholesterol concentrations the phase transition is no longer detectable. At low temperatures, corresponding to the DMPC gel phase, the addition of cholesterol causes an increase of the excitation GP value and a small decrease of the emission GP value.

Excitation and emission GP values versus temperature were calculated from Laurdan spectra obtained in samples composed of an equimolar mixture of DLPC and DPPC with increasing cholesterol concentrations, from 5 to 60 mol %, and some representative curves are reported in Fig. 2, *C* and *D*. For these samples, blank spectra were acquired and subtracted from the excitation and emission Laurdan spectra. The effect of blank subtraction is relatively small (GP value difference of about 3%) and the major effect of the blank subtraction was observed at the edges of both excitation and emission GP spectra. For the construction of Fig. 2, *C* and *D*, an excitation wavelength of 370 nm rather than 340 nm and an emission wavelength of 490 nm rather than 440 nm were chosen as the wavelengths less affected by the blank subtraction. Similar to the results obtained using DMPC vesicles, by adding cholesterol to this phospholipid mixture, both excitation and emission GP values increase. At temperatures above 25°C the increase of the GP value is particularly relevant, and the effect on the GP variation with temperature is smoothed. Similar results (data not shown) were also obtained for DLPC, where the GP follows the high temperature behavior observed with DMPC.

In Fig. 3 the difference between the excitation (Fig. 3, *A* and *C*) and the emission (Fig. 3, *B* and *D*) GP values obtained

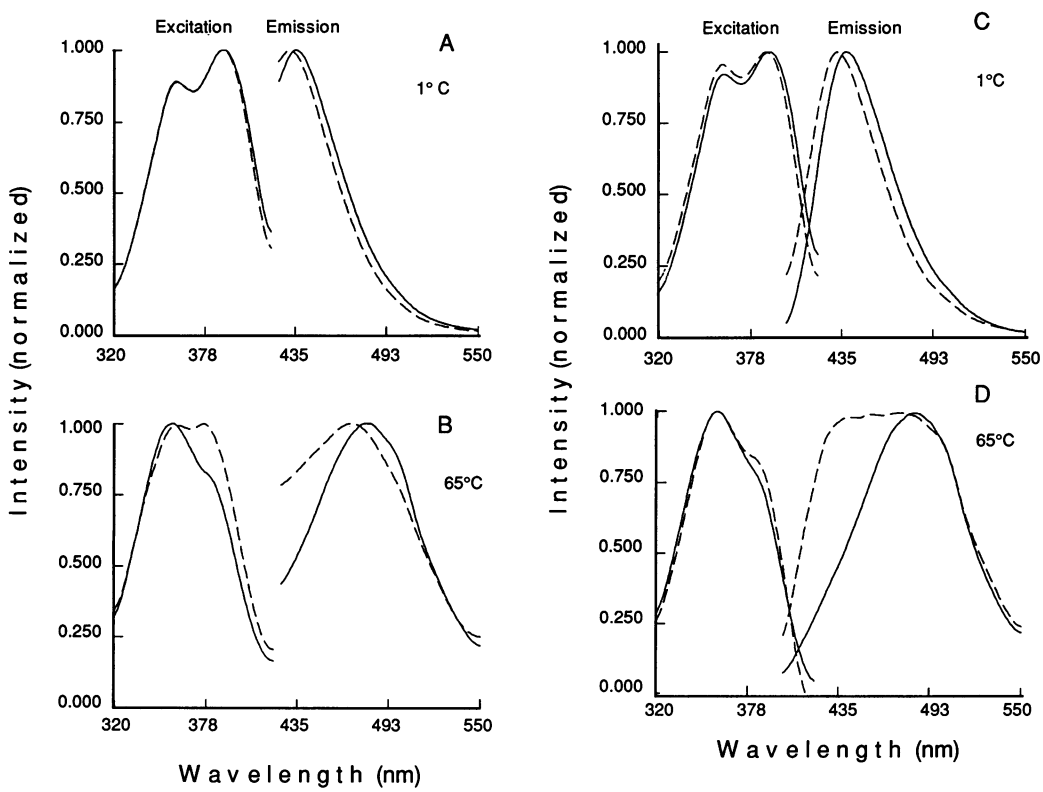

FIGURE 1 Laurdan normalized excitation and emission spectra obtained in multilamellar vesicles composed of DMPC (*A* and *B*) and of an equimolar DLPC-DPPC mixture (*C* and *D*) with no cholesterol (——) and with 30 mol % cholesterol (– – –) at 1°C (*A* and *C*) and at 65°C (*B* and *D*).

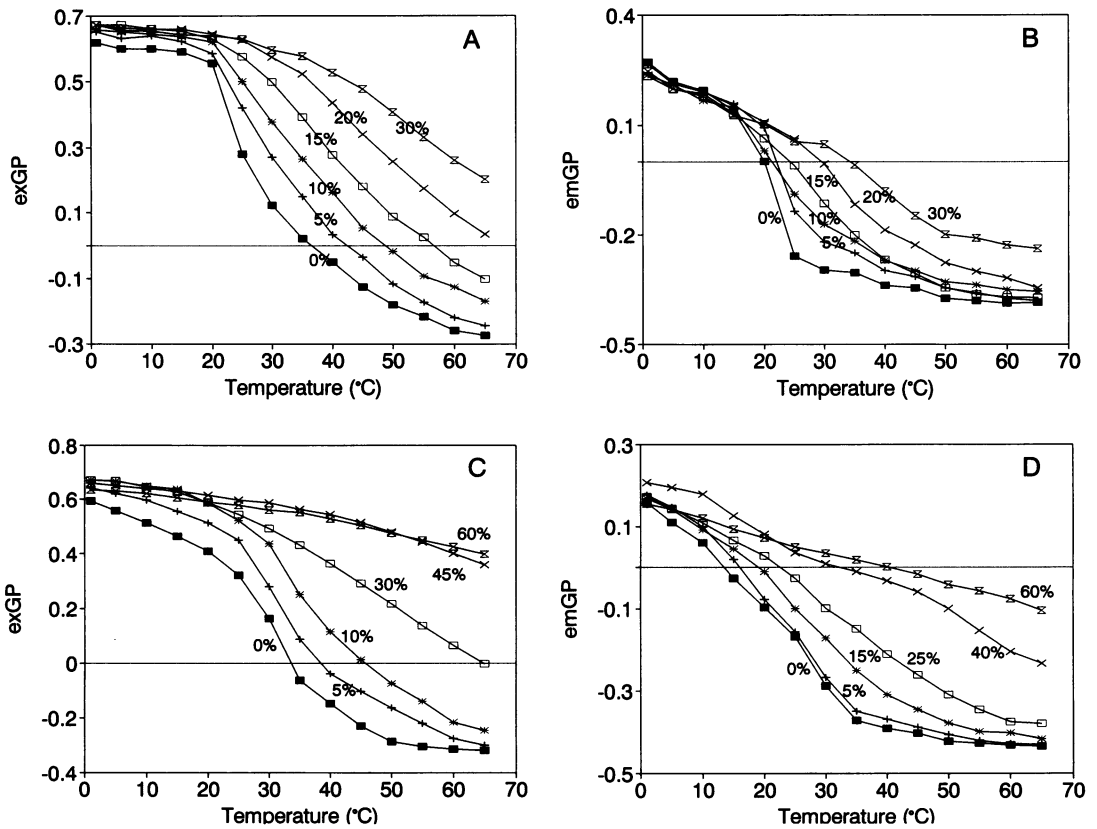

FIGURE 2 Laurdan excitation (*A* and *C*) and emission (*B* and *D*) GP values versus temperature obtained in multilamellar vesicles at various cholesterol concentrations. The percent values represent mol % of cholesterol in phospholipids. The vesicle composition was DMPC (*A* and *B*) and equimolar DLPC-DPPC (*C* and *D*).

at various cholesterol concentrations and without cholesterol is reported for DMPC vesicles (Fig. 3, *A* and *B*) and for vesicles composed of equimolar DLPC-DPPC (Fig. 3, *C* and *D*). Fig. 3 shows that (*a*) major variations of the emission GP value occur at a temperature a few degrees lower with respect to variations of the excitation GP value; (*b*) the increase of both excitation and emission GP values with the increase of cholesterol concentration is particularly relevant in the liquid-crystalline phase; (*c*) when cholesterol is added, the excitation GP values generally show larger variations than the emission GP values, i.e., the variation of the emission spectra are larger than those of the excitation spectra; (*d*) for the DMPC sample, the variation of emission GP at low temperature is negative; and (*e*) a partial saturation effect can be observed at high cholesterol concentrations. In DMPC vesicles, small variations of emission and excitation GP values are observed by increasing cholesterol concentration above 20 mol % (Fig. 3, *A* and *B*). In the equimolar DLPC-DPPC sample, the excitation GP value shows larger variations for cholesterol addition up to 30 mol % and the emission GP value for cholesterol addition up to 35 mol % (Fig. 3, *C* and *D*). A similar behavior was observed for DLPC vesicles at different cholesterol concentrations (data not shown).

Wavelength dependence of Laurdan GP spectra

Some representative Laurdan excitation and emission GP spectra in DMPC vesicles with various cholesterol concentrations and at different temperatures are reported in Fig. 4. Both excitation and emission GP at 1°C do not show a relevant wavelength dependence, and this behavior is not affected by cholesterol addition. This wavelength independence of GP value is characteristic of the gel phase (Parasassi et al., 1993). At 65°C, excitation GP spectra show decreasing values as the excitation wavelength increases up to 10 mol % cholesterol (Fig. 4 *A*), while emission GP spectra show increasing values as the emission wavelength increases (Fig. 4 *B*). At high cholesterol concentration, the slope due to the wavelength dependence is decreased. This behavior of the GP versus wavelength observed at 65°C is characteristic of phospholipids in the liquid-crystalline phase (Parasassi et al., 1993). The temperature at which the behavior of GP versus wavelength changes from that characteristic of the gel to that characteristic of the liquid-crystalline phase depends on cholesterol concentration and increases with the increase of cholesterol concentration, in agreement with the shift of the transition temperature reported in Figs. 2 and 3.

Similarly, in vesicles composed of an equimolar mixture of DLPC and DPPC, at low and at high temperatures, the wavelength dependence of excitation and emission GP is not affected by cholesterol addition up to 30 mol % (Fig. 5). At low temperature, the effect of cholesterol only consists of a slight increase of the GP value, without affecting the wavelength independence (Fig. 5). At high temperature, the addition of cholesterol causes a relevant increase of both absolute excitation and emission GP values (Fig. 5, *A* and *B*,

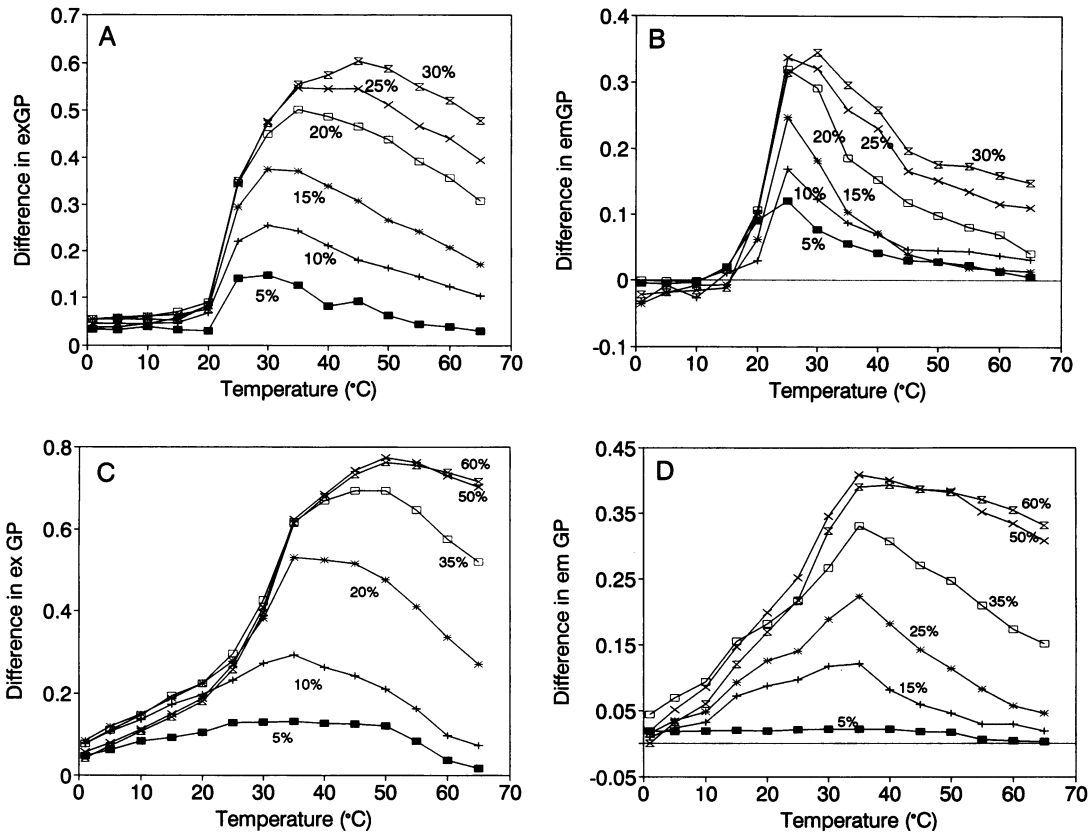

FIGURE 3 Difference between Laurdan excitation (*A* and *C*) and emission (*B* and *D*) GP values obtained in phospholipid multilamellar vesicles with various cholesterol concentrations and without cholesterol as a function of temperature. The percent values represent mol % of cholesterol in phospholipids. The vesicle composition was DMPC (*A* and *B*) and eqimolar DLPC-DPPC (*C* and *D*).

respectively, and Figs. 2 and 3), with little change of the wavelength dependence. Above 30 mol % cholesterol, very little wavelength dependence of both emission and excitation GP values can be observed also at high temperatures. At intermediate temperatures, particularly between 10 and 30°C, the major variation caused by cholesterol concerns the change of the typical wavelength dependence of GP spectra, which is observed when coexisting domains of gel and liquid-crystalline phases are present. In Fig. 6 the GP spectra obtained in the equimolar DLPC-DPPC mixture at 25°C are shown. In the absence of cholesterol, the equimolar mixture of DLPC and DPPC at temperatures from about 10°C to about 30°C gives increasing values of the excitation GP spectrum and decreasing values of the emission GP spectrum with the increase of excitation and emission wavelength, respectively. Of note is the fact that this wavelength dependence is opposite to that observed in the pure liquid-crystalline phase. Indeed, without cholesterol, the type of wavelength dependence of emission and excitation GP values can be used to ascertain the coexistence of phospholipid phase domains (Parasassi et al., 1993). This behavior can still be observed in the phospholipid mixture after cholesterol addition up to 10 mol % for the excitation GP spectrum (Fig. 6) and up to 15 mol % for the emission GP spectrum (data not shown). At these cholesterol concentrations, the temperature range at which the wavelength dependence typical of coexisting domains can be observed is restricted. Above 15 mol % cholesterol, the behavior of GP values changes from the absence of an appreciable wavelength dependence observed at low temperatures, to the wavelength dependence typical of the liquid-crystalline phase. This last behavior is observed regardless of the absolute GP values.

In DLPC vesicles, the wavelength dependence of the GP value typical of the liquid-crystalline phase is not modified by increasing cholesterol concentration (data not shown). The absolute excitation and emission GP values increase with the addition of cholesterol, but, regardless to cholesterol concentration, the excitation GP spectra show decreasing values with the increase of excitation wavelength, and the emission GP spectra show increasing values with the increase of emission wavelength. This behavior is similar to that reported in Fig. 4, *A* and *B*, for DMPC at high temperature. Similarly to the DLPC-DPPC mixture, above 30 mol % cholesterol very little variation of GP values as a function of wavelength was observed (data not shown).

To verify the possibility that Laurdan preferentially associates with cholesterol or that the probe will partition in possible domains with high cholesterol concentration, the determination of the partition coefficient of the probe between phospholipids and cholesterol must be obtained. The equipartition of Laurdan between the two phospholipid phases has been already demonstrated (Parasassi et al., 1993). Excitation GP values obtained using excitation wavelengths of 340 or 410 nm and emission wavelengths of 440

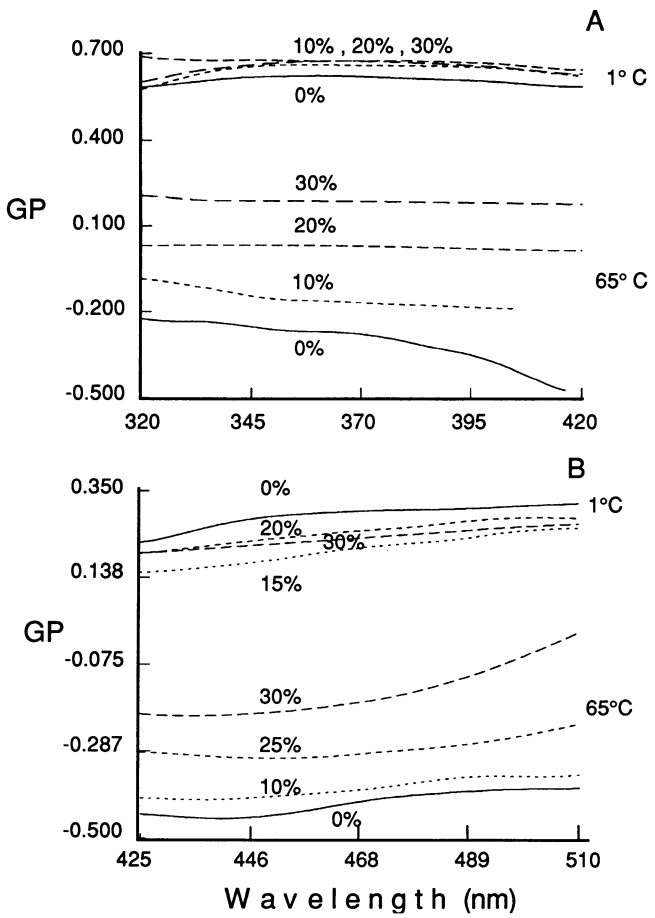

FIGURE 4 Laurdan excitation (*A*) and emission (*B*) GP spectra obtained in DMPC vesicles at various cholesterol concentrations, at 1°C and at 65°C. The percent values represent mol % of cholesterol in phospholipids.

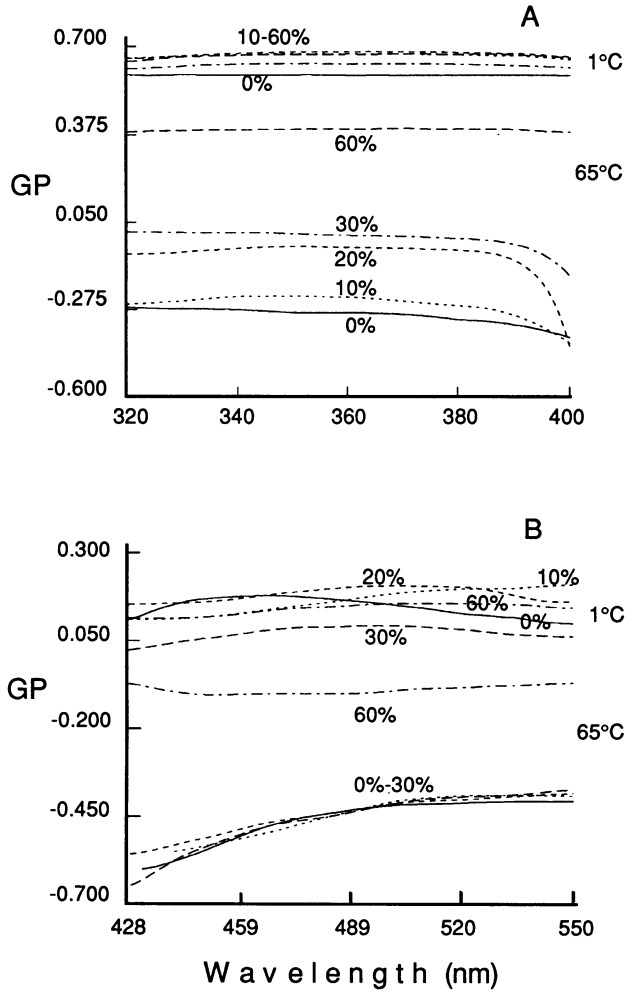

FIGURE 5 Laurdan excitation (*A*) and emission (*B*) GP spectra obtained in the equimolar DLPC-DPPC mixture at various cholesterol concentrations, at 1°C and at 65°C. The percent values represent mol % of cholesterol in phospholipids.

and 490 nm in DLPC vesicles at 20°C with cholesterol concentrations varying from 1 to 55 mol % are reported in Fig. 7. In Fig. 7 *A*, the straight line segments from 0 to 30 mol % cholesterol, obtained by joining the GP values at 0 and 30 mol % cholesterol, represent the GP values expected from a mixture of DLPC and cholesterol in which Laurdan shows ideal homogeneous partitioning. The small deviation of the experimental GP values from the straight line is to be attributed to dipolar relaxation and to interconversion between domains, as previously reported (Parasassi et al., 1993). A saturation can be observed in the excitation GP above 30 mol % cholesterol (Fig. 7 *A*) and in the emission GP above 40 mol % cholesterol (Fig. 7 *B*). From preliminary experiments (data not shown) performed using a dynamic fluorescence technique for the measurement of time-resolved emission spectra of Laurdan in DLPC at various cholesterol concentrations, we observed a modification of Laurdan relaxation kinetics due to the presence of cholesterol. Nevertheless, no specific relaxation kinetics due to the presence of cholesterol and indicative of a preferential partitioning of Laurdan in separated cholesterol domains was observed. From previous oxygen-quenching experiments of Laurdan fluorescence (Parasassi and Gratton, 1992), a linear Stern-Volmer plot was obtained using phospholipids in a single phase. In the equimolar DLPC-DPPC mixture, at temperature where the coexistence of separate domains of different phases occurs, the Stern-Volmer plot was biphasic. By adding 10 mol % cholesterol to vesicles in the gel phase, a biphasic Stern-Volmer plot was also obtained (Parasassi and Gratton, 1992). Here we performed oxygen-quenching experiments of Laurdan fluorescence in DLPC vesicles at 20°C (Fig. 8). In this pure liquid-crystalline phase the Stern-Volmer plot is linear, as already reported (Parasassi and Gratton, 1992). By adding cholesterol up to 55 mol %, no deviation from linearity was observed (Fig. 8), although by increasing cholesterol concentration the quenching constants are quite different, by more than a factor of 2 at 55 mol % cholesterol.

Influence of cholesterol on bilayer polarity and on Laurdan dipolar relaxation

Using emission and excitation GP values and the temperature of their measurement obtained with each phospholipid

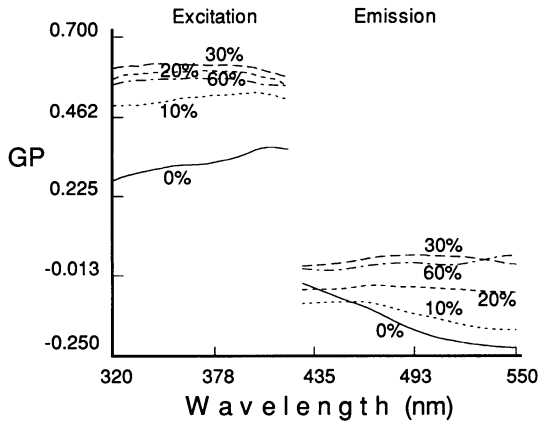

FIGURE 6 Laurdan excitation and emission GP spectra obtained in the equimolar DLPC-DPPC mixture at various cholesterol concentrations at 25°C. The percent values represent mol % of cholesterol in phospholipids.

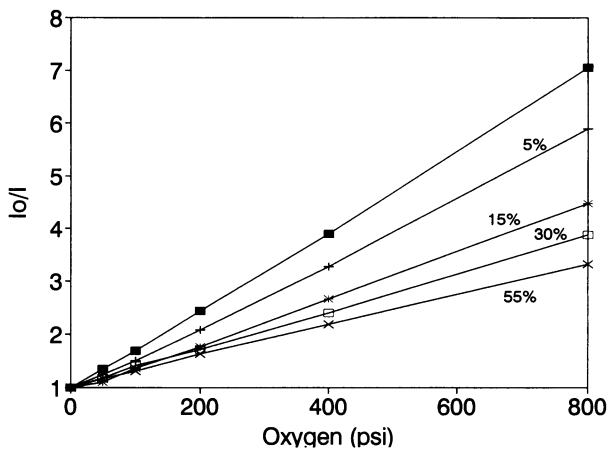

FIGURE 8 Stern-Volmer plot obtained by oxygen-quenching experiments of Laurdan fluorescence in vesicles composed of DLPC without cholesterol and with various cholesterol concentrations at 20°C. Percent values represent mol % of cholesterol in DLPC.

sample with different cholesterol concentrations, a three-dimensional surface was constructed. In Fig. 9 the surface obtained with DMPC and cholesterol concentrations of 0, 3, 5, 10, 15, 20, 25, and 30 mol % are shown. In Fig. 10 *A*, the surface obtained with the equimolar DLPC-DPPC mixture and cholesterol concentrations of 0, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mol % is shown. In Fig. 11 *A*, the surface obtained with DLPC and cholesterol concentrations of 0, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mol % is shown. On each surface, the individual three-dimensional curves of samples at each cholesterol concentration are superimposed. To better display the area of the high cholesterol concentration, in Fig. 10 *B* the surface obtained with the equimolar DLPC-DPPC mixture in the presence of 25, 30, 35, 40, 45, 50, 55, and 60 mol % cholesterol is reported, and in Fig. 11 *B* the surface obtained using DLPC and cholesterol concentrations of 25, 30, 35, 40, 45, 50, 55, and 60 mol % is reported.

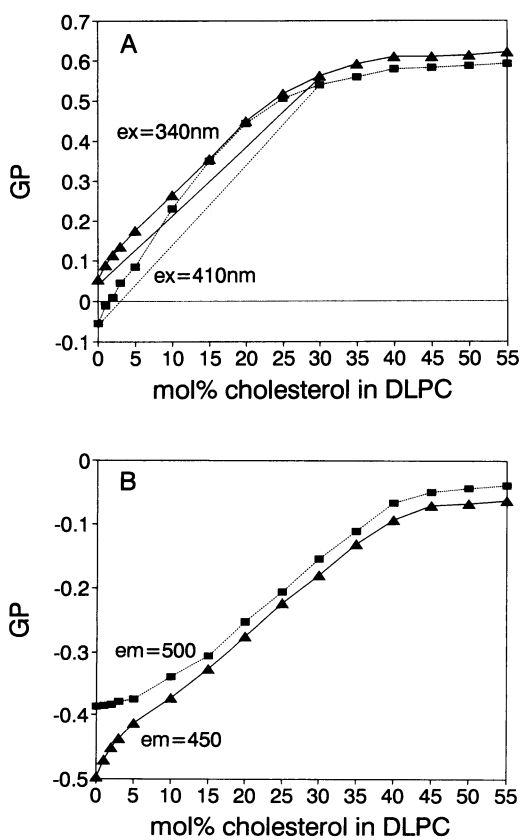

FIGURE 7 Laurdan excitation (*A*) and emission (*B*) GP values in DLPC vesicles with increasing cholesterol concentrations at 20°C. GP values were obtained using excitation wavelengths of 340 and 410 nm (*A*) and emission wavelengths of 450 and 500 nm (*B*).

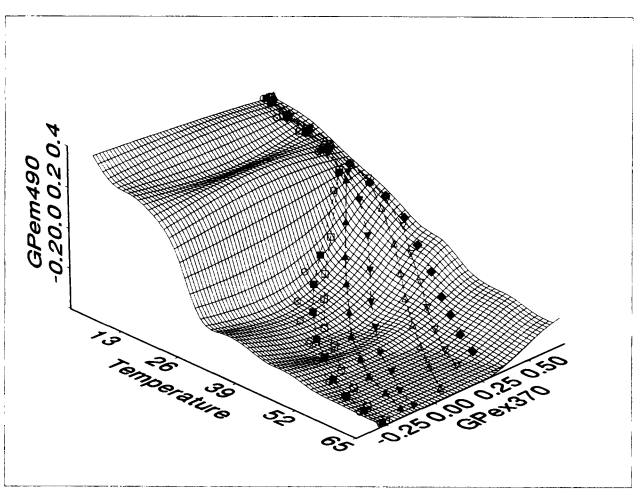

FIGURE 9 Three-dimensional surface obtained from Laurdan excitation and emission GP values and from the temperature of their measurement in DMPC vesicles with cholesterol concentrations of 0, 3, 5, 10, 15, 20, 25, and 30 mol %. The excitation GP values were measured using excitation at 370 nm and the emission GP values using emission at 490 nm. The surface was generated using all the data by the three-dimensional spline option of the Axum software, as reported in Materials and Methods. The individual three-dimensional curves of samples at each cholesterol concentration are superimposed.

In general, by adding cholesterol to phospholipids, Laurdan excitation GP value varies by a different amount with respect to the variation of emission GP value; i.e., for each phospholipid, the three-dimensional plots obtained at various cholesterol concentrations do not overlap.

In these surfaces different areas can be characterized. High values of both excitation and emission GP at low temperatures define an area of gel-like phase where little relaxation occurs (Figs. 10 *B* and 11 *B*). At high temperatures, low excitation and emission GP values characterize the area of the liquid-crystalline-like phase, where dipolar relaxation occurs. At intermediate temperature, intermediate excitation and emission GP values are observed, and this area could be characterized by the coexistence of gel and liquid-crystalline domains. By increasing cholesterol concentration, the plots of the individual samples are shifted to higher excitation and emission GP values.

DISCUSSION

To understand at a molecular level the effect of cholesterol on phospholipid bilayers, we discuss first how the properties of Laurdan are influenced by the nature and dynamics of its local surroundings. Laurdan spectral properties are dependent on at least two major factors: 1) the polarity of the environment; and 2) the rate of relaxation of molecules or molecular residues that can reorient around Laurdan fluorescent moiety during its excited-state lifetime.

In apolar solvents, such as hexane or cyclohexane, Laurdan shows very blue emission and excitation spectra and a very low quantum yield (Parasassi et al., 1991). In these apolar solvents, Laurdan fluorescence lifetime is of the order of 100 ps (Parasassi et al., 1993). Hydrogen bonding solvents cause a red shift of emission and excitation spectra. Dipolar reorientation of solvent during Laur-

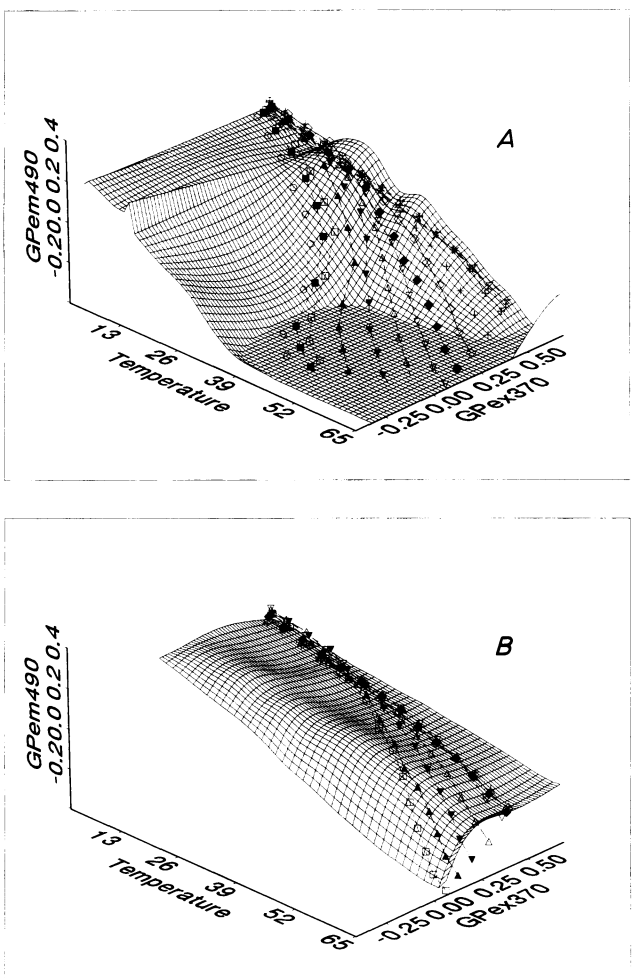

FIGURE 10 Three-dimensional surface obtained as reported in Fig. 9 from Laurdan excitation and emission GP values and from the temperature of their measurement in vesicles composed of the equimolar DLPC-DPPC mixture with cholesterol concentrations of (*A*): 0, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mol %; (*B*): 25, 30, 35, 40, 45, 50, 55, and 60 mol %. The excitation GP values were measured using excitation at 370 nm and the emission GP values using emission at 490 nm. Intensities of the blanks were subtracted. In both figures the individual three-dimensional curves of samples at each cholesterol concentration are superimposed.

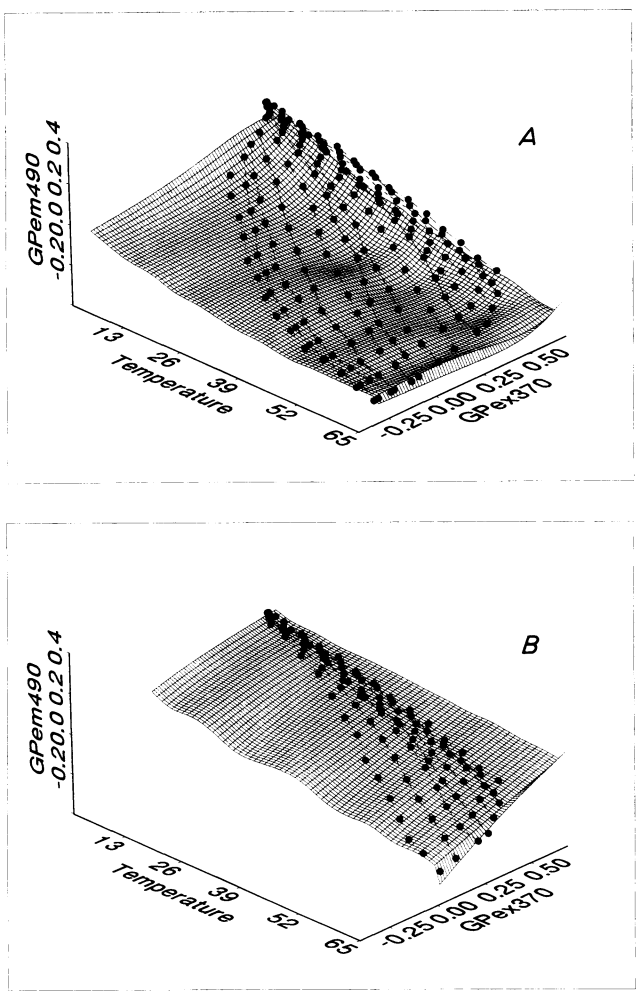

FIGURE 11 Three-dimensional surface obtained as reported in Fig. 9 from Laurdan excitation and emission GP values and from the temperature of their measurement in vesicles composed of DLPC with cholesterol concentrations of (*A*): 0, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mol %; (*B*): 25, 30, 35, 40, 45, 50, 55, and 60 mol %. The excitation GP values were measured using excitation at 370 nm and the emission GP values using emission at 490 nm. In both figures the individual three-dimensional curves of samples at each cholesterol concentration are superimposed.

dan excited-state lifetime can cause an additional time-dependent red shift of the emission (Parasassi et al., 1990). If the rate of relaxation is very small, such as in the phospholipid gel phase, this additional emission spectral shift is small (Parasassi et al., 1993). In the phospholipid liquid-crystalline phase, relaxation processes can occur at a much faster rate, and we can observe a very large emission red shift (Parasassi et al., 1993).

One important consideration relates to the nature of the molecule that reorients during the exited-state lifetime. We have demonstrated that the rate and extent of the relaxation process are independent on the nature of the phospholipid polar residue and are also independent on the pH value of the medium (Parasassi et al., 1991). This observation rules out the motion of phospholipid polar heads as the molecular entity responsible for reorientation. Measurements of the polarization value across the emission band also rules out the possibility that the Laurdan molecule itself rotates to better orient with respect to dipoles or charges of the membrane polar surface (Parasassi et al., 1990). To rule out a further possibility that residues of Laurdan molecules rotate and reorient during the probe excited state, we performed measurements on Laurdan analogues. Our results (Parasassi and Gratton, unpublished observations) excluded the hypothesis that this intermolecular relaxation was the source of the relaxation process. Instead, the possibility exists that a few water molecules present in the hydrophilic-hydrophobic interface of the bilayer, close to the naphthalene moiety of Laurdan, can reorient during Laurdan excited-state lifetime. The fluorescent moiety of Laurdan is located at the level of the phospholipid glycerol backbone. The dynamic properties of these water molecules in the membrane are reduced with respect to the bulk water molecules of the solvent. The orientational rate must be on the order of nanoseconds in the phospholipid liquid-crystalline phase. We have proposed that a few water molecules present in the bilayer close to Laurdan naphthalene moiety are the molecular entities that produce the dipolar relaxation process responsible for the red emission spectral shift displayed by Laurdan (Parasassi et al., 1991). In the gel phase this process is strongly reduced either because of the absence of water or because the phospholipid tight packing prevents a rapid relaxation, in the nanosecond scale, of water molecules.

A peculiar feature of Laurdan excitation spectrum is related to the red excitation band, the intensity of which increases in polar solvents and in hydrogen bonding solvents. This effect has been attributed to the stabilization of the L_α conformation transitions. In phospholipids, this red excitation band corresponds also to a blue-emitting population, especially intense in gel phase phospholipids (Parasassi et al., 1990), where very little relaxation occurs.

Laurdan fluorescence spectra in gel phase and in the presence of cholesterol

At low temperature, when cholesterol is added to DMPC in the gel phase, at all the concentrations used in this work, from 1 to 30 mol %, and to the equimolar DLPC-DPPC mixture with cholesterol concentrations from 3 to 60 mol %, the excitation spectrum slightly shifts toward the blue, indicating a less polar environment (Fig. 1, *A* and *C*) and in agreement with the reported effect of cholesterol of reducing the hydration of bilayers (Levine and Wilkins, 1971). The emission spectrum shows a blue shift, also indicating a less polar environment. After the addition of cholesterol, both Laurdan ground and excited states appear less polar. Besides the small blue shift of the excitation spectrum in the pure phospholipid gel phase, i.e., in the DMPC samples at low temperature, the peculiar behavior of the excitation red band, centered at about 390 nm, must be described. In apolar solvents, the blue shift of the excitation spectrum is also accompanied by the decrease of this red band. By adding cholesterol to the gel phase, this red band also slightly decreases in its relative intensity, in agreement with a decrease of polarity of Laurdan environment. Variations of the ratio between the two excitation bands are well represented by the emission GP value. The decreased polarity of Laurdan environment after cholesterol addition is clearly displayed by the decrease of the emission GP value at low temperatures (Figs. 2 *B* and 3 *B*). In this case, the small decrease in the intensity of the excitation band at 410 nm is not indicative of a more liquid-crystalline-like environment of Laurdan fluorescent moiety, but only of a reduced stabilization of the ground state L_α population which is typical of Laurdan molecules surrounded by dipoles with a favorable orientation. The same blue shift of both Laurdan excitation and emission spectra are observed in the equimolar DLPC-DPPC mixture (Fig. 1 *C*). In this mixture, the emission GP value slightly increases instead of decreasing as in the DMPC sample (Fig. 3 *D*). In the case of the mixture of the two phospholipids, the decrease in polarity induced by cholesterol is masked by the dipolar relaxation, even in small amounts, due to the presence of DLPC with a T_m of $\cong$ 0°C.

Laurdan fluorescence spectra in the liquid-crystalline phase and in the presence of cholesterol

Cholesterol addition to DLPC vesicles, to the liquid-crystalline phase of DMPC vesicles, and to the equimolar DLPC-DPPC mixture has a larger effect than on the gel phase. In this phase state the emission spectrum shows a blue shift. Both the decreased polarity at the level of the glycerol backbone of phospholipids in the bilayer and the ordering effect of cholesterol (Vist and Davis, 1990; Ipsen et al., 1987), that renders the acyl chains conformation in the bilayers similar to that of the gel phase, reduce the amount of dipolar relaxation and cause a blue shift of the emission spectrum, similar to that observed in gel phase phospholipids. On the contrary, the excitation spectrum shows a red shift, with a relevant increase of the red excitation band (Fig. 1, *B* and *D*). After addition of cholesterol, the prevailing effect on the excitation spectrum is due to the decreased amount of relaxation rather than to decreased polarity that should cause

a blue shift. Laurdan excitation spectrum seems quite sensitive to the ordering effect of cholesterol on the acyl chain conformation. Modifications of Laurdan emission due to cholesterol are also quite relevant, both the reduced polarity and the increased conformational order contributing to the blue shift of the emission.

At low temperature and when cholesterol is present, the variations of Laurdan emission and excitation spectra in phospholipids are largely due to the decrease of polarity, probably because of the decreased water concentration at the level of the fluorescent moiety of Laurdan molecules in the bilayer (Levine and Wilkins, 1971), with the consequence of a further decrease of the dipolar relaxation and of a decreased stabilization of the L_α conformations. In DLPC at all temperatures, in DMPC, and in the equimolar DLPC-DPPC mixture at high temperatures, where dipolar relaxation prevails, the major consequence of cholesterol addition is the reduction of the molecular motion of water molecules rather than decreasing the water concentration itself, thus rendering Laurdan spectra closer to those observed in gel phase phospholipids where very little relaxation of water dipoles occurs.

Coexisting gel and liquid-crystalline phospholipid domains

In vesicles composed of the equimolar DLPC-DPPC mixture and at temperatures between 10 and 30°C, Laurdan spectra, which are intermediate between those characteristic of the gel and of the liquid-crystalline phase, are observed. In this temperature range, the above phospholipid mixture is known to display coexistence of gel and liquid-crystalline domains (Shimshick and McConnel, 1973; Parasassi et al., 1984). The effect of increasing cholesterol concentration in this sample is similar to that observed in pure liquid-crystalline samples. The Laurdan emission spectrum is blue-shifted and the excitation spectrum shows the increase in intensity of the red band.

Laurdan excitation and emission GP in the presence of cholesterol

To explain the wavelength dependence of the excitation and emission GP value, we first analyzed the expected behavior of a homogeneous liquid-crystalline phase in the absence of cholesterol. It is characteristic of the dipolar relaxation process that by exciting in the blue part of the spectrum we photoselect those molecules with an energetically unfavorable ground state, i.e., those molecules surrounded by randomly oriented dipoles. As a consequence, by moving the excitation toward the blue, more blue emitting molecules are excited. The excitation GP value, which depends on the difference between the emission intensities at 440 and 490 nm (Eq. 3), should increase toward shorter excitation wavelengths. Of course, this effect is only visible in a homogeneous liquid-crystalline phase where dipolar relaxation occurs (Figs. 4 and 5, spectra at 65°C).

The emission GP spectra are obtained by measuring the difference between the excitation intensities at 410 nm and 340 nm at all emission wavelengths (Eq. 2). Excitation at 340 nm slightly selects Laurdan molecules with energetically unfavorable orientation of the surrounding dipoles (Parasassi et al., 1990), only a part of which will relax during the probe excited state. Excitation at 410 nm preferentially selects Laurdan molecules mostly surrounded by favorably oriented dipoles, which will show a relaxed emission. Consequently, by observing the red emission at 490 nm, higher intensity will originate from the 410 nm band of excitation. By moving the emission toward the blue, the intensity arising from 410 nm excitation will decrease, and the GP value will also decrease (Parasassi et al., 1993). Again, this trend can only be observed in a homogeneous liquid-crystalline phase in the presence of the dipolar relaxation process (Figs. 4 and 5, spectra at 65°C).

The coexistence of gel and liquid-crystalline phases, in the absence of cholesterol, gives rise to an opposite wavelength dependence of the excitation and emission GP spectra, as previously discussed (Parasassi et al., 1993). Laurdan molecules, in a domain of phospholipids in gel phase, preferentially absorb in the 390 nm region and emit in the 440 nm region. Laurdan molecules in a domain of liquid-crystalline phase preferentially absorb in the 360 nm region and emit in the 490 nm region. In the presence of both coexisting phases (sample of equimolar mixture of DLPC and DPPC, at temperatures between 10 and 30°C) the excitation GP spectrum should then decrease toward shorter wavelengths, while the emission GP spectrum should increase. Therefore, phase domains coexistence has an opposite trend with respect to that observed only in the presence of the dipolar relaxation, with the excitation GP spectrum showing increasing values with the increase of the excitation wavelength, and with the emission GP spectrum showing decreasing values with the increase of the emission wavelength (Parasassi et al., 1990, 1991, 1993) (Fig. 6). Finally, in the homogeneous gel phase, no appreciable wavelength dependence of excitation and emission GP values is observed (Figs. 4 and 5, spectra at 1°C).

The behavior of the GP spectra as a function of wavelength in the presence of coexisting domains is modified by the presence of cholesterol, and the type and amount of modification depends on cholesterol concentration. In the equimolar DLPC-DPPC mixture, at low cholesterol concentrations up to about 10 mol %, the wavelength dependence of excitation and emission GP is similar to that observed without cholesterol. Differences are observed only for the temperature at which the wavelength dependence of excitation and emission GP typical of the coexistence of both phases can be observed. In correspondence to the shift of the phase transition temperature reported in Figs. 2 and 3, emission and excitation GP spectra with the characteristic wavelength dependence observed when phase domains coexist are observed at temperatures progressively higher with the increase of cholesterol concentration (Fig. 6). At cholesterol concentrations higher than 10 mol %, the above described behavior of GP values is no longer observed. From low to high temperatures both excitation and emission GP spectra

pass from a wavelength dependence typical of the pure gel to that typical of the pure liquid-crystalline phase. No intermediate behavior indicative of gel and liquid-crystalline domain coexistence was observed (Fig. 6).

The absence of rapid mixing

Our experiments failed to detect separate spectroscopic signals in the regions in which liquid phase or gel phase immiscibility has been postulated. Separated and resolvable Laurdan spectroscopic properties indicative of the coexistence of domains with different polarity or different molecular order could not be seen. As we noted before, the same failure occurred for NMR and ESR experiments (Vist and Davis, 1990). Since the time scale of the fluorescent phenomenon is on the order of 10^{-9} s, if an averaging of emission properties should be invoked to explain the absence of distinct signals, this argument set a limit on the size and lifetime of domains. The lifetime of the domain should be shorter that 5 ns and the size should be no larger than 50 lipid molecules, based on an estimate of the diffusion rate of small molecules in the membrane plane (Parasassi and Gratton, 1992). Similar estimates on the dimension of domains in vesicles composed of equimolar mixtures of gel and liquid-crystalline phases, without cholesterol, were obtained on the basis of time-resolved measurements of Laurdan emission (Parasassi et al., 1993). From the diffusion coefficient in the two phases and from the rate of interconversion of Laurdan between the phases, an average area of 20–50 $Å^2$ has been calculated for the domains (Parasassi et al., 1993). It is quite puzzling that at least three spectroscopic techniques with quite different characteristic time scales (NMR, ESR, and fluorescence) have failed to detect distinct environments in a region of phase diagram where separated domains are expected to exist. The possibility remains that these theoretical domains are very small, composed of a few phospholipid molecules, and/or fluctuating more rapidly than previously anticipated. Moreover, the different Laurdan spectroscopic properties, specific for each phase domain (Parasassi et al., 1991 and 1993), are progressively closer by increasing cholesterol concentration. In the case of pure phospholipid separate domains, the results showed that each phase is profoundly modified by the presence of the other (Parasassi et al., 1993). By adding cholesterol to phospholipid mixtures showing domain segregation, as in the present work, the modification of each phase is even more significant, so that separate spectroscopic Laurdan parameters cannot be distinguished.

Analysis of the GP excitation-emission surfaces

In general, from our results we can conclude that Laurdan sensitivity to the polarity and to the kinetics of dipolar relaxation of its environment detects a decreased polarity and a lower amount of relaxation in phospholipids after addition of cholesterol. The decrease of the amount of dipolar relaxation produced by cholesterol in the liquid-crystalline phase is too large to be attributed to a decreased polarity, i.e., to the substitution of water molecules with the hydroxyl residue of cholesterol. Instead, the increase in the phospholipid acyl chain conformational order or a tight packing of the bilayer close to that of the gel phase must be invoked. Since the excitation spectrum shows opposite response to the polarity or to the order, while the emission spectrum is shifted to the same direction by modification of these two quantities, the emission GP values do not vary of the same amount as the excitation GP. Indeed, by plotting the excitation GP values versus the emission GP values obtained at each temperature, the curves do not overlap. Using the temperature as the third axis, a surface is obtained where different areas can be characterized (Figs. 9–11). High values of both excitation and emission GP at low temperature characterize an area of gel-like phase. Low excitation and emission GP values at high temperature characterize an area where a large amount of dipolar relaxation occurs, such as in the liquid-crystalline phase. The intermediate areas characterize the possible coexistence of separate phases. Noticeably, the sharp temperature range of DMPC phase transition is well enclosed in the intermediate area of phase coexistence, while at 30 mol % cholesterol in DMPC the liquid-crystalline valley is never reached, even at 65°C (Fig. 9). Similarly, plots of DLPC vesicles with cholesterol concentrations $\geq$30 mol % are not contained in the liquid-crystalline area.

A further intermediate area can be observed in the surface obtained using the equimolar DLPC-DPPC mixture, where a peak is present, at intermediate temperature, from about 10°C to about 30°C (Fig. 10 *A*). Nevertheless, when the surface is obtained with the data from the same phospholipid mixture but in the presence of cholesterol concentrations from 25 to 60 mol %, the intermediate peak disappears (Fig. 10 *B*).

From Figs. 10 *B* and 11 *B* we can observe that at cholesterol concentrations $\geq$30 mol % a plateau is reached and that the surface is flat. The concentration of cholesterol in most biological membranes is $\geq$30 mol %. Both from our results and from the reported phase diagrams (Vist and Davis, 1990; Sankaram and Thompson, 1990b; Tampé et al., 1991), this is the region of homogeneous liquid-ordered phase. Different concentrations of cholesterol affect differently Laurdan dipolar relaxation and its response to polarity, so that instead of overlapping curves a surface can be obtained.

At low cholesterol concentration, the intermediate area between the gel and the liquid-crystalline regions can be representative both of coexisting domains of liquid-ordered and liquid-disordered phases, i.e., liquid-phase immiscibility, and of a homogeneous environment of intermediate properties. Our analysis based on the wavelength dependence of excitation and emission GP values rules out the possibility of coexisting domains, as reported above. Instead, evidence of solid-phase immiscibility can be found in already reported oxygen quenching experiments (Parasassi and Gratton, 1992) where 10 mol % cholesterol was added to gel phase phospholipids. In this case a biphasic behavior of the Stern-Volmer plot was observed, indicative of different Laurdan

environments. However, when experiments are performed in the liquid-crystalline phase with various cholesterol concentrations linear Stern-Volmer plots were always obtained, with no indications of liquid-phase immiscibility (Fig. 8).

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REFERENCES

Ben-Yasar, V., and Y. Barenholz. 1989. The interaction of cholesterol and cholest-4-en-3-one with dipalmitoylphosphatidyl-choline. Comparison based on the use of three fluorophores. *Biochim. Biophys. Acta*. 985: 271–278.

Ipsen, J. H., G. Karlstrom, O. G. Mouritsen, H. Wennerstrom, and M. H. Zuckermann. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta*. 905:162–172.

Keough, K. M, B. Giffin, and P. L. Matthews. 1989. Phosphatidylcholine-cholesterol interactions: bilayers of heteroacid lipids containing linoleate lose calorimetric transitions at low cholesterol concentration. *Biochim. Biophys. Acta*. 983:51–55.

Lakowicz, J. R., and G. Weber. 1973. Quenching of fluorescence by oxygen. A probe for structural fluctuations in macromolecules. *Biochemistry*. 12: 4161–4170.

Levine, Y. K., and M. H. F. Wilkins. 1971. Structure of oriented lipid bilayers. *Nature New Biol.* 230:69–72.

Melchior, D. L., F. J. Scavitto, and J. M. Steim. 1980. Dilatometry of dipalmitoyl-lecithin-cholesterol bilayers. *Biochemistry*. 19:4828–4834.

Mortensen, N. K., W. Pfeiffer, E. Sackmann, and W. Knoll. 1988. Structural properties of a phosphatidylcholine–cholesterol system as studied by small-angle neutron scattering: ripple structure and phase diagram. *Biochim. Biophys. Acta*. 945:221–245.

Mouritsen, O. G. 1991. Theoretical models of phospholipid phase transitions. *Chem. Phys. Lipids*. 57:179–194.

Nemecz, G., and F. Shroeder. 1988. Time-resolved fluorescence investigation on membrane cholesterol heterogeneity and exchange. *Biochemistry*. 27:7740–7749.

Parasassi, T., F. Conti, M. Glaser, and E. Gratton. 1984. Detection of phospholipid phase separation. A multifrequency phase fluorimetry study of 1:6-diphenyl-1,3,5-hexatriene fluorescence. *J. Biol. Chem.* 259:14011–14017.

Parasassi, T., G. De Stasio, A. d'Ubaldo, and E. Gratton. 1990. Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. *Biophys. J.* 57:1179–1186

Parasassi, T., G. De Stasio, G. Ravagnan, R. M. Rusch, and E. Gratton. 1991. Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. *Biophys. J.* 60:179–180.

Parasassi, T., M. Di Stefano, G. Ravagnan, O. Sapora, and E. Gratton. 1992. Membrane aging during cells growth ascertained by Laurdan generalized polarization. *Exp. Cell Res.* 202:432–439.

Parasassi, T., and E. Gratton, 1992. Packing of phospholipid vesicles studied by oxygen quenching of Laurdan fluorescence. *J. Fluorescence*. 2: 167–174.

Parasassi, T., G. Ravagnan, R. M. Rusch, and E. Gratton. 1993. Modulation and dynamics of phase properties in phospholipid mixtures detected by Laurdan fluorescence. *Photochem. Photobiol.* 57:403–410.

Rubenstein, J. L., B. A. Smith, and H. D. McConnell. 1979. Lateral diffusion in binary mixtures of cholesterol and phosphatidylcholines. *Proc. Natl. Acad. Sci. USA*. 76:15–18.

Sankaram, M. B., and T. E. Thompson. 1990a. Modulation of phospholipid acyl chain order by cholesterol. A solid-state ^2H nuclear magnetic resonance study. *Biochemistry*. 29:10676–10684.

Sankaram, M. B., and T. E. Thompson. 1990b. Interaction of cholesterol with various glycerophospholipids and sphingomyelin. *Biochemistry*. 29: 10670–10675.

Schroeder, F., J. R. Jefferson, A. B. Kier, J. Knittel, T. J. Scallen, W. Gibson Wood, and I. Hapala. 1991. Membrane cholesterol dynamics: cholesterol domains and kinetic pools. *Proc. Soc. Exp. Biol. Med.* 196:235–252.

Shimshick, E. J., and H. M. McConnel. 1973. Lateral phase separation in phospholipid membranes. *Biochemistry*. 12:2351–2360.

Subczynski, W. K., W. E. Antholine, J. S. Hyde, and A. Kusumi. 1990. Microimmiscibility and three-dimensional dynamic structure of phosphatidylcholine-cholesterol membranes: translational diffusion of a copper complex in the membrane. *Biochemistry*. 29:7936–7945.

Tampé, R., A. von Lukas, and H. J. Galla. 1991. Glycophorin-induced cholesterol-phospholipid domains in dimyristoylphosphatidylcholine bilayer vesicles. *Biochemistry*. 30:4909–4916.

van Langen, H., G. van Ginkel, D. Shaw, and Y. K. Levine. 1989. The fidelity response by 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene in time-resolved fluorescence anisotropy measurements on lipid vesicles. *Eur. Biophys. J.* 17:37–48.

Vist, M. R., and J. D. Davis. 1990. Phase equilibria of cholesterol/dipalmitoyl-phosphatidylcholine mixtures; ^2H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry*. 29:451–464.