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Cholesterol Modifies Water Concentration and Dynamics in Phospholipid Bilayers: A Fluorescence Study Using Laurdan Probe

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ABSTRACT The effect of cholesterol on the gel, the liquid-crystalline, and mixed phospholipid phases has been studied using the fluorescence properties of 2-dimethylamino-6-lauroylnaphthalene (Laurdan). Laurdan sensitivity to the polarity and to the dynamics of its environment reveals that cholesterol affects phospholipid bilayers in the gel phase by expelling water and by increasing the amount of dipolar relaxation. In the liquid-crystalline phase, the effect of cholesterol is of a reduction of both water concentration and amount of dipolar relaxation. Detailed studies of Laurdan excitation and emission spectral contours as a function of cholesterol concentration show that there are some cholesterol concentrations at which Laurdan spectral properties changes discontinuously. These peculiar cholesterol concentrations are in agreement with recent observations of other workers showing the formation of local order in the liquid-crystalline phase of phospholipids upon addition of phospholipid derivatives of pyrene. A local organization of phospholipids around cholesterol molecule seems to be produced by the presence of peculiar concentrations of cholesterol itself. This local organization is stable enough to be observed during the excited state lifetime of Laurdan of approximately 5–6 ns.

INTRODUCTION

The relevance of cholesterol for the structural and dynamic properties of cell membranes of most eucaryotic organisms is well recognized. Cholesterol concentration in cell plasma membrane can vary up to 50 mol % with respect to phospholipids and, averaged over various cell types, is of about 30 mol % (Yeagle, 1985). In other cell membranes cholesterol concentration is lower, about 10–20% of the total, so that its structural and dynamic relevance would be expected to affect mainly plasma membrane functions.

The influence of cholesterol on the properties of phospholipid bilayers at a molecular level has been investigated using a variety of spectroscopic techniques such as nuclear magnetic resonance (Vist and Davis, 1990; Sankaram and Thompson, 1990a), electron-spin resonance (Subczynski et al., 1990; Sankaram and Thompson, 1990b), fluorescence (van Langen et al., 1989; Nemecz and Shroeder, 1988), calorimetry (Tampé et al., 1991; Keough et al., 1989), and small-angle neutron scattering (Mortensen et al., 1988), as well as theoretical studies (Ipsen et al., 1987; Kumar, 1991). In particular, the two phospholipid phases are modified by cholesterol. The gel phase is disordered by cholesterol addition, while the liquid-crystalline phase is ordered (Vist and Davis, 1990). As a consequence, in bilayers composed of a single phospholipid the phase transition is no longer detectable when cholesterol is present at concentrations ≥20 mol % (Vist and Davis, 1990; Keough et al., 1989). Moreover, depending on cholesterol concentration, the liquid-crystalline phase can acquire new properties. At low cholesterol concentrations, up to about 25 mol %, the properties of the modified phase have been described as liquid-disordered, whereas at higher concentrations a liquid-ordered phase occurs (Ipsen et al., 1987; Mouritsen, 1991). The liquid-disordered phase is characterized by a decreased rotational motion of phospholipids. In the liquid-ordered phase the lateral diffusion is also decreased (Ipsen et al., 1987; Mouritsen, 1991). Other properties of the phospholipid bilayer are modified by the presence of cholesterol. The permeability of liposomes to small molecules and ions is decreased, and the bilayer thickness and compressibility are increased (Yeagle, 1985).

In this work we utilized the spectral sensitivity of the membrane fluorescent probe 2-dimethylamino-6-lauroylnaphthalene (Laurdan) to the phase state and to the polarity of phospholipid bilayers to investigate the effect of various cholesterol concentrations from 1 to 60 mol % on the dynamic properties of the membrane. Laurdan is anchored to the membrane by its lauric acid tail, and its fluorescent naphthalene residue is located at the level of the glycerol backbone (Parasassi et al., 1990; Chong, 1988). Fluorescence excitation and emission spectra of Laurdan differ in the two phospholipid phases. A 50-nm red shift is observed in Laurdan emission by passing from the gel to the liquid-crystalline phase, with the maximum emission varying from 440 nm to 490 nm. Of the two excitation bands, the blue, centered at about 350 nm, shows the maximum excitation intensity in the liquid-crystalline phase, while the red, centered at about 390 nm, has the maximum excitation intensity in the gel phase (Parasassi et al., 1990). These Laurdan properties depend on the rate of dipolar relaxation (Weber and Farris, 1979) and to the polarity of the environment (Parasassi et al., 1990). In apolar solvents, both the emission and the excitation spectra are blue-shifted. Moreover, by increasing the polarity of solvents a second, red excitation band

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appears (Parasassi et al., 1991). Using Laurdan steady-state excitation and emission spectral properties we can monitor the influence of cholesterol on the bilayer hydration and on the molecular dynamics of phospholipids in the presence of various cholesterol concentrations.

MATERIALS AND METHODS

Multilamellar phospholipid vesicles were prepared by mixing chloroform solutions of phospholipids (dilauroyl-, dimyristoyl-, and dipalmitoylphosphatidylcholine; 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) and then evaporating the solvent by nitrogen flow. The dried samples were resuspended in phosphate-buffered saline solution (PBS, Sigma Chemical Co.), 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.

Laurdan excitation and emission spectra were obtained using a GREG 200 fluorometer, equipped with photon counting electronics (PX01, ISS Inc., Champaign, IL) and the accompanying software. A xenon arc lamp was used as the light source. Monochromator bandpass was 4 nm. The spectra were only corrected for lamp intensity variations. The temperature in the sample compartment was controlled to $\pm$0.1°C by a water circulating bath.

RESULTS

Laurdan excitation and emission spectra were measured in multilamellar vesicles composed of DLPC, DMPC, and an equimolar mixture of DLPC and DPPC, with cholesterol concentrations varying from 0 to 60 mol %, at temperatures from 1°C to 65°C. In vesicles composed of phospholipids in the liquid-crystalline phase, such as DLPC, DMPC above 25°C, and in the equimolar DLPC and DPPC mixture above 35°C, without cholesterol, Laurdan excitation and emission spectra display the typical features of this liquid-crystalline phase (Parasassi et al., 1990). The excitation spectrum shows a maximum at about 350 nm, corresponding to the blue excitation band, and the emission spectrum shows an increased intensity at 490 nm. As previously reported (Parasassi et al., 1990), the ratio between the intensity at 440 nm and 490 nm depends on the phospholipid phase state and, in the liquid-crystalline phase, depends slightly also on temperature. The same dependence on the phospholipid phase state and on temperature influences the ratio of the excitation intensity between 390 and 350 nm. In vesicles composed of phospholipids in the gel phase, such as DMPC below about 20°C and DLPC/DPPC equimolar mixture below 5°C, Laurdan typically displays a maximum excitation at 390 nm, the red excitation band, and a maximum emission at 440 nm with very little intensity at 490 nm (Parasassi et al., 1990). In the phospholipid gel phase, where the dipolar relaxation phenomenon is negligible, the effect of polarity can be isolated from the effect of dipolar relaxation. A decrease in polarity induces a blue shift of both the excitation and emission spectra and the progressive disappearance of the red excitation band (Parasassi et al., 1991).

The addition of cholesterol to phospholipids produces modifications of both the excitation and emission spectra of Laurdan. The type and amount of these changes depend on the phospholipid phase state and on cholesterol concentration. In the pure gel phase, such as DMPC vesicles at 1°C, the addition of cholesterol causes a small decrease of the red excitation band, centered at about 390 nm, and a consequent blue shift of the spectrum center of mass (Fig. 1 *A*). Instead, in the liquid-crystalline phase and in the mixture of the two phases, the addition of cholesterol induces an increase of the red excitation band. As a representative example, Fig. 2 shows Laurdan excitation and emission spectra in DLPC vesicles at 25°C with increasing cholesterol concentrations up to 25 mol %. As for Laurdan emission spectra, in both phospholipid phases the addition of cholesterol causes a blue shift (Fig. 1 *B*), particularly relevant in the liquid-crystalline phase (Fig. 2). In general, the effects of cholesterol on the excitation and emission spectra of Laurdan in phospholipid vesicles are much more relevant in the liquid-crystalline phase than in the gel phase. Moreover, these effects are not a linear function of cholesterol concentration.

The absence of proportionality between the amount of spectral shift and cholesterol concentration is particularly evident when the results are shown as contour plots in which,

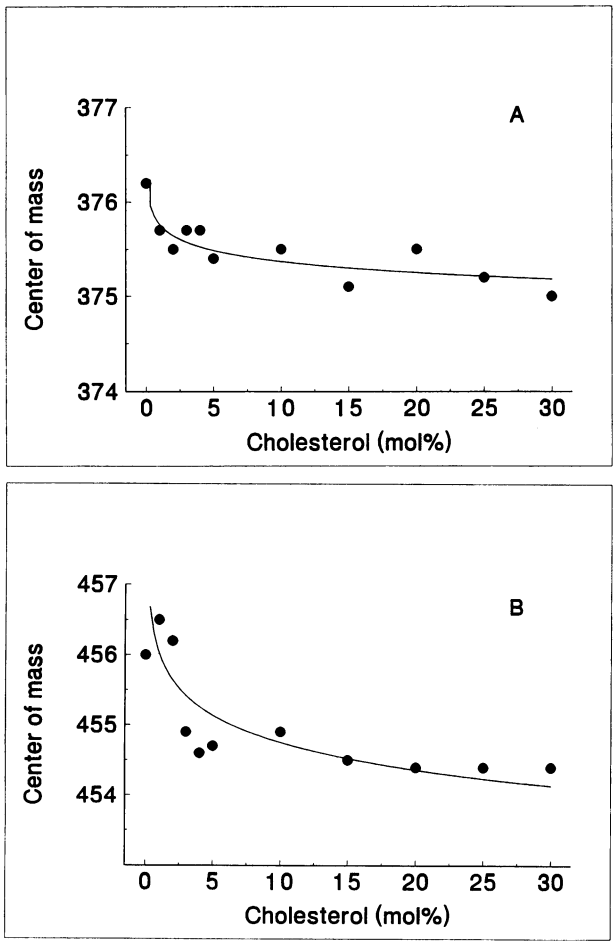

FIGURE 1 Variation of Laurdan spectral center of mass in DMPC vesicles at 1°C as a function of cholesterol concentration in DMPC. (*A*) excitation spectra; (*B*) emission spectra. The solid line has been drawn to better highlight the variation of the center of mass and does not represent a curve fitting.

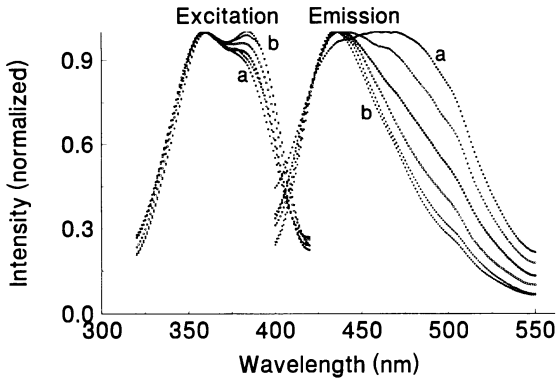

FIGURE 2 Normalized Laurdan excitation and emission spectra in DLPC vesicles at 25°C at different cholesterol concentrations. Cholesterol mol % with respect to DLPC, from *a* to *b*: 0, 5, 10, 15, 20, and 25.

at a given temperature, the points with the same excitation or emission intensities obtained in samples of different cholesterol concentration and at the different wavelengths are joined by a line (Figs. 3–5). These contour plots can be seen

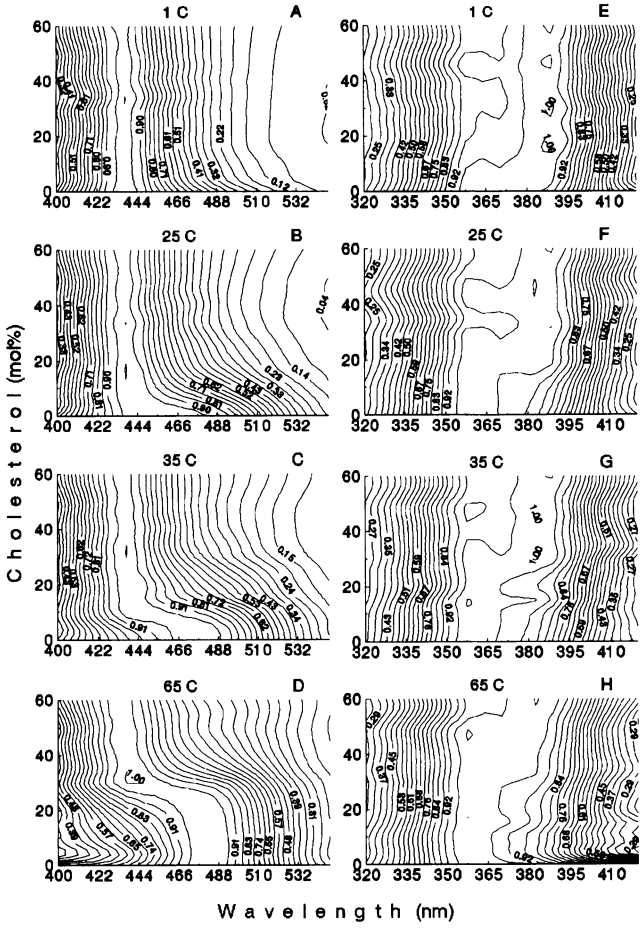

FIGURE 3 Contour plots of the normalized emission (*A* to *D*) and excitation (*E* to *H*) spectra of Laurdan in DLPC vesicles with cholesterol concentrations of 0, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 mol %. Spectra acquired at 1°C (*A* and *E*), 25°C (*B* and *F*), 35°C (*C* and *G*), and 65°C (*D* and *H*).

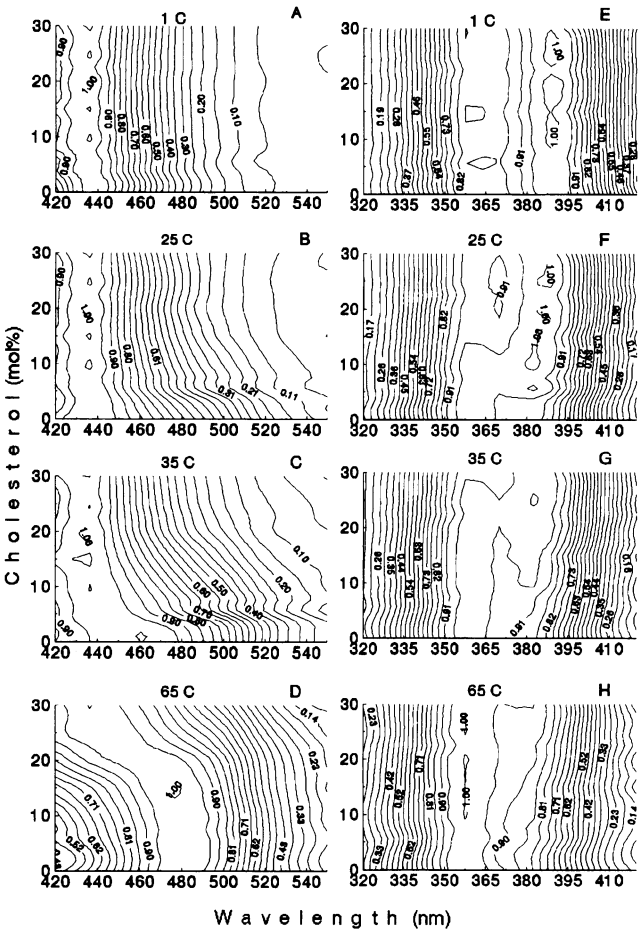

FIGURE 4 Contour plots of the normalized emission (*A* to *D*) and excitation (*E* to *H*) spectra of Laurdan in DMPC vesicles with cholesterol concentrations of 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 mol %. Spectra acquired at 1°C (*A* and *E*), 25°C (*B* and *F*), 35°C (*C* and *G*), and 65°C (*D* and *H*).

as the plane projection of a three-dimensional plot of intensity, wavelength, and cholesterol concentration. Focusing our attention to the contour plots obtained at high temperature, ≥35°C, we can observe that major modifications of the emission shape are observed for the DLPC sample at 15 mol % cholesterol at 35°C (Fig. 3 *C*) and at 30 mol % cholesterol at 65°C (Fig. 3 *D*). In the DMPC sample, major modifications are observed at 5 mol % cholesterol at 35°C (Fig. 4 *C*) and at about 25 mol % cholesterol at 65°C (Fig. 4 *D*). The equimolar DLPC and DPPC sample shows major modifications of the emission maximum at 5 mol % cholesterol at 35°C (Fig. 5 *C*) and at 30 mol % cholesterol at 65°C (Fig. 5 *D*). The excitation spectra show a more homogeneous behavior, with major modifications of the relative intensity of the two excitation bands at 30 mol % cholesterol both for the experiments at 35°C and 65°C for all samples (Figs. 3–5, *G* and *H*). At lower temperatures these discontinuity points are less evident. Nevertheless, critical modifications of both excitation and emission spectra can be identified for the DLPC (Fig. 3) and DMPC (Fig. 4) samples at 1°C at ~5 mol % cholesterol, as for the DLPC/DPPC sample emission spectra

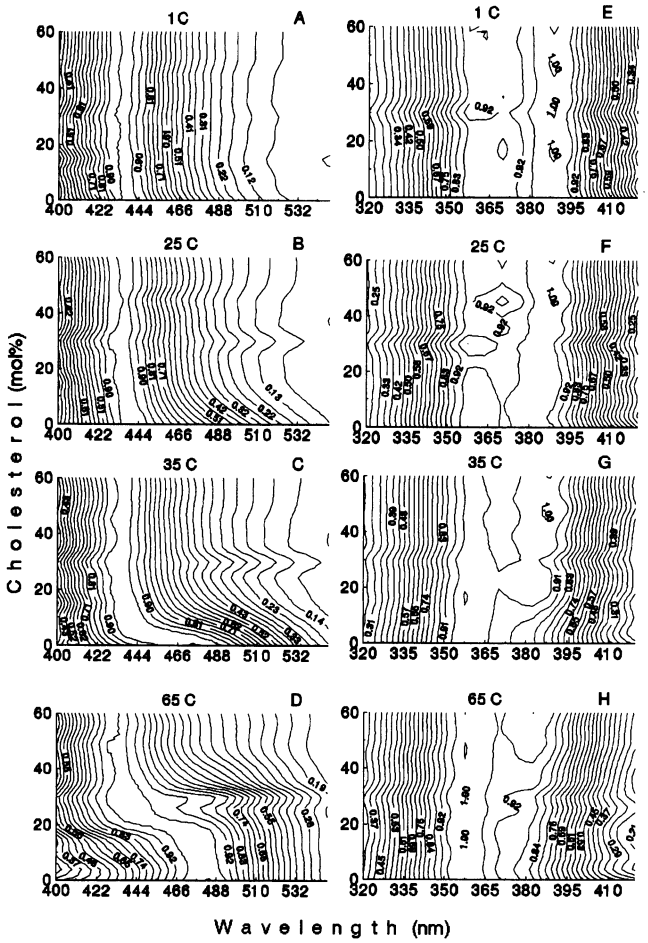

FIGURE 5 Contour plots of the normalized emission (*A* to *D*) and excitation (*E* to *H*) spectra of Laurdan in vesicles composed of the equimolar DLPC and DPPC mixture with cholesterol concentrations of 0, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 mol %. Spectra acquired at 1°C (*A* and *E*), 25°C (*B* and *F*), 35°C (*C* and *G*), and 65°C (*D* and *H*).

(Fig. 5 *A*). Instead, this last sample excitation shows a discontinuity point at 30 mol % cholesterol (Fig. 5 *E*). At 25°C discontinuities can be observed at about 5–10 mol % cholesterol for the emission spectra of the three samples, both at 15 and 30 mol % cholesterol for the DLPC excitation (Fig. 3 *F*), at 15 mol % cholesterol for the DMPC excitation (Fig. 4 *F*), and at 30 mol % cholesterol for the DLPC/DPPC excitation (Fig. 5 *F*). This last sample shows a peculiar behavior of both excitation and emission shape at 30 mol % cholesterol at the four examined temperatures (Fig. 5).

DISCUSSION

Laurdan spectroscopic properties reflect the probe sensitivity to the polarity and to the dynamics of its environment. In apolar solvents, such as hexane or cyclohexane, Laurdan emission and excitation spectra are blue-shifted with respect to more polar solvents (Parasassi et al., 1991). Along with a red shift of its excitation and emission spectra, in hydrogen bonding solvents a second excitation band, centered at about 390 nm, appears. This second red excitation band has been reported to originate from the stabilization of a Laurdan ground state conformation, Lα, characterized by a favorable orientation of the solvent molecular dipoles surrounding the probe dipole and is particularly intense in phospholipid bilayers in the gel phase (Parasassi et al., 1991). A further process that affects Laurdan fluorescence is due to the molecular dynamics of solvent molecules in the vicinity of the probe. Laurdan excited state dipole, several Debyes greater than its ground state dipole, can orient solvent dipoles possessing molecular dynamics of the same order of magnitude as its excited state lifetime. This process is known as dipolar relaxation, and the energy required for the reorientation of solvent molecules is reflected in the red shift of Laurdan emission. If the rate of relaxation is very small, such as in the phospholipid gel phase, the emission spectral shift is small (Parasassi et al., 1990). In the phospholipid liquid-crystalline phase, relaxation processes can occur at a much faster rate, and this process gives rise to a very large emission red shift (Parasassi et al., 1990). Briefly, Laurdan excitation spectrum is strongly influenced by the polarity of its local environment, with bluer spectrum in apolar solvents and with an additional excitation band due to probe molecules stabilized in the Lα conformation (Parasassi et al., 1991). A small blue shift of the emission is also observed in apolar solvents, but major modifications of Laurdan emission spectrum are due to the dipolar relaxation phenomenon.

In the gel phase of phospholipid vesicles the dipolar relaxation rate is very small so that its emission maximum is at about 440 nm. In this gel phase the fraction of Laurdan molecules stabilized in the Lα ground state conformation is relevant, so that the maximum excitation is at 390 nm. Increasing cholesterol concentration in phospholipid bilayers in the gel phase, i.e., in the absence of dipolar relaxation, modify Laurdan excitation and emission by changing the local polarity in the vicinity of the fluorescent moiety of the probe. A decrease in Laurdan excitation and emission center of mass as a function of cholesterol concentration is shown in Fig. 1. By comparison with experiments performed in organic solvents (Parasassi et al., 1991), this blue shift of Laurdan spectra indicates a decreased polarity of its environment. This result is in agreement with previous observations (Levine and Wilkins, 1971) reporting a decreased hydration of the bilayer in the presence of cholesterol.

In the liquid-crystalline phase of phospholipid bilayers and without cholesterol, the rate of dipolar relaxation is high, and Laurdan emission displays a maximum at about 490 nm (Parasassi et al., 1990). The population of the second excitation band is decreased and the excitation maximum is at about 350 nm. The addition of cholesterol decreases the amount of dipolar relaxation (Fig. 2), producing a relevant blue shift of Laurdan emission and an increase in the intensity of the red excitation band. Because of the effect of the variation induced by cholesterol on the rate of Laurdan relaxation, the effects on the polarity cannot be isolated.

The dipolar relaxation phenomenon depends upon the molecular dynamics of the environment of the naphthalene moiety of the Laurdan molecule. The chemical nature of phospholipid polar heads does not influence Laurdan relaxation, as shown by experiments performed using different phospholipids at pH values between 4.0 and 10.0 (Parasassi et al., 1991). The molecular origin of the dipolar relaxation has been attributed to a few water molecules present at the hydrophobic-hydrophilic interface of the bilayer, close to Laurdan fluorescent moiety, whose dynamics changes in the different phases. Specifically, the relaxation time of these water molecules is of the same order of magnitude of Laurdan fluorescence lifetime if the bilayer is in the liquid-crystalline phase. In the gel phase, water rotation is slower, so that the dipoles cannot reorient during Laurdan excited state. Also relevant for the discussion of the presented data is the fact that the fluorescence properties of Laurdan do not depend upon a preferential partition of the probe in one phospholipid phase or upon a selective association with cholesterol. Laurdan equally partitions between the two phospholipid phases, and no evidence of its association with cholesterol have been found (Parasassi et al., 1993, 1994).

Our results indicate that cholesterol reduces the polarity of phospholipid bilayers and decreases the relaxation rate of water molecules present at the hydrophobic-hydrophilic interface of the bilayer. From the behavior of Laurdan relaxation in the two phospholipid phases (Parasassi et al., 1990) we showed that decreased molecular dynamics of these water molecules must be related to decreased dynamics of phospholipid molecules in the bilayer.

Additional insights on the influence of cholesterol on phospholipid bilayers can be achieved by observing the contour plots where the points with the same spectral intensity are joined for samples of different cholesterol concentration and for wavelength (Figs. 3–5). Variations in the wavelength excitation and emission maximum and in the spectral shape reveal that the effect of cholesterol is not proportional to its concentration. Continuous modification of the spectral shape can be observed up to certain critical cholesterol concentrations, after which a plateau is reached. In some samples more than one critical cholesterol concentration is present, which depends on temperature. In general, at least three different cholesterol concentrations can be identified at the various temperatures and in the different samples, producing abrupt changes in the spectral maximum and shape. These concentrations are about 5, 15, and 30 mol % cholesterol with respect to phospholipids. These cholesterol concentrations correspond to critical points in reported phase diagrams of DMPC (Tampé et al., 1991) and DPPC (Vist and Davis, 1990; Sankaram and Thompson, 1990b) and cholesterol that have been used to determine the separation between the different phases, namely the liquid-crystalline, the liquid-disordered, and the liquid-ordered phases.

Of particular interest are the results obtained using vesicles composed of the equimolar mixture of DLPC and DPPC (Fig. 5). In this sample, the presence of 30 mol % cholesterol produces a broadening of the emission spectrum and a blue shift of the excitation spectrum. From our understanding of Laurdan spectroscopy, a blue shift of excitation spectrum indicates a less polar environment. The interpretation of the broadening of emission spectra is not immediate. The increase in the intensity in the blue part of the emission spectrum can be observed in cases of decreased polarity, in agreement with the blue shift of the excitation spectrum. Instead, the increase in intensity in the red part of the emission spectrum indicates an increased rate of dipolar relaxation. When dipolar relaxation rate increases, all of the emission spectrum is red-shifted, so that also the intensity in its blue part decreases. Then, an increased rate of dipolar relaxation, i.e., an increased molecular motion of phospholipid molecules in the presence of 30 mol % cholesterol, cannot explain our results. We propose that this particular value of cholesterol concentration decreases the phospholipid molecular motion. The equimolar DLPC/DPPC mixture, in the absence of cholesterol, is composed of coexisting phases, one with properties close to the gel and the other close to the liquid-crystalline, each slightly modified by the presence of the other (Parasassi et al., 1993). Fluctuation between these two phases has been determined, with a rate of about 20×10^9 sec^{-1} (Parasassi et al., 1990; Parasassi et al., 1993). This fluctuation rate must be decreased by the presence of 30 mol % cholesterol, since the emission spectrum is broader both in the blue and in the red part. At the same time, water concentration in the hydrophobic-hydrophilic interface of the bilayer is decreased due to a tighter packing of phospholipid molecules, and the Laurdan excitation spectrum is blue-shifted.

The critical cholesterol concentrations, identified by the analysis of Laurdan emission and excitation spectral shifts, are in close agreement with concentration values determined by two different kinds of observations. First, by constructing phospholipid/cholesterol phase diagrams (Vist and Davis, 1990; Sankaram and Thompson, 1990b; Tampé et al., 1991), the same cholesterol concentrations have been identified as the points were the phase properties change. Second, by measuring the excimer/monomer ratio of pyrene-PC in various phospholipid bilayers, a hexagonal super-lattice organization of the phospholipid/pyrene-PC has been proposed to explain discontinuities observed in the excimer/monomer ratio at peculiar pyrene-PC concentrations (Somerharju et al., 1985; Tang and Chong, 1992). These concentrations are surprisingly similar to the critical cholesterol concentrations observed in this work. In these two-component systems, composed of a single phospholipid and pyrene-PC (Somerharju et al., 1985; Tang and Chong, 1992) or of a single phospholipid with cholesterol (Vist and Davis, 1990; Sankaram and Thompson, 1990b; Tampé et al., 1991), the introduction of a molecule perturbing the lateral arrangement of the bilayer induces the formation of a new arrangement that minimizes the free energy of the interaction between the components. Particularly at about 30 mol % of cholesterol or of pyrene-PC, the hexagonal super-lattice appears to be particularly stable, so that the kinetics of lateral diffusion of

phospholipids is decreased. From our results, also phospholipid molecular packing appears to be tighter, with a concomitant reduced axial motion, as indicated by the decreased water concentration at the level of Laurdan naphthalene moiety. The hexagonal super-lattice organization can be hardly expected in natural membranes, for the heterogeneity of their lipid composition and for the presence of proteins. Nevertheless, the results of our study can help to clarify, at a molecular level, the importance of cholesterol in regulating the lateral diffusion and the permeability of the bilayer and confirm previous observations on critical host molecule concentrations on the organization and dynamics of lipid bilayers. The observation of ripples in the contour plots provides a useful method for the identification of critical cholesterol concentrations.

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