Differential Scanning Calorimetric Studies of the Interaction of Cholesterol with Distearoyl and Dielaidoyl Molecular Species of Phosphatidylcholine, Phosphatidylethanolamine, and Phosphatidylserine†

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ABSTRACT: We have carried out a comparative study of the effect of cholesterol on the thermotropic phase behavior of the distearoyl and dielaidoyl molecular species of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine using high-sensitivity differential scanning calorimetry. For both molecular species of phosphatidylcholine, cholesterol incorporation produces bimodal endotherms at lower and unimodal endotherms at higher sterol concentrations. In both cases, heating and cooling endotherms are identical, and high concentrations of cholesterol (50 mol %) completely abolish the gel to liquid-crystalline phase transition. For the distearoyl molecular species of phosphatidylserine and phosphatidylethanolamine, heating and cooling endotherms are not identical, and cholesterol exhibits a considerably reduced miscibility in the gel as compared to the liquid-crystalline phase, particularly in the latter case. Thus, in neither case does the addition of 50 mol % cholesterol completely abolish the cooperative hydrocarbon chain-melting phase transition. However, the dielaidoyl molecular species of phosphatidylserine and phosphatidylethanolamine exhibit much closer correspondence in the heating and cooling modes than do the distearoyl species, and 50 mol % cholesterol is sufficient to almost or completely abolish the gel to liquid-crystalline phase transition of dielaidoylphosphatidylethanolamine and dielaidoylphosphatidylserine. In general, there is an inverse correlation between the strength of intermolecular phospholipid−phospholipid interactions, as manifested by the relative gel to liquid-crystalline phase transition temperatures of the pure phospholipids, and the miscibility of cholesterol in bilayers, particularly gel-state bilayers, formed from these phospholipids. These results indicate that the nature of cholesterol−phospholipid interactions, and thus the miscibility of cholesterol in the bilayer, depends on both the structure of the phospholipid polar headgroup and the hydrocarbon chains, as well as on the temperature and phase state of the phospholipid bilayer.

The occurrence of substantial quantities of cholesterol (or of a structurally similar sterol) in the plasma membranes of virtually all eukaryotic cells has prompted many investigations into the role of cholesterol in the structure and function of cell membranes [for reviews, see Dahl and Dahl (1988), Yeagle (1988), and McElhaney (1992a,b,c)]. Although cholesterol and related sterols appear to have several different functions in eukaryotic cells, one of its primary and apparently essential roles is as a modulator of the physical properties of the phospholipid bilayer of the plasma membrane. Thus, numerous studies of the interactions of cholesterol with single-component phospholipid model membranes have been carried out using many different physical techniques [for reviews, see Demel and de Kruijff (1976), Razin and Rottem (1978), Yeagle (1988), Finean (1990), McElhaney (1992a,b,c), and McMullen and McElhaney (1996)]. There is now a consensus that the incorporation of increasing levels of cholesterol broadens and eventually eliminates altogether the cooperative gel/liquid-crystalline phase transition of the host lipid bilayer. In addition, cholesterol increases the degree of orientational order and reduces the rate of motion of the phospholipid hydrocarbon chains in the biologically relevant liquid-crystalline state, condensing the bilayer laterally, increasing its mechanical strength, and decreasing its permeability, while maintaining the relatively high rates of lateral and rotational diffusion characteristic of the fluid phospholipid bilayers. Although much fewer in number, similar studies of the interactions of cholesterol with the complex mixtures of lipids found in natural membranes seem to yield similar results. However, despite an impressive body of experimental data derived from both model and biological membranes, our detailed understanding of the molecular basis for the effects of cholesterol on phospholipid bilayers remains incomplete.

The vast majority of studies of cholesterol−phospholipid interactions have utilized binary mixtures of cholesterol with a phosphatidylcholine (PC)[1] containing two identical linear saturated hydrocarbon chains, especially DPPC. Although

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[1] Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DEPC, dielaidoylphosphatidylcholine; PE, phosphatidylethanolamine; DSPE, distearoylphosphatidylethanolamine; DEPE, dielaidoylphosphatidylethanolamine; PS, phosphatidylserine; DSPS, distearoylphosphatidylserine; DEPS, dielaidoylphosphatidylserine; DGDG, diglucosyldiacylglycerol; MGDG, monoglucosyldiacylglycerol; LS/HS-DSC, low-sensitivity or high-sensitivity differential scanning calorimetry; FTIR, Fourier transform infrared; ^{31}P, phosphorus-31; ^{2}H, deuterium; ^{13}C, carbon-13; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; PPM, parts per million; L_c, crystalline phase; L_β, lamellar gel phase; $L_{\beta'}$, lamellar gel phase with tilted phospholipid hydrocarbon chains; $P_{\beta'}$, rippled gel phase with tilted hydrocarbon chains; L_α, lamellar liquid-crystalline phase; H_{II}, inverted hexagonal phase.

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it has been suggested that the thermotropic phase behavior of cholesterol/DPPC mixtures is generally representative of all molecular species of PC's (Ipsen *et al*., 1987; Thewalt & Bloom, 1992; Linseisen *et al*., 1993), in fact considerable evidence exists that there are significant variations in such behavior, depending on the fatty acid composition of the host PC bilayer. For example, the miscibility of cholesterol, or various side chain truncated cholesterol analogues, with various PC molecular species, and the effect of the incorporated sterol on the phase transition temperature and organization of the host PC bilayer, has been shown to be markedly dependent on hydrocarbon chain length (McMullen *et al*., 1993, 1994, 1995; McMullen & McElhaney, 1996; Vilchèze *et al*., 1996). Moreover, the thermotropic phase behavior of binary mixtures of cholesterol or cholesterol analogues with PC's containing one or two unsaturated hydrocarbon chains seems to be quite different from that of binary mixtures of cholesterol with linear saturated PC's (Davis & Keough, 1983; Keough *et al*., 1989; Kariel *et al*., 1991; Vilchèze *et al*., 1996). As well, the thermotropic phase behavior of mixtures of cholesterol with synthetic or naturally occurring phospholipids such as PE's (Blume, 1980; Epand & Bottega, 1987; Cheetham *et al*., 1989; McMullen *et al*., 1996a), PS's (Bach, 1984; Wachtel & Bach, 1987; Wachtel *et al*., 1991; Bach *et al*., 1992), or PG's (Borochov *et al*., 1995), as well as with the neutral glyceroglycolipids MGDG and DGDG from the *Acholeplasma laidlawii* B membrane (McMullen *et al*., 1996b), may differ significantly from that of binary mixtures of cholesterol with the homologous PC's. In addition, the interactions of cholesterol with phospho- and glycosphingolipids seem to vary with respect both to each other and to the related glycerophospho- and glyceroglycolipids (Bach, 1984). Thus, the detailed nature of cholesterol−lipid interactions may vary appreciably with the structure of the polar headgroup and interfacial region, and with the structure and length of the hydrocarbon chains, of the phospho- or glycolipid molecules which make up the host lipid bilayer [see McMullen and McElhaney (1996)]. However, the majority of prior studies examining the effect of phospholipid polar headgroup structure on cholesterol−phospholipid interactions have utilized phospholipids of varying hydrocarbon chain length and saturation. Consequently, the conclusions of these studies are not always consistent, and many of these studies have employed LS-DSC and experimental protocols which do not reveal the more subtle aspects of cholesterol−phospholipid interactions [see McMullen *et al.* (1993)].

The PC's and PE's are generally the two major zwitterionic phospholipid classes, and the PS's are the major anionic phospholipid class found in eucaryotic plasma membranes. In the present comparative study, we have investigated the effects of cholesterol incorporation on the thermotropic phase behavior of aqueous dispersions of these three important phospholipid classes, in order to assess the effect of variations in lipid polar headgroup structure on the nature of cholesterol−phospholipid interactions. Within each phospholipid class, we have also compared the distearoyl and dielaidoyl molecular species in order to assess the influence of *trans*-unsaturation on the interactions of cholesterol in the absence of any hydrocarbon chain length mismatch between the host phospholipid bilayers. [The effects of variations in hydrocarbon chain length on the thermotropic phase behavior of cholesterol/PC (McMullen *et al.*, 1993), cholesterol/PE (McMullen *et al.*, 1997a), and cholesterol/PS (McMullen *et al.*, 1997c) are reported elsewhere.] In these studies, we employed HS-DSC and an experimental protocol designed to accurately monitor phospholipid thermotropic phase behavior over a wide range of cholesterol concentrations, and we performed cooling as well as heating runs to assess cholesterol miscibility in both the gel and liquid-crystalline states. We do indeed find that variations in phospholipid polar headgroup structure and the degree of unsaturation of the fatty acyl chains can both have significant effects on the thermotropic phase behavior of cholesterol/phospholipid binary mixtures in water.

MATERIALS AND METHODS

The PC's, PE's, and PS's used in these experiments were purchased from Avanti Polar Lipids (Alabaster, AL) and checked for purity by TLC using chloroform/methanol/ammonia (50:50:4, by volume) (PC's and PE's) or chloroform/methanol/glacial acetic acid/water (60:40:10:4, by volume) (PS's) as the developing solvent followed by spraying with 2% K_2CrO_4 in 60% sulfuric acid and charring. Each phospholipid gave a single spot on the developed TLC plates. The cholesterol was also purchased from Avanti Polar Lipids and recrystallized from ethanol before use. We found that the best procedure for producing homogeneous mixtures of phospholipid and cholesterol required chloroform/methanol (2:1, v/v) stock solutions (or lyophilization from benzene) which, after mixing, were heated to approximately 40−50 °C under N_2 to remove the solvent, and then dried under vacuum for at least 18 h. When carefully followed, both protocols provide fully reproducible thermograms [see also McMullen *et al*. (1997a,c)]. Failure to lyophilize from benzene or to heat the chloroform/methanol/cholesterol/phospholipid mixtures would often require repeated heating and cooling DSC runs upon hydration to achieve consistent thermogram profiles.

For the HS-DSC experiments, the dried phospholipid/cholesterol mixtures were dispersed and suspended in a buffer containing 50 mM Tris, 100 mM NaCl, and 10 mM EDTA (pH 7.4), heated to approximately 10−20 °C above the phase transition of the mixture, and then vortexed for at least 30 min to give a multilamellar suspension. When we examined variations in buffer (Tris and phosphate) and ionic strength (NaCl, 0.100−0.400 M) on the thermotropic phase behavior of these phospholipid/cholesterol mixtures, we observed only small shifts in the transition temperature and no changes in the qualitative behavior of the sample. For all of the HS-DSC samples containing cholesterol, the mixtures were hydrated and suspended as detailed, and then stored for 1 day at 2 °C before the HS-DSC experiment. The HS-DSC thermograms for the cholesterol/phospholipid suspensions were recorded with a Hart 7701 high-sensitivity differential scanning calorimeter (Provo, UT). The scan rates used were 10 °C/h unless otherwise noted. In addition, the amount of phospholipid used for the DSC analyses was progressively increased from 0.5 mg for pure phospholipid bilayers to 20 mg for phospholipid samples containing 45 or 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 Hart calorimeter was calibrated using solid standards from Hart Scientific, as well as aqueous lipid samples synthetically prepared and

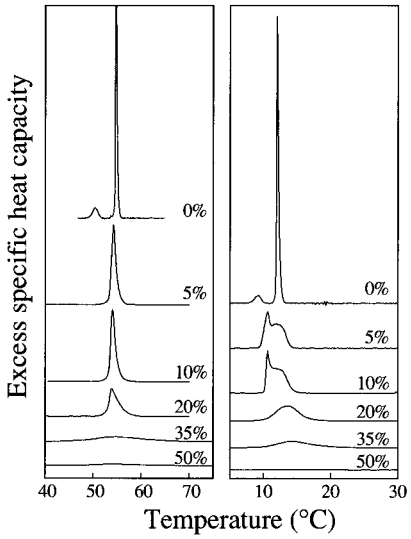

FIGURE 1: Representative DSC heating scans of aqueous dispersions of DSPC (left panel) and DEPC (right panel) bilayers containing various concentrations of cholesterol. The scans are corrected for mass and scan rate. Cooling scans are essentially identical and are not reproduced here.

purified within this laboratory using methods previously shown to provide highly pure samples (Lewis *et al*., 1985). Sample runs were repeated at least 3 times to ensure reproducibility. Low-temperature-annealed samples typically involved holding the temperature at 2 °C anywhere from 2 to 48 h, depending on the sample, but may also involve more complicated regimes as detailed in Lewis and McElhaney (1993). For all mixtures, phospholipid and cholesterol degradation was monitored by TLC, 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. The analysis and the decomposition of the HS-DSC endotherms were done using Microcal's Origin (Northhampton, MA) and DA-2 software.

RESULTS

Thermotropic Phase Behavior of Cholesterol/Phosphatidylcholine Mixtures. Representative high-sensitivity DSC heating thermograms of DSPC and DEPC, alone and in the presence of various amounts of cholesterol, are presented in Figure 1. In the absence of cholesterol, DSPC and DEPC bilayers exhibit two endotherms on heating: a lower temperature, lower enthalpy pretransition and a higher temperature and higher enthalpy main transition. The pretransition arises from the conversion of a lamellar gel ($L_{\beta'}$) phase to the rippled gel ($P_{\beta'}$) phase and the main transition from a conversion of the $P_{\beta'}$ phase to the lamellar liquid-crystalline (L_α) phase. These transitions are fully reversible on cooling (results not shown). As expected, both the pretransition (9.5 °C) and main transition (12 °C) temperatures (Figure 2) of DEPC are considerably lower than for DSPC (50.5 °C and 54.0 °C, respectively), due to the presence of a *trans*-double bond in each hydrocarbon chain of the former, and the enthalpy of the main transition of DEPC (7.9 kcal/mol) is also somewhat lower than that of DSPC (9.3 kcal/mol) (Figure 3). The pretransition of both PC's is abolished upon the incorporation of 5 mol % or more of cholesterol.

The DSC heating scans in Figure 1 also illustrate the overall effect of the incorporation of increasing quantities

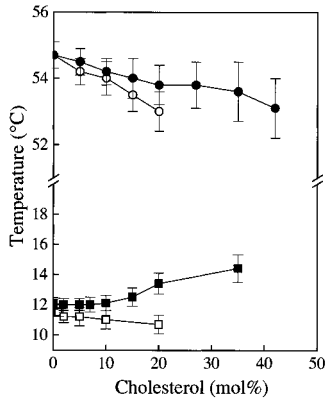

FIGURE 2: Representative plots of the temperatures of the DSPC (heating, ●) (cooling, ○) and DEPC (heating, ■) (cooling, □) chain-melting transition. Transition temperatures were corrected for differences in heating scan rates between the various samples analyzed.

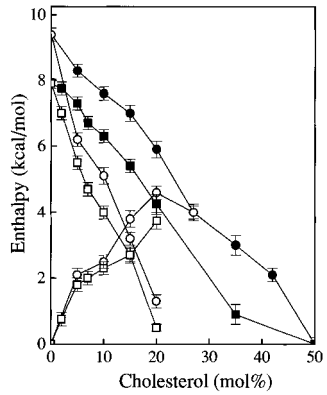

FIGURE 3: Representative plots of the total enthalpy for the DSPC (●) and DEPC (■) chain-melting transitions as a function of cholesterol concentration in the heating mode (the enthalpy values in the cooling mode are essentially identical). The enthalpies of the sharp and broad components of the DSPC (○) and DEPC (□) chain-melting transitions are also shown.

of cholesterol on the main phase transition of DSPC and DEPC. At cholesterol concentrations of less than about 20 mol %, the asymmetric DSC endotherms clearly consist of two overlapping components [see McMullen *et al*. (1993)]. We and others have presented evidence previously that the sharp component of the DSC endotherm corresponds to the chain melting of cholesterol-poor and the broad component to the chain melting of cholesterol-rich phospholipid domains [see McMullen *et al.* (1993) and reference cited therein]. As illustrated in Figure 2, the phase transition temperature of the sharp component decreases moderately with increasing cholesterol incorporation and its cooperativity decreases moderately (Figure 1) for both cholesterol/PC mixtures. Moreover, the enthalpy of the sharp component decreases markedly with increasing cholesterol concentration, becoming zero between 20 and 25 mol % cholesterol (Figure 3). In contrast, the phase transition temperature of the broad component of the DSC endotherm either decreases more markedly than that of the sharp component (DSPC) or actually increases slightly (DEPC) (Figure 2), and in both cases, the cooperativity of the broad transition decreases markedly as cholesterol concentration increases. As a result of the difference in the relative temperature shifts of the sharp and broad components for cholesterol/DSPC and cholesterol/DEPC mixtures, the multicomponent nature of the chain-melting transition of cholesterol/DEPC mixtures is more

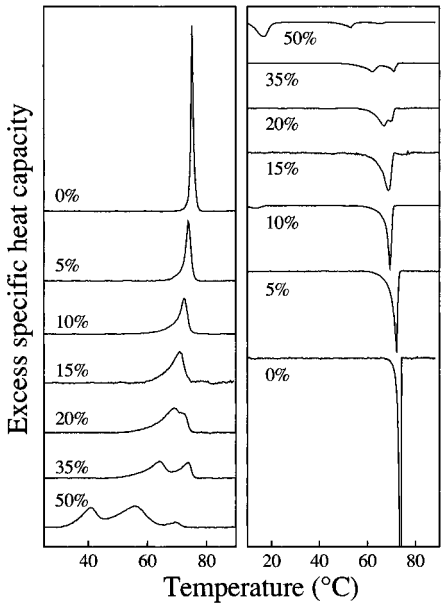

FIGURE 4: Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DSPE bilayers containing various concentrations of cholesterol. The scans are adjusted for mass and scan rate.

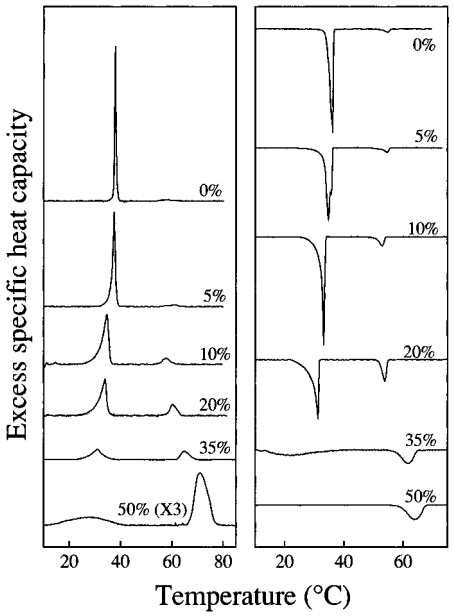

FIGURE 5: Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DEPE bilayers containing various concentrations of cholesterol. The scans are adjusted for mass and scan rate.

evident. The enthalpy of the broad component of both the DSPC and DEPC endotherms first increases with increasing cholesterol concentration, reaching a local maximum at 20−25 mol %, and then decreases again to zero at 50 mol % cholesterol. Thus, in both cases the total enthalpy of the PC chain-melting phase transition enthalpy decreases more or less linearly from 0 to 50 mol % cholesterol (Figure 3). It is important to note that, except for the direction of the shift in the temperature of the broad component, the thermotropic phase behaviors of DSPC and DEPC are influenced in exactly the same way by the incorporation of various quantities of cholesterol. One should also note that the DSC endotherms obtained upon heating and the DSC exotherms obtained upon cooling are essentially identical (data not presented), indicating that cholesterol is equally and fully miscible in both the gel and liquid-crystalline phases of these compounds.

Thermotropic Phase Behavior of Cholesterol/Phosphatidylethanolamine Mixtures. Representative DSC heating and cooling thermograms of DSPE and DEPE, alone and in the presence of various amounts of cholesterol, are presented in Figures 4 and 5, respectively. In the absence of cholesterol, DSPE and DEPE bilayers exhibit a single, highly energetic and cooperative chain-melting (L_β/L_α) phase transition. The chain-melting phase transition of DSPE is centered at 74.5 °C and has an enthalpy value of 11.2 kcal/mol, while that of DEPE is centered at 37.5 °C and the phase transition enthalpy is 8.0 kcal/mol. In addition, DEPE bilayers also exhibit a higher temperature, lower enthalpy lamellar liquid-crystalline to reversed hexagonal (L_α/H_{II}) phase transition centered at 57.3 °C. The main phase transition of both compounds is reversible on heating and cooling as is the L_α/H_{II} phase transition of DEPE, albeit with some hysteresis for the latter process.

The influence of cholesterol incorporation on the thermotropic phase behavior of aqueous dispersions of DSPE is illustrated in Figure 4. The effect of the incorporation of increasing quantities of cholesterol into DSPE bilayers is to

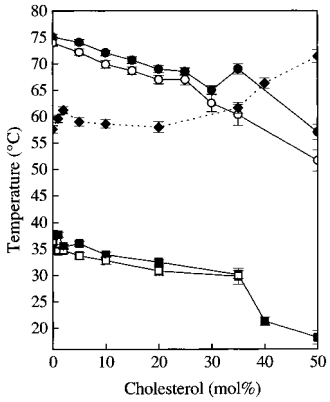

FIGURE 6: Representative plots of the temperatures of the phase transition of DSPE (heating, ●) (cooling, ○) and DEPE (heating, ■) (cooling, □) bilayers as a function of increasing cholesterol levels. In addition, the temperature of the L_α/H_{II} transition (◆) of DEPE bilayers is also shown as a function of increasing cholesterol concentration. Transition temperatures were corrected for differences in heating and cooling scan rates between the various samples analyzed.

progressively decrease the temperature (Figure 6), enthalpy (Figure 7), and cooperativity (Figure 4) of the main phase transition in both the DSC heating and cooling modes. Note that in contrast to the cholesterol/PC binary mixtures, the DSC thermograms are not readily resolvable into sharp and broad components at low cholesterol concentrations, although this does not necessarily mean that cholesterol-poor and cholesterol-rich domains do not exist, only that their chain-melting phase transitions may not be resolved for this particular phospholipid. At cholesterol concentrations of 20 mol % and above, multiple components are present in the DSC thermograms. The endotherm near 60 °C corresponds to a L_c/L_β phase transition, and the endotherm near 70 °C corresponds to a L_β/L_α phase transition (McMullen *et al.*, 1997a). Cholesterol also produces a much greater depression of the phase transition temperature and enthalpy at high cholesterol concentrations when monitored in the cooling

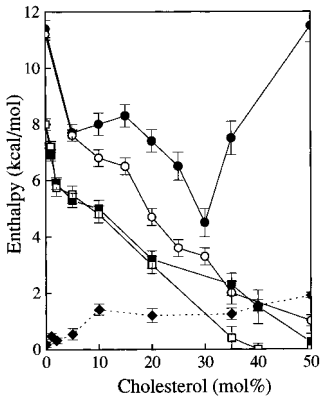

FIGURE 7: Representative plots of the enthalpy of the phase transition of DSPE (heating, ●) (cooling, ○) and DEPE (heating, ■) (cooling, □) bilayers as a function of increasing cholesterol levels. In addition, the enthalpy of the L_α/H_{II} transition (◆) of DEPE bilayers is also shown as a function of increasing cholesterol concentration.

mode. Moreover, the enthalpy of the main phase transition of DSPE is not reduced to zero at 50 mol % cholesterol in either the heating or cooling modes. These results indicate that cholesterol is not fully miscible with DSPE bilayers in either the gel or the liquid-crystalline states, but is considerably more miscible in the latter than the former. Also, in contrast to DSPC, cholesterol potentiates L_c formation in DSPE bilayers exposed to low temperatures.

For cholesterol/DEPE mixtures, the incorporation of cholesterol progressively reduces the temperature (Figure 6), enthalpy (Figure 7), and cooperativity (Figure 5) of the lamellar chain-melting transition such that by 50 mol % cholesterol, this phase transition is virtually abolished on both heating and cooling. Conversely, the effect of cholesterol on the nonlamellar L_α/H_{II} transition is to markedly increase both the temperature (Figure 6) and enthalpy (Figure 7) of this transition while only moderately affecting its cooperativity (Figure 5).

Thermotropic Phase Behavior of Cholesterol/Phosphatidylserine Mixtures. Representative DSC heating and cooling thermograms of DSPS and DEPS, alone and in the presence of various amounts of cholesterol, are presented in Figures 8 and 9, respectively. In the absence of cholesterol, both DSPS and DEPS bilayers exhibit a single, highly energetic and cooperative chain-melting (L_β/L_α) phase transition. For DSPS, the chain-melting phase transition occurs at 62 °C and has an enthalpy value of 12.4 kcal/mol, while for DEPS the phase transition is centered at 25 °C and has an enthalpy value of 8.0 kcal/mol. The main phase transition of both compounds is reversible on heating and cooling. In addition, DSPS bilayers also exhibit a lower temperature (44.5 °C) and lower enthalpy (1.7 kcal/mol) L_c/L_β phase transition when monitored on heating.

The influence of cholesterol incorporation on the thermotropic phase behavior of aqueous dispersions of DSPS is illustrated in Figure 8. The effect of the incorporation of increasing quantities of cholesterol into DSPS bilayers is to progressively decrease the temperature (Figure 10), enthalpy (Figure 11), and cooperativity (Figure 8) of the main phase transition in both the heating and cooling modes. Note that in contrast to the cholesterol/PC binary mixtures, the DSC thermograms are again not readily resolvable into sharp and broad components at low cholesterol concentrations. Moreover, from 20 to 50 mol % cholesterol, an additional, low-temperature endotherm is apparent in these mixtures which increases in size with increasing cholesterol levels. This endotherm results from cholesterol dissolution in the DSPS bilayer and from the conversion from the L_c to the L_β phase (McMullen *et al.*, 1997c). The relative sizes of these components, one centered at approximately 45 °C and the other at 35 °C, also vary with the cholesterol content of the mixture, the low-temperature component dominating at cholesterol levels exceeding 20 mol %. By 50 mol % cholesterol, the chain-melting transition at 61 °C is almost abolished on heating, but the low-temperature transition becomes progressively more energetic. Thus, increasing the level of cholesterol actually increases the overall enthalpy of the DSPS phase transitions (Figure 11). In contrast, when monitored in the cooling mode, these mixtures exhibit only

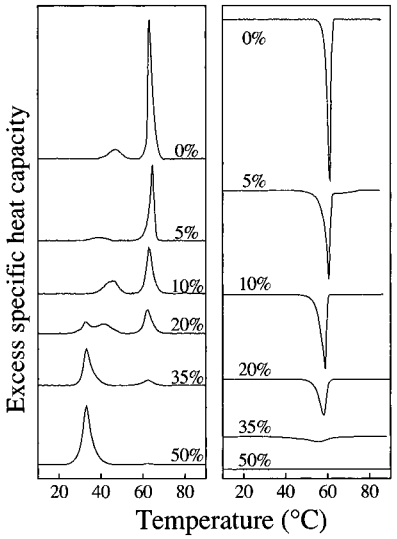

FIGURE 8: Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DSPS bilayers containing various concentrations of cholesterol. The scans are adjusted for mass and scan rate.

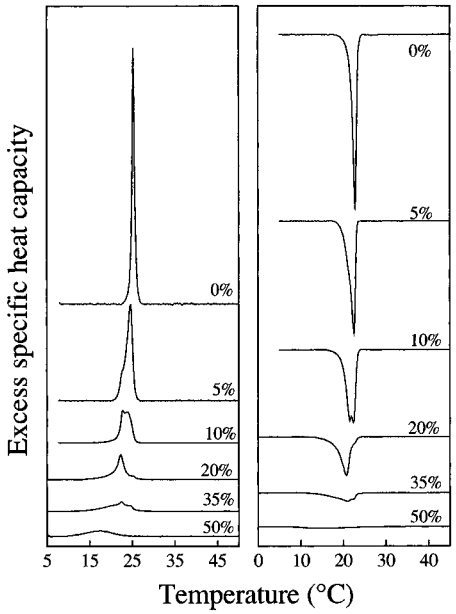

FIGURE 9: Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DEPS bilayers containing various concentrations of cholesterol. The scans are adjusted for mass and scan rate.

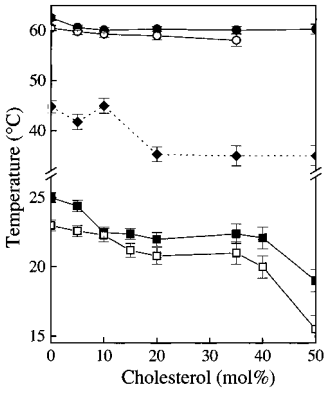

FIGURE 10: Representative plots of the temperatures of the phase transition of DSPS (heating, ●) (cooling, ○) and DEPS (heating, ■) (cooling, □) bilayers as a function of increasing cholesterol level. In addition, the temperature of the L_c/L_β transition (◆) of DSPS bilayers is also shown as a function of increasing cholesterol concentrations. Transition temperatures were corrected for differences in heating and cooling scan rates between the various samples analyzed.

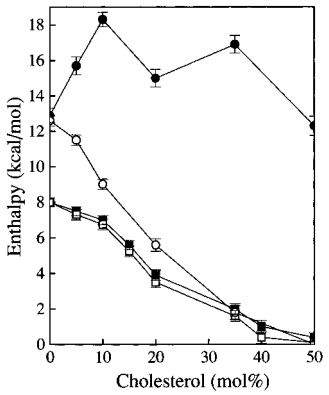

FIGURE 11: Representative plots of the total enthalpy of the phase transitions of DSPS (heating, ●) (cooling, ○) and DEPS (heating, ■) (cooling, □) bilayers as a function of increasing cholesterol level.

the chain-melting phase transition at 59 °C, and cholesterol produces a much greater depression of the phase transition temperature (Figure 10) and enthalpy (Figure 11) at high cholesterol concentrations. Moreover, the enthalpy of the main phase transition of DSPS is reduced to zero at 50 mol % cholesterol in the cooling mode. These results indicate that cholesterol is almost fully miscible with DSPS bilayers in the gel state and completely miscible in DSPS bilayers in the liquid-crystalline state. Also noteworthy is the fact that the presence of cholesterol also potentiates L_c phase formation in this system.

When monitored on heating, the incorporation of cholesterol into DEPS bilayers (Figure 9) results in small but progressive decreases in the temperature (Figure 10) but relatively large decreases in the enthalpy (Figure 11) and cooperativity (Figure 9) of the chain-melting phase transition. Interestingly, the chain-melting phase transition clearly exhibits at least two, and sometimes three, different components. As with the PC's, these components appear to correspond to the hydrocarbon chain-melting phase transitions of cholesterol-poor and cholesterol-rich domains. These three components do not exhibit the same cholesterol-dependent shifts in temperature, enthalpy, or cooperativity as those observed with the cholesterol-rich or -poor phospholipid endotherms in the corresponding cholesterol/DEPC mixtures. By 50 mol % cholesterol, the phase transition of DEPS bilayers is almost, but not completely, abolished. When monitored on cooling, the phase behavior of cholesterol/DEPS mixtures is virtually identical to that observed on heating. Thus, cholesterol is almost completely miscible with DEPS in both the gel and liquid-crystalline states.

DISCUSSION

The effect of cholesterol on the thermotropic phase behavior of aqueous dispersions of the three eukaryotic phospholipids studied here clearly depends on both the structure of the polar headgroup and the degree of unsaturation of the phospholipid hydrocarbon chains, even when the effective lengths of these chains are comparable. For both DSPC and DEPC, cholesterol incorporation produces clearly bimodal endotherms at low concentrations and unimodal endotherms at high concentrations. In both cases, heating and cooling endotherms are identical, and concentrations of cholesterol of 50 mol % completely abolish the gel to liquid-crystalline phase transition. For both molecular species of PE, bimodal main phase transition endotherms are not obvious at low cholesterol concentrations, but multiple endotherms are observed on both heating and cooling in the DSPE but not in the DEPE sample. Moreover, concentrations of cholesterol of 50 mol % do not abolish the chain-melting phase transition in DSPE bilayers on either heating or cooling, but do abolish this transition in DEPE bilayers only on cooling. Finally, for DSPS and DEPS dispersions, bimodal gel to liquid-crystalline phase transition endotherms are noted for DEPS but not for DSPS bilayers, and the chain-melting phase transition is abolished by 50 mol % cholesterol in the cooling but not in the heating mode. In addition, the presence of increasing quantities of cholesterol actually potentiates L_c phase formation in DSPS and DSPE bilayers while inhibiting L_c phase formation in DSPC bilayers. Thus, the miscibility of cholesterol in both the gel and liquid-crystalline states of these phospholipid bilayers and the effect of cholesterol on the temperature, enthalpy, and cooperativity of the gel to liquid-crystalline phase transition depend rather significantly on phospholipid structure.

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 (Hauser *et al.*, 1981, 1988; Boggs, 1980, 1986, 1987; Lewis & McElhaney, 1993). Although these differences are most pronounced in the gel state, the higher gel to liquid-crystalline phase transition temperature of the PE's is also manifest as a greater degree of order in the liquid-crystalline state, even

at comparable reduced temperatures, at least relative to the PC's (Lafleur *et al*., 1990; Senak *et al*., 1991; Davies *et al*., 1992; Tuchtenhagen *et al*., 1994). 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 therefore conclude that the miscibility of cholesterol with any particular phospholipid is 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*., 1992a,b, 1995a,b), PE (Zhang *et al*., 1995c), and PS (unpublished observations from this laboratory) bilayers. Although other, more subtle and specific interactions may occur in particular phospholipid/cholesterol binary systems, it seems that, in general, the relative strength of phospholipid−cholesterol interactions, as manifest 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. 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 MGDG, DGDG, and PG components of the *A. laidlawii* membrane, where the higher-melting neutral glycolipids exhibit a more limited ability to mix with cholesterol (Monck *et al*., 1993; McMullen *et al*., 1996b). 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 lengths, or by increasing the temperature of the system. Finally, it is important to note that we do not observe the presence of solid phase cholesterol in any of the cholesterol/phospholipid mixtures examined here, in contrast to the results of some of the prior studies of cholesterol/PE and cholesterol/PS mixtures (Bach, 1984; Bach & Wachtel, 1987; Wachtel *et al*., 1991; Bach *et al*., 1992). Differences in the hydrocarbon chain structure and length of the PE and PS molecular species used in these prior studies and the present one may be partly responsible for this discrepancy. However, the care which we took in the present work to ensure that all cholesterol/phospholipid mixtures were well mixed and fully hydrated may also account for our findings.

Although we have emphasized here the dominant effect of the strength of phospholipid−phospholipid interactions on the effect of cholesterol on lipid thermotropic phase behavior and miscibility, we do not wish to imply that specific interactions of cholesterol with different classes of phospholipids do not occur or could not be of biological relevance. However, these effects are difficult to study mechanistically with a thermodynamic technique such as DSC which we have applied here. Further studies using various spectroscopic and other techniques will be required in the future to address this issue in detail and to explain more fully the general features of cholesterol−phospholipid interactions revealed in this study.

This study, as well as a similar study of the interaction of cholesterol with the individual phospho- and glycolipids of the *A. laidlawii* membrane (McMullen *et al*., 1997c), has confirmed that the strength and nature of lipid/cholesterol interactions can vary markedly with the detailed chemical structure of the lipid molecule, and thus that the cholesterol/PC systems are not an adequate general model for cholesterol interactions in biological membranes, with their generally very complex lipid compositions.

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