Differential Scanning Calorimetric Study of the Effect of Cholesterol on the Thermotropic Phase Behavior of a Homologous Series of Linear Saturated Phosphatidylcholines†

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ABSTRACT: We have studied the effects of cholesterol on the thermotropic phase behavior of aqueous dispersions of a homologous series of linear saturated phosphatidylcholines, using high-sensitivity differential scanning calorimetry and an experimental protocol which ensures that broad, low-enthalpy phase transitions are accurately monitored. We find that the incorporation of small amounts of cholesterol progressively decreases the temperature and the enthalpy, but not the cooperativity, of the pretransition of all phosphatidylcholines exhibiting such a pretransition and that the pretransition is completely abolished at cholesterol concentrations above 5 mol % in all cases. The incorporation of increasing quantities of cholesterol also alters the main or chain-melting phase transition of these phospholipid bilayers in both hydrocarbon chain length-dependent and hydrocarbon chain length-independent ways. At cholesterol concentrations of from 1 to 20–25 mol %, the DSC endotherms of all phosphatidylcholines studied consist of a superimposed sharp and broad component, the former ascribed to the melting of cholesterol-poor and the latter to the melting of the cholesterol-rich phosphatidylcholine domains. The temperature and cooperativity of the sharp component are reduced only slightly and in a chain length-independent manner with increasing cholesterol concentration, an effect we ascribe to the colligative effect of the presence of small quantities of cholesterol at the domain boundaries. Moreover, the enthalpy of the sharp component decreases and becomes zero at 20–25 mol % cholesterol for all of the phosphatidylcholines examined. In contrast, the broad component exhibits a chain length-dependent shift in temperature and a chain length-dependent decrease in cooperativity, but a chain length-independent increase in enthalpy over this same range of cholesterol concentrations. Specifically, cholesterol incorporation progressively increases the phase transition temperature of the broad component in phosphatidylcholines having hydrocarbon chains of 16 or fewer carbon atoms, while decreasing the broad-component phase transition temperature in phosphatidylcholines having hydrocarbon chains of 18 or more carbon atoms. We attribute this behavior to the effects of hydrophobic mismatch between the cholesterol molecule and its host phosphatidylcholine bilayer [see Mouritsen, O. G., & Bloom, M. (1984) *Biophys. J. 46*, 141–153] and propose that the best match between the effective length of the cholesterol molecule and the mean hydrophobic thickness of the phospholipid bilayers is obtained with the diheptadecanoylphosphatidylcholine molecule. Moreover, cholesterol decreases the cooperativity of the broad component more rapidly and to a greater extent in the shorter chain as compared to the longer chain phosphatidylcholines. At cholesterol concentrations above 20–25 mol %, the sharp component is abolished, and the broad component continues to manifest the chain length-dependent effects on the temperature and cooperativity described above. However, the enthalpy of the broad component decreases linearly and reaches zero at about 50 mol % cholesterol, regardless of the chain length of the phosphatidylcholine. This latter finding does not agree with a previous study [Singer, M. A., & Finegold, L. (1990) *Biophys. J. 57*, 153–156], which found that the cholesterol concentration required to reduce the enthalpy of the main phase transition to zero appeared to increase steeply and approximately linearly with phosphatidylcholine hydrocarbon chain length. We ascribe these previous results to an experimental artifact arising from the use of a low-sensitivity calorimeter and an experimental protocol not optimized to detect broad, low-enthalpy phase transitions.

The occurrence of high concentrations of cholesterol in the plasma membranes of higher organisms has prompted numerous investigations into its role in the structure and function of cell membranes [for reviews, see Dahl and Dahl (1988) and Yeagle (1988)]. Although cholesterol appears to have several different functions in cells, one of its primary roles is as a modulator of the physical properties of the plasma membrane phospholipid bilayer. Thus, a large number of studies of the interactions of cholesterol with phospholipid monolayers and bilayers have been carried out utilizing a wide range of physical techniques [for reviews, see Demel and de Kruijff (1976), Razin and Rottem (1978), Yeagle (1985, 1988), and Finean (1990)]. Some of the major effects of cholesterol incorporation observed in such studies include (i) a broadening and eventual elimination of the cooperative gel to liquid-crystalline phase transition of the phospholipid bilayer, (ii) a marked increase (decrease) in the orientational order of the phospholipid hydrocarbon chains above (below) the phase transition, (iii) a decrease in the phospholipid acyl chain tilt angle in the gel phase, and (iv) the abolition of the phospholipid pretransition at low cholesterol contents. In addition, cholesterol decreases (increases) the passive per-

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meability of liquid-crystalline (gel) phospholipid bilayers and condenses (expands) the fluid (solid) state of phospholipid monolayers, as well as altering the mechanical properties of the phospholipid bilayers. Despite this impressive body of experimental data, our understanding of the molecular basis of cholesterol/phospholipid interactions remains incomplete.

Differential scanning calorimetry (DSC)[1] is a thermodynamic technique which has proven of great value in studies of lipid thermotropic phase behavior in model phospholipid bilayers and biological membranes [see Mabrey and Sturtevant (1976), McElhaney (1982, 1984, 1989), and Keough (1984)]. A considerable number of DSC studies of cholesterol/phospholipid binary mixtures have been carried out over the last 25 years, by far the majority on cholesterol/DPPC or, to a lesser extent, cholesterol/DMPC systems. Although all earlier studies, which used relatively low-sensitivity calorimeters, agreed that the progressive addition of cholesterol broadens the gel to liquid-crystalline phase transition of these phospholipids and progressively reduces the transition enthalpy, there was considerable disagreement about the details of this interaction. For example, the addition of cholesterol was reported either to considerably reduce (Ladbrooke et al., 1968), to have little effect on (Hinz & Sturtevart, 1972; Gershfeld, 1978), or to increase (Calhoun & Shipley, 1979) the phospholipid phase transition temperature. Also, the cooperative phospholipid phase transition was reported to be completely abolished at cholesterol concentrations ranging from 33 to 43 mol %. Similar results were reported for chemical analogues of DPPC (De Kruyff et al., 1973) and for the interaction of cholesterol with various binary mixtures of linear saturated PC's (De Kruyff et al., 1974). Moreover, only a single endothermic transition was detected in all of these studies at all cholesterol concentrations examined.

More recent high-sensitivity DSC studies of cholesterol/PC interactions, however, have revealed a more consistent but also more complex picture of cholesterol/DPPC and cholesterol/DMPC interactions (Estep et al., 1978; Mabrey et al., 1978; Genz et al., 1986; Vist & Davis, 1990). At cholesterol concentrations from 0 to 20–25 mol %, the DSC endotherm consists of two components. The sharp component exhibits a phase transition temperature and cooperativity only slightly reduced from those of the pure phospholipid, and the enthalpy of this component decreases linearly with increasing cholesterol content, becoming zero at 20–25 mol %. In contrast, the broad component exhibits a progressively increasing phase transition temperature and enthalpy and a progressively decreasing cooperativity over this same range of cholesterol content. Above cholesterol levels of 20–25 mol %, the broad component becomes progressively less cooperative, the phase transition midpoint temperature continues to increase, and the transition enthalpy continues to decrease, eventually approaching zero only at cholesterol concentrations near 50 mol %. These results suggest that at low cholesterol concentrations, cholesterol-poor and cholesterol-rich domains coexist, with the former decreasing in proportion to the latter as cholesterol concentrations increase. In fact, a cardinal point in the cholesterol/DPPC phase diagram at about 22 mol % had been predicted from earlier model-building studies of Engleman and Rothman (1972), who calculated that the cholesterol molecule could interact with a maximum of 7 adjacent phospholipid hydrocarbon chains (or 3.5 phospholipid molecules) and thus that free phospholipid would exist only at cholesterol concentrations below this value. This model also explains the decreasing enthalpy of the broad component observed above 22 mol % cholesterol, since an increasing proportion of phospholipid molecules would interact with more than one cholesterol molecule rather than with the more flexible hydrocarbon chains of adjacent phospholipids, thus progressively decreasing and eventually abolishing the cooperative chain-melting phase transition. However, this model may not be valid for all PC's, since DSC studies of PC's containing one or two unsaturated fatty acyl chains can apparently exhibit quite different cholesterol/PC stoichiometries (Davis & Keough, 1983; Keough et al., 1989; Kariel et al., 1991).

The only systematic DSC study on the effect of variations in bilayer thickness on cholesterol/phospholipid interactions is that of Singer and Finegold (1990). In studying the effect of increasing cholesterol levels on linear saturated PC's containing 12–20 carbon atoms, these investigators reported that the enthalpy of the main transition of all these phospholipids decreased with increasing cholesterol levels as reported previously. However, they also reported the rather surprising finding that the cholesterol concentration required to reduce the transition enthalpy to zero also increases linearly with the length of the PC hydrocarbon chains. We believe that the validity of this latter observation is suspect for several reasons. First, these workers report that cholesterol concentrations of only about 17 and 26–27 mol % are sufficient to completely abolish the cooperative main transitions of DMPC and DPPC, respectively, whereas all recent high-sensitivity DSC studies find that a cooperative phase transition persists until cholesterol concentrations reach 50 mol %. Second, a linear and marked dependence of cholesterol/PC interaction stoichiometry on hydrocarbon chain length cannot be explained by any of the current models of cholesterol/PC interactions. Finally, the use of a low-sensitivity DSC instrument with constant and relatively small phospholipid samples raises the possibility that the less energetic and less cooperative phase transitions, characteristic particularly of the shorter chain length PC's and higher cholesterol concentrations, will not be detected. For these reasons, we have reinvestigated the effect of the incorporation of increasing quantities of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated PC's, utilizing high-sensitivity DSC and an experimental protocol ensuring that broad, low-enthalpy phase transitions can be accurately monitored. Indeed, using such an approach, we find that the amount of cholesterol required to completely abolish the cooperative chain-melting transition of all PC's studies is 50 mol %, regardless of hydrocarbon chain length. Moreover, we also demonstrate that the phase transition temperature of the broad component of the DSC endotherm shifts either to lower or to higher temperatures in accordance with the direction and degree of mismatch between the thickness of the hydrophobic core of the PC bilayer and the length of the cholesterol molecule.

MATERIALS AND METHODS

The PC's in this experiment were synthesized in this laboratory by methods previously shown to ensure highly pure samples (Lewis & McElhaney, 1985). The cholesterol was purchased from Fisher Chemicals and recrystallized twice

[1] Abbreviations: DSC, differential scanning calorimetry; PC, phosphatidylcholine (specific phosphatidylcholines are designated by the notation n:0 PC, where n is the number of carbon atoms per hydrocarbon chain and zero indicates the absence of double bonds); DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; ^{2}H-NMR, deuterium nuclear magnetic resonance spectroscopy; $\Delta T_{1/2}$, width of the phase transition at half-height, inversely related to the cooperativity of the phase transition; $L_{\beta'}$, lamellar gel phase with tilted hydrocarbon chains; $P_{\beta'}$, rippled gel phase with tilted hydrocarbon chains; L_{α}, lamellar liquid-crystalline phase.

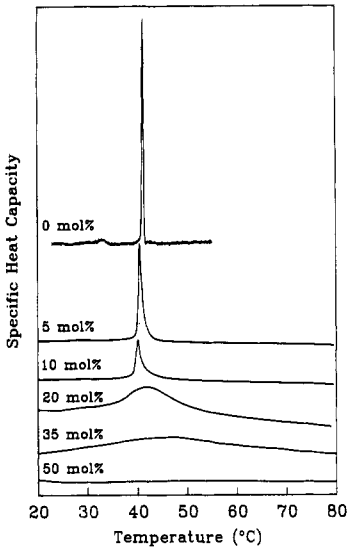

FIGURE 1: Representative DSC scans of unannealed DPPC bilayers of various cholesterol molar concentrations. DSC scans of DPPC vesicles containing 1, 2, 3, 6, 15, 25, 30, and 40 mol % cholesterol were also performed but are not illustrated here. Endotherms are not adjusted for scan rate or mass of sample.

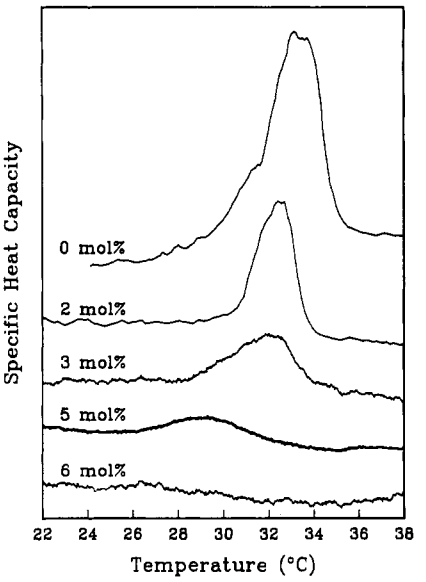

FIGURE 2: Optimized scans of the pretransition ($L\beta'/P_{\beta'}$) of DPPC vesicles containing various molar concentrations of cholesterol. Scans shown are corrected for mass and scan rate to show the relative effect of cholesterol on the pretransition enthalpy (1 mol % cholesterol not shown).

from ethanol before use. Both cholesterol and the PC's were dissolved in chloroform and mixed from those stock solutions. Mixtures were then dried under N_2 and evaporated to dryness in a vacuum overnight. The dried mixtures were than dispersed and resuspended with deionized water, heated to approximately 10–20 °C above the phase transition temperature of the PC studied, and then vortexed to give a multilamellar suspension. The amounts of PC used varied from 0.5 to 2.0 mg for pure lipid samples to 10–12 mg for samples containing high concentrations of cholesterol. The calorimetric analysis was performed on a high-sensitivity instrument manufactured by Hart Scientific. Transition signal strength was also enhanced via scan rate variation depending on cholesterol concentration. Scan rates of 5 °C/h was used for pure lipid samples, and these were progressively increased to 40 °C/h for samples approaching 50 mol % cholesterol. To eliminate the possibility of instrumental artifacts in calorimetric measurements, control experiments involving lipid and nonlipid standards were used to test the dependence of transition temperature, enthalpy, and cooperativity on scan rate and sample size. No significant differential effects were noted in any experimental parameter. This experimental protocol ensured that the broad, low-enthalpy endotherms observed at high cholesterol concentrations, particularly with the shorter chain PC's, are accurately recorded. Sample runs were repeated at least 3 times and as many as 15 times to ensure reproducibility. After calorimetric analysis, quantification of the phospholipids was achieved by gas chromatographic analysis of the PC methyl esters after transesterification with acidic methanol using a known amount of appropriate PC as an internal standard. The methyl esters were quantified as described previously (Lewis & McElhaney, 1985). Cholesterol quantification was done using the assay developed by Watson (1960). Thin-layer chromatography was used to check if sample degradation had occurred during calorimetric analysis. No degradation was found in any of the samples.

RESULTS

Thermotropic Phase Behavior of Pure Phosphatidylcholines. Representative high-sensitivity DSC scans for a typical member of the PC homologous series studied, DPPC, are presented in Figure 1 as a function of increasing cholesterol content. In the absence of cholesterol, unannealed DPPC bilayers exhibit two endotherms on heating, a lower temperature, lower enthalpy pretransition and a higher temperature, 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 phase transition from a conversion of the $P_{\beta'}$ phase to the lamellar liquid-crystalline (L_α) phase. Since the pretransition increases in temperature more steeply with increasing hydrocarbon chain length than does the main transition, the temperature interval between these two events decreases as the length of the PC chains increases. Thus, for 21:0 PC, a discrete pretransition is not observed, and the main transition results from a $L_{\beta'}/L_\alpha$ conversion. Similarly, 13:0 PC (and 12:0 PC, not studied here) is unique in this homologous series of PC's in not exhibiting a pretransition but in exhibiting instead a higher temperature shoulder on the main phase transition endotherm. Although the physical basis of this behavior is not fully understood, both thermal events are known to involve chain melting (Morrow & Davis, 1987; Lewis and McElhaney, unpublished observations) and will thus be considered together to constitute the main phase transition in the following analyses. For a more thorough discussion of the thermotropic phase behavior of the linear saturated PC series, the reader is referred to Lewis et al. (1987) and to the references cited therein.

Effect of Cholesterol on the Pretransition. The DSC scans presented in FIgure 1 suggest that the pretransition of DPPC is abolished at cholesterol concentrations between 5 and 10 mol %. In order to illustrate the effect of cholesterol on the pretransition more clearly, in Figure 2 we present a series of DSC scans of DPPC containing 0–6 mol % cholesterol obtained under conditions optimal for monitoring the pretransition. From these scans, it is clear that cholesterol incorporation progressively decreases the pretransition temperature and enthalpy in an approximately linear manner without significantly altering the cooperativity and that the pretransition becomes undetectable at a cholesterol concentration of 6 mol %. Similar results were obtained for all of the other linear saturated PC's (14:0–20:0 PC) exhibiting discrete pretransitions (data not presented). Thus, the effect of cholesterol

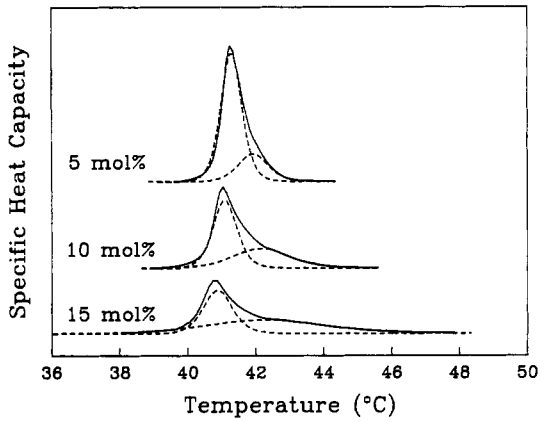

FIGURE 3: Sample deconvolution of the DSC endotherm for three concentrations of cholesterol in DPPC vesicles. The lower melting curve represents the sharp component, and the higher melting curve represents the broad component. Curves are corrected for mass and scan rate.

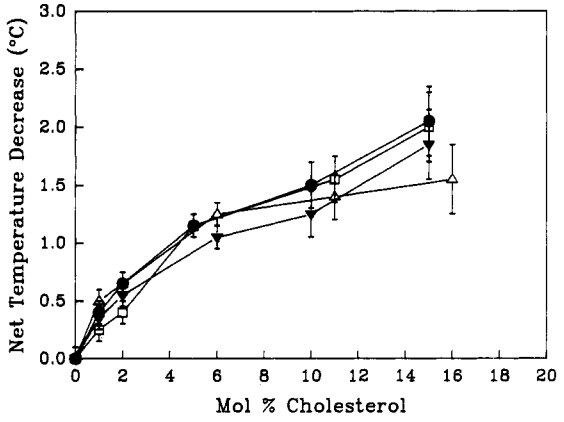

FIGURE 4: Representative plot of the net decrease in the temperature of the sharp component of the DSC endotherm as a function of cholesterol concentration. Represented PC's are 14:0 (△), 16:0 (□), 18:0 (▼), and 20:0 (●). Odd chains (13:0, 15:0, 17:0, 19:0, and 21:0 PC) are not plotted but are very similar to the even-chain PC's plotted above. Transition temperatures are corrected for scan rate.

on the pretransition is not dependent on the hydrocarbon chain length of the host PC bilayer.

Effect of Cholesterol on the Main Phase Transition. The DSC scans in Figure 1 illustrate the overall effect of the incorporation of increasing quantities of cholesterol on the DPPC main phase transition. As shown in Figure 3, the asymmetric DSC endotherms observed at cholesterol concentrations of 1–20 mol % can be well fit by deconvolution into two components. The phase transition temperature of the sharp component decreases slightly with cholesterol incorporation (see Figure 4), and its cooperativity decreases moderately. Moreover, as illustrated in Figure 5, the enthalpy of the sharp component of DPPC decreases with increases in cholesterol content and becomes zero between 20 and 25 mol %. Similar behavior was exhibited by all chain lengths of PC investigated. In contrast, the phase transition temperature of the broad component of the DPPC endotherm increases fairly markedly with increasing cholesterol incorporation, and its cooperativity decreases markedly. Thus, the broad component encompasses a very large temperature range at high cholesterol levels. As shown in Figure 6, the enthalpy of the broad component of DPPC first increases with increases in cholesterol incorporation to a level of 20–25 mol % and then decreases to zero at cholesterol concentrations near 50 mol %. Again, similar behavior is noted for all the other PC's studied.

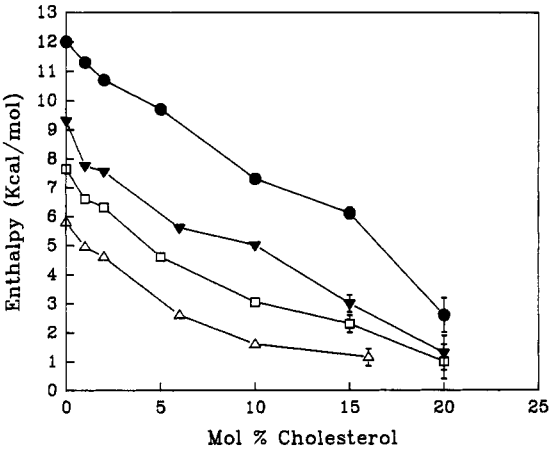

FIGURE 5: Representative plots of the enthalpy of the sharp component of the DSC endotherm as a function of cholesterol concentration for the PC homologous series. Represented PC's are 14:0 (△), 16:0 (□), 18:0 (▼), and 20:0 (●). Data were obtained from the deconvolution of overall endotherms for each chain length (see Figure 3) and averaged. Chain lengths not plotted (13:0, 15:0, 17:0, 19:0, and 21:0 PC) shown the same trends as those illustrated in the figure.

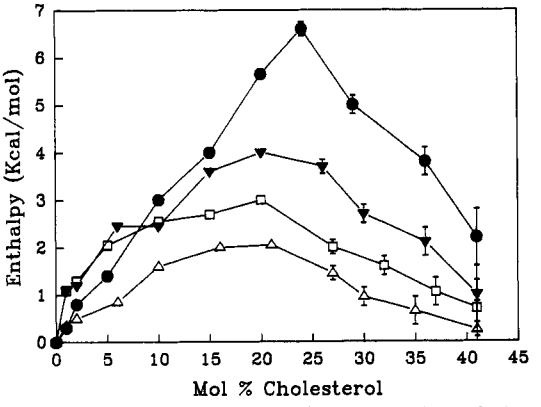

FIGURE 6: Representative plots of the enthalpy of the broad component of the DSC endotherm as a function of cholesterol concentration for the PC homologous series. Represented PC's are 14:0 (△), 16:0 (□), 18:0 (▼), and 20:0 (●). Data were obtained from deconvolution of the overall endotherms for each chain length (see Figure 3) and averaged. The chain lengths not plotted (13:0, 15:0, 17;0, 19:0, and 21:0 PC) again show the same trends as those shown above.

In order to compare directly our results with those of Singer and Finegold (1990), in Figure 7 we have plotted the overall enthalpy of the main phase transition (broad plus sharp component, when present) as a function of cholesterol content for representative members of the homologous series of PC's studied. As expected from the analysis of the individual components just presented, the total enthalpy decreases in an approximately linear fashion with increasing cholesterol levels for all the PC's studied. Moreover, in all cases, the enthalpy does not approach zero until cholesterol levels of near 50 mol % are reached. In particular, no marked dependence of the apparent cholesterol/PC interaction stoichiometry is evident, in contrast to the results of Singer and Finegold (1990). We thus conclude that, with the exceptions to be discussed below, the overall effects of cholesterol on PC thermotropic phase behavior are qualitatively and quantitatively similar for all members of this homologous series and thus that hydrocarbon chain length, and hence bilayer thickness, is not a major determinant of the nature of cholesterol/PC interactions.

In these studies, we did identify two thermodynamic parameters that do appear to be dependent on the hydrocarbon chain length of the PC molecule, and these are the phase

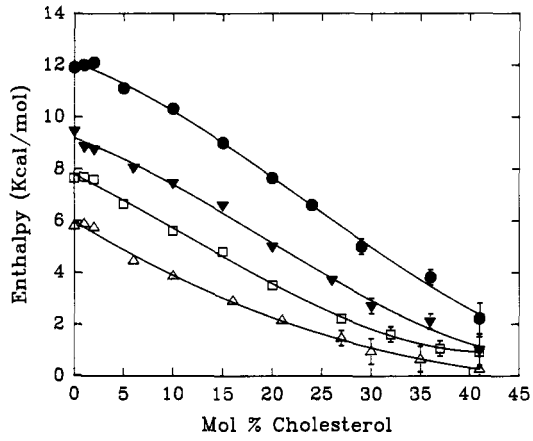

FIGURE 7: Representative plots of the overall main phase transition enthalpies of PC's as a function of cholesterol concentration [PC's shown are 14:0 (△), 16:0 (□), 18:0 (▼), and 20:0 (●)]. Each point represents the average of at least three independent runs. Odd-chain PC's, as the Figures 4 and 5, are not plotted but exhibit the same trends as the even-chain PC's.

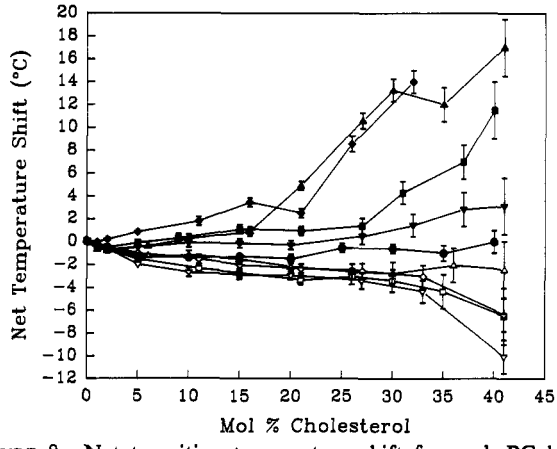

FIGURE 8: Net transition temperature shift for each PC broad component is shown as a function of cholesterol concentration. Legend for PC's shown is as follows: 13:0 (♦); 14:0 (▲); 15:0 (■); 16:0 (▼); 17:0 (●); 18:0 (△); 19:0 (◊); 20:0 (□); 21:0 (▽). Each point is the average of at least three runs, and all chain lengths are compared to their respective pure chain-melting phase transition temperatures. All transition temperatures are scan-rate-corrected.

transition temperature and the cooperativity of the broad component of the main phase transition. As illustrated in Figure 8, we find that phase transition midpoint temperature of the broad component is progressively shifted to higher temperatures (relative to the phase transition temperature of the pure PC) as the hydrocarbon chain length decreases below 17 carbons and vice versa as the chain length increases above 17 carbons. Furthermore, this effect is asymmetric in that it is of greater magnitude and is manifest at lower cholesterol concentrations in the shorter chain PC's as compared to the longer chain PC's. The width of the broad component, shown in Figure 9, also displays a differential response with respect to chain length. At chain lengths <18 carbons, the $\Delta T_{1/2}$ of the broad component increases with cholesterol incorporation more rapidly and to a larger extent than observed for PC's with chain lengths >18 carbons. Although we do not at present fully understand the mechanism behind the chain length-dependent effect of cholesterol on the cooperativity of the main phase transition, we do believe that the characteristic shift in phase transition temperature can be explained on the basis of a mismatch between the thickness of the hydrocarbon core of the PC bilayer and the effective hydrophobic length of the cholesterol molecule (see Discussion).

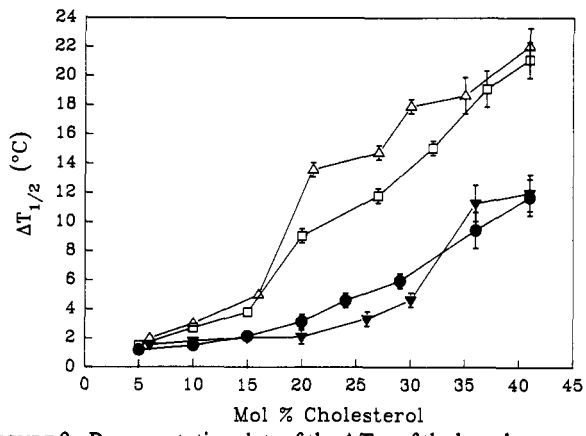

FIGURE 9: Representative plots of the $\Delta T_{1/2}$ of the broad component of the main phase transition as a function of cholesterol concentration. Legend for PC's in figure: 14:0 (△); 16:0 (□); 18:0 (▼); 20:0 (●). Odd-chain PC's were not plotted, but the $\Delta T_{1/2}$ values of the 13:0, 15:0, and 17:0 PC's were found to behave similarly to the 14:0 and 16:0 PC's, while 19:0 and 21:0 PC's behaved much like the 18:0 and 20:0 PC's.

DISCUSSION

Our finding that cholesterol concentrations of greater than 5 mol % abolish the pretransition of the 14:0–20:0 PC's is in good agreement with previous high-sensitivity studies of DPPC (Mabrey et al., 1978; Genz et al., 1986; Vist & Davis, 1990) and DMPC (Mabrey et al., 1978), although Estep et al. (1978) claimed that as little as 3.6 mol % cholesterol abolishes the pretransition of DPPC. However, our results clearly show that the pretransition persists until 6 mol % cholesterol. The progressive reduction in the temperature and enthalpy of the pretransition with increasing cholesterol concentration is a new finding and may account for the inability of Estep et al. (1978) to detect a discrete pretransition at cholesterol levels above 3 mol %. The abolition of the pretransition by low levels of cholesterol is presumably due to the conversion of the $P_{\beta'}$ phase of these PC bilayers into an L_{β}-like phase, in which the hydrocarbon chains become more perpendicular to the bilayer plane and the bilayer initially thickens [see Finean (1990) for further discussion].

Our results on the effect of increasing concentrations of cholesterol on the thermotropic phase behavior of the entire homologous series of linear saturated PC's are also in good agreement with previous high-sensitivity DSC studies of DPPC and DMPC (Estep et al., 1978; Mabrey et al., 1978; Genz et al., 1986; Vist & Davis, 1990) although, unlike Mabrey et al. (1978) and Tampe et al., (1991), we did not require three components to fit our DMPC curves at cholesterol concentrations between 10 and 20 mol %. In contrast, our results do not agree with the recent low-sensitivity DSC study of Singer and Finegold (1990), who claim that the amount of cholesterol required to completely abolish the main phase transition of members of the same homologous series of linear saturated PC's varies linearly with chain length from as little as 8.5 mol % for 12:0 PC to as much as 43.3 mol % for 20:0 PC. On the contrary, our data show that the main phase transition of all the PC's studied persists until cholesterol levels of 50 mol % are reached, regardless of hydrocarbon chain length. Therefore, the stoichiometry of cholesterol/PC interactions seems largely independently of the thickness of the host PC bilayer. Our findings thus support the qualitative applicability of the cholesterol/DPPC phase diagrams derived experimentally (Vist & Davis, 1990; Sankaram & Thompson, 1990a) and theoretically (Ipsen et al., 1987; Sperotto et al.,

1989) to other members of the linear saturated PC homologous series.

The explanation for the discrepancy in our DSC results as compared to those of Singer and Finegold (1990) is relatively straightforward. The ability to detect a discrete endothermic event using DSC is limited by the sensitivity of the calorimeter employed and by both the intrinsic enthalpy and the cooperativity of that event. Thus, even a low-sensitivity DSC instrument can detect the highly cooperative, relatively energetic gel to liquid-crystalline phase transitions of pure PC's but may be unable to detect broad phase transitions of comparable total enthalpy. Since the incorporation of increasing amounts of cholesterol into a PC bilayer decreases the enthalpy of the main transition linearly and also decreases the cooperativity even more markedly, it is clear that a low-sensitivity DSC instrument may lose the ability to discriminate the broad low-enthalpy phase transition from the base line as cholesterol concentrations increase, particularly if the amount of PC in the sample is kept constant. This effect will be more pronounced as the chain length of the PC decreases, because the initial enthalpy before cholesterol addition decreases markedly with chain length and because cholesterol decreases the cooperativity of the broad component of the main phase transition more rapidly and to a greater extent as the chain length of the PC decreases (see Figure 9). For these reasons, it can appear that cholesterol abolishes the main phase transition of the shorter chain PC's well before that transition actually disappears. In fact, using a low-sensitivity DSC (a Perkin-Elmer DSC-2C) and the experimental protocol of Singer and Finegold, we can reproduce their results, at least qualitatively. However, if a high-sensitivity DSC instrument is employed with the sample size and scan rate maximized to compensate for the effect of cholesterol on the main transition, as in the present study, then clearly defined (albeit broad and poorly energetic) phase transitions can be detected for all the PC's studied here at cholesterol concentrations approaching 50 mol %.

A new and important finding to arise from this study is the observation of a characteristic shift in the temperature of the broad-component main phase transition of cholesterol/PC mixtures, dependent on the length of the phospholipid hydrocarbon chain. This effect was neither seen nor predicted to occur in any prior study or theoretical treatment. The biphasic nature of the temperature shift suggests that cholesterol stabilizes the gel phase of shorter chain PC's (<17:0 PC) while destabilizing the gel phase of bilayers composed of PC's with acyl chains longer than 18:0 carbons. This characteristic shift can be explained, at least qualitatively, by reference to the hydrophobic mismatch between the effective length of the cholesterol molecule compared to that of the PC hydrocarbon chains normal to the bilayer plane [see Mouritsen and Bloom (1984) and reference cited therein]. The key point here is that the hydrophobic thickness of PC bilayers decreases by approximately one-third at the gel to liquid-crystalline phase transition due to the introduction of gauche conformers into the all-trans hydrocarbon chains. Thus, the introduction of another type of molecule, e.g., a transmembrane α-helical peptide also oriented with the long axis perpendicular to the bilayer plane, can affect the relative stabilities of the gel and liquid-crystalline phases according to the degree of mismatch between the effective hydrophobic length of the peptide and the phospholipid hydrocarbon chains. At least to a first approximation, the relative stabilities of gel and liquid-crystalline phases, and thus the chain-melting phase transition temperature, should be least affected when the hydrophobic length of the peptide equals the mean hydrophobic thickness of the bilayer (i.e., has a hydrophobic length midway between that of the gel and liquid-crystalline phases). Peptides with a greater hydrophobic length would tend to differentially stabilize the gel phase of the host phospholipid bilayer, thus increasing the phase transition temperature, and vice versa. In fact, there is now good experimental evidence for the existence of this hydrophobic mismatch effect in reconstituted synthetic peptide–PC or synthetic peptide–cholesterol–PC bilayer systems (Zhang et al., 1992; Nezil & Bloom, 1992).

One can apply this analysis to the cholesterol/PC systems studied here to good effect. As a first approximation, we assume that the cholesterol molecule is arranged with the long axis perpendicular to the PC bilayer with the 3-OH at the bilayer interface and that the effective length of the cholesterol molecule is temperature-invariant, which is equivalent to assuming that the conformation of the cholesterol alkyl group does not change with temperature but remains fully extended. This leads to an effective hydrophobic length of the cholesterol molecule of about 20 Å. Further assuming for the moment that the presence of cholesterol does not affect the conformation of the hydrocarbon chains in the gel and liquid-crystalline states, one could then calculate that the optimum match should occur between cholesterol and 19:0 PC, which has a mean hydrophobic length of about 19.8 Å. However, there is experimental evidence that the alkyl side chain of the cholesterol molecule does possess some restricted conformational freedom in PC bilayers (Kroon et al., 1975; Opella et al., 1976; Dufourc et al., 1984; Duax et al., 1988). If this is so, then the effective hydrophobic length of the cholesterol molecule may be somewhat less than 20 Å, particularly at higher temperatures or in shorter chain PC's (Wu & Chi, 1991). Moreover, although cholesterol induces an "intermediate state" in PC bilayers at temperatures near the phase transition of the pure phospholipid, both X-ray diffraction (Finean, 1990) and ^{2}H-NMR (Vist & Davis, 1990; Sankaram & Thompson, 1990b) data indicate that the hydrocarbon thickness and degree of orientational order of the hydrocarbon chains in this intermediate phase are more similar to the gel than to the liquid-crystalline phase of the pure phospholipid, at least at high cholesterol concentrations. Also, evidence has recently been presented that increases in temperature may drive cholesterol from the center toward the polar headgroup region of DPPC bilayers (Reinl et al., 1992). These considerations would all suggest that in fact the optimal degree of hydrophobic mismatch might well occur in a PC with a chain length shorter than 19 carbons. The results presented here, which suggest that 17:0 PC, with a mean hydrophobic chain length of 17.5 Å, provides the best match to the cholesterol molecule, are thus entirely reasonable. Moreover, McIntosh et al. (1978) have shown that, at temperatures below the phase transition temperatures of the pure PC's, cholesterol incorporation increases the thickness of 12:0 PC and 16:0 PC bilayers while decreasing the bilayer thickness of 18:0 PC. These observations support our predictions about the differential effect of cholesterol on the gel state of PC's having hydrocarbon chain lengths shorter or longer than 17 carbons.

It is clear from this and other recent studies that the interaction of cholesterol with phospholipid bilayers is a complex process which can be influenced by temperature, by cholesterol concentration, and by the structure of the phospholipid molecules themselves. Clearly, additional studies, utilizing modern structural, thermodynamic, and spectroscopic techniques, and cholesterol analogues and phospholipid mol-

ecules whose polar headgroup and fatty acyl chain structures are systematically varied, will be required to fully understand these interactions. Such studies are currently underway in this laboratory.

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