Differential Scanning Calorimetric and Fourier Transform Infrared Spectroscopic Studies of the Effects of Cholesterol on the Thermotropic Phase Behavior and Organization of a Homologous Series of Linear Saturated Phosphatidylserine Bilayer Membranes

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ABSTRACT We have examined the effects of cholesterol on the thermotropic phase behavior and organization of aqueous dispersions of a homologous series of linear disaturated phosphatidylserines by high-sensitivity differential scanning calorimetry and Fourier transform infrared spectroscopy. We find that the incorporation of increasing quantities of cholesterol progressively reduces the temperature, enthalpy, and cooperativity of the gel-to-liquid-crystalline phase transition of the host phosphatidylserine bilayer, such that a cooperative chain-melting phase transition is completely or almost completely abolished at 50 mol % cholesterol, in contrast to the results of previous studies. We are also unable to detect the presence of a separate anhydrous cholesterol or cholesterol monohydrate phase in our binary mixtures, again in contrast to previous reports. We further show that the magnitude of the reduction in the phase transition temperature induced by cholesterol addition is independent of the hydrocarbon chain length of the phosphatidylserine studied. This result contrasts with our previous results with phosphatidylcholine bilayers, where we found that cholesterol increases or decreases the phase transition temperature in a chain length-dependent manner (1993. *Biochemistry*, 32:516–522), but is in agreement with our previous results for phosphatidylethanolamine bilayers, where no hydrocarbon chain length-dependent effects were observed (1999. *Biochim. Biophys. Acta,* 1416:119–234). However, the reduction in the phase transition temperature by cholesterol is of greater magnitude in phosphatidylethanolamine as compared to phosphatidylserine bilayers. We also show that the addition of cholesterol facilitates the formation of the lamellar crystalline phase in phosphatidylserine bilayers, as it does in phosphatidylethanolamine bilayers, whereas the formation of such phases in phosphatidylcholine bilayers is inhibited by the presence of cholesterol. We ascribe the limited miscibility of cholesterol in phosphatidylserine bilayers reported previously to a fractional crystallization of the cholesterol and phospholipid phases during the removal of organic solvent from the binary mixture before the hydration of the sample. In general, the results of our studies to date indicate that the magnitude of the effect of cholesterol on the thermotropic phase behavior of the host phospholipid bilayer, and its miscibility in phospholipid dispersions generally, depend on the strength of the attractive interactions between the polar headgroups and the hydrocarbon chains of the phospholipid molecule, and not on the charge of the polar headgroups per se.

INTRODUCTION

Cholesterol is a major and essential lipid component of the plasma membranes of the cells of higher animals and is also found in lower concentrations in certain intracellular membranes in vesicular communication with the plasma membrane (Nes and McKean, 1977; Yeagle, 1988; Liscum and Munn, 1999). Although cholesterol has a number of different functions in animal cells, one of its primary roles is as a modulator of the physical properties of the plasma membrane lipid bilayer. Thus, many studies of the interaction of cholesterol with phospholipid monolayer and bilayer model membranes have been performed, utilizing a wide range of physical techniques (Demel and de Kruijff, 1976; Yeagle, 1988; Vist and Davis, 1990; McMullen and McElhaney, 1996). These studies, most of which have utilized symmetrical chain linear saturated phosphatidylcholines (PCs), have established that the major effects of cholesterol incorporation on phospholipid monolayer and bilayer model membranes are a broadening and eventual elimination of the cooperative gel-to-liquid-crystalline phase transition, a decrease (increase) in the cross-sectional area occupied by the phospholipid molecule in the liquid-crystalline (gel) state, an increase (decrease) in hydrocarbon chain orientational order in the liquid-crystalline (gel) state, and a decrease (increase) in the passive permeability in the liquid-crystalline (gel) state. As well, the presence of cholesterol increases both in the thickness and mechanical strength of the phospholipid monolayer or bilayer in the physiologically relevant fluid phase. The relatively high rates of intramolecular and intermolecular motion characteristic of phospholipid model membranes in the presence of high levels of cholesterol, coupled with an increased hydrocarbon chain order and a decreased area compressibility, have prompted several workers to postulate the existence of a discrete liquid-ordered state in model and biological membranes with cholesterol levels above about 25 mol % (Ipsen et al., 1987; Vist and Davis, 1990; Thewalt and Bloom, 1992). However, whether or not such a discrete thermodynamic state actually exists is controversial (Reinl et al., 1992; McMullen and McElhaney, 1995, 1996).

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Although the thermotropic phase behavior of mixtures of cholesterol with the major zwitterionic phospholipids of animal plasma membranes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), have been relatively well studied (Demel and de Kruijff, 1976; Yeagle, 1988; Vist and Davis, 1990; McMullen and McElhaney, 1996), that of mixtures of cholesterol with the major anionic phospholipid, phosphatidylserine (PS), has been less extensively investigated (Bach, 1984; Bach and Wachtel, 1989; Bach et al., 1992, 1998; Wachtel and Bach, 1987; Wachtel et al., 1991). Moreover, some of the results obtained with cholesterol/PS mixtures appear to be quite different from those obtained with the corresponding PC/cholesterol or PE/cholesterol mixtures. For example, low-sensitivity differential scanning calorimetry (DSC) and x-ray diffraction results indicate that in binary mixtures of cholesterol with the sodium salts of dimyristoylphosphatidylserine (DMPS), dipalmitoylphosphtidylserine (DPPS), 1-palmitoyl-2-oleoyl-PS, and bovine brain PS, cholesterol crystallites can be detected at cholesterol levels above 30–36 mol %, in both the gel and liquid-crystalline phases, and as low as 20 mol % in 1-stearoyl-2-oleoyl-PS/cholesterol mixtures. This lateral phase separation of crystalline cholesterol monohydrate was manifested both as an endotherm located about 37°C in the DSC traces and as two additional peaks at 17 and 34 Å in the x-ray diffraction pattern. As well, it was reported that the gel-to-liquid-crystalline phase transition of some of the PSs studied persists even at remarkably high cholesterol contents (70–80 mol %). Such behavior has not been noted previously in similar studies of cholesterol-containing zwitterionic PC and PE bilayers, but has been reported for a number of phosphatidylglycerols (PGs; Borochov et al., 1995), leading to the suggestion that the presence of a negative charge on the phospholipid headgroup reduces the lateral miscibility of cholesterol in phospholipid bilayers. Indeed, cholesterol has been reported to exhibit a greater solubility in bovine spinal cord PS bilayers at lower pH, where the carboxyl group of the serine moiety is protonated, although cholesterol crystallites were still observed at about 37 mol % cholesterol as compared to 30 mol % at neutral pH (Wachtel et al., 1991). Thus, a net negative charge on the PS headgroup may not be the major factor responsible for the apparently limited solubility of cholesterol in these systems.

We have recently carried out a comparative high-sensitivity DSC study of the effect of cholesterol on the distearoyl and dielaidoyl molecular species of PC, PE, and PS (McMullen and McElhaney, 1997). In both the distearoylphosphatidylserine (DSPS) and dielaidoylphosphatidylserine (DEPS) systems, we observed that a very weak gel-to-liquid-crystalline phase transition persists at a cholesterol concentration of 50 mol % upon heating but was abolished at 50 mol % upon cooling, indicating that cholesterol does indeed exhibit a slightly limited solubility in gel but not in liquid-crystalline DSPS and DEPS bilayers. This latter result is at variance with the results of previous work, which indicate appreciable cholesterol immiscibility in liquid-crystalline as well as gel state PS bilayers (Bach, 1984; Bach and Wachtel, 1989; Bach et al., 1992, 1998; Wachtel and Bach, 1987; Wachtel et al., 1991). Moreover, in our experiments, no DSC endotherm corresponding to the melting of crystalline cholesterol monohydrate could be detected in either system, even at the highest cholesterol concentration tested (50 mol %). In fact, in our study, cholesterol exhibited a greater miscibility in anionic DSPS and DEPS bilayers than in the corresponding zwitterionic DSPE and DEPE bilayers, again calling into question the proposed role of an anionic phospholipid polar headgroup in limiting cholesterol solubility in lipid bilayers.

In order to resolve these apparently discrepant results, we have carried out the present high-sensitivity DSC and Fourier transform infrared (FTIR) spectroscopic studies of the interaction of cholesterol with three members of the homologous series of linear saturated PEs. This study was also designed to complement our earlier investigations of the effects of variations in phospholipid hydrocarbon chain length on the interactions of cholesterol with zwitterionic disaturated PC (McMullen et al., 1993) and PE (McMullen et al., 1999) bilayers. Our present results indicate that the strength of phospholipid polar headgroup electrostatic and hydrogen-bonding interactions, rather than the headgroup change per se, is the primary determinant of cholesterol miscibility in phospholipid bilayers, although the strength of the van der Waals and hydrophobic interactions of the phospholipid hydrocarbon chains also plays a role. Moreover, we find no evidence for the existence of cholesterol crystallites in either gel or liquid-crystalline PS bilayers having cholesterol levels up to 50 mol %.

MATERIALS AND METHODS

The PS sodium salts used in these experiments were purchased from Avanti Polar Lipids (Alabaster, AL) and checked for purity by thin layer chromatography using chloroform:methanol:glacial acetic acid:water (60:40:10:4, by volume) as the developing solvent, followed by spraying with 2% K_2CrO_4 in 60% sulfuric acid and charring. The cholesterol was also purchased from Avanti Polar Lipids and recrystallized from ethanol before use. For cholesterol/DMPS and cholesterol/DPPS mixtures, stock solutions of PS and cholesterol in chloroform/methanol (2:1, vol:vol) were used to prepare the cholesterol/PS mixtures. After addition of the appropriate amounts of PS and cholesterol from the respective stock solutions, the samples were vortexed to ensure thorough mixing and the solvent was then removed with a stream of nitrogen at temperatures between 40 and 50°C before drying under vacuum for at least 18 h. (Note that the removal of solvent at elevated temperatures was required to maintain sample homogeneity.) Due to the relatively low solubility of DSPS in mixtures of chloroform and methanol, we prepared cholesterol/DSPS mixtures by weighing out the appropriate amounts of DSPS and cholesterol, dissolving the powders in benzene, gently heating, and then lyophilizing. The samples were dried under vacuum for at least 18 h to ensure that all traces of the benzene were removed.

For the high-sensitivity DSC experiments, the dried cholesterol/PS mixtures were dispersed in a buffer containing 50 mM Tris, 100 mM NaCl,

and 10 mM EDTA (pH 7.4) and heated to approximately 10–20°C above the phase transition of the mixture for at least 30 min with repeated vortexing to give a multilamellar suspension. When we examined the effect of variations in buffers (Tris and phosphate) and ionic strength (NaCl, 0.100 to 0.400 M) on the thermotropic phase behavior of cholesterol/PS mixtures, we observed only small shifts in the transition temperature and no changes in overall qualitative phase behavior. For all of the DSC samples, the mixtures were hydrated and suspended as detailed, then stored for 1 day at 2°C before the DSC experiment, to ensure that these samples were in thermodynamic equilibrium before analysis. The DSC thermograms for the cholesterol/PS suspensions were recorded with a high-sensitivity multi-cell differential scanning calorimeter (Calorimetry Sciences Corporation, Provo, UT). The scan rates used were 10°C/h unless otherwise noted. In addition, the amount of PS used for DSC was progressively increased from 0.5 mg/ml for pure PS bilayers to 20 mg/ml for PS samples containing 50 mol % cholesterol. We have shown previously that this protocol is required to accurately monitor the broad, low-enthalpy phase transitions observed at higher cholesterol concentrations (McMullen et al., 1993). The calorimeter was calibrated using solid standards from Calorimetry Sciences Corporation as well as aqueous dispersions of highly pure disaturated PCs prepared in this laboratory (Lewis et al., 1987). Sample runs were repeated at least three times to ensure reproducibility. For all mixtures phospholipid and cholesterol degradation was monitored by thin layer chromatography and no degradation products were observed. Moreover, sequential HS-DSC runs were completely reproducible, supporting the absence of chemical degradation in our samples after HS-DSC or spectroscopic analysis and the homogeneity of our lipid dispersions. Data were analyzed and plotted using the Origin software package obtained from Microcal Software Inc. (Northampton, MA).

For FTIR spectroscopic analysis, the cholesterol/PS dispersions were prepared as described above for the HS-DSC experiments except that these samples were suspended in D_2O buffered with 100 mM sodium phosphate, 100 mM NaCl, and 10 mM EDTA (pH 7.4). The samples were placed between CaF_2 windows containing a 25-μm spacer and mounted in a cell holder attached to a computer-controlled circulating water bath. FTIR spectra were recorded with a Digilab (Cambridge, MA) FTS-40 Fourier transform infrared spectrometer. Data acquisition and processing protocols similar to those described earlier were employed (Mantsch et al., 1985). FTIR spectra were analyzed using computer programs developed by the National Research Council of Canada, and were plotted using the Origin software package.

RESULTS

DSC studies

Previous studies of the thermotropic phase behavior of aqueous dispersions of synthetic symmetrical-chain disaturated PSs have revealed only a single gel-to-liquid-crystalline phase transition for these compounds. However, we found that after prolonged incubation at low temperature, these compounds all exhibit a minor lower temperature as well as a major higher temperature phase transition (see accompanying paper). We present spectroscopic evidence in the accompanying paper that the lower temperature transition consists of a conversion from a lamellar crystalline (L_c) to a lamellar gel (L_β) phase, whereas the higher temperature transition is a conventional L_β to lamellar liquid-crystalline (L_α) phase transition. It is also important to note that under the experimental conditions employed in the DSC studies described below (overnight incubation at 2°C), the L_c phase does not have sufficient time to form in DMPS, DPPS, or DSPS bilayers in the absence of cholesterol; thus, a subtransition is not observed in heating scans of the pure phospholipid bilayers.

Our previous studies of the interactions of cholesterol with PC (McMullen and McElhaney, 1995, 1997; McMullen et al., 1993) and PE (McMullen and McElhaney, 1997; McMullen et al., 1999) bilayers have established that the thermotropic phase behavior of these mixtures depends on hydrocarbon chain length and structure of the phospholipid and on whether the heating or cooling mode is employed. As well, in some cases the thermal history of the sample can be important. Thus, we present DSC heating and cooling scans below for each individual PS studied, so that cholesterol miscibility with both gel and liquid-crystalline PS bilayers of various hydrophobic thicknesses can be monitored. The effects of the thermal history of the sample are also discussed where appropriate.

Illustrated in Fig. 1 are representative high-sensitivity DSC heating and cooling thermograms of DMPS bilayers containing cholesterol concentrations ranging from 0 to 50 mol % annealed at low temperatures. In both the heating and cooling modes, the overall effects of increasing concentrations of cholesterol are to lower the temperature of the L_β/L_α phase transition slightly (Fig. 2), to decrease the

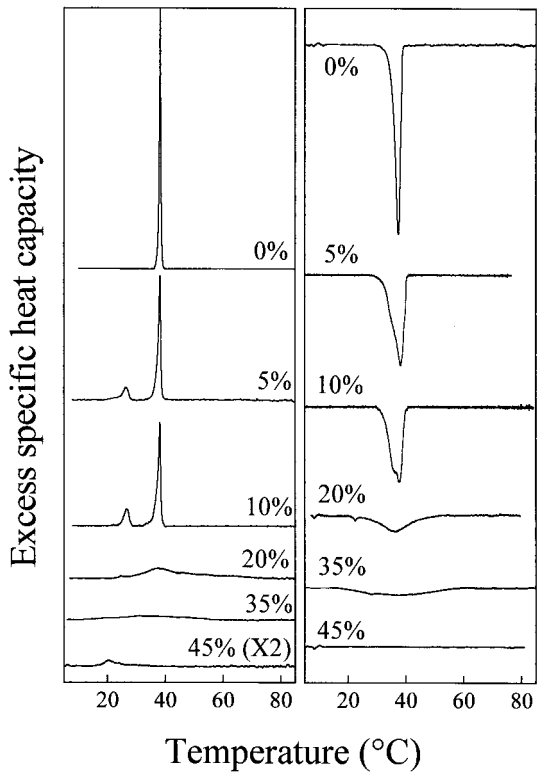

FIGURE 1 Representative heating (*left panel*) and cooling (*right panel*) thermograms of DMPS bilayers acquired at 10°C/h. The data presented are for the cholesterol contents indicated and have been normalized with respect to the mass of DMPS used.

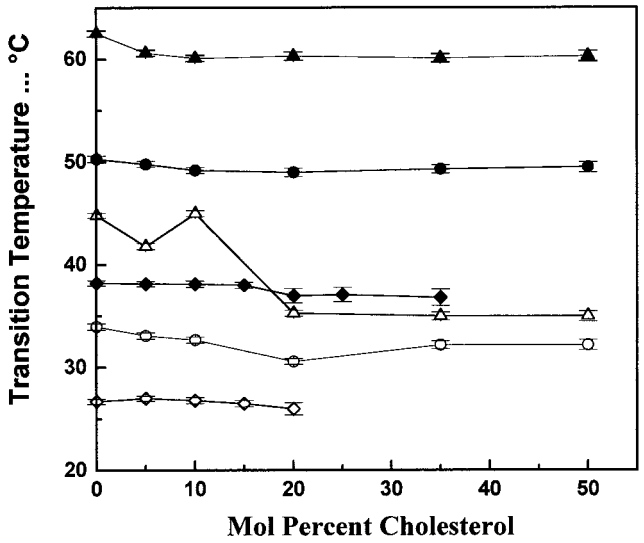

FIGURE 2 Effect of cholesterol content on the L_α/L_β (*solid symbols*) and L_c/L_β (*open symbols*) phase transition temperatures of the N-saturated 1,2,diacylphosphatidylserines. The data shown were acquired in the heating mode (10°C/h) and plotted as a function of cholesterol content thus: (—♦— and —◇—) DMPS/cholesterol samples; (—●— and —○—) DPPS/cholesterol samples; (—▲— and —Δ—) DSPS/cholesterol samples

cooperativity of this transition markedly (Fig. 1), and to progressively reduce the transition enthalpy to zero at 50 mol % (Fig. 3). Note that a sharp phase transition near 37°C, due to the melting of cholesterol monohydrate crystals, is not observed in either the heating or cooling runs, even at the highest cholesterol level tested (50 mol %). The complete abolition of a cooperative gel-to-liquid-crystalline phase transition by 50 mol % cholesterol, in both the heating and cooling modes, and the absence of an endotherm arising from a separate cholesterol phase, indicate that cholesterol is miscible with DMPS bilayers up to concentration of 50 mol % sterol in both the gel and liquid-crystalline states, in contrast to previous reports (Bach, 1984; Bach and Wachtel, 1989; Bach et al., 1992, 1998; Wachtel and Bach, 1987; Wachtel et al., 1991).

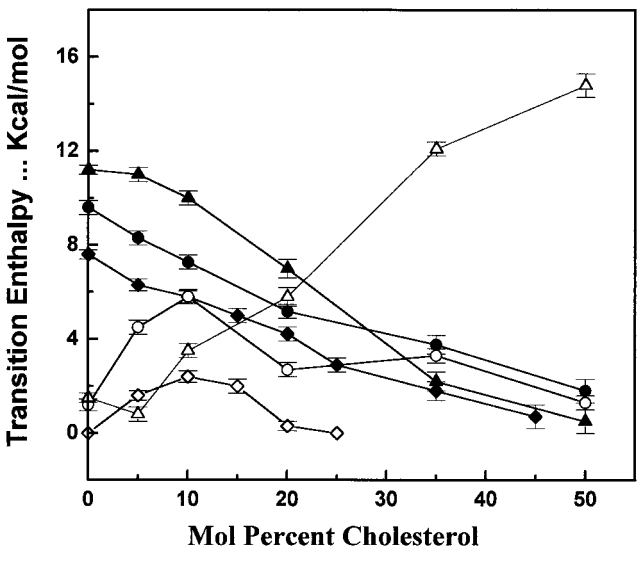

FIGURE 3 Effect of cholesterol content on the enthalpy of the L_β/L_α (*solid symbols*) and L_c/L_β (*open symbols*) phase transitions exhibited by the N-saturated 1,2-diacyl phosphatidylserines. The data shown were acquired in the heating mode (10°C/h) and plotted as a function of cholesterol content thus: (—♦— and —◇—) DMPS/cholesterol samples; (—●— and —○—) DPPS/cholesterol samples; (—▲— and —Δ—) DSPS/cholesterol samples. The enthalpy values quoted for the phase transitions of the DSPS/cholesterol mixtures includes the additional thermotropic event observed at high (35 mol %) cholesterol concentrations.

In the DSC heating scans of DMPS bilayers containing relatively low cholesterol levels (5–20 mol %), an additional minor lower temperature endotherm appears (Fig. 1). This endotherm increases in prominence from 0 to 10 mol % cholesterol and decreases in prominence from 10 to 20 mol % cholesterol, being absent entirely at higher sterol concentrations. Moreover, this endotherm is absent from cooling scans and also disappears if the sample is immediately reheated after cooling rather than being held overnight at 2°C. Note also that this minor endotherm is centered near 27°C, the same temperature as the lower temperature endotherm observed in DMPS bilayers not containing cholesterol that have undergone extensive low temperature annealing (see accompanying paper). We thus ascribe this lower temperature endotherm to an L_c/L_α phase transition, as confirmed by the FTIR results presented below. Thus, the presence of low levels of cholesterol actually facilitates the formation of the L_c phase in DMPS bilayers. Moreover, because the tightly packed DMPS L_c phase is unlikely to contain significant amounts of cholesterol, low temperature incubation of DMPS bilayers containing low concentrations of cholesterol must result in the formation of cholesterol-poor L_c and cholesterol-enriched L_β domains in this system.

DSC heating and cooling scans illustrating the thermotropic phase behavior of low temperature-annealed DPPS bilayers containing various concentrations of cholesterol from 0 to 50 mol % are presented in Fig. 4. As before, the overall effects of the presence of increasing amounts of cholesterol are to slightly lower the L_α/L_α phase transition temperature (Fig. 2), decrease the cooperativity of this transition (Fig. 4), and progressively reduce the transition enthalpy (Fig. 3). However, in this case, the reduction in both the cooperativity and the enthalpy of the main phase transition is not quite as pronounced as in the case of DMPC bilayers, and in fact a weak chain-melting phase transition persists even at a cholesterol concentration of 50 mol % in both the heating and cooling modes. This result suggests that cholesterol is largely but not completely miscible in either gel or liquid-crystalline bilayers of DPPS when cholesterol levels approach 50 mol %. However, a phase transition near 37°C due to the presence of a separate cholesterol monohydrate phase was again not observed in either the heating or cooling runs.

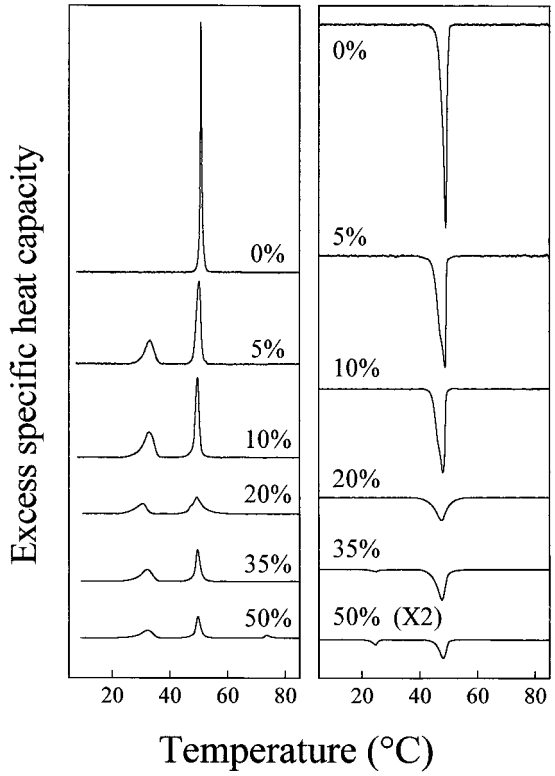

FIGURE 4 Representative heating (*left panel*) and cooling (*right panel*) DSC thermograms of cholesterol-containing DMPS bilayers acquired at 10°C/h. The data presented are for the cholesterol contents indicated and have been normalized with respect to the mass of DPPS used.

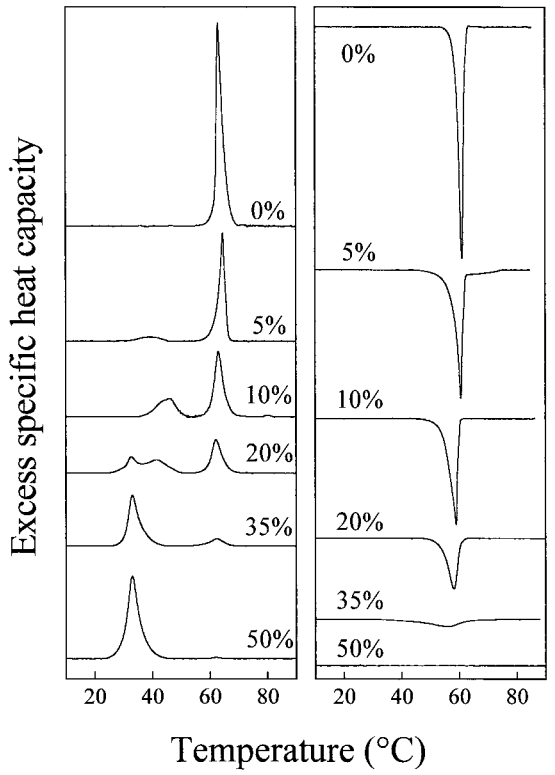

FIGURE 5 Representative heating (*left panel*) and cooling (*right panel*) DSC thermograms of cholesterol-containing DMPS bilayers acquired at 10°C/h. The data presented are for the cholesterol contents indicated and have been normalized with respect to the mass of DSPS used.

An additional lower temperature endotherm again appears in the heating runs of DPPS/cholesterol mixtures (Fig. 4). However, in contrast to the DMPS/cholesterol mixtures, the subtransition induced by the presence of cholesterol did not disappear at higher cholesterol concentrations, although the enthalpy of this transition was again maximal at 10 mol % cholesterol and decreased progressively at higher sterol concentrations (Fig. 3). As well, the subtransition enthalpy in DPPS/cholesterol mixtures is higher than that noted in the DMPS/cholesterol mixtures at comparable cholesterol concentrations, implying that cholesterol is more effective at inducing L_c phase formation in this longer chain PS.

DSC heating and cooling thermograms illustrating the thermotropic phase behavior of low temperature-annealed DSPS bilayers containing from 0 to 50 mol % cholesterol are shown in Fig. 5. Again, in both the heating and cooling modes, the overall effects of cholesterol are to reduce the temperature of the main phase transition slightly (Fig. 2), to decrease the cooperativity of this transition markedly (Fig. 5), and to progressively reduce the transition enthalpy to zero (cooling mode) or almost zero (heating mode) at 50 mol % (Fig. 3). Thus, cholesterol seems to be completely miscible with liquid-crystalline and almost completely miscible with gel state DSPS bilayers at levels up to 50 mol %.

The low-temperature thermotropic phase behavior of DSPS/cholesterol mixtures in the heating mode, however, appears to be more complex than with mixtures of cholesterol with DMPS and DPPS, at least at the higher sterol concentrations (Fig. 5). At low cholesterol concentrations, an additional minor endotherm near 45°C is observed, which becomes more prominent as cholesterol concentration increases from 5 to 10 mol %. As this is the temperature at which the subtransition occurs in pure DSPS bilayers annealed at low temperatures, we again assign this endotherm to a L_c/L_β phase transition. However, at higher cholesterol concentrations, a second, lower temperature endotherm appears near 32°C and grows rapidly in enthalpy with further increases in cholesterol levels, while the original low temperature endotherm appears to decrease in temperature until it merges with the new and progressively more prominent endotherm centered near 32°C. A possible molecular interpretation of this aspect of the thermotropic phase behavior of DSPS bilayers containing higher concentrations of cholesterol will be offered below. For the present, it is important only to note that the presence of this additional endotherm at 32°C does not alter the characteristic effect of increasing cholesterol levels on the L_β/L_α phase transition.

FTIR spectroscopic studies

FTIR spectroscopic analyses of PS and cholesterol/PS mixtures were performed to determine the effect of cholesterol on the phase state and conformational order of the cholesterol/PS bilayers as a function of temperature and to correlate this information with the thermotropic phase behavior observed by DSC. The polymorphic phases of these lipids each exhibit distinctive patterns of infrared absorption, which are interpretable in terms of variations in hydrocarbon chain conformational order, hydrocarbon chain packing, and hydration and hydrogen bonding interactions in the headgroup and bilayer polar/apolar interfacial regions (for details, see Lewis and McElhaney, 2000). Moreover, as illustrated in Fig. 6, the infrared spectroscopic signatures of each of these lipid phases are quite distinct and can therefore be used to differentiate between the various lipid phases and to assign the nature of the phase transitions observed in our DSC studies. Detailed structural interpretations of the data shown in Fig. 6 are not the focus of this paper and are presented in the accompanying paper (Lewis and McElhaney, 2000).

Illustrated in Fig. 7 are representative FTIR spectra exhibited by cholesterol-containing (5–20 mol %) DMPS samples at temperatures bracketing the two thermotropic transitions observed in our DSC studies (see Fig. 1). A comparison of the spectroscopic features shown therein with those of the reference data shown in Fig. 6 reveals the following. First, the spectroscopic signatures observed at temperatures just below and just above the higher-temperature phase transition exhibited by these DMPS/cholesterol mixtures (Fig. 7, spectra *B* and *C*, respectively) are consistent with the existence of the L_β and L_α phases at those temperatures, indicating that the relatively cooperative higher temperature transition exhibited by these DMPS/cholesterol mixtures is a L_β/L_α phase transition. These spectroscopic changes are comparable to those observed at temperatures below and above the single highly cooperative transition exhibited by cholesterol-free PS preparations that have not been annealed at low temperatures (Lewis and McElhaney, 2000) and at temperatures below and above the single broad thermotropic phase transition exhibited by the DMPS/cholesterol preparations containing more than 20 mol % cholesterol (spectra not shown). The latter observation indicates that the structural changes occurring at the single broad transition exhibited by the cholesterol-rich DMPS preparations is also qualitatively comparable to that occurring at the L_β/L_α phase transitions observed with preparations of lower cholesterol content. Second, at temperatures below the onset of the lowest temperature phase transition exhibited by preparations containing relatively low (5–20 mol %) amounts of cholesterol, DMPS/cholesterol mixtures exhibit FTIR spectra comparable to the spectra *A* shown in Fig. 7. A comparison of these spectroscopic features with the reference set shown in Fig. 6 indicates that they are similar to but not identical with those of the L_c phase (designated here as L_c1) formed by the shorter chain PSs (for details, see Lewis and McElhaney, 2000). Our analyses also indicate that the spectra shown in Fig. 7 *A* can be approximated by a summation of the L_c1 (~70–90%) and L_β (~30–10%) phase spectra illustrated in Fig. 6 (data not shown). We conclude from these observations that,

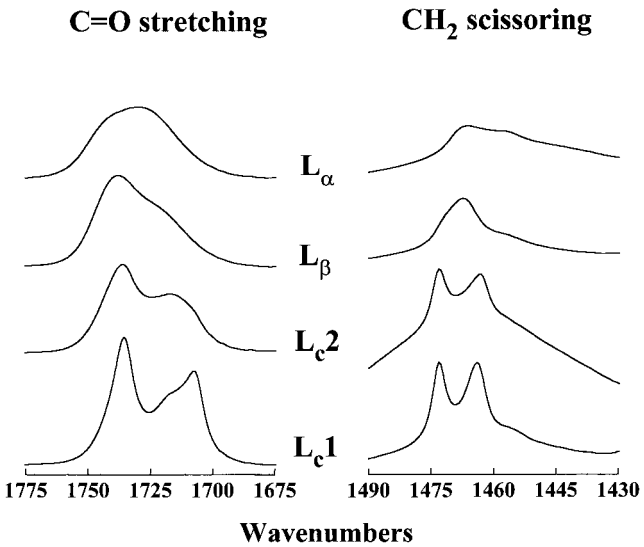

FIGURE 6 The C = O stretching (*left panel*) and CH_2 scissoring (*right panel*) infrared absorption bands exhibited by the polymorphic phases of the n-saturated 1,2-diacyl phosphatidylserines. The data shown are representative of L_α, the lamellar-liquid-crystalline phase of all PSs; L_β, the lamellar gel phase of all PSs; L_c1, the lamellar crystalline phases of DMPS; and L_c2, the lamellar crystalline phases of DPPS and DSPS.

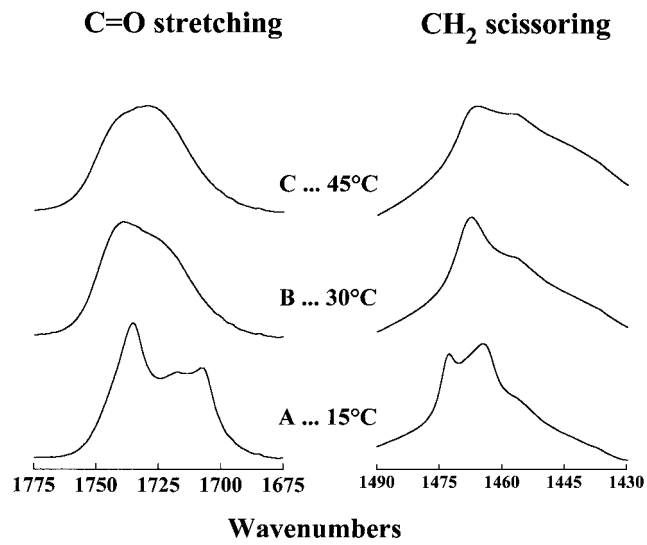

FIGURE 7 Representative C = O stretching (*left panel*) and CH_2 scissoring (*right panel*) infrared absorption bands exhibited by cholesterol-containing (5–20 mol %) DMPS bilayers. The data shown were acquired in the heating mode with a sample containing 10 mol % cholesterol.

under the experimental conditions indicated, a significant fraction of the PS of these DMPS/cholesterol mixtures forms a lamellar crystalline phase that is spectroscopically indistinguishable from that normally formed in the absence of cholesterol. Therefore, the low-temperature thermotropic event exhibited by these mixtures is a transition between a lamellar crystalline and lamellar gel phase. That L_c phases can be formed from these cholesterol-containing bilayers indicates that essentially cholesterol-free DMPS domains must exist at temperatures well below the L_β/L_α phase transition temperature. These observations suggest, therefore, that cholesterol may not be very miscible with the gel phases of DMPS at temperatures well below T_m. Finally, we note that the kinetics of forming the L_c phase from these DMPC/cholesterol mixtures, as well as the comparable DPPS/cholesterol and DSPS/cholesterol mixtures (see below), are considerably faster than in pure PS bilayers. Under our experimental conditions significant amounts of the L_c phase are formed upon overnight incubation of the cholesterol-containing PS bilayers at temperatures near 0–2°C, whereas significant L_c phase formation in pure PS bilayers typically requires incubation periods of at least 1 week under comparable conditions (Lewis and McElhaney, 2000). Comparable phenomena have been observed in cholesterol-containing PE bilayers (McMullen et al., 1999).

FTIR spectra representative of those exhibited by cholesterol-containing (5–20 mol %) DPPS and DSPS mixtures are shown in Fig. 8. As illustrated in Fig. 4 and 5, these mixtures exhibit also two thermotropic transitions. The FTIR spectroscopic signatures exhibited by these mixtures at temperatures just below and above the higher-temperature transition (Fig. 8, spectra *B* and *C*, respectively), are comparable to those of L_β and L_α phases of the pure PS, respectively (see reference data shown in Fig. 6), again indicating that those thermotropic events are qualitatively comparable to the L_β/L_α phase transitions of the pure PS bilayers. Moreover, as is also observed in the DMPS system, comparable spectra are observed at temperatures just below and above the broad, weakly energetic, higher temperature phase transition exhibited by cholesterol-rich (>20 mol %) DPPS and DSPS preparations (spectra not shown), indicating that this thermotropic event is also qualitatively similar to a L_β/L_α type of phase transition. The FTIR spectra exhibited by DPPS/cholesterol and DSPS/cholesterol mixtures (<20 mol %) at temperatures below the onset of their lower-temperature transition (Fig. 8, spectra *A*) appear to be similar to though not identical with the type of L_c phase (designated here as L_c2) formed by the longer chain PSs (see reference spectra in Fig. 6). However, our analyses also indicate that the observed spectra can be approximated by a summation of the L_c2 (~50–70%) and L_β (~50–30%) phase spectra illustrated in Fig. 6 (data not shown). Thus, as observed with the DMPS-based mixtures described above, a significant fraction of the PS of these DPPS/cholesterol and DSPS/cholesterol mixtures forms a lamellar crystalline phase that is spectroscopically indistinguishable from that normally formed by the pure lipid. Also, as observed with the DMPS mixtures, the low-temperature transition can be identified as a transition between the lamellar crystalline and lamellar gel forms of DPPS and DSPS.

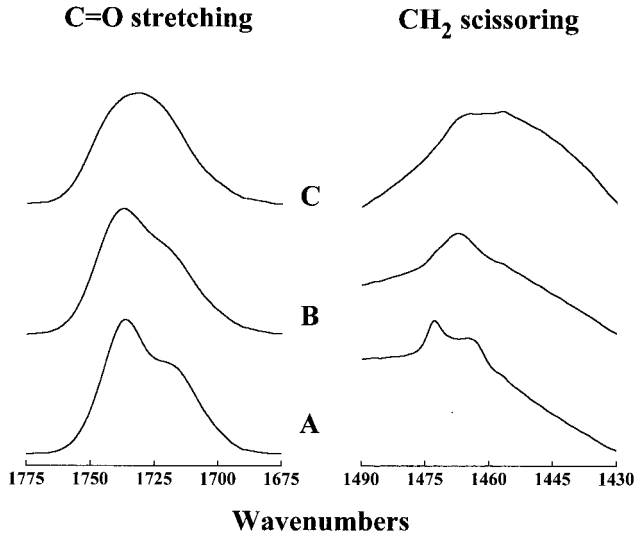

FIGURE 8 Representative FTIR C = O stretching (*left panel*) and CH_2 scissoring (*right panel*) infrared absorption bands exhibited by cholesterol-containing (5–20 mol %) DPPS and DSPS bilayers. The data shown were acquired in the heating mode with a DSPS sample containing 10 mol % cholesterol at temperatures bracketing the two thermotropic transitions resolved by DSC. (*A*) The lamellar crystalline phase, $T < T_s$. (*B*) The lamellar gel phase, $T_s < T < T_m$. (*C*) The lamellar liquid-crystalline phase, $T > T_m$.

Finally, as illustrated in Fig. 5, cholesterol-rich (>35 mol %) DSPS mixtures exhibit another low-temperature thermotropic event different that is distinct from the L_c/L_β phase transition alluded to above (Fig. 5). We find, however, that the FTIR spectra acquired at temperatures below and just above this thermotropic transition (Fig. 9, spectra *A* and *B*, respectively) are similar to each other and to the spectra of the L_β phase formed by pure PS bilayers (see reference spectra shown in Fig. 6). Therefore, we conclude that the anomalous thermotropic event exhibited by the cholesterol-rich DSPS preparations does not involve a lamellar crystalline phase. Interestingly, however, a close inspection of Fig. 9 also shows that the CH_2 scissoring bands observed at low temperature (spectrum *A*) are broader than those observed upon completion of this new thermotropic transition (spectrum *B*). Our analyses of these bands indicate that the increase in line width observed at low temperatures is attributable to a slight splitting of the CH_2 scissoring of the absorption band into components centered near 1470 and 1466 cm^{-1} (data not shown). This splitting collapses upon completion of the thermotropic transition, and the CH_2 scissoring band appears as a single peak centered near 1468 cm^{-1}. Similar phenomena have been observed at tempera-

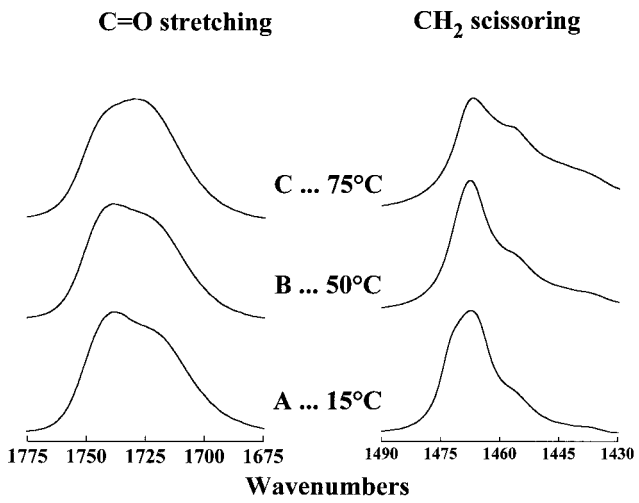

FIGURE 9 Representative FTIR C = O stretching (*left panel*) and CH_2 scissoring (*right panel*) infrared absorption bands exhibited by cholesterol-containing DSPS bilayers. The data shown were acquired in the heating mode at the temperatures indicated with a DSPS sample containing 35 mol % cholesterol.

tures bracketing a high-enthalpy solid phase transition exhibited by androstenol-rich DSPC mixtures and have been ascribed to a solid phase thermally-induced mixing/demixing of that sterol analog with the phospholipid matrix (McMullen et al., 1994). We believe that a comparable phenomenon is occurring with these cholesterol-rich DSPS mixtures.

DISCUSSION

The results of our present high-sensitivity DSC and FTIR spectroscopic study of the thermotropic phase behavior of aqueous dispersions of binary mixtures of DMPS, DPPS, and DSPS with cholesterol do not agree in many respects with the results of prior low-sensitivity DSC and x-ray diffraction studies of cholesterol/DMPS and cholesterol/DPPS binary mixtures (Bach and Wachtel, 1989; Bach et al., 1998). Although all three studies report that the addition of cholesterol broadens and shifts the temperature of the gel-to-liquid-crystalline phase transition and reduces its enthalpy, there are many quantitative differences in the results obtained. For example, Bach et al. (1998) report that the addition of 50 mol % cholesterol shifts the phase transition temperature of DMPS bilayers upward by several degrees, whereas Bach and Wachtel (1989) report that this level of cholesterol shifts the phase transition temperature of DPPS bilayers downward by about 3°C. However, we find a similar downward shift in the phase transition temperature of all three PSs studied by 2–3°C at similar cholesterol levels. Moreover, Bach and coworkers (1998) report that a cooperative gel-to-liquid-crystalline phase transition is still prominent in DMPS and DPPS bilayers at 50 mol % cholesterol, whereas our results indicate that this phase transition is abolished or nearly abolished when equimolar phospholipid/cholesterol ratios are reached. Finally, a major qualitative difference in these studies is that Bach and coworkers (1998) report the existence of cholesterol phase separation in both the gel and liquid-crystalline DMPS- and DPPS-cholesterol mixtures at cholesterol concentrations above about 30 mol %, whereas we find no calorimetric or spectroscopic evidence for the existence of a separate cholesterol phase at sterol concentrations of up to 50 mol %.

We propose that all or most of the discrepancies in experimental results summarized above arise from differences in the preparation and treatment of the PS/cholesterol mixtures utilized in this and the previous studies. We find, for example, that if chloroform alone is used to dissolve the PS and cholesterol, and if the temperature of the binary mixture is not maintained above the transition temperature of the PS component when the organic solvents are evaporated, then cholesterol appears to be much less miscible in PS bilayers, and DSC endotherms corresponding to the melting of cholesterol monohydrate crystals are observed. Moreover, Huang et al. (1999) have recently examined the maximum solubility limit of cholesterol using an x-ray diffraction technique that is very sensitive to the formation of cholesterol monohydrate crystals. These workers found that the maximum solubility limit of cholesterol in four different PCs of different hydrocarbon chain length and degree of unsaturation falls near 67 mol % sterol, whereas that for 1-palmitoyl-2-oleoyl PE falls near 50 mol %. Moreover, these workers also showed that the previous reports of the existence of cholesterol crystals at lower cholesterol concentrations in these systems were due to the artifactual demixing of cholesterol that can occur during conventional sample preparation, particularly when the cholesterol/phospholipid mixtures pass through an intermediary solid state. Moreover, neither rehydration, heating, nor mechanical agitation can bring about the complete remixing of this demixed cholesterol once crystals of cholesterol are formed. In addition, the results of recent x-ray diffraction studies indicate that cholesterol is soluble in 1-palmitoyl-2-oleoyl-PS bilayers to levels of about 67 mol % sterol (G. W. Feigenson, personal communication), as is the case for the corresponding PC bilayer. This latter result, and the absence of calorimetric and spectroscopic evidence for a separate cholesterol phase in our present experiments with PS bilayers, strongly suggest that an artifactual demixing of cholesterol occurred in these earlier studies of PS/cholesterol (Bach, 1984; Bach and Wachtel, 1989; Bach et al., 1992, 1998; Wachtel and Bach, 1987; Wachtel et al., 1991) and PG/cholesterol (Borochov et al., 1995) systems. In this regard, it would be instructive to repeat these investigations of the thermotropic phase behavior of binary mixtures of the anionic phospholipids and cholesterol using the two novel preparation methods developed by Buboltz and Feigenson (1999), which were specifically designed to prevent cholesterol demixing during sample preparation.

A comparison of the thermotropic phase behavior of mixtures of cholesterol with a homologous series of symmetrical chain disaturated PCs (McMullen et al., 1993, 1995; McMullen and McElhaney, 1995; 1994, Vilcheze et al., 1996), PEs (McMullen et al., 1999) and PSs (this study) is instructive, as it permits a comparison of the effects of variations in polar headgroup structure and charge and variations in hydrocarbon chain length on phospholipid-cholesterol interactions. In the PC series, the shift in the temperature of the broad component induced by the addition of cholesterol depends on hydrocarbon chain length, with this temperature shifting upward by progressively greater amounts as hydrocarbon chain length decreases from 17 carbon atoms, and shifting downward by progressively greater amounts as hydrocarbon chain length increases from 17 carbon atoms (a hydrophobic mismatch effect). In contrast, in the PS homologous series, this shift in temperature is always slightly downward (about 2–3°C at 50 mol % cholesterol) and is essentially independent of hydrocarbon chain length. A similar hydrocarbon chain length-independent behavior is observed in the PE series, except that shift to lower temperature is much more pronounced (about 15°C at 50 mol % cholesterol). Thus, at moderate hydrocarbon chain lengths (14–16 carbon atoms), the overall effect of cholesterol is to stabilize the PC gel state relative to the liquid-crystalline state, whereas cholesterol incorporation slightly or strongly destabilizes the gel state relative to the liquid-crystalline state in PS and PE bilayers, respectively. Similarly, cholesterol appears to be miscible in gel and liquid-crystalline PC bilayers of a range of hydrocarbon chain lengths to levels of up to 50 mol %, abolishing the cooperative gel-to-liquid-crystalline phase transition at 50 mol % cholesterol in both cases, except for very long chain compounds. Moreover, cholesterol is at least largely miscible in gel and liquid-crystalline PS bilayers, so that the gel-to-liquid-crystalline phase transition is completely abolished or at least markedly attenuated at 50 mol % cholesterol on heating or cooling. In contrast, in the PE series, a significantly reduced miscibility of cholesterol in both the gel and liquid-crystalline states is observed, particularly with the longer chain compounds, since a gel-to-liquid-crystalline phase transition persists at 50 mol % sterol. Finally, the presence of cholesterol inhibits the formation of the L_c phase in PC bilayers incubated at low temperatures, whereas it facilitates L_c phase formation in PS and especially in PE bilayers. Thus, in all aspects of their thermotropic phase behavior, these three classes of phospholipids form a graded series from PC to PS to PE. The fact that the behavior of mixtures of the two zwitterionic phospholipids, PC and PE, with cholesterol are very different, and that the anionic lipid PS/cholesterol mixtures occupy an intermediate position in this series, argues against a unique general role for polar headgroup charge per se in determining the strength and nature of phospholipid-cholesterol interactions. This conclusion is supported by earlier work on the effect of limiting amounts of cholesterol on the thermotropic behavior of binary phospholipid mixtures, which indicated that anionic phospholipids exhibit greater affinity for cholesterol than do the zwitterionic phospholipids PC and PE (van Dijck, 1979), and by our recent calorimetric study, which showed that cholesterol exhibits greater miscibility in anionic PG than in uncharged glycolipid bilayers (McMullen et al., 1996).

The results discussed above can be explained, at least qualitatively, by considering the strength of the intermolecular interactions characteristic of the various phospholipid molecular species studied, as manifested, for example, in their relative gel-to-liquid-crystalline phase transition temperatures. The relative phase transition temperatures of the three phospholipid classes studied here increase in the order PC $<$ PS $<$ PE, and, for any given phospholipid class, the dielaidoyl species undergoes the chain-melting phase transition at a considerably lower temperature than does the distearoyl molecular species. Moreover, the gel-to-liquid-crystalline phase transition temperature increases with hydrocarbon chain length in all three lipid classes. The high phase transition temperature of the PE molecular species is due to the strong attractive electrostatic and hydrogen-bonding interactions characteristic of the polar headgroup of this phospholipid relative to PS and especially to PC. Although these difference are most pronounced in the gel state, the higher gel-to-liquid-crystalline phase transition temperature of the PEs is also manifested as a greater degree of order in the liquid-crystalline state, even at comparable reduced temperatures, at least relative to the PCs. As well, the stronger van der Waals forces characteristic of saturated and longer-chain phospholipids increase the tightness of packing of these molecules in both gel and liquid-crystalline bilayers. We conclude, therefore, that the miscibility of cholesterol with any particular phospholipid is in general inversely related to the degree of order or the tightness of packing characteristic of that phospholipid at a given temperature and phase state. Interestingly, this also seems to be true of the miscibility of hydrophobic transmembrane peptides with PC (Zhang et al., 1992), PE (Zhang et al., 1995), and PS (unpublished observations from this laboratory) bilayers. Although more subtle and specific cholesterol-phospholipid interactions may occur in particular binary systems, it seems that, in general, the relative strength of phospholipid-cholesterol interactions, as manifested in the ability of cholesterol to maximize phospholipid-cholesterol and to minimize cholesterol-cholesterol interactions, is determined primarily by the strength of phospholipid-phospholipid interactions in most phospholipid bilayer systems. Thus, phospholipids with strong intermolecular interactions with one another tend to exclude cholesterol from the bilayer above certain critical concentrations. This finding seems also to hold for the monoglucosyl diacylglycerol, diglucosyldiacylglycerol, and PG components of the *Acholeplasma laidlawii* membrane, where the higher-melting

neutral glycolipids exhibit a more limited ability to mix with cholesterol than the anionic phospholipid PG (McMullen et al., 1996). Conversely, the tendency for phospholipid-cholesterol lateral phase separation can be reduced by decreasing the strength of the intermolecular phospholipid-phospholipid interactions. This can be accomplished by decreasing the phospholipid phase transition temperature by changes in polar headgroup structure, by introducing hydrocarbon chain unsaturation, by decreasing hydrocarbon chain length, or by increasing the temperature of the system.

We are presently investigating the effect of cholesterol on the thermotropic phase behavior of anionic PG and phosphatidic acid bilayers in order to further delineate the role of polar headgroup change in phospholipid-cholesterol interactions.

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