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New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry

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Abstract

We have investigated the effects of cholesterol on the thermotropic phase behavior of annealed and unannealed aqueous dispersions of dipalmitoylphosphatidylcholine (DPPC) using high-sensitivity differential scanning calorimetry (DSC), concentrating particularly on the cholesterol concentration range from 0 to 20 mol%. We find that the incorporation of cholesterol into low-temperature annealed DPPC bilayers decreases the enthalpy of the subtransition without affecting the transition temperature, such that the subtransition is abolished by 20 mol% cholesterol. Similarly, the incorporation of cholesterol progressively decreases the temperature and enthalpy of the pretransition and abolishes it entirely at cholesterol concentrations above 5 mol%. The incorporation of increasing quantities of cholesterol also alters the main or chain-melting phase transition. At cholesterol concentrations of 2 to 20 mol% cholesterol, the DSC endotherm arising from the main transition consists of superimposed sharp and broad components, the former due to the melting of cholesterol-poor and the latter to the melting of the cholesterol-rich DPPC domains. The temperature and cooperativity of the sharp component decreases slightly with increasing cholesterol concentration whereas the enthalpy decreases markedly, becoming zero at 20–25 mol% cholesterol. In contrast, the temperature and enthalpy of the broad component increases, and the cooperativity decreases markedly over this same range of cholesterol concentrations. An apparent increase in cooperativity of the overall DPPC endotherm at 7 mol% cholesterol is shown to arise because of a convergence in the transition temperatures of the sharp and broad components of the DSC endotherms. Some of our experimental findings, particularly the absence of any evidence for the existence of a triple point near 7.5 mol% cholesterol, do not accord with a recently proposed DPPC/cholesterol phase diagram derived from DSC and ^{2}H-NMR data (see Vist, M.R. and Davis, J.H. (1990) Biochemistry 29, 451–464). In addition, we examined the effect of cholesterol on phosphatidylcholines (PCs) of different chain lengths and confirm that a eutectic point does not exist for any of these PC/cholesterol mixtures. We then propose a new, more complete DPPC/cholesterol phase diagram based on our high-sensitivity DSC data as well as some recent spectroscopic data on PC/cholesterol mixtures and explore some of its biological implications.

Keywords: Cholesterol; Dipalmitoylphosphatidylcholine; DSC; Bilayer

1. Introduction

Cholesterol, or a closely related sterol, is an essential component of most eucaryotic cellular membranes [1]. Consequently there have been many investigations into the interactions of cholesterol with phospholipid monolayers and bilayers employing a wide variety of physical techniques (for reviews, see [2–6]). Although we currently know a considerable amount about the manner in which cholesterol alters the organization and mechanical properties of phospholipid bilayers, our understanding of the molecular basis of cholesterol/phospholipid interactions remains incomplete.

Abbreviations: DSC, differential scanning calorimetry; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; PC, phosphatidylcholine; DSPC, distearoylphosphatidylcholine; DAPC, diarachidoylphosphatidylcholine; ^{2}H-NMR, deuterium nuclear magnetic resonance spectroscopy; T_m, gel to liquid-crystalline phase transition (measured as the temperature of the peak in the DSC endotherm); ΔH, transition enthalpy; $\Delta T_{1/2}$, transition half-width (measured as the width at half-height of the DSC endotherm and inversely related to cooperativity by the equation $\Delta H_{vh} = 4RT_m^2 / \Delta T_{1/2}$).

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Differential scanning calorimetry (DSC) is a sensitive and non-perturbing thermodynamic technique which has been extensively used to study lipid thermotropic phase transitions in model and biological membranes [7–12]. The application of high-sensitivity DSC to DMPC/cholesterol or DPPC/cholesterol binary mixtures has revealed the complex effects of cholesterol incorporation on phospholipid thermotropic phase behavior [13–17]. The incorporation of small amounts of cholesterol progressively decreases the temperature and enthalpy of the pretransition and abolishes it entirely at cholesterol concentrations above 5 mol%. At cholesterol concentrations below 20–25 mol%, the main or chain-melting phase transition endotherm consists of a superimposed sharp and broad component, the former due to the melting of cholesterol-poor and the latter to the melting of cholesterol-rich phospholipid domains. The temperature and cooperativity of the sharp component are reduced slightly with increasing cholesterol incorporation while the enthalpy is reduced markedly and becomes zero at 20–25 mol% cholesterol. In contrast, the broad component increases in temperature and enthalpy but decreases markedly in cooperativity over the same range of cholesterol concentrations. At cholesterol concentrations above 20–25 mol%, the sharp component is abolished and the broad component continues to increase in temperature but to decrease in enthalpy and cooperativity until it is virtually eliminated at about 50 mol% cholesterol (but see [17]). We have recently shown that a qualitatively similar behavior is manifested by the entire homologous series of linear n-saturated PCs [16]. McMullen et al. [16] have also shown that in addition to the hydrocarbon chain length-independent effects discussed above, cholesterol incorporation has hydrocarbon chain length-dependent effects on the main phase transition of PC bilayers. Specifically, cholesterol incorporation progressively increases the phase transition temperature of the broad component of the DSC endotherm in PCs having hydrocarbon chain lengths of 16 or fewer carbon atoms while progressively decreasing the phase transition temperature in PCs having hydrocarbon chains of 18 or more carbon atoms. This behavior is attributed to the effects of hydrophobic mismatch between the cholesterol molecule and the hydrophobic core of the host PC bilayer (see [18]).

There have been a number of attempts to study the phase equilibria of two-component cholesterol/PC systems and to construct partial phase diagrams [6,7,17,19–29]. One notable recent attempt is that of Vist and Davis [6], who employed DSC and ^{2}H-NMR spectroscopy to determine the phase boundaries of mixtures of cholesterol and chain-perdeuterated DPPC at cholesterol concentrations of 0–25 mol% (see Fig. 1). These workers identified three distinct phases: the liquid-crystalline or L_α phase, the gel or L_β phase, and the β phase. The former phases are pure phospholipid phases while the latter phase contains both phospholipid and cholesterol. The liquid-crystalline phase is characterized by flexible, disordered DPPC

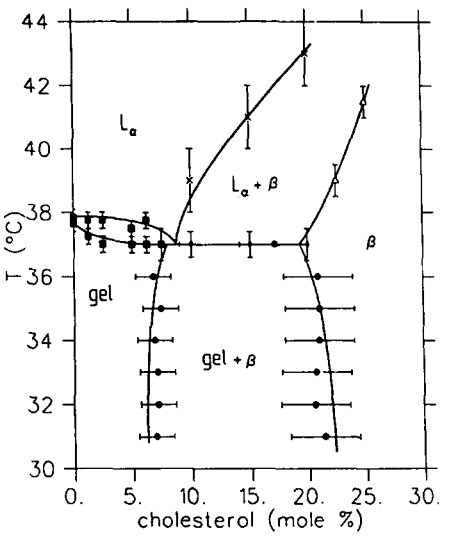

Fig. 1. The DPPC/cholesterol phase diagram proposed by Vist and Davis [6]. Used with permission.

hydrocarbon chains undergoing rapid axially symmetric reorientation while the gel phase is characterized by rigid, ordered hydrocarbon chains whose motions are no longer axially symmetric on the ^{2}H-NMR spectroscopic timescale. The cholesterol-rich β phase is characterized by relatively ordered, rigid hydrocarbon chains but rapid axially symmetric reorientation. In addition, these workers identified three regions of two-phase coexistence. The first is a narrow L_α/L_β phase coexistence region between 0 and about 7 mol% cholesterol occurring just below the chain-melting temperature of the pure phospholipid. The second boundary occurs between 7.5 and 22 mol% cholesterol and runs from 37°C down to at least 30°C; within this region the gel and β phases coexist. The third two-phase region lies above 37°C, beginning at a eutectic point between 7.5 and 10 mol% cholesterol and ending at about 20 mol% cholesterol; in this region the L_α and β phases are in equilibrium. As well, a three-phase line at 37°C extends from the eutectic point up to at least 20 mol% cholesterol. The phase diagram constructed by Vist and Davis [6] is widely quoted and has received both experimental and theoretical support [28–30]. Moreover, this same diagram is thought to describe the phase behavior of other PC/cholesterol systems varying in PC hydrocarbon chain length and unsaturation.

The existence of a eutectic or triple point near 7.5 mol% cholesterol was deduced by Vist and Davis [6] from an apparent sharpening of the DSC endotherm corresponding to the main phase transition of chain-perdeuterated DPPC. However, in our previous study of DPPC/cholesterol mixtures, we [16] and others [9,13–15] did not detect any discontinuity in the behavior of the sharp and broad components of the DSC endotherm between 0 and 20–25 mol% cholesterol. To clarify this point, we have carefully reinvestigated the thermotropic phase behavior of both annealed and unannealed DPPC/cholesterol mixtures con-

taining 0 to 20 mol% cholesterol using very low scan rates and 1 mol% intervals over the cholesterol concentration range of the presumed eutectic point. Although the apparent cooperativity of the overall DSC endotherm, measured as $\Delta T_{1/2}$, does appear to reach a local minimum at approx. 7 mol% cholesterol, this behavior is actually an artifact due to characteristic alterations in the temperature, enthalpy, and cooperativity of the sharp and broad components of the DSC endotherm as cholesterol concentrations in the bilayer progressively increase. We confirm that this is the case by comparative studies of cholesterol interactions with DMPC, DSPC, and DAPC bilayers. We also point out other features of our DSC data which are not in accord with the phase diagram constructed by Vist and Davis [6] and describe the effects of cholesterol incorporation on the various gel phases of DPPC. Based on our DSC results as well as recent spectroscopic studies [17,31], we then propose a modified DPPC/cholesterol temperature/composition diagram and indicate how changes in PC chain length alter the temperature/composition plot. Finally, we interpret our results for the CnPC/cholesterol phase equilibria in light of recent models for the maintenance of the non-uniform distribution of cholesterol within eucaryotic cells [32] and non-protein-mediated PC/cholesterol vesicular budding [33,34].

2. Materials and methods

The PCs used in this experiment were synthesized and purified by the methods used by Lewis and McElhaney [35] which have been shown to yield highly pure samples. The cholesterol was purchased from Fisher Chemicals (Fairlawn, NJ) and recrystallized twice from ethanol before use. PC/cholesterol mixtures were prepared from stock solutions of chloroform and dried under N_2 and evaporated to dryness under vacuum overnight. The dispersions were hydrated with milli-Q water with repeated vortexing and heating to approx. 20°C above the bilayer main phase transition. No significant differences were observed in the thermotropic behavior of these mixtures when different combinations of chloroform and methanol stock solvents were used to prepared the mixtures or different buffers were used to hydrate the bilayers. The calorimetry was done on a Hart Scientific high-sensitivity differential scanning calorimeter (Pleasant Grove, UT). For all samples a scan rate of 5°C/h was used. This slow scan rate ensures that there is a minimum of endotherm curve distortion due to instrumental time lag. Sample runs were repeated at least three times to ensure reproducibility. We also analyzed the thermotropic phase behavior of these same samples using a Microcal MC-2 calorimeter with a scan rate of 11.0°C/h. The results were essentially identical to those obtained with the Hart calorimeter. Decomposition of the multicomponent melting curves was done using Microcal's Origin and DA-2 software. Briefly, this computer program approximates the multicomponent melting thermograms as a linear combination of multiple, independent, two-state transitions. The curve broadening is expressed in terms of the van 't Hoff enthalpy, which is evaluated by the equation $\Delta H_{vH} = 4RT_m^2(c_{max}/\Delta q)$ where c_{max} is the excess specific heat capacity and Δq is the area under the curve. This protocol accurately reproduces the experimental DSC endotherms and has been used in previous studies (see also [13–17]). Although other methods of estimating the temperature, enthalpy and cooperativity of the components of these DSC endotherms could be employed, these would yield qualitatively similar results. In addition to our curve decomposition protocol, we analyzed the differences between pure DPPC and DPPC/cholesterol (2–5 mol%) endotherms using a simple thermogram subtraction routine. This procedure involves the subtraction of a pure DPPC endotherm from the endotherms of DPPC/cholesterol mixtures containing the same amount of DPPC scanned at the same scan rate and matched precisely for T_m. This routine allows us to directly observe qualitative differences in DPPC curve shape upon cholesterol incorporation.

3. Results

Some representative high-sensitivity DSC plots of low-temperature annealed, aqueous, multilamellar dispersions of DPPC containing 0 to 20 mol% cholesterol are shown in Fig. 2. In the absence of cholesterol, annealed DPPC bilayers exhibit three endotherms upon heating, a subtransition at approx. 18°C, a pretransition at 34°C, and the main transition at 41.4°C. The subtransition arises from the conversion of a crystalline gel ($L_{c'}$) phase to the lamellar gel ($L_{\beta'}$) phase, the pretransition from the conversion of the $L_{\beta'}$ 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. The enthalpy of the subtransition decreases progressively with increases in cholesterol content with little change in the transition temperature and is abolished completely at 20 mol% cholesterol. FTIR analysis of the CH_2 bending band of cholesterol-containing DPPC bilayers indicates that the $L_{c'}$ phase observed in these mixtures is structurally identical to that observed in pure DPPC bilayers [36] (data not presented). For the pretransition, both the enthalpy and the temperature of the transition decrease in a linear manner with increasing cholesterol concentration and the pretransition completely disappears above 5 mol% cholesterol, as shown previously [16]. The enthalpy of the overall main phase transition decreases with increases in cholesterol concentration (see Fig. 4a) while the transition temperature decreases slightly (see Fig. 4b). Interestingly, the apparent cooperativity of the overall chain-melting phase transition (inversely related to the $\Delta T_{1/2}$ value), appears to decrease from 0 to 3–4 mol%, to increase from 4–7 mol%, and then

to decrease again at cholesterol concentrations above 7 mol% (see Fig. 4c). This result would appear to confirm the observations of Vist and Davis [6] and to support the existence of a eutectic point near 7 mol% cholesterol. Note, however, that the cooperativity of the overall main phase transition at 7 mol% cholesterol is considerably less than that of pure DPPC. The effects of cholesterol on the behavior of the pretransition and main transition of unannealed DPPC/cholesterol mixtures is exactly the same as for the corresponding annealed samples as described above (data not presented).

We [16] and others [13–17] have established that the asymmetric DSC endotherm corresponding to the main phase transition of DPPC (and other linear saturated PCs) consists of superimposed sharp and broad melting components at cholesterol concentrations of approx. 1–2 mol% to 20–25 mol%. Moreover, the sharp component has been assigned to the chain-melting phase transition of cholesterol-poor PC domains and the broad component to the melting of cholesterol-rich PC domains. Accordingly, we have decomposed the DSC endotherms presented in Fig. 2 into sharp and broad components and the results of the decomposition are presented in Fig. 3. As illustrated in Fig. 4a–c, the sharp component enthalpy decreases substantially, the phase transition temperature decreases slightly (about 1.5°C), and the cooperativity decreases moderately with increases in cholesterol concentration from 0 to 15 mol%. In contrast, the broad component enthalpy

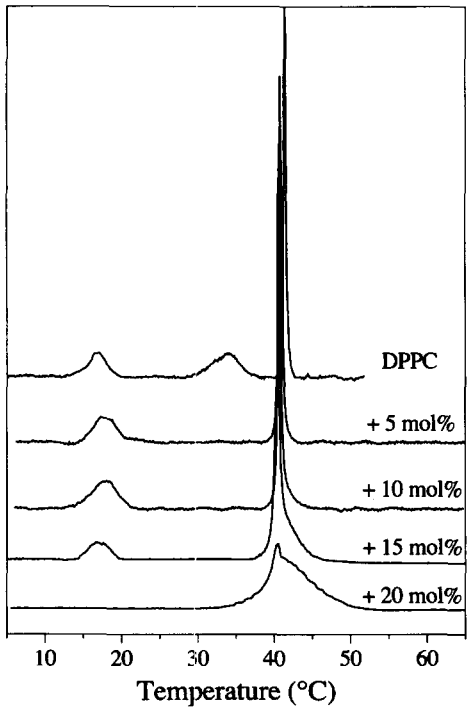

Fig. 2. Raw representative DSC scans of annealed DPPC bilayers of various cholesterol molar concentrations. DPPC vesicles containing 1, 2, 3, 4, 6, 7, 8, 9, 11, 25, 30 and 50 mol% were also performed but are not illustrated here.

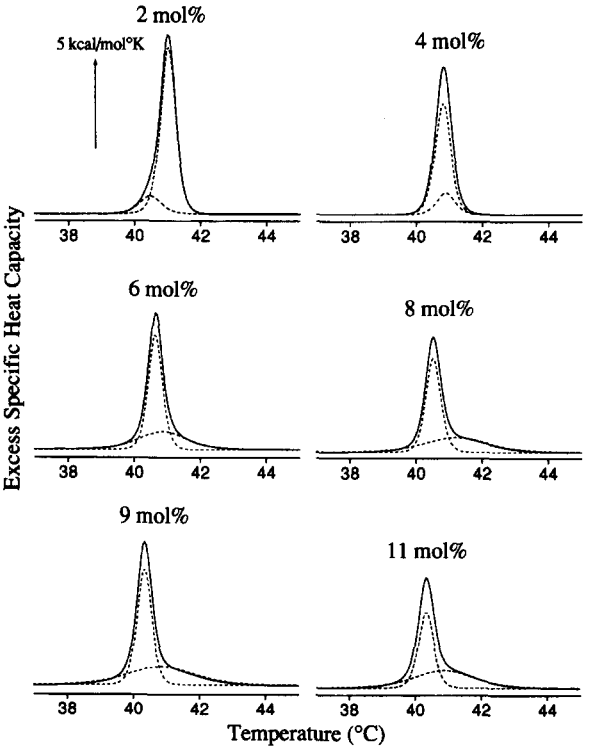

Fig. 3. Representative decomposed endotherms of the main phase transition of DPPC with various amounts of cholesterol. The solid line represents the overall endotherm while the dotted lines represent the sharp and broad components which are discernable by their transition temperatures and cooperativity (see text for discussion). Decompositions for 1, 3, 5, 7, 10, 15 and 20 mol% cholesterol were performed but are not shown here.

increases markedly, the phase transition temperature increases slightly (about 2.0–2.5°C), and the cooperativity decreases to a greater extent than for the sharp component. Clearly, each of the above thermodynamic parameters for both the sharp and broad phase transitions changes monotonically with increases in the level of cholesterol incorporation over the range 1–2 to 20 mol%. Thus there is no evidence for a eutectic point or any discontinuity in phase behavior near 7.5 mol% cholesterol. In addition, our results clearly show that the broad melting component is observed at temperatures both above and below the T_m of pure lipid domains, as illustrated in Fig. 3 for DPPC.

To ensure that the decomposition procedure of the DPPC/cholesterol endotherms is an accurate description of the changes in the overall endotherm curve shape with increasing cholesterol, we also used a thermogram-subtraction technique to identify changes in endotherm contour. This method does not assume a two-state transition, nor does it apply curve-fitting routines to analyze the curve profile, it simply demonstrates the differences between the curves matched for T_m. This routine is based on the fact that the $\Delta T_{1/2}$ value of the pure lipid endotherm is very sensitive to the addition of an added 'impurity' and that changes in curve shape are indicative of the nature of the interaction of the impurity with the lipid bilayer [10,37].

Shown in Fig. 5 is an example of the results for the subtraction of a pure DPPC endotherm from endotherms with the same quantity of DPPC but containing 2 or 5 mol% cholesterol. In accord with our decomposition results, cholesterol-induced differences in the shape as well as in the cooperativity of the DPPC endotherms are clearly observed even at these low cholesterol concentrations. Note that at the lower cholesterol concentration (2 mol%), the DPPC endotherm is broadened primarily on the low-temperature side while at the higher cholesterol concentration (5 mol%), this endotherm is broadened primarily on the high-temperature side. This asymmetric broadening of the endotherm induced by these low concentrations of cholesterol supports the existence of two components in the DSC chain-melting transition whose peak temperature change in a differential fashion with increasing cholesterol concentration. These results contrast with those of Vist and Davis [6], who reported symmetric, one-component DSC endotherms for DPPC/cholesterol mixtures containing less than 7.5 mol% cholesterol.

We have carried out a parallel series of high-sensitivity DSC experiments in which the effect of low concentrations of cholesterol on the thermotropic phase behavior of DMPC, DSPC, and DAPC bilayers were also investigated

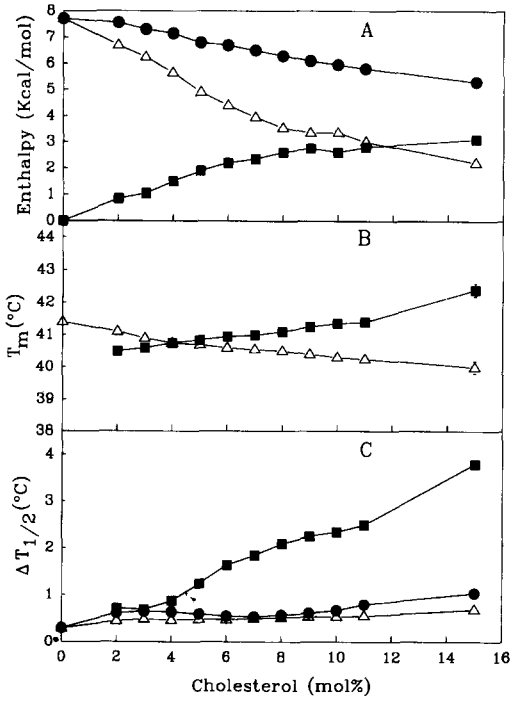

Fig. 4. (A) Effect of increasing amounts of cholesterol on the overall as well as broad and sharp component DPPC bilayer main transition chain melting enthalpies. Legend for figure is as follows: overall (●); broad component (■), sharp component (Δ). (B) Plot of the DPPC/cholesterol bilayer sharp and broad melting component transition temperatures as a function of increasing cholesterol concentration up to 15 mol% cholesterol. Legend as in (A). (C) Effect of increasing levels of cholesterol on the DPPC bilayer main transition broad and sharp component $\Delta T_{1/2}$ values. Legend as in (A).

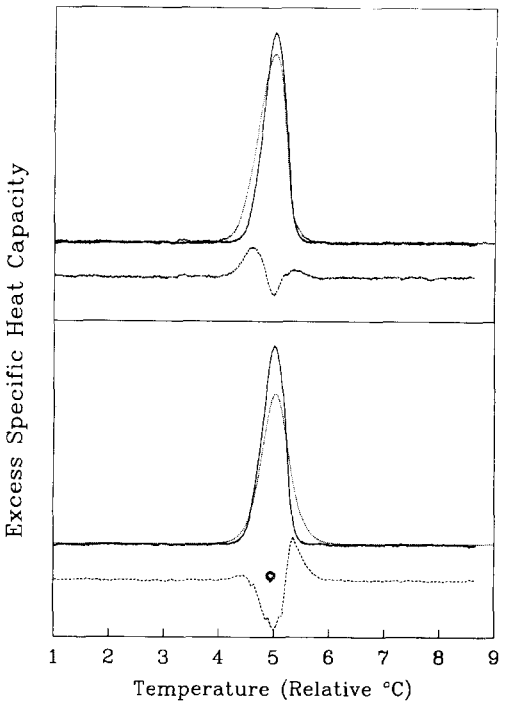

Fig. 5. Representative plots of the endotherm subtraction of pure DPPC from cholesterol-containing DPPC bilayers. The solid line represents the pure DPPC bilayer endotherm, the dotted line the cholesterol-containing DPPC bilayer (top 2 mol%, bottom 5 mol%), and the difference between the curves is plotted by the dashed line at the bottom of each plot. DPPC/cholesterol samples analyzed but not shown are 3 and 4 mol% cholesterol.

so that the effect of variations in PC hydrocarbon chain could be ascertained. The original and decomposed DSC thermograms for a series of progressively increasing cholesterol concentrations in bilayers of these three PCs are presented in Fig. 6. In all cases the T_m of the sharp component of the DSC endotherms decreases slightly as the concentration of cholesterol incorporated into the PC bilayer increases. However, the T_m of the broader component shifts upward relative to that of the sharp component for DMPC and for DPPC, while the opposite is the case for DSPC and DAPC. In all cases the T_m of the broad component at very low cholesterol concentrations is slightly less than that of the sharp component, such that crossing over of the T_m values will be observed for DMPC and DPPC but not for DSPC and DAPC. Moreover, since the upward shift of the T_m of the broad component of DMPC is more pronounced than that of DPPC at comparable cholesterol concentrations, one would predict that the crossing over of the T_m values of the sharp and broad components would occur at lower cholesterol concentrations in DMPC than in DPPC, and this indeed is observed. That this phenomenon of T_m crossover is in turn related to the apparent decrease in the overall $\Delta T_{1/2}$ of the overall DSC endotherm at modest cholesterol concentrations is amply illustrated in Fig. 7. These results clearly establish that the local minimum in $\Delta T_{1/2}$ values at cholesterol

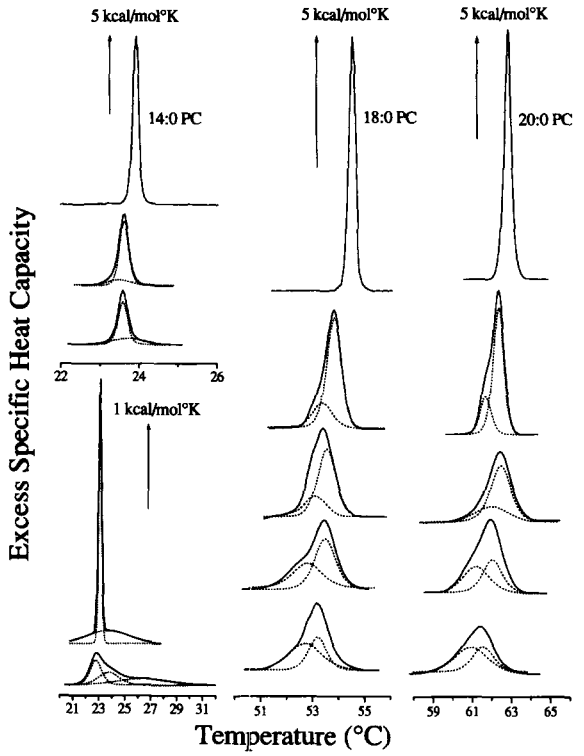

Fig. 6. Overall and decomposed plots of DMPC, DSPC and DAPC endotherms with various levels of cholesterol. The sharp and broad melting components are designated by the dotted lines, see text for a more detailed description. For DMPC/cholesterol mixtures the broadening due to cholesterol is such that the two scales are required to provide the resolution necessary to observe the sharp and broad components with increasing cholesterol. Cholesterol concentrations for each PC are as follows starting at the top; 14:0 PC, 0, 2, 5, 10, 14 mol%; 18:0 PC, 0, 2, 5, 10, 13 mol%; 20:0 PC 0, 2, 5, 10, 13 mol%. With DMPC, between 14 and 17 mol% cholesterol, a third peak is evident which is not seen in any other PC (see also [13,14]). The origin of this peak is unclear.

concentrations of 5–7 mol% is observed only with the shorter chain PCs, and that this local minimum is the result of a superposition of the sharp and broad components of the DSC endotherm.

4. Discussion

Our investigation of the thermotropic phase behavior of DPPC/cholesterol mixtures clearly shows that the sharpening of the DSC endotherm observed by Vist and Davis [6] arises because of the divergent effects of cholesterol on the various thermodynamic parameters of the sharp and broad components of the main phase transition. At low cholesterol concentrations (1 to 3 mol%), the cooperativity of the unresolved endotherm decreases because the cooperativities of both the sharp and broad components are decreasing. However, because the phase transition temperature of the broad component is initially slightly lower (about 1°C) than the sharp component but the two components are converging in temperature with increasing cholesterol concentration, the initial decrease in the cooperativity of the unresolved endotherm is somewhat attenuated. Moreover, as the transition temperature of the sharp component continues to decrease and that of the broad component to increase, these component transitions superimpose on the temperature scale. This results in a slight sharpening of the unresolved endotherm in the cholesterol concentration range of 3 to 7 mol%, despite the fact that the cooperativities of both the sharp and broad components continue to decrease. At higher cholesterol concentrations the increasing divergence of the sharp and broad components on the temperature scale, as well as the increasingly dominant contribution of the broad component, again cause the cooperativity of the unresolved endotherm to decrease, now quite rapidly. We confirmed this interpretation of our results by studies of longer chain length PC/cholesterol mixtures (DSPC and DAPC), in which the sharp and broad component T_m values do not cross at all, resulting in no apparent increase in endotherm cooperativity at any cholesterol concentration. This explains why other investigators have reported that the multicomponent melting of PC/cholesterol mixtures is less obvious in longer chain PCs [17,38,39]. We stress that the cooperativity of both the sharp and broad components decrease continuously with increases in cholesterol concentration, which is incompatible with the existence of a eutectic point near 7.5 mol% cholesterol for any CnPC/cholesterol mixture.

Another aspect of the present and previous [13–16] DSC studies of cholesterol/PC mixtures which does not appear to be compatible with the phase diagram proposed

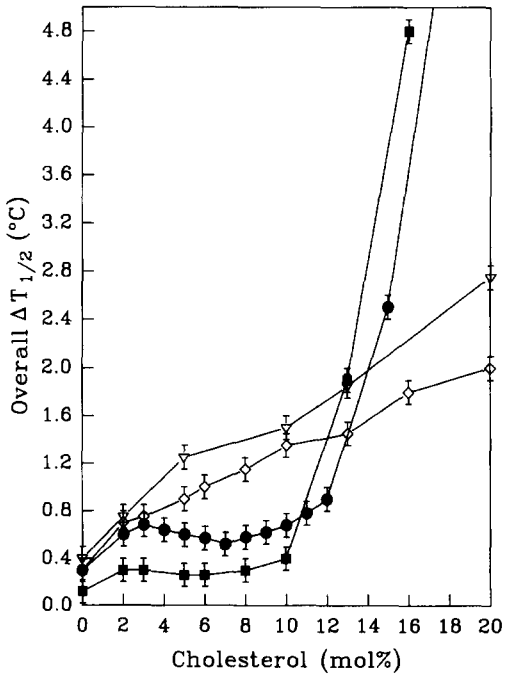

Fig. 7. Overall $\Delta T_{1/2}$ values of DMPC, DPPC, DSPC and DAPC plotted as a function of cholesterol concentration. Legend for the figure is as follows; DMPC (■); DPPC (●); DSPC (◇); DAPC (▽).

by Vist and Davis [6] relates to the cholesterol concentration at which the cholesterol-rich β phase first arises. Vist and Davis [6] report that the β phase exists only at concentrations above the proposed eutectic point at approx. 7.5–10 mol% cholesterol. This conclusion arises from the asymmetric shape of their DSC endotherms, which appeared to show that the broad component exists exclusively as a higher temperature shoulder on the sharp component. However, the asymmetric endotherms for DPPC/cholesterol mixtures which we observe over the cholesterol concentration range 2 to 20 mol% are very well fit by a superposition of a single sharp and a single broad component, even at very low cholesterol concentrations. Moreover, the limits of the broad component clearly extend to temperatures below as well as above those of the sharp component. We also note that Huang et al. [17] indicate that their ^{2}H-NMR spectra did not exhibit clearly separable components which could be attributed to the gel or L_α phase, nor did they observe a characteristic sharpening of the ^{2}H-NMR resonances that Vist and Davis [6] reported. Huang et al. [17] also state that ^{13}C-NMR spectroscopic data are crucial to correctly interpreting the ^{2}H-NMR spectral data. Thus, the rationale used by Vist and Davis [6] in support of the existence of the L_α/β two-phase coexistence region only at cholesterol levels greater than 7.5 mol% is not supported by our results or those of Huang et al. [17].

The phase diagram of Vist and Davis [6] is also incompatible with the present and previous DSC results in its identification of only a single gel phase. In annealed DPPC bilayers without cholesterol, three different gel phases (the $L_{c'}$, $L_{\beta'}$ and $P_{\beta'}$) clearly exist. Our DSC data indicate that the $L_{\beta'}/P_{\beta'}$ phase transition is eventually abolished by 5 mol% cholesterol while the $L_{c'}/L_{\beta'}$ phase transition persists until approx. 20 mol% cholesterol (see also [40]). The persistence of the $L_{c'}$ phase up to approx. 20 mol% cholesterol indicates that extended arrays of pure lipid exist and that their size and/or number is inversely proportional to the level of cholesterol incorporation. This is important evidence directly demonstrating the existence of a phase-separated mixture containing pure DPPC and DPPC-cholesterol domains at cholesterol concentrations between 0 and 20 mol%. In addition, the incorporation of cholesterol results in the loss of the rippled structure characteristic of the $P_{\beta'}$ phase [41] and of the characteristic chain tilt of all three gel phases of pure DPPC. Thus, the $L_{\beta'}$ and $P_{\beta'}$ (and possibly also the low-temperature annealed $L_{c'}$) phases of DPPC which exist in the absence of cholesterol are converted to a new, untilted gel phase (the L_β phase) upon the incorporation of relatively small amounts of cholesterol (5 mol%) (see [42]).

Using our DSC data as well as information from more recent PC/cholesterol spectroscopic studies [17,31] we propose a revised form of the DPPC/cholesterol 'phase diagram' (or perhaps more accurately a temperature/composition diagram) in Fig. 8. Similar to the majority of prior

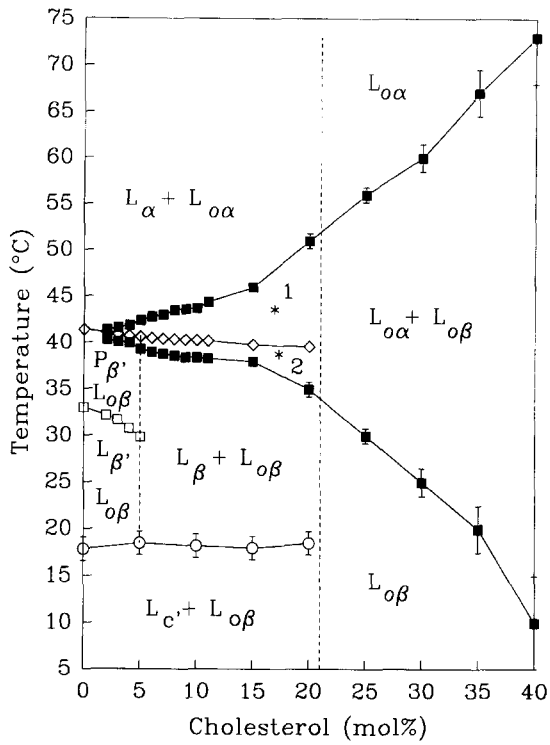

Fig. 8. Temperature composition diagram for DPPC/cholesterol mixtures as a function of temperature and cholesterol concentration. A detailed description of the phase diagram is provided in the text. Briefly, the phase designations are as follows: $L_{c'}$, the pure DPPC crystalline phase; L_β, the pure DPPC gel phase; $L_{o\beta}$, the DPPC/cholesterol liquid ordered gel-like phase; $L_{o\alpha}$, the DPPC/cholesterol liquid ordered liquid crystalline-like phase; $P_{\beta'}$, the pure DPPC rippled phase, and L_α, the liquid-crystalline phase. The ' designation indicates the lipids in a particular phase are tilted to the bilayer normal. The transitions are marked as follows; the subtransition (○); pretransition (□); pure lipid phase transition (◇); broad component phase transition boundaries (■). For ^{*1} and ^{*2} refer to text for description of phase coexistence. If no error bars are shown, the degree of variability between runs is within the range of the symbol. Data for this phase diagram come from this manuscript as well as [16].

PC/cholesterol phase diagrams [6,19–26,43], our temperature/composition diagram can be described in two segments, DPPC with less than 20 mol% cholesterol and DPPC with greater than 20 mol% cholesterol. Below 20 mol% cholesterol the simultaneous existence of pure lipid as well as lipid-sterol domains accounts for the thermotropic behavior of the bilayer, while above 20 mol% cholesterol the pure lipid domains are abolished. This interpretation is based on a lipid/sterol interaction stoichiometry of approx. 3.5 lipid molecules to one cholesterol molecule [44], which is universally accepted for DPPC and has been shown to apply to different chain length PC/cholesterol mixtures by McMullen et al. [16]. For cholesterol concentrations of 0 to 20 mol% in annealed DPPC/cholesterol bilayers, the first transition observed with increasing temperature is the subtransition ($L_{c'}/L_{\beta'}$) which persists up to 18–20 mol% cholesterol. The $L_{\beta'}$

phase is stable until approx. 33°C, after which the $L_{\beta'}$ phase converts to the $P_{\beta'}$ phase, but only at cholesterol concentrations less than 5 mol%. Finally, the $P_{\beta'}$ and L_β phases convert to the L_α phase to a limit of 20 mol% cholesterol. However, unlike Vist and Davis [6], we do not expand the $P_{\beta'}/L_\alpha$ phase boundaries to show a large phase coexistence region which then proceeds to a eutectic point and a single L_o or β phase at cholesterol concentrations above 7.5 mol%. Instead, we describe two distinct L_o regions, the liquid-crystalline-like $L_{o\alpha}$ and gel-like $L_{o\beta}$ region. Our classification of two different L_o regions is based on the experimentally observable chain-melting transition seen by FTIR and DSC [16,17,31,45,46], as well as the ^{13}C-NMR and ^{2}H-NMR spectroscopic data of Reinl et al. [31] and Huang et al. [17], which demonstrate that the L_o or β phase is not homogeneous with respect to temperature. In fact, the ^{2}H-NMR and ^{13}C-NMR data of Huang et al. [17] is in close agreement to the DSC study of McMullen et al. [16] regarding the chain length-dependent transition temperature and boundaries of the melting of cholesterol-rich DPPC and DSPC domains. The differences in $L_{o\alpha}$ and $L_{o\beta}$ thermotropic phase behavior may be manifest structurally as changes in the orientational order of the PC hydrocarbon chains and the cholesterol C17 side chain as well as the relative position of the cholesterol molecule in the host PC bilayer [16,26,31,47,48]. We also indicate that the origin of these regions is at very low cholesterol concentrations and that only at very low, experimentally inaccessible cholesterol concentrations does one observe ideal miscibility and possibly the existence of a eutectic point [49]. Within the boundaries of the broad component transition (denoted by ■) but above or below the sharp component phase transition, three different phases apparently coexist; (^{*1}) $L_\alpha + L_{o\alpha} + L_{o\beta}$ (above) or (^{*2}) $L_\beta + L_{o\alpha} + L_{o\beta}$ (below). At approx. 20–25 mol% cholesterol only the $L_{o\alpha}$ and $L_{o\beta}$ phases persist and as the level of cholesterol increases beyond 20–25 mol%, so does the temperature range for the coexistence of these two regions. At 50 mol% cholesterol pure cholesterol domains form and a cooperative lipid transition is no longer observable. Moreover, while the stoichiometry of cholesterol/PC interactions does not vary with chain length, it is clear that there is some specificity to cholesterol/PC interactions, primarily due to the phenomena of hydrophobic mismatch (see [16]). Thus, variations in PC hydrocarbon chain length affect both the direction and magnitude of the temperature shift as well as the cooperativity of the broad component DSC endotherm. As a result, the $L_{o\alpha}$ and $L_{o\beta}$ region boundaries of the DPPC/cholesterol temperature/composition plot (denoted ■) will change with changes in PC chain length. This contradicts previous theoretical predictions and ^{2}H-NMR spectroscopic evidence that PC/cholesterol interactions demonstrate no specificity regarding the PC hydrocarbon chain composition [6,28–30]. It should be also noted that sterol/phospholipid thermotropic phase behavior can be profoundly altered by subtle structural changes in the sterol molecule or in the polar headgroup and hydrocarbon chains of the host phospholipid bilayer [2,5,10–12,45].

Our DSC results indicate that phase separation of cholesterol-rich and cholesterol-poor domains occurs even at very low cholesterol concentrations, such as exist in various intracellular membranes. Interestingly, this finding has implications regarding the maintenance of a non-uniform distribution of cholesterol in eucaryotic cell membranes and possibly also in the process of protein sorting. Recently, Bretscher and Munro [32] proposed that the formation of cholesterol-rich phospholipid or sphingolipid domains could serve as a sorting mechanism for membrane proteins destined for the plasma membrane. This process was postulated to occur as a direct consequence of the greater hydrophobic thickness of the more ordered cholesterol/lipid domains. For such a mechanism to be effective, the phase separation of cholesterol/lipid domains would have to occur at low cholesterol concentrations to allow for selective concentration of cholesterol from the endoplasmic reticulum through the Golgi network towards the plasma membrane. Our work clearly demonstrates that cholesterol-induced phase separation is possible even at very low cholesterol concentrations. In addition, theoretical and experimental studies have shown that phase-separated PC/cholesterol vesicles may undergo dramatic shape fluctuations which can lead to vesicular fission even without the assistance of membrane-associated proteins [33,34]. This budding process was related to the formation of liquid-ordered ($L_{o\alpha}$ in our phase diagram) and liquid-disordered (L_α in our phase diagram) DMPC/cholesterol phase-separated regions [34]. We believe that cholesterol may in fact be required for the domain-induced budding process due to its ability to form phase-separated liquid-ordered domains even in the heterogeneous and fluid environment of biological membranes [33]. Thus, changes in bilayer lipid/sterol composition via phase separation may be selectively driving the vesicular transport of the relatively thicker cholesterol-enriched domains and their associated proteins. Although proteins may also induce or facilitate vesicular budding in biological membranes, the physical properties of the bilayer may still be crucial for this process in vivo. Clearly much work remains to be done to understand the complex interactions of cholesterol with the great diversity of the lipids found in natural membranes.

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