Cholesterol at Different Bilayer Concentrations Can Promote or Antagonize Lateral Segregation of Phospholipids of Differing Acyl Chain Length†

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Received June 26, 1996; Revised Manuscript Received September 13, 1996⊗

ABSTRACT: Fourier-transform infrared-spectroscopic and fluorescence measurements have been combined to examine the effect of cholesterol on the intermixing of short-chain dilauroyl phosphatidylcholine (DLPC) and its bromo-substituted derivative (12BrPC) with longer-chain (C16- or C18-) phosphatidylcholines (PCs) in hydrated lipid bilayers. Infrared spectroscopy of mixtures combining protonated DLPC or 12BrPC with chain-perdeuterated dipalmitoyl PC reveals that cholesterol at lower concentrations in the bilayer modifies the resolved thermal melting profiles for both phospholipid components and, at high bilayer concentrations, produces a convergence of the thermal transitions for the two PC species. Fluorescence-quenching measurements using a short-chain fluorescent PC (1-dodecanoyl-2-[8-[*N*-indolyl]octanoyl] PC) in ternary mixtures combining 12BrPC, dipalmitoyl or distearoyl PC, and cholesterol confirm that very high cholesterol levels (50 mol %) abolish the lateral segregation of the PC components at 25 °C, a temperature where the phospholipids extensively phase-separate in the absence of sterol. By contrast, under these same conditions cholesterol at lower concentrations in the bilayer is found to *enhance* the tendency of the PC components to exhibit lateral segregation. We show that these seemingly contradictory effects of cholesterol can be readily explained in the light of a ternary phase diagram that is fully consistent with our current understanding of the nature of cholesterol−phospholipid interactions in binary mixtures.

INTRODUCTION

Cholesterol is a major component of the plasma and certain intracellular membranes of mammalian cells, where it coexists with a variety of phospho- and sphingolipid species with diverse structures and physical properties (Yeagle, 1985). An understanding of the physical behavior of heterogeneous lipid systems including cholesterol is essential to elucidate various important properties of cholesterol-containing biological membranes, including such aspects as membrane mechanical and thermotropic properties (Needham et al., 1988), lateral diffusion of membrane components (Almeida et al., 1993), and the possible existence of segregated membrane domains with distinct lipid compositions (Simons & van Meer, 1988).

The interaction between phospholipids and cholesterol has been extensively studied using various physical methods [reviewed in Yeagle (1985), Vist and Davis (1990), Finegold (1993)]. A variety of previous studies have endeavoured to elucidate the phase diagrams for binary mixtures combining cholesterol with different saturated and unsaturated phosphatidylcholines (PCs) (Recktenwald & McConnell, 1981; Lentz et al., 1980; Yeagle, 1985; Vist & Davis, 1990; Sankaram & Thompson, 1991; Thewalt & Bloom, 1992; Huang et al., 1993; McMullen & McElhaney, 1995). A particularly interesting feature of most such phase diagrams is the coexistence of a cholesterol-enriched "intermediate" phase (often called the liquid-ordered [l_o] phase) that coexists with a cholesterol-depleted liquid-crystalline ("liquid-disordered" [l_d]) or a gel phase over a wide range of temperatures and sterol contents (Ipsen et al., 1987, 1989). Several previous studies have also examined the phase behavior of cholesterol-containing ternary mixtures (de Kruyff et al., 1973, 1974; Verkleij et al., 1974; van Dijck et al., 1976; Demel et al., 1977; Cullis et al., 1978; Cullis & de Kruijff, 1978; van Dijck, 1979; Tilcock et al., 1982; Finegold & Singer, 1991; Silvius, 1992; Almeida et al., 1993; Parasassi et al., 1994). These studies have shown that cholesterol at high levels eliminates the cooperative thermotropic "melting" of both phospholipid components in such mixtures, similar to the results obtained for binary cholesterol/phospholipid systems. However, only a few of these studies have employed methods that can assess whether cholesterol promotes a microscopically homogeneous intermixing of the components in ternary (or higher-order) systems, an important issue for systems where the nonsterol components exhibit lateral segregation in the absence of sterol.

Previous work has shown that when present in the bilayer at high levels (ca. 50 mol %), cholesterol can promote microscopically homogeneous intermixing of monounsaturated and disaturated phosphatidylcholines at temperatures where the two phospholipids exhibit extensive (solid/fluid) lateral phase separation in the absence of sterol (Silvius, 1992). However, this and previous studies (Ruocco & Shipley, 1984; Johnston & Chapman, 1988) also revealed that even high levels of cholesterol cannot induce complete miscibility of unsaturated phosphatidylcholines with saturated galactosylsphingolipids, demonstrating that cholesterol is not invariably able to suppress the tendency of different polar membrane lipids to undergo lateral segregation.

In the present study we have combined infrared-spectroscopic and fluorescence-quenching methods to examine the ability of cholesterol to favor homogeneous lipid mixing in

† This work was supported by grants from les Fonds FCAR du Quebec (to J.R.S. and M.L.) and from the Natural Sciences and Engineering Research Council of Canada (to M.L.).

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⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

S0006-2960(96)01550-4 CCC: $12.00 © 1996 American Chemical Society

a different type of phase-separating system, consisting of mixtures of saturated phosphatidylcholines that exhibit extensive lateral phase separations based on differences in acyl chain length. Our results indicate that very high bilayer levels of cholesterol (ca. 50 mol %) can promote homogeneous intermixing of such phospholipids. However, we also find that in these systems cholesterol at lower bilayer concentrations can actually promote *demixing* of the phospholipid components. We propose that the latter effect reflects the unequal partitioning of the different phospholipid components between liquid-disordered (sterol-depleted) and liquid-ordered (sterol-enriched) phases that coexist at intermediate cholesterol concentrations in the bilayer.

MATERIALS AND METHODS

Materials. Dilauroyl, dipalmitoyl, and distearoyl PC (DLPC, DPPC, and DSPC),[1] 1-palmitoyl-2-oleoyl-PC (POPC), 1-dodecanoyl-*lyso*-PC, and perdeuterated DPPC (DPPC-d_{62}) were obtained from Avanti Polar Lipids (Alabaster, AL). 1-Lauroyl-2-(12-bromododecanoyl) phosphatidylcholine (12BrPC) was synthesized by acylation of 1-dodecanoyl-*lyso*-PC with the anhydride of 12-bromododecanoic acid using 4-pyrrolidinopyridine as catalyst (Mason et al., 1981). 1-Dodecanoyl-2-(8-[*N*-indolyl]octanoyl)-PC ((12/8-Ind)-PC) was synthesized as described previously (Shin et al., 1991). Cholesterol was obtained from Sigma Chemicals (St. Louis, MO) or NuChek Prep (Elysian, MN).

Infrared Spectroscopy. Mixed-lipid samples were prepared by mixing appropriate volumes of stock solutions (in benzene/methanol, 95/5 v/v); the samples were then rapidly frozen and the solvent removed by freeze-drying under vacuum overnight. Lipid bilayers were prepared by hydrating the lyophilized lipid mixtures in 100 mM NaCl, 10 mM HEPES, 5 mM EDTA, pH 7.4, to obtain a final concentration of 20% (w/w) lipid. The samples were heated and vortexed while in the fluid phase to ensure complete dispersion. FT-IR spectra were recorded with samples freshly hydrated as just described. Alternatively, 12BrPC-containing samples initially hydrated as just described were cooled to 4 °C, incubated at this temperature for 14 days, warmed to 20 °C, and incubated at the latter temperature for 72 h. For spectral collection lipid dispersions were placed between two CaF_2 windows separated by a 5 μm Teflon spacer. Spectra were recorded on a Bio-Rad FTS-25 infrared spectrometer, equipped with a globar source, a KBr beam splitter, and a mercury cadmium telluride broad band detector. Two hundred and fifty-six interferograms, with a resolution of 2 cm^{-1}, were collected, added, and Fourier transformed to yield spectra. Sample temperature was controlled by thermopumps (Pézolet et al., 1983) and monitored with a thermodetector next to the windows.

Original spectra were corrected for the contribution of the hydrating buffer by subtraction of water bands. In the case of the C−H bands, a polynomial fitted along the shoulder of the ν_{O-H} water band at 3395 cm^{-1} was subtracted. The C−D bands, superimposed on the water association band near 2120 cm^{-1}, were corrected by subtracting a buffer spectrum taken at an equivalent temperature. Spectra were also corrected for a cholesterol contribution, by subtracting a spectrum of pure cholesterol in a KBr pellet. Cholesterol bands at 1388 and 1365 cm^{-1} were used as an internal standard for the subtraction, as described elsewhere (Kodati & Lafleur, 1993).

Fluorescence Quenching. Lipid samples containing 2 mol % (12/8-Ind)-PC (440 nmol of total lipid in 200 μL of chloroform or 4:1 chloroform/methanol)[2] were rapidly dried down under nitrogen with warming to 45 °C. The samples were freed of residual solvent under high vacuum for a minimum of 6 h and then hydrated by vortexing in 440 μL of sterile 150 mM NaCl, 2 mM Tes, 0.1 mM EDTA, pH 7.4, at 48 °C except for samples containing DSPC (hydrated at 58 °C). The samples were then cooled at ca. 1 °C/min to 40 °C and at 0.2 °C/min from this temperature to 4 °C. Samples were maintained at the latter temperature for 14 days and then warmed to 25 °C (at ca. 0.2 °C/min) and incubated further for 3 or 7 days (with equivalent results). The fluorescence of replicate aliquots of such samples was read on a Perkin-Elmer LS-5 spectrofluorimeter (excitation/emission wavelengths 281/321 nm, slit settings 10 nm/10 nm) in 3 mL of either buffer or methanol; after appropriate blank corrections the former readings were normalized to the latter, which provided a measure of the amount of probe in each sample.

RESULTS

Infrared-Spectroscopic Studies. DLPC/DPPC-d_{62} Binary Mixtures. The system DLPC/DPPC system is a relatively simple one, whose phospholipid components differ only in acyl chain length. Previously reported phase diagrams (obtained by calorimetry or by probe techniques) indicate significant non-ideality in the mixing of saturated phospholipids whose chains differ in length by four or more carbon atoms (Mabrey & Sturtevant, 1976; van Dijck et al., 1977; Lee, 1977). This non-ideality can be readily characterized using vibrational spectroscopy to study the thermotropism of the pure lipids and of their mixtures, monitoring the transition from a more rigid gel phase to a more fluid liquid-crystal phase. For lipids with protonated acyl chains an abrupt shift of about 4 cm^{-1} in the C−H stretching frequency is observed at the phase transition temperature (T_m), reflecting mainly the increase in conformational disorder of the acyl chains at the transition (Mantsch & McElhaney, 1991). An analogous behavior at the phase transition is observed in the C−D stretching region for chain-perdeuterated lipids (Mendelsohn & Koch, 1980). However, as illustrated in Figure 1, the isotopic substitution moves the C−H stretching frequencies from 2920 and 2850 to 2195 and 2090 cm^{-1} for

[1] Abbreviations: 12BrPC, 1-dodecanoyl-2-(12-bromododecanoyl) phosphatidylcholine; (12/8-Ind)-PC, 1-dodecanoyl-2-(8-[*N*-indolyl]octanoyl) phosphatidylcholine; DLPC, 1,2-dilauroyl phosphatidycholine; DPPC, 1,2-dipalmitoyl phosphatidylcholine; DPPC-d_{62}, 1,2-di(perdeuteropalmitoyl) phosphatidylcholine; DSPC, 1,2-distearoyl phosphatidylcholine; PC, phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid trisodium salt; HEPES, *N*-(2−hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) sodium salt; (FT-)IR, (Fourier transform) infrared spectroscopy; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; Tes, (*N*-tris[hydroxymethyl]methyl)-2-aminoethanesulfonic acid sodium salt.

[2] For practical reasons it was not possible to prepare the large numbers of samples required for the fluorescence studies by lyophilization. However, using the protocol described in Materials and Methods it was verified that the fluorescence quenching curves measured for cholesterol-containing DPPC/12BrPC or POPC/12BrPC samples were not altered either by prolonging the time of sample incubation at 25 °C (from 3 to 7 days) or by changing the solvent from which the lipids were initially dried down (chloroform, 4:1 chloroform/methanol or 1:1 chloroform/methanol).

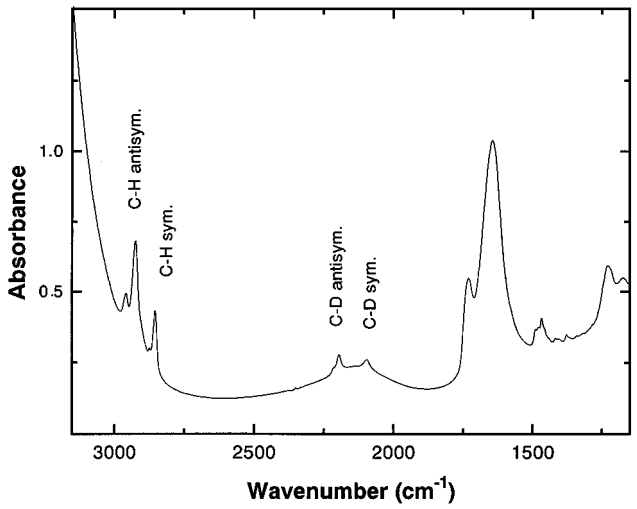

FIGURE 1: Infrared spectrum of a binary (80:20) mixture of DLPC and DPPC-d_{62} at 20 °C, showing C−H and C−D symmetric and antisymmetric methylene stretching bands.

the asymmetric and symmetric stretching bands, respectively, such that the C−H and C−D bands in a mixture of protonated and deuterated components can be monitored separately in a single spectrum. Using a binary mixture of protonated and chain-perdeuterated phospholipids (here, DLPC and DPPC-d_{62}) it is thereby possible to monitor simultaneously and independently the behavior of each component, allowing assessment of the miscibility of the components based on the (dis)similarity of their thermotropic behavior. This approach has previously been proven useful to examine binary lipid mixtures in the presence of peptides, proteins, or ions (Mendelsohn & Taraschi, 1978; Kouaouci et al., 1985; Laroche et al., 1988; Lafleur et al., 1989).

IR-derived temperature profiles of acyl chain melting are shown in Figure 2 for a series of DLPC/DPPC-d_{62} mixtures spanning the entire composition range, plotting both the C−H (non-deuterated, low-melting DLPC) and C−D (perdeuterated, high-melting DPPC-d_{62}) symmetric stretching frequencies as a function of temperature. The sharp phase transitions of the two pure components (Figures 2A and 2F) correspond to the reported T_m's for these species (Marsh, 1990). The melting profile for pure DLPC (Figure 2A) reflects the unusual thermotropic behavior reported previously for this species: its passage from an ordered gel to a liquid-disordered phase appears by calorimetry to be a bimodal phase transition, with a sharp peak at −2 °C and a broad endotherm near 5 °C (Mabrey & Sturtevant, 1976; Finegold et al., 1990). While the nature of this complex transition is still under investigation (Morrow & Davis, 1987; Finegold et al., 1990), vibrational spectroscopy seems to be sensitive to both parts of the transition (Huang et al., 1982), and our results are consistent with this. For the mixtures of DLPC and DPPC-d_{62} (20, 40, 60, and 80 mol % DPPC-d_{62}, Figures 2B−E), the melting profiles for the DLPC and DPPC-d_{62} components have been superimposed to better illustrate how IR spectroscopy monitors the thermotropic behavior of each lipid. While the curves for the mixed-lipid samples show the broadened transition that is expected for any mixture, the effect is markedly different for the two components. The transition of the higher-melting (DPPC-d_{62}) component appears to remain relatively sharp in the mixed-lipid samples, while that of the lower-melting (DLPC) component rapidly becomes spread out over a larger range of temperatures as increasing amounts of DPPC-d_{62} are incorporated. As discussed below, the distinct behaviors of the melting profiles

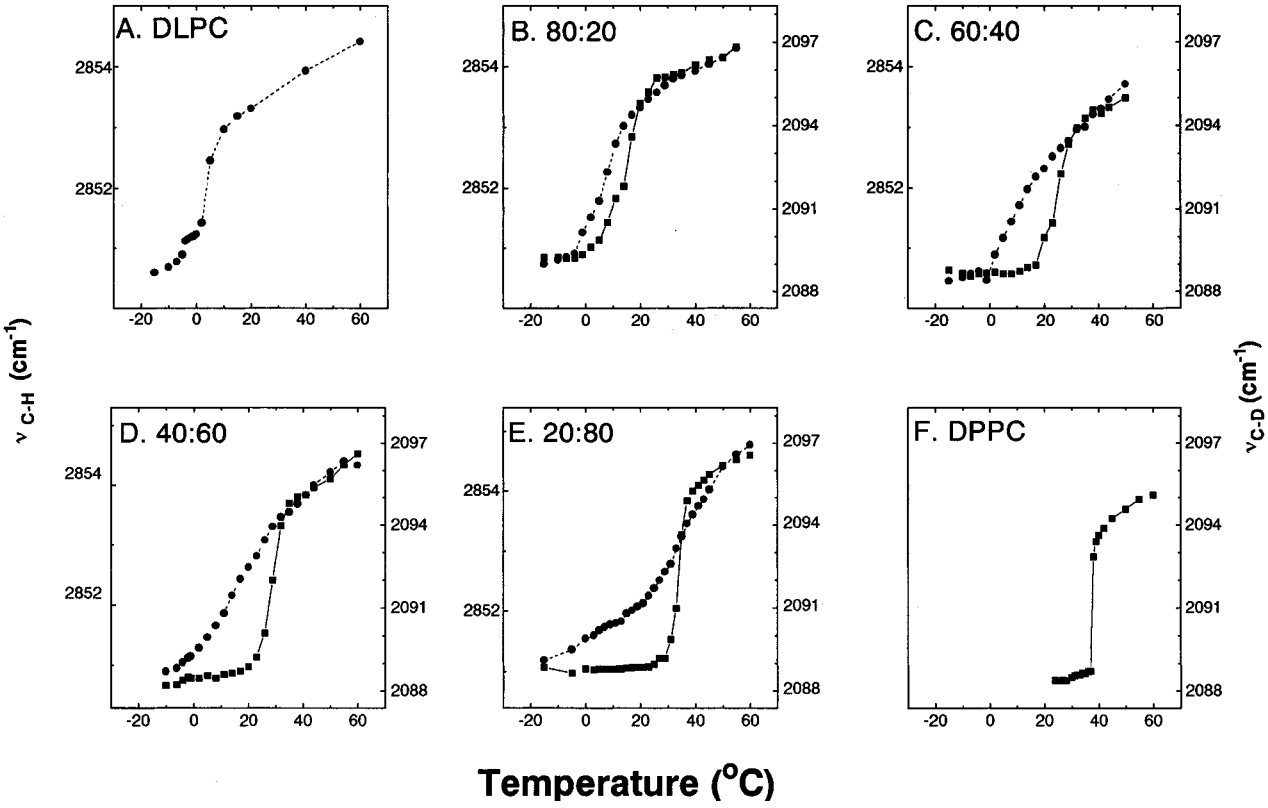

FIGURE 2: Frequency shift of the methylene symmetric stretching mode with temperature for mixtures of DLPC and DPPC-d_{62} in different proportions: (A) pure DLPC; (B) 80:20, (C) 60:40, (D) 40:60, and (E) 20:80 (molar proportions) DLPC/DPPC-d_{62}; (F) pure DPPC-d_{62}. The left-hand axes correspond to the C−H (DLPC) stretching frequency (●), while the right-hand axes indicate the C−D (DPPC-d_{62}) stretching frequency (■).

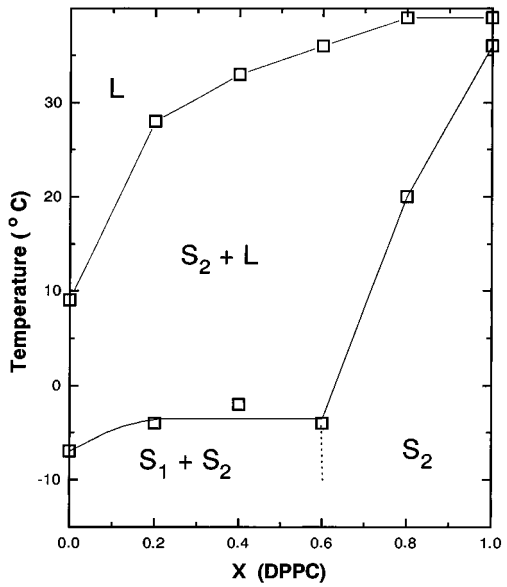

FIGURE 3: IR-derived phase diagram for the binary system DLPC/DPPC-d_{62}. Phase boundaries were deduced as described in the text. S_1 and S_2 represent distinct solid phases coexisting below the horizontal portion of the solidus curve; a region where solid phase S_1 alone exists is expected to be present below the solidus at very low DPPC contents but has been omitted for clarity.

for DLPC and DPPC-d_{62} in the mixed samples reflect the non-ideal mixing indicated by the phase diagram for this system, where the onset of melting of the short-chain lipid remains essentially constant over a large part of the concentration range.

By plotting the temperatures of onset and completion of lipid melting as a function of composition, we could use the above results to derive the phase diagram for the DLPC/DPPC-d_{62} system (Figure 3). To obtain the overall lipid-melting transition profile for the lipid mixtures, the temperature profiles for ν_{C-H} and ν_{C-D} were summed. The temperatures of onset and completion of melting for each mixture were then readily determined from the first derivatives of these overall transition profles. The phase diagram shown in Figure 3 agrees well with that reported previously for the system DLPC/DPPC (van Dijck et al., 1977) confirming the reliability of the approach outlined above and clearly illustrating the non-ideal mixing behavior noted in the transition profiles for individual lipid mixtures (Figure 2): while mixing in the fluid phase is near-ideal, the essentially flat solidus line indicates the occurrence of solid−solid phase separation up to roughly 60 mol % DPPC-d_{62}.

Two points should be noted in regard to the experimental phase diagram shown in Figure 3. First, for simplicity we have considered only one solid phase for each phospholipid component, designated S_1 for DLPC and S_2 (= $L_{\beta'}$) for DPPC-d_{62} , thereby ignoring the reported bimodal phase transition of DLPC as well as the pretransition of DPPC-d_{62} (near 30 °C). Second, the change in frequency of the methylene stretching band has been used as a direct probe of the progress of the transition. This is a simplification, since it has been shown that changes in frequency are not linearly correlated with the extent of melting of the lipids (Dluhy et al., 1985). However, a more sophisticated approach based on a two-state model (Dluhy et al., 1985) could not be used for the cholesterol-containing systems discussed below due to the formation of a third phase, the liquid-ordered phase (Vist & Davis, 1990). It is, however, clear that the phase diagram determined for the DLPC/DPPC-d_{62} system using our simplified approach exhibits the major features observed previously in the phase diagram for DLPC/DPPC mixtures (van Dijck et al., 1977).

Cholesterol-Containing Ternary Mixtures. The IR melting profiles shown in Figure 2 for DLPC/DPPC-d_{62} mixtures, particularly for mixtures containing near-equimolar proportions of these two species, clearly indicate distinct melting behavior and limited solid-phase miscibility of the phospholipid components. By introducing varying amounts of cholesterol into a 40:60 DLPC/DPPC-d_{62} mixture, for which these tendencies are quite pronounced, it is possible to examine sensitively the effects of the sterol on the intermixing of the two phospholipids. The results of such experiments are presented in Figure 4 and yield two major conclusions. First, it can be seen that cholesterol progressively reduces the amplitude of the chain-melting transition for both lipid species, as indicated by a smaller increase in both ν_{C-H} and ν_{C-D} as a function of temperature. This result is in agreement with previous observations on the effect of cholesterol on the main transition of DPPC (Umemura et al., 1980; Cortijo & Chapman, 1981). Second, our results show that when increasing mole fractions of cholesterol are added to DLPC/DPPC-d_{62} mixtures, the large difference in the thermotropic behavior of the two lipids is gradually reduced, reflecting notably a gradual shift in the DLPC transition midpoint to higher temperatures, a substantial broadening of the DPPC-d_{62} melting profiles and, as just noted, a gradual decrease in the apparent transition amplitude for both phospholipid components. At 40 mol % cholesterol these trends have produced a virtual convergence of the two transitions, as indicated by the nearly superimposable melting profiles observed for DLPC and for DPPC-d_{62} at this cholesterol content (Figure 4F). In a region where the phospholipid components alone exhibit pronounced immiscibility and quite distinct melting profiles, the presence of 40 mol % cholesterol thus causes the two components to melt in a very similar fashion. At lower cholesterol concentrations, by contrast, the distinct melting profiles observed for the two phospholipid components indicate that they are not homogeneously intermixed, although both PCs show some modification of their thermotropic melting due to the presence of cholesterol.

As discussed below, the mixing behavior of the DLPC/DPPC/cholesterol system was also examined by a fluorescence technique employing a monobrominated analogue of DLPC (12BrPC), which differs from the parent lipid only by the presence of a bromo substituent at the methyl teminus of the 2-position acyl chain. Because the samples used in these fluorescence studies can contain large mole fractions of brominated lipid, it was important to demonstrate that 12BrPC resembles DLPC in its thermotropic behavior, both alone and when combined with DPPC and cholesterol. Figure 5 shows the IR melting curve of hydrated 12BrPC in comparison with that of normal DLPC. The gel-to-liquid crystalline phase transition for 12BrPC is centered near −4 °C, slightly lower than the value obtained for DLPC. While the melting profile for 12BrPC does not clearly show the two-part transition of DLPC, the overall similarity of the two melting profiles (including their very similar overall amplitudes) suggests that the behavior of the brominated analogue reasonably approximates that of the parent lipid.

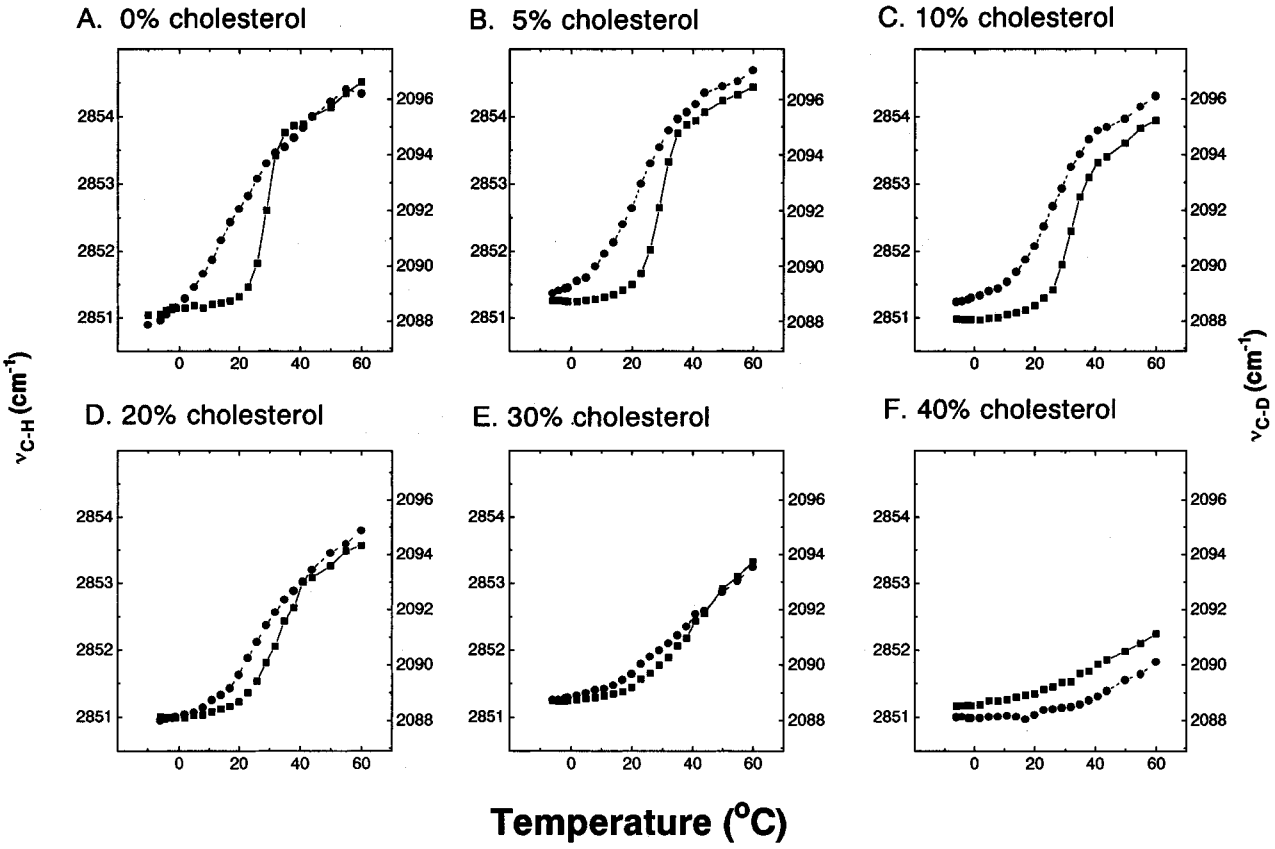

FIGURE 4: Frequency shift of the methylene symmetric stretching mode with temperature, for DLPC/DPPC-d_{62} mixtures (40:60 molar ratio) containing increasing molar percentages of cholesterol as indicated for each panel: (●) C−H stretch; (■) C−D stretch.

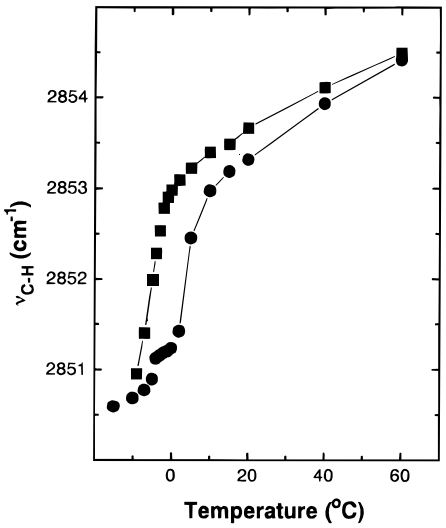

FIGURE 5: Comparison of the phase transition temperature for 12BrPC (■) and DLPC (●) dispersions as detected by the shift of the methylene symmetric stretching mode.

The thermal behavior of a 40:60 (mol/mol) 12BrPC/DPPC-d_{62} mixture incorporating various amounts of cholesterol is shown in Figure 6. Similarly to what is observed with the analogous DLPC/DPPC-d_{62} mixture, in the absence of cholesterol the two phosphatidylcholines display clearly inhomogeneous mixing, the low-melting component (12BrPC) showing a gradual increase in ν_{C-H} over more than a 20 °C interval while the melting transition of DPPC-d_{62} remains relatively cooperative. The addition of cholesterol likewise affects the thermotropism of the 12BrPC/DPPC-d_{62} mixture in a similar manner as for the DLPC/DPPC-d_{62} system. As in the latter system, cholesterol substantially reduces the amplitude of the observed frequency shift due to chain melting for both lipid components over the temperature range examined. In addition, increasing concentrations of cholesterol gradually reduce the large difference in the thermotropic behavior of the two lipids, producing an upward shift in the melting profile for 12BrPC and a substantial broadening of the melting profile for DPPC-d_{62}. At 30 mol % cholesterol, the melting profiles for the two phospholipids are much more similar than in the absence of sterol, although the melting curve for 12BrPC still shows a more gradual increase in frequency than does that for DPPC-d_{62} between 10 and 30 °C. At 40 mol % cholesterol, the melting curves for the two phospholipid components have become nearly coincident over the temperature range examined. These effects of cholesterol are very similar to those observed above for DLPC/DPPC-d_{62}/cholesterol mixtures of similar composition (Figure 4).

Fluorescence-Quenching Studies. The fluorescence of indolyl-labeled phospholipid probes can be quenched by brominated phospholipids when the two species are in close proximity. As a result, when an indolyl−phospholipid probe is incorporated into bilayers consisting of mixed brominated and nonbrominated phospholipids, the measured fluorescence intensity is a sensitive function of the concentration of brominated lipid molecules in the immediate environment of the probe (Silvius, 1990, 1992). As we have shown previously and as discussed below, by observing the variation of the normalized probe fluorescence intensity with the content of brominated lipids in a given lipid system (referred to hereafter as a "quenching curve"), it is possible to assess the homo- or heterogeneity of lipid mixing at a microscopic level. The experiments described here employed 12BrPC

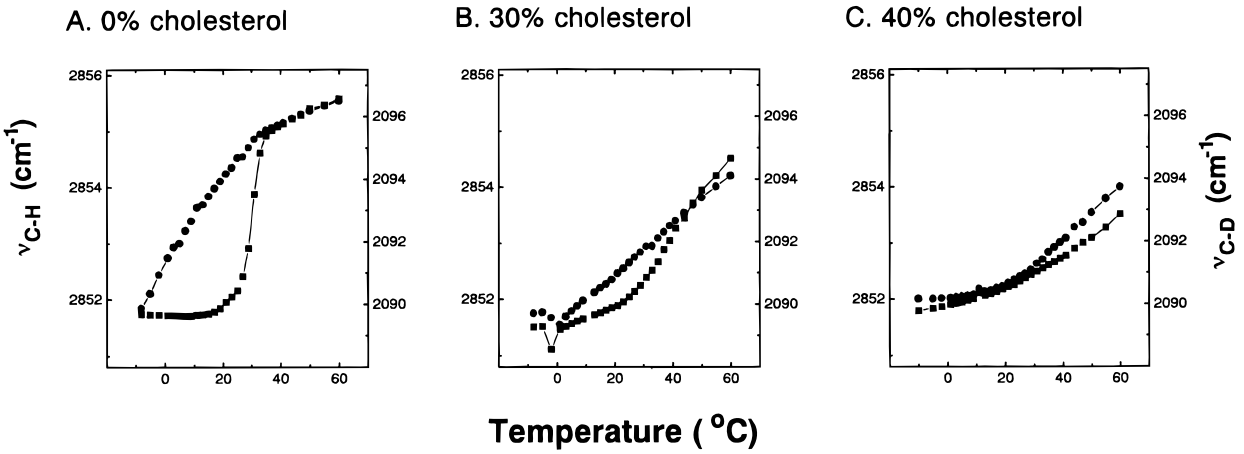

FIGURE 6: Frequency shift of the methylene symmetric stretching mode with temperature, for 12BrPC/DPPC-d_{62} mixtures (40:60 molar ratio) containing the indicated molar percentages of cholesterol: (●) C−H stretch; (■) C−D stretch.

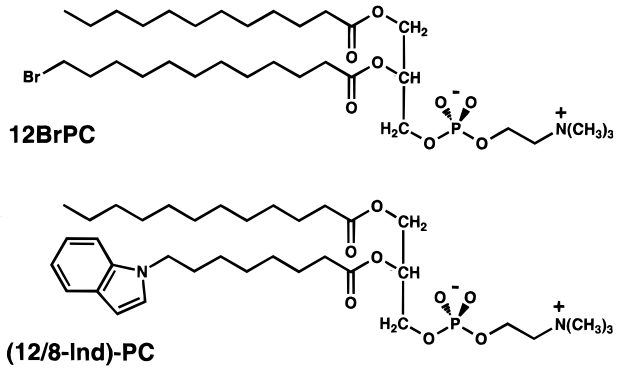

FIGURE 7: Structures of 12BrPC and (12/8-Ind)-PC.

as the brominated component and (12/8−Ind)-PC as the fluorescent probe (see Figure 7 for structures), in mixtures with different nonbrominated PCs with or without varying proportions of cholesterol. As the FT-IR results in the previous section demonstrate, the bromo substituent in 12BrPC is not a major determinant of the interactions of this species with either DPPC-d_{62} or cholesterol. All experiments described in this section were carried out at 25 °C, a temperature at which the above FT-IR experiments indicated extensive phase separation of 12BrPC from DPPC-d_{62} over a wide range of cholesterol contents.

Given the similarity of their structures and transition temperatures (see above), DLPC and 12BrPC are expected to form homogeneous fluid bilayers at 25 °C. In agreement with this expectation, as shown in Figure 8A the quenching curve for (12/8-Ind)-PC in mixtures of DLPC and 12BrPC is smooth and monophasic, with no abrupt changes in slope, consistent with homogeneous intermixing of the lipid components. The quenching curve for (12/8-Ind)-PC in mixtures of 12BrPC with POPC is likewise smooth and monophasic, again suggesting homogeneous mixing at all molar ratios of the phospholipid components. However, the quenching curves for the two systems are not identical in form, notably in that the quenching curve for the DLPC/12BrPC system shows a stronger concavity than does that for the POPC/12BrPC system. This difference is readily explainable given that the bromo group of 12BrPC and the indolyl group of (12/8-Ind)-PC are both expected to lie deep in the lipid bilayer (Abrams et al., 1992; Weiner & White, 1991) and therefore can exhibit a significant amount of quenching of indolyl probes in one bilayer leaflet by brominated lipids in the opposite leaflet (Chattopadhyay & London, 1987). The relative magnitude of this "trans"-quenching (compared to that of "cis"-quenching) is expected to decrease, due to an increase in bilayer thickness, when 12BrPC is diluted with a longer-chain (nonbrominated) PC (McIntosh, 1978; Chattopadhyay and London, 1987) but not when 12BrPC is diluted with DLPC, a species of similar acyl chain length. It is straightforward to predict from these considerations that the quenching curve for the 12BrPC/DLPC system will be more concave (exhibiting greater quenching at intermediate mole fractions of 12BrPC) than that for a homogeneous system combining 12BrPC with a longer-chain non-brominated lipid such as POPC, consistent with our experimental results.

The addition of cholesterol to DLPC/12BrPC mixtures labeled with (12/8-Ind)-PC alters the absolute fluorescence of the indolyl-phospholipid probe at any given phospholipid composition but not the general form of the quenching profile, which indicates homogeneous mixing of the phospholipid components at 45 mol % as well as at 0 mol % cholesterol (Figure 8A). Quenching curves for the POPC/12BrPC system at either 35 mol % or 45 mol % cholesterol (Figure 8B) likewise indicate homogeneous intermixing of the components. The quenching curves shown in Figure 8 (and all other quenching curves shown in this paper), are plotted as a function of the molar percentage of brominated lipid *in the phospholipid fraction*. Interestingly, when the curves shown in Figure 8B are replotted as a function of the molar percentage of brominated PC in the *total* lipid (including sterol), the curves at 0, 35, and 45 mol % cholesterol become effectively superimposable when scaled to a common left-hand intercept (not shown). In this system cholesterol and POPC molecules are thus essentially equivalent in their ability to attenuate fluorescence quenching when mixed with 12BrPC.

At 25 °C mixtures of 12BrPC with DPPC exhibit markedly inhomogeneous mixing, as illustrated by the quenching curve shown in Figure 9A (filled circles). With increasing mole fractions of DPPC the normalized fluorescence of (12/8-Ind)-PC initially increases smoothly, in a manner very similar to that seen for the miscible binary systems described above (e.g., POPC/12BrPC, crosses in Figure 9A). However, the DPPC/12BrPC quenching curve shows an abrupt change in slope and a pronounced flattened region, indicating the onset of phase separation, beginning at ca. 15 mol % DPPC in 12BrPC (arrow in Figure 9A). This behavior agrees well

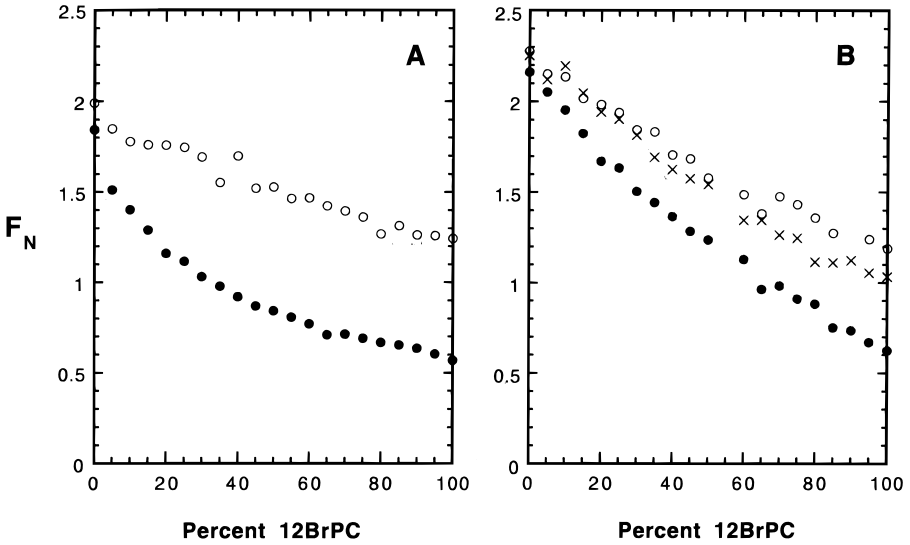

FIGURE 8: Quenching curves for mixtures of 12BrPC with non-brominated PCs at 25 °C. (A) 12BrPC plus DLPC in the presence of 0 (●) or 45 mol % cholesterol (○). (B) 12BrPC plus POPC in the presence of 0 (●), 35 (×), or 45 mol % (○) cholesterol. The normalized fluorescence (F_N) represents the ratio of (12/8-Ind)-PC fluorescence determined for equal aliquots of a given sample in buffer and in methanol. Samples were prepared and incubated, and the normalized fluorescence was measured, as described in Materials and Methods. Data shown represent the means of duplicate determinations for samples prepared and incubated in parallel.

with that expected on the basis of the IR-derived phase diagram described above for DLPC/DPPC-d_{62} mixtures. The pronounced flattening of the quenching curve at higher mole fractions of DPPC indicates that the (12/8-Ind)-PC probe partitions preferentially into the DLPC-rich fluid phase (Silvius, 1990, 1992), as might be anticipated given the bulky substituent on its 2-position acyl chain and the fact that its acyl chains are comparable in length to those of DLPC.

In the presence of 20% cholesterol, mixtures of 12BrPC and DPPC show the onset of phase separation at a lower mole fraction of DPPC than is observed in the absence of sterol (Figure 9B). This is evidenced by the fact that the quenching curve for such mixtures exhibits an abrupt decrease in slope above ca. 5 mol % DPPC (arrow). Qualitatively similar behavior is observed in the quenching curves for DPPC/12BrPC mixtures containing 30−35 mol % sterol, which moreover exhibit a pronounced, broad local minimum at higher DPPC contents (Figures 9B and 9C). The form of the quenching curves observed for these systems (specifically, the fact that the normalized fluorescence values fall *below* those expected for homogeneous mixing) indicates that the (12/8-Ind)-PC probe partitions preferentially into domains enriched in 12BrPC within the region of phase separation (Silvius, 1990, 1992), as it does in cholesterol-free samples. A local minimum is still observed in the quenching curve for mixtures containing 40 mol % cholesterol (Figure 9C), but the onset of phase separation now appears shifted to ca. 10−15 mol % DPPC. This latter trend progresses in the quenching curve for mixtures containing 45 mol % sterol, for which the onset of phase separation appears shifted to ca. 30 mol % DPPC (Figure 9D). Nonetheless, the form of the quenching curve clearly indicates that even in the presence of 45 mol % cholesterol, mixing of the phospholipid components is not homogenous across the full range of DPPC/12BrPC molar ratios. At 50 mol % cholesterol (Figure 9D) the quenching curve for DPPC/12BrPC mixtures becomes monophasic and very similar in shape to that observed for POPC/12BrPC mixtures of similar sterol content (Figure 8B), indicating that for this cholesterol content 12BrPC and DPPC have become truly miscible at the microscopic level. Samples containing high levels of cholesterol (particularly 40−50 mol %) gave coarser dispersions, causing somewhat greater data scatter, than did samples of lower cholesterol content. Nonetheless, the individual quenching curves obtained for independent sets of samples with these cholesterol contents consistently exhibited the features discussed above and shown in the averaged quenching curves of Figures 9C and 9D.

To map more completely the solubility of DPPC in the 12BrPC-enriched fluid phase at 25 °C as a function of cholesterol content, we examined the region of the quenching curve from 0 to 40 mol % DPPC (again, expressed as a percentage of total *phospholipid*) for DPPC/12BrPC mixtures in which the cholesterol content was varied from 0 to 50 mol % in increments of 5 mol %. The approach used to determine the limit of solubility of DPPC in the 12BrPC-rich fluid phase can be illustrated with reference to the quenching curves shown in Figure 9A. At low DPPC contents the quenching curve for DPPC/12BrPC mixtures (closed circles) closely parallels that measured for analogous POPC/12BrPC mixtures (crosses),[3] indicating miscibility of the lipid components. However, the two curves diverge sharply, indicating the onset of phase separation in the DPPC/12BrPC system, above a threshold molar percentage of DPPC (arrow in Figure 9A) that was estimated from repeated determinations to be 16 ± 2 mol %. In an analogous manner, by comparing the appropriate portions of the quenching curves for DPPC/12BrPC and POPC/12BrPC mixtures containing a fixed mole fraction of cholesterol (varying the relative proportions of brominated and nonbrominated PC in increments as fine as 1% where necessary) we determined

[3] POPC/12BrPC mixtures with different cholesterol contents provided "homogeneous-mixing" reference quenching curves useful in determining the presence and the point of onset of phase separation in DPPC/12BrPC mixtures of the same cholesterol content, notably for samples containing ≥40 mol % sterol. POPC/12BrPC mixtures were preferred to DLPC/12BrPC mixtures for this purpose because (as discussed in the text) when combined with 12BrPC in "liquid" phases POPC is expected to significantly modify the bilayer thickness (and hence the efficiency of transbilayer quenching of [12/8-Ind]-PC fluorescence by 12BrPC), in a similar manner as does DPPC, while DLPC should not.

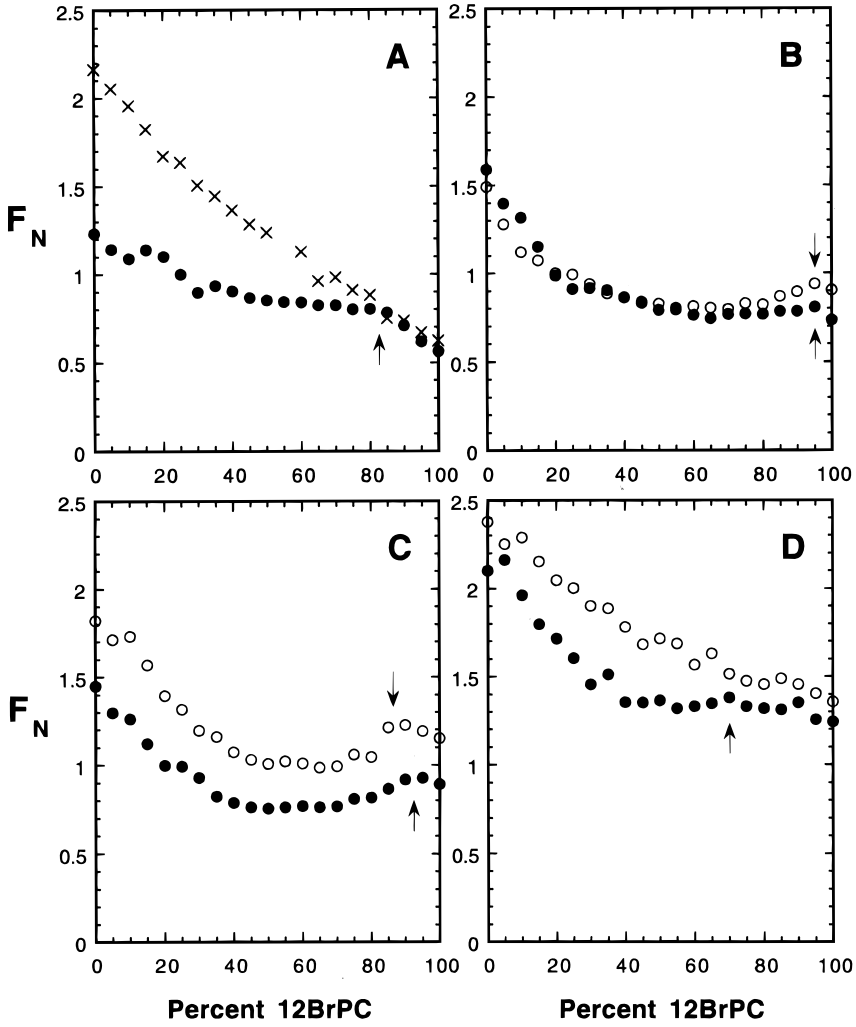

FIGURE 9: (A) Quenching curves for mixtures of 12BrPC plus DPPC (●) or POPC (×) at 25 °C. (B) Quenching curves measured at 25 °C for mixtures of 12BrPC plus DPPC in the presence of 20 (●) or 30 mol % (○) cholesterol. (C) Quenching curves measured at 25 °C for mixtures of 12BrPC plus DPPC in the presence of 35 (●) or 40 mol % (○) cholesterol. (D) Quenching curves measured at 25 °C for mixtures of 12BrPC plus DPPC in the presence of 45 (●) or 50 mol % (○) cholesterol. Data shown represent the average of duplicate determinations (for sets of samples prepared and incubated in parallel) for samples containing 0−40 mol % cholesterol, and of six determinations (for three sets of duplicate samples prepared and incubated independently) for samples containing 45−50 mol % cholesterol. Arrows represent the apparent limits of solubility of DPPC in a homogeneous 12BrPC-enriched fluid phase, determined as described in the text using data additional to those plotted here. Other experimental details were as for Figure 8.

the limiting solubility of DPPC in a homogeneous 12BrPC-enriched fluid phase as a function of the cholesterol concentration, obtaining the results summarized in Figure 10. The solubility of DPPC in the 12BrPC-enriched fluid phase decreases strongly from 0 to ca. 25 mol % cholesterol, then rises again at higher cholesterol contents until, as discussed above, complete miscibility of the phospholipid components in all proportions is attained at 50 mol % cholesterol. Cholesterol at 50 mol % (but not at lower levels) was also found to promote complete intermixing of 12BrPC with DSPC at 25 °C, while in the absence of sterol DSPC was soluble in the fluid 12BrPC-rich phase only to a level of ca. 10 mol % (data not shown), in agreement with the calorimetric DLPC/DSPC phase diagram reported by Mabrey and Sturtevant (1976).

The data just presented can be combined with information regarding the phase diagrams for the binary systems DPPC/cholesterol (Vist & Davis, 1990) and DPPC/DLPC [this paper and van Dijck et al. (1977)] to derive the proposed ternary phase diagram shown in Figure 11 for the system 12BrPC/DPPC/cholesterol at 25 °C. As noted in the introduction, a key element in most currently proposed phase diagrams for PC/cholesterol binary systems is the existence of a region of fluid-fluid phase separation, with a cholesterol-depleted "liquid-disordered" (l_d) and a cholesterol-enriched "liquid-ordered" (l_o) phase coexisting over a finite range of temperatures (Vist & Davis, 1990; Ipsen et al., 1987, 1989; Sankaram & Thompson, 1991; Thewalt & Bloom, 1992; Almeida et al., 1992, 1993). This feature accordingly appears in the proposed ternary phase diagram shown in Figure 11A, which is the simplest that is consistent with both the experimental data obtained here and the behavior of the corresponding binary systems (Ricci, 1966) The left-hand boundary of the ($l_o + l_d$) region in the ternary phase diagram can be mapped rather accurately using the data summarized in Figure 10 (open circles in Figure 11A). The orientation of the tie lines in this two-phase region has been inferred by considering the expected topology of the four-dimensional (temperature/composition) phase diagram for the 12BrPC/DPPC/cholesterol system, as outlined in the Discussion. The broad, shallow minima (within the region of phase separation) observed in the quenching curves for samples containing 25−40 mol % cholesterol (Figures 9B and 9C) are predicted from the phase diagram shown in Figure 11A given

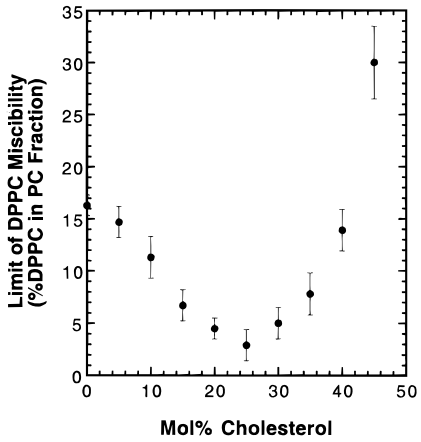

FIGURE 10: Effect of cholesterol on the miscibility of DPPC with 12BrPC at 25 °C. The molar percentage of DPPC marking the onset of ($[l_d + P_{\beta'}]$ or $[l_d + l_o]$) phase separation was determined from quenching curves for DPPC/12BrPC mixtures at various cholesterol contents as described in the text. Values plotted represent the mean of three independent determinations; for each cholesterol concentration, the error limits indicated are based on the uncertainty of determination estimated for each individual data set as well as the variation between the three data sets. Other experimental details were as described in Materials and Methods.

the expected orientation of the tie lines in the ($l_d + l_o$) two-phase region and the apparent preferential partitioning of (12/8-Ind)-PC in favor of the 12BrPC-enriched (l_d) phase.

DISCUSSION

Previous studies of the phase behavior of ternary mixtures containing cholesterol and phospholipids (Cullis & de Kruijff, 1978; Cullis et al., 1978; de Kruyff et al., 1973, 1974; Verkleij et al., 1974; van Dijck et al., 1976; van Dijck, 1979; Demel et al., 1977; Tilcock et al., 1982; Finegold & Singer, 1991; Silvius, 1992; Almeida et al., 1993; Parasassi et al., 1994) have led to two general conclusions. First, in certain ternary systems cholesterol at high levels appears to promote the intermixing of lipids that exhibit pronounced phase separations in the absence of sterol (Tilcock et al., 1982, 1984; Silvius, 1992). Second, however, in a variety of ternary (phospholipid/phospholipid/sterol) systems cholesterol has been suggested to interact nonrandomly with different phospholipid components within the same bilayer (de Kruyff et al., 1974; Verkleij et al., 1974; van Dijck et al., 1976; van Dijck, 1979; Demel et al., 1977; Almeida et al., 1993). As already discussed, cholesterol has likewise been shown to exhibit markedly nonideal interactions in the fluid state with various individual phosphatidylcholines in binary cholesterol/PC mixtures (Vist & Davis, 1990; Shin & Freed, 1989; Subczynski et al., 1990; Pasenkiewicz-Gierula et al., 1990; Sankaram & Thompson, 1991) and markedly nonrandom partitioning between vesicles with distinct (saturated *vs* unsaturated) phospholipid compositions (Lange et al., 1979; Nakagawa et al., 1979; Wattenberg & Silbert, 1983; Yeagle & Young, 1986; Rujanavech & Silbert, 1986), demonstrating the potential of cholesterol to discriminate between different phospholipid species.

In the ternary systems examined here, combining saturated di-C12 with di-C16 or -C18 PCs, which exhibit extensive phase separation in the absence of sterol, the contrasting properties of cholesterol just noted lead to opposite consequences at very high and at lower bilayer sterol contents. At very high bilayer levels of cholesterol, the shorter- and

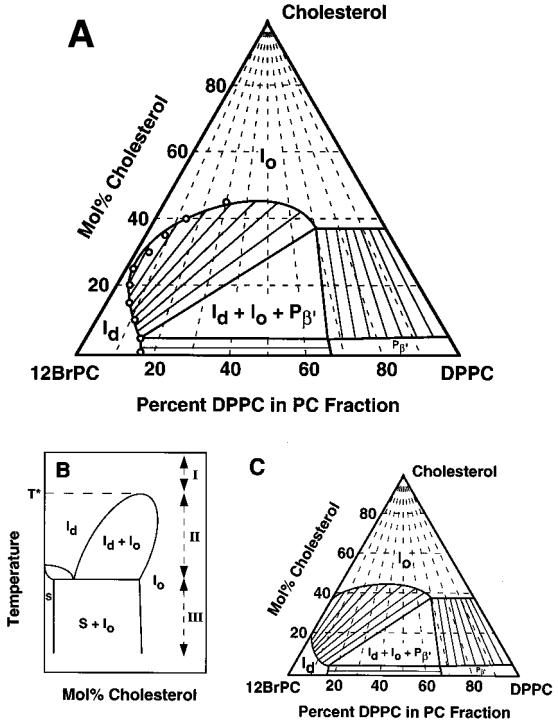

FIGURE 11: (A) Ternary phase derived for the system 12BrPC/DPPC/cholesterol at 25 °C as described in the text. (B) Generic binary phase diagram for a PC/cholesterol mixture, after Ipsen et al. (1987, 1989) and Vist and Davis (1990). (C) Hypothetical phase diagram for the 12BrPC/DPPC/cholesterol system at a temperature falling below T^* for the 12BrPC/cholesterol binary system (and within region II in the 12BrPC/cholesterol phase diagram) as illustrated in Figure 11B. In the ternary phase diagrams A and C, for clarity regions of two-phase coexistence are unlabeled, but the phases present can be deduced from the designations of the adjacent single-phase regions.

longer-chain phospholipid components exhibit convergent thermotropic behavior (assessed by IR) and at 25 °C exhibit complete miscibility at the microscopic level (assessed by fluorescence-quenching measurements). At lower levels in the bilayer, by contrast, cholesterol does not promote truly homogeneous intermixing of the phospholipid components in these PC mixtures, as indicated by both our IR and our fluorescence results.[4] In fact, fluorescence-quenching results indicate that at 25 °C cholesterol at intermediate concentrations in the bilayer *reduces* the mutual miscibility of the phospholipid components below the level observed in the absence of sterol (Figures 10 and 11A). In the region of ($l_d + l_o$) phase coexistence, 12BrPC is enriched in the l_d phase while cholesterol and DPPC are co-enriched in the l_o phase.

The conclusions just described can readily be rationalized on both a molecular and a thermodynamic level. On the molecular level, previous work has suggested that the degree of hydrophobic length mismatch between sterols and saturated PCs can strongly affect the nature of their interactions (Mouritsen & Bloom, 1984; McMullen et al., 1993, 1995)

[4] It may be noted that the IR results suggest a convergence of the melting profiles for DLPC (or 12BrPC) and DPPC-d_{62} at 40 mol % cholesterol in 40:60 mixtures of the phospholipid components, while the fluorescence-quenching results suggest that 50 mol % cholesterol is required to promote homogeneous intermixing of these phospholipid species at 25 °C. There is no inherent discrepancy in these results. While the observation of distinct IR melting profiles for DLPC (or 12BrPC) and DPPC-d_{62} in a given sample provides clear evidence that these species do *not* intermix homogeneously within the temperature range of the thermal transition, the converse cannot be directly inferred.

and in the case of cholesterol should be considerably larger for a di-C12 than for a di-C16 PC (McIntosh, 1978; McMullen et al., 1995). The preferential distribution of DPPC into the cholesterol-enriched liquid-ordered phase, and of 12BrPC into the cholesterol-depleted liquid-disordered phase, within the (l_o + l_d) phase-coexistence region is consistent with this expectation.

Our conclusion of a preferential association of cholesterol and DPPC within the (l_d + l_o) phase-coexistence region in 12BrPC/DPPC/cholesterol mixtures is somewhat at variance with that of a previous calorimetric study (de Kruyff et al., 1974). In this latter study, cholesterol at low levels appeared to perturb preferentially the calorimetric endotherm attributed to DLPC melting in thermograms for DLPC/DPPC/cholesterol mixtures and was thus suggested to interact preferentially with DLPC over DPPC. However, in binary mixtures the thermotropic phase transitions of shorter-chain PCs have been shown to be inherently more susceptible to broadening by low levels of cholesterol than is the transition of DPPC (Singer & Finegold, 1990; McMullen et al., 1993). As well, as shown in the present study (Figure 4), the thermal transition of the DLPC component in DLPC/DPPC-d_{62}/cholesterol mixtures shifts upward, increasingly overlapping that of the DPPC component, as the cholesterol concentration increases. Both of these effects complicate the interpretation of the calorimetric observations noted above. As well, previous conclusions that cholesterol interacts preferentially with unsaturated over saturated PCs, based on the same type of calorimetric observations (de Kruyff et al., 1974), were contradicted by subsequent direct measurements of cholesterol partitioning between vesicles of different phospholipid compositions (Lange et al., 1979; Nakagawa et al., 1979), suggesting that the interpretation of such calorimetric results may not be straightforward. It is thus not clear that the previous calorimetric observations for the DLPC/DPPC/cholesterol system are necessarily incompatible with our conclusion that DPPC and cholesterol interact preferentially in 12BrPC/DPPC/cholesterol mixtures in the region of (l_d + l_o) phase coexistence. It is interesting to note that the present FT-IR data, in which the thermal melting profiles of DLPC (or 12BrPC) and DPPC-d_{62} are separately determined, suggest that intermediate levels of cholesterol produce a greater relative broadening of the melting profile for DPPC-d_{62} than for the DLPC component. This result is consistent with the conclusion just noted.

On a thermodynamic level, the phase diagram deduced for the 12BrPC/DPPC/cholesterol phase diagram at 25 °C (Figure 11A) is a logical extension of the corresponding binary phase diagrams. To appreciate this point, we present in Figure 11B a generic binary phase diagram proposed to characterize diverse PC/cholesterol systems on the basis of previous theoretical (Ipsen et al., 1987, 1989) and experimental results (Vist & Davis, 1990; Sankaram & Thompson, 1991; Thewalt & Bloom, 1992; Almeida et al., 1993). At 25 °C the DPPC/cholesterol binary system is expected to fall in region III of this phase diagram (Vist & Davis, 1990). By contrast, the 12BrPC/cholesterol system will exist in either region I or region II depending on whether the experimental temperature of 25 °C lies above or below the critical temperature (T *) for the 12BrPC/cholesterol system. If at 25 °C the 12BrPC/cholesterol system existed in region II, the ternary phase diagram would be of the form shown in Figure 11C, with the region of (l_d + l_o) phase coexistence intersecting the left-hand (12BrPC/cholesterol) axis and with the tie lines in this region running roughly as indicated. If instead at 25 °C the 12BrPC/cholesterol binary system exists just above the critical temperature T * indicated in Figure 11B, the region of (l_d + l_o) phase separation in the ternary phase diagram will have pulled slightly away from the left-hand axis of the ternary phase diagram, but the orientation of the tie lines in the (l_d + l_o) region should still resemble that observed just below T *. Our fluorescence-quenching results (Figures 10 and 11A) suggest that at 25 °C the 12BrPC/DPPC/cholesterol system exists in the latter regime, i.e., just above the critical temperature T * for the 12BrPC/cholesterol binary system. In this regime, given the predicted orientation of the tie lines in the (l_d + l_o) phase-separation region, the addition of intermediate levels of cholesterol to a DPPC/12BrPC mixture will lead to significant demixing of the phospholipid components in the (l_d + l_o) phase-coexistence region, as is observed experimentally.

The phase equilibria of multicomponent systems including cholesterol have attracted considerable interest in the light of recent suggestions that various nonlipid membrane components, including both membrane-spanning and phosphatidylinositol/glycan-linked proteins, may distribute differentially between liquid-ordered and liquid-disordered domains in membranes where the two phases coexist (Simons & van Meer, 1988; Schroeder et al., 1991, 1994, 1995; Brown, 1992; Pelham & Munro, 1993; Bretscher & Munro, 1993; Dontchev et al., 1994; Munro, 1991, 1995). While the systems examined here are of course nonphysiological ones, they serve to demonstrate that the presence of cholesterol in bilayers combining polar lipids with differing physical properties can increase the overall complexity of the system's phase behavior and preserve or even augment the tendency of the system to exhibit segregation of domains of distinct composition. It will be of interest to investigate to what extent such effects of cholesterol may be observed in the heterogeneous lipid mixtures that characterize the plasma and other sterol-containing membranes of eukaryotic cells.

ACKNOWLEDGMENT

We thank Dr. Martin Zuckermann for useful discussions during the course of this work.

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BI9615506