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Calorimetric and spectroscopic studies of the effects of cholesterol on the thermotropic phase behavior and organization of a homologous series of linear saturated phosphatidylethanolamine bilayers

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Abstract

Aqueous dispersions of cholesterol-containing phosphatidylethanolamine (PE) bilayers were examined by a combination of high-sensitivity differential scanning calorimetry (DSC), Fourier transform infrared (FTIR) and ^{31}P-nuclear magnetic resonance spectroscopy. Regardless of hydrocarbon chain length, the incorporation of low levels of cholesterol into these bilayers causes progressive reductions in the temperature, enthalpy and overall cooperativity of the lipid hydrocarbon chain-melting phase transition. Moreover, at low cholesterol levels, the heating and cooling thermograms observed for the cholesterol/PE binary mixtures are similar, indicating comparable levels of lateral miscibility of cholesterol with PE bilayers in the gel and liquid-crystalline states. However, at higher levels of cholesterol incorporation, marked differences between the heating and cooling thermograms are noted. Upon heating, complex multicomponent thermograms are observed in PE bilayers containing large amounts of cholesterol, and the temperature and overall enthalpy values *increase* discontinuously from the pattern of monotonic decrease observed at lower cholesterol levels. Moreover, these discontinuities begin to emerge at progressively lower cholesterol concentrations as PE hydrocarbon chain length increases. Upon cooling, a simpler pattern of thermotropic behavior is observed, and the measured temperature and enthalpy values continue to decrease monotonically with increases in cholesterol content. These results suggest that at higher concentrations cholesterol exhibits a decreased degree of lateral miscibility in the gel or crystalline as compared to the liquid-crystalline states of PE bilayers, particularly in the case of the longer-chain PEs. Our FTIR and ^{31}P-nuclear magnetic resonance spectroscopic studies also show that the thermotropic events observed with mixtures of low cholesterol content are analogous to the gel/liquid-crystalline phase transitions exhibited by the pure PEs. However, lamellar crystalline phases readily form when mixtures of high cholesterol content are cooled to low temperatures. Moreover, these crystalline phases are spectroscopically indistinguishable from those formed by the pure PEs, indicating that cholesterol is excluded from such phases. Upon subsequent heating, the melting of these crystalline phases gives rise to the complex thermograms detected by DSC and to the discontinuities in the phase transition temperature and enthalpy noted above. This pattern of behavior differs markedly from that observed with the corresponding phosphatidylcholines (PCs), where comparable degrees of cholesterol miscibility are observed in the gel and

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Abbreviations: DEPE, dielaidoylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DSC, differential scanning calorimetry; EYPE, egg yolk phosphatidylethanolamine; FTIR, Fourier transform infrared; GalCer, galactoceramide; L_α, lamellar liquid-crystalline phase; L_β, lamellar gel phase; L_c, lamellar crystalline phase; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; ppm, parts per million; SPM, sphingomyelin; TLC, thin-layer chromatography

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liquid-crystalline states even at high cholesterol concentrations, and where cholesterol inhibits rather than facilitates the formation of lamellar crystalline phases. We also find that the presence of cholesterol does not result in the hydrophobic mismatch-dependent shifts in the phase transition temperature in PE bilayers previously observed in PC bilayers of varying thickness. We attribute these differences in the effects of cholesterol on phospholipid thermotropic phase behavior to stronger electrostatic and hydrogen bonding interactions at the surfaces of PE and compared to PC bilayers.

Keywords: Cholesterol; Phosphatidylethanolamine; Thermotropic phase behavior; Differential scanning calorimetry; Fourier transform infrared spectroscopy; ^{31}P-nuclear magnetic resonance spectroscopy

1. Introduction

Cholesterol, or a closely related sterol, is an essential structural component of the plasma membrane of virtually all eukaryotic cells [1]. Cholesterol is a potent modulator of the bulk physical properties of biological membranes [2–5] and the presence of cholesterol or cholesterol-rich phospholipid domains in membranes can modify protein conformation and function [4,6–13]. In addition, differences in the affinity of cholesterol for different phospholipids may govern the lateral distribution of cholesterol within biological membranes, and cholesterol-rich phospholipid or sphingolipid domains may be important for the sorting and localization of membrane proteins within eukaryotic cells [13–17]. However, while it is clear that cholesterol is vital to the structure and function of biological membranes, our understanding of the molecular basis of cholesterol-phospholipid interactions remains incomplete [5].

Since the pioneering work of Ladbrooke et al. [18], the phase behavior and organization of cholesterol-containing dimyristoylphoshatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) mixtures have been the subject of extensive study (see [4,5,19]). Conversely, studies of the phase behavior and organization of cholesterol/phosphatidylethanolamine (PE) mixtures are limited in scope and number, despite the fact that PEs are a major component of the plasma membranes of eukaryotic cells (see [20,21]). Of the relatively few reports that do address cholesterol-PE interactions, the results vary markedly. For example, the addition of cholesterol was reported either to markedly reduce (1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and dielaidoylphosphatidylethanolamine (DEPE)), moderately reduce (dipalmitoylphosphatidylethanolamine (DPPE), egg yolk phosphatidylethanolamine (EYPE)) or have little effect on (dimyristoylphosphatidylethanolamine (DMPE)) the chain-melting phase transition temperature of the host PE bilayer [22–25]. The levels of cholesterol required to abolish the cooperative chain-melting phase transition of these same PE bilayers have also been reported to vary from approx. 25 mol% to more than 50 mol%. Moreover, evidence has been presented for the formation of domains of solid cholesterol in cholesterol-containing PE bilayers at cholesterol levels as low as 20–30 mol% [22–25]. However, there does not appear to be any correlation between the miscibility of cholesterol and effect of cholesterol on the thermotropic phase behavior of the host bilayer, because high concentrations of cholesterol have been reported to abolish the gel to liquid-crystalline phase transition of those PE bilayers with which it is supposedly sparingly miscible [25]. Furthermore, in studies of monotectic dioleoylphosphatidylethanolamine (DOPE)/DPPC and dioleoylphosphatidylcholine (DOPC)/DMPE mixtures, cholesterol was reported to abolish the chain-melting phase transition of the phosphatidylcholine (PC) component at cholesterol levels considerably lower than for the PE component, thus indicating that cholesterol preferentially associates with PCs [22,26–28]. However, cholesterol does not appear to have a preference for either PC or PE in non-monotectic DMPE/DMPC mixtures [23]. Finally, the observed thermotropic phase behavior and organization of cholesterol/PE mixtures also appears to vary when studied by either calorimetry or nuclear magnetic resonance (NMR) spectroscopy. When Blume and Griffin [29] utilized both differential scanning calorimetry (DSC) and ^{2}H-NMR spectroscopy to develop a temperature/composition diagram for cholesterol/DPPE mixtures, they found that the apparent phase boundaries reported by these two techniques varied by as much as 25°C. Moreover,

^{2}H-NMR spectroscopy revealed metastable phase behavior not observed by DSC, especially at high cholesterol concentrations. These differences were ascribed to the inability of DSC to monitor the poorly cooperative phase transitions of the small cholesterol-rich DPPE domains which were postulated to exist in this system.

Recently, we demonstrated that the effect of cholesterol or cholesterol analogues on the phase behavior and organization of PC bilayers varies considerably with changes in PC hydrocarbon chain length and degree of unsaturation as well as with the length of the sterol C17 alkyl side chain [30–34]. The observed differences in the phase behavior and organization of the various sterol/PC mixtures were attributed primarily to differences in the mean hydrophobic lengths of the sterol and the mean hydrophobic thickness of the host PC bilayer [30–33]. Similarly, the interactions of sphingomyelin (SPM) and galactoceramide (GalCer) with cholesterol, and subsequent changes in the molecular area of cholesterol/SPM or cholesterol/GalCer mixtures, depend on the hydrocarbon chain composition and phase state of the host phosphosphingolipid or glycosphingolipid bilayer [35,36]. Thus, to properly address the effects of both the phospholipid headgroup and hydrocarbon chain structure on the phase behavior of cholesterol/PE mixtures, we have systematically investigated the effects of cholesterol on the thermotropic phase behavior and organization of linear saturated PE bilayers with hydrocarbon chain lengths varying from 14 to 20 carbons using HS-DSC, Fourier transform infrared (FTIR) and ^{31}P-NMR spectroscopy. This study was designed to complement our prior study of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated PCs [30]. Briefly, we find that the effect of cholesterol on the thermotropic phase behavior of PE bilayers differs both qualitatively and quantitatively from that reported for cholesterol/PC mixtures [30]. We find that 50 mol% cholesterol does not abolish the chain-melting transition of PE bilayers of progressively increasing chain lengths, nor do cholesterol/PE mixtures exhibit chain length-dependent shifts in transition temperature consistent with the hydrophobic mismatch effect as documented for the corresponding cholesterol/PC mixtures. Moreover, we also report the existence of cholesterol-rich gel and cholesterol-poor crystalline PE domains in cholesterol/PE systems exposed to low temperatures. In summary, we demonstrate that the lateral miscibility of cholesterol in PE bilayers varies with temperature, the level of cholesterol, and the hydrocarbon chain length of the host PE bilayer to a much greater extent than for PC bilayers. The differences in the miscibility of cholesterol in PC and PE bilayers may have important consequences for the lateral organization of cholesterol in both model and biological membranes.

2. Materials and methods

The PEs used in these experiments were obtained from Avanti Polar Lipids (Alabaster, AB) and used without further purification. Cholesterol was also obtained from Avanti Polar Lipids and recrystallized from ethanol before use. The preparation of experimental samples proceeded as follows. With short chain compounds such as DMPE, stock solutions of phospholipid and of cholesterol were prepared by dissolving known amounts of the materials in chloroform:methanol (2:1, v/v) and appropriate volumes of these solutions were mixed to obtain the cholesterol/PE mixtures required. Next, the solvent was removed with a stream of nitrogen at temperatures between 60 and 70°C. Removal of the solvent at such high temperatures is essential for maintaining sample homogeneity. The material so obtained was then redissolved in warm benzene and lyophilized. With the longer chain PEs the preparation of stock solutions was not practical because of solubility limitations. Thus, gravimetrically determined amounts of those lipids were dissolved in chloroform:methanol (2:1, v/v) and appropriate volumes of the stock solution of cholesterol were added to obtain the required PE/cholesterol mixtures. After removal of the solvent as described above, the mixture was redissolved in warm benzene and lyophilized. Finally, the dry cholesterol/PE mixtures were dispersed in deionized water, by vigorous vortexing at temperatures some 10–20°C above the gel/liquid-crystalline phase transition of the mixture. In the DSC experiment, the amount of PE used was progressively increased from 1.0 mg with pure PE bilayers to approx. 15.0 mg with PE samples containing 45–50 mol% cholesterol. This is necessary to ensure accurate monitoring the broad, low-enthalpy phase transitions which are commonly observed at higher cholesterol concentrations (see [30]). In our experience this overall sample preparation methodology consistently produces homogenous cholesterol/PE mixtures from which reproducible DSC thermograms can be obtained.

DSC thermograms were recorded with a Hart high-sensitivity differential scanning calorimeter (Provo, UT). Typically, samples were kept overnight at 2°C prior to initial data acquisition. Unless otherwise stated, the DSC thermograms were recorded at a scan rate of 10°C/h, and a minimum of three heating and cooling cycles were performed with each sample. At the end of the DSC experiment, samples were routinely checked by thin-layer chromatography (TLC) to determine whether sample degradation had occurred. There was no evidence for significant sample degradation during the DSC experiments. The analysis and the deconvolution of the DSC endotherms was done using Microcal's (Northampton, MA) Origin and DA-2 software. Briefly, the procedure for DSC deconvolution is based on the assumption that the observed thermograms can be approximated as a linear combination of multiple, independent two-state transitions as shown in Estep et al. [37] and Mabrey et al. [38] for PC/sterol mixtures. The curve broadening is expressed in terms of the van't Hoff enthalpy, which is evaluated by the equation $\Delta H_{\mathrm{vH}} = 4\mathrm{RT}_{\mathrm{m}}^{2}\ (c_{\max}/\Delta q)$, where $c_{\max}$ is the excess specific heat capacity, and Δq is the area under the curve. We have shown previously that this approach provides a complete and consistent description of the thermotropic phase behavior of PC/sterol binary mixtures [30–34].

Cholesterol/PE mixtures (2–3 mg) were prepared for FTIR spectroscopy using the sample protocols outlined above and were dispersed in 50–75 μl of $^{2}H_2O$. The samples were placed between the CaF_2 windows of a demountable liquid cell equipped with a 25 μm teflon spacer. When mounted in the cell holder of the instrument, the temperature of the sample could be controlled between −20 and 90°C by an external, computer-controlled circulating water bath. FTIR spectra were recorded with a Digilab (Cambridge, MA) FTS-40 Fourier transform infrared spectrometer using data acquisition and data processing protocols similar to those described by Mantsch and McElhaney [39]. The spectra were analyzed using computer programs provided by Digilab and by the National Research Council of Canada, and were plotted using the Origin software package obtained from Microcal Software.

Samples (30–50 mg) were prepared for ^{31}P-NMR spectroscopy using the same protocols outlined for the DSC experiments and were dispersed in 0.9 ml of water. Spectra were acquired as a function of temperature with a Varian (Palo Alto, CA) Unity-300 spectrometer operating at 121.42 MHz for ^{31}P using the single-pulse, direct-excitation methods described by Lewis et al. [40]. Typically 12 000–15 000 transients were acquired and processed as described by Lewis et al. [40]. These samples were routinely analyzed by DSC at the end of the NMR measurements and they were also checked for sample degradation by TLC. We found no evidence for significant sample degradation or for any changes in the thermotropic phase behavior of the sample.

3. Results

A detailed study of the polymorphic phase behavior of the homologous series of *n*-saturated 1,2-diacyl PEs has recently been published by Lewis and McElhaney [41]. Using a combination of DSC, FTIR and ^{31}P-NMR spectroscopy, these authors demonstrated that the *n*-saturated 1,2-diacyl PEs exhibit a complex pattern of polymorphic phase behavior that is strongly dependent upon the hydrocarbon chain length and the thermal history of the sample. Fully hydrated samples of these lipids exhibit highly energetic transitions between the lamellar gel (L_β) and lamellar liquid-crystalline (L_α) phases which increase in temperature and enthalpy with increasing hydrocarbon chain length. However, after prolonged low-temperature incubation, these compounds may form one or more lamellar crystalline (L_c) phases. These phases are partially dehydrated structures in which the hydrocarbon chains are tightly packed and the polar headgroups are involved in an extended hydrogen bonding network at the surface of the lipid bilayer (for more structural details about these L_c phases, see [41]). It is also important to note that, depending on hydrocarbon chain length, the L_c

phases of these compounds may undergo chain melting at temperatures that are either above or below the gel/liquid-crystalline phase transition observed with the freshly dispersed sample. The L_c phases of the PEs with hydrocarbon chains equal to or less than 17 carbons melt to form the L_α phase at temperatures above the L_β/L_α phase transition temperature, whereas the L_c phases of the longer chain compounds convert to the L_β phase at temperatures below the L_β/L_α phase transition temperature of the lipid (see [41], and references cited therein). It is also important to note that under the experimental conditions employed here, the L_c phase does *not* form in DMPE, DPPE or DSPE bilayers which do not contain cholesterol. An understanding of the polymorphic phase behavior of these phospholipids in the absence of cholesterol is crucial to the interpretation of the calorimetric and spectroscopic data presented below.

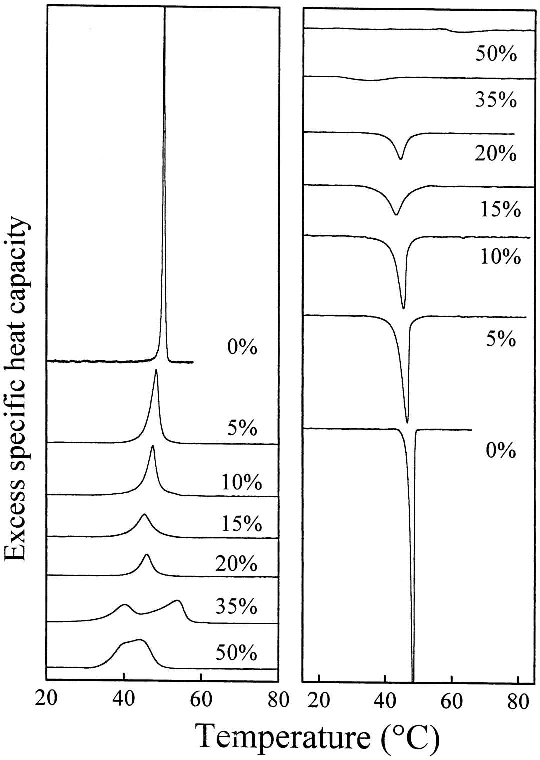

Fig. 1. Representative DSC heating (left panel) and cooling (right panel) thermograms of cholesterol-containing DMPE bilayers. Thermograms are shown for mixtures of the cholesterol concentrations indicated. All data shown were acquired at scan rates 10°C/h and are normalized with respect to the mass of DMPE.

3.1. *Differential scanning calorimetric studies*

Illustrated in Fig. 1 are representative high-sensitivity DSC heating and cooling thermograms exhibited by DMPE and cholesterol-containing DMPE bilayers. In the absence of cholesterol, fully hydrated DMPE bilayers exhibit a reversible, fairly energetic ($\Delta H = 6.2$ kcal/mol) and highly cooperative phase transition near 50°C. This event is the well characterized transition between the L_β and L_α phases of this lipid (see [41]). It is clear that the incorporation of cholesterol markedly alters the calorimetric behavior of DMPE bilayers. At cholesterol concentrations of up to 30 mol%, cholesterol produces progressively more marked decreases in the temperature (Fig. 4) and enthalpy (Fig. 5) of the lipid hydrocarbon chain-melting process, and significant increases in the widths of the observed heating and cooling thermograms (Fig. 1). Also, in the lower range of cholesterol concentration (5–20 mol%), the observed DSC thermograms appear to be a summation of two components (one sharp and one broad), the proportions of which are cholesterol concentration dependent. Such behavior has been observed in previous studies of cholesterol/PC mixtures and has been interpreted in terms of the existence of cholesterol-rich and cholesterol-poor lipid domains (see [5,30–34,37,38] and references cited therein). Fig. 1 also shows that the heating and cooling thermograms observed at low (≤ 30 mol%) cholesterol contents are essentially similar. This behavior differs markedly from that observed at higher cholesterol contents (>30 mol% cholesterol). As shown in Fig. 1, DMPE bilayers with high levels of cholesterol exhibit broad, multicomponent heating endotherms which are not observed in the cooling mode. The temperature and enthalpy of the lower temperature transition is similar to that of the L_α/L_β phase transition observed upon cooling the cholesterol-containing DMPC system, while the temperature and enthalpy of the higher temperature transition more closely resembles that of the L_c/L_α phase transition of the pure DMPE (see [41]). Moreover, our spectroscopic studies confirm that such behavior is attributable to the sequential hydrocarbon chain melting of an L_β phase enriched in cholesterol and of one or more of the cholesterol-poor, quasi-crystalline L_c phases of DMPE (see below). Thus the accelerated formation of the crystal-

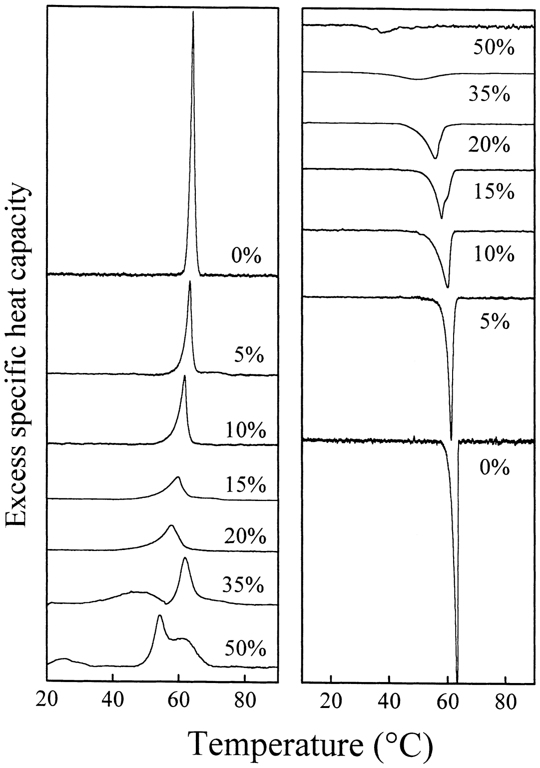

Fig. 2. Representative DSC heating (left panel) and cooling (right panel) thermograms of cholesterol-containing DPPE bilayers. Thermograms are shown for mixtures of the cholesterol concentrations indicated. All data shown were acquired at scan rates 10°C/h and are normalized with respect to the mass of DPPE.

line and/or quasi-crystalline forms of DMPE in mixtures of high cholesterol content is responsible for the apparently discontinuous *increases* in the temperature and enthalpy of the 'main' phase transition observed upon heating, since the temperature (approx. 57°C) and enthalpy (approx. 19 kcal/mol) of the L_c/L_α phase transition of DMPE is greater than that of the L_β/L_α phase transition (approx. 6 kcal/mol), particularly when the temperature and enthalpy of the latter transition have already been reduced by the presence of cholesterol (see Figs. 4 and 5). These results differ markedly from those observed with cholesterol/DMPC mixtures, where the presence of cholesterol has been shown to inhibit L_c phase formation (see [30]), and have important implications with regard to the overall miscibility of cholesterol in the gel or crystalline phases of PEs which will be discussed later.

DSC heating and cooling thermograms illustrating the thermotropic phase behavior of DPPE and the cholesterol/DPPE mixtures are shown in Fig. 2. As expected, fully hydrated DPPE bilayers exhibit a reversible, highly cooperative and highly energetic ($\Delta H = 8.7$ kcal/mol) L_β/L_α phase transition near 64.5°C (see [41]). With the incorporation of low levels of cholesterol, the cooperativity (Fig. 2), temperature (Fig. 4) and enthalpy (Fig. 5) of the hydrocarbon chain-melting phase transition all decrease markedly. The DSC thermograms of cholesterol/DPPE mixtures at low (≤ 20 mol%) cholesterol concentrations also appear to be a summation of sharp and broad components. However, unlike the cholesterol/DMPE system, the observed contours are more complex and cannot be adequately approximated by the summation of the chain-melting of cholesterol-rich and cholesterol-poor PE domains (data not shown). We also find that cholesterol/DPPE mixtures of lower cholesterol content exhibit comparable ther-

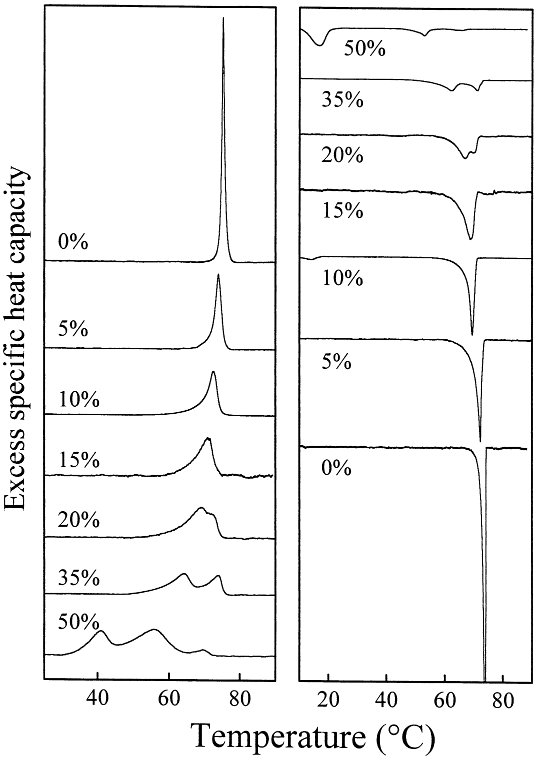

Fig. 3. Representative DSC heating (left panel) and cooling (right panel) thermograms of cholesterol-containing DSPE bilayers. Thermograms are shown for mixtures of the cholesterol concentrations indicated. All data shown were acquired at scan rates 10°C/h and are normalized with respect to the mass of DSPE.

mograms in both the heating and cooling modes, while notably different heating and cooling thermograms are observed with mixtures of higher cholesterol contents (> 25 mol% cholesterol) (see Fig. 2). This pattern of behavior is qualitatively similar to what was observed with the cholesterol/DMPE mixtures described above and, likewise, is attributable to the formation of both cholesterol-enriched L_β and highly ordered arrays of cholesterol-free L_c phases of DPPC at low temperatures (see the spectroscopic data presented below). The formation and hydrocarbon chain melting of the L_c phases of these mixtures again account for the discontinuous increases in the enthalpy and temperature measured from heating endotherms observed at high cholesterol content (see Fig. 5).

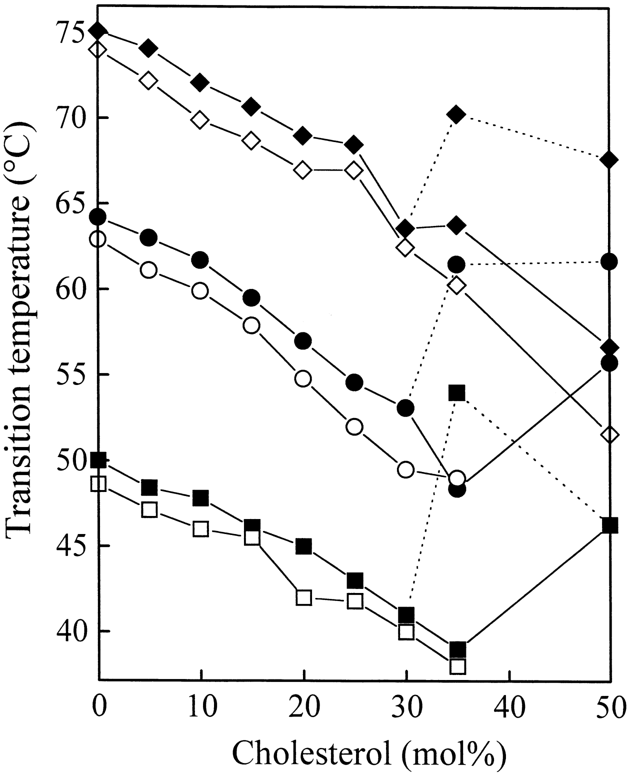

Fig. 4. Plots of the transition temperature as a function of cholesterol concentration for cholesterol/DMPE (■,□), cholesterol/DPPE (●,○) and cholesterol/DSPE (◆,◇) mixtures. Transition temperatures obtained upon heating are represented by solid symbols and upon cooling by open symbols. The dotted lines represent the midpoint temperatures of the additional components (L_c/L_α or L_c/L_β observed by DSC) in these cholesterol/PE mixtures. The estimated error of the phase transition temperature determinations is ± 1°C.

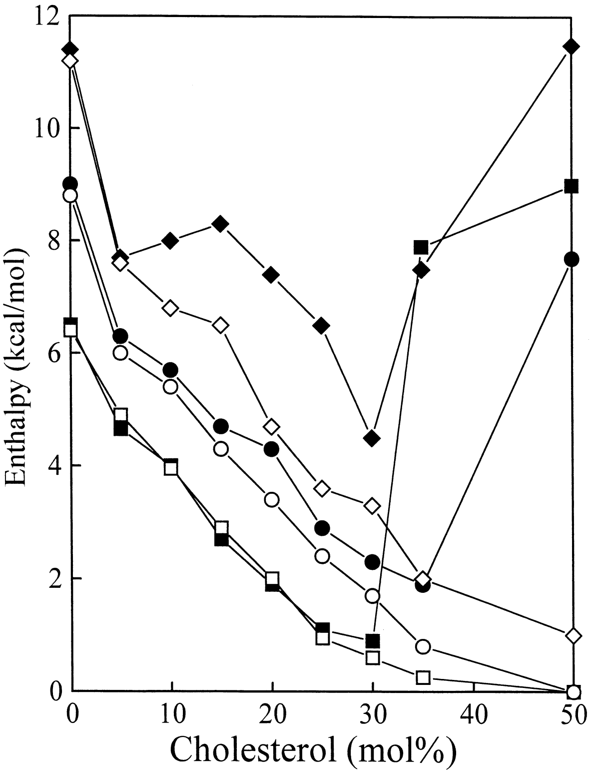

Fig. 5. Plots of the transition enthalpy as a function of cholesterol concentration for cholesterol/DMPE (■,□), cholesterol/DPPE (●,○) and cholesterol/DSPE (◆,◇) mixtures obtained from both heating and cooling runs. Enthalpy values obtained upon heating are represented by solid symbols and upon cooling by open symbols. The estimated errors of these transition enthalpy determinations are ± 0.4 kcal/mol at cholesterol concentrations or 25 mol% or less and ± 0.8 kcal/mol for cholesterol concentrations of 30 mol% or more.

DSC thermograms illustrating the calorimetric behavior of DSPE and cholesterol/DSPE mixtures are shown in Fig. 3. In the absence of cholesterol, the transition between L_β and L_α phases of fully hydrated DSPE is observed at temperatures near 74.3°C. This is a freely reversible, highly cooperative phase transition with an enthalpy of 11.0 kcal/mol. As observed with the other PEs, the incorporation of cholesterol markedly alters all aspects of the calorimetric behavior of this lipid. At lower levels (≤15 mol%), the effects of cholesterol on the calorimetric behavior of DSPE are essentially similar to that exhibited by cholesterol/DMPE and cholesterol/DPPE mixtures. On both heating and cooling scans (see Fig. 3) the cooperativity, temperature and enthalpy of the main transition decrease significantly (see Figs. 4 and 5). However, at cholesterol contents in excess

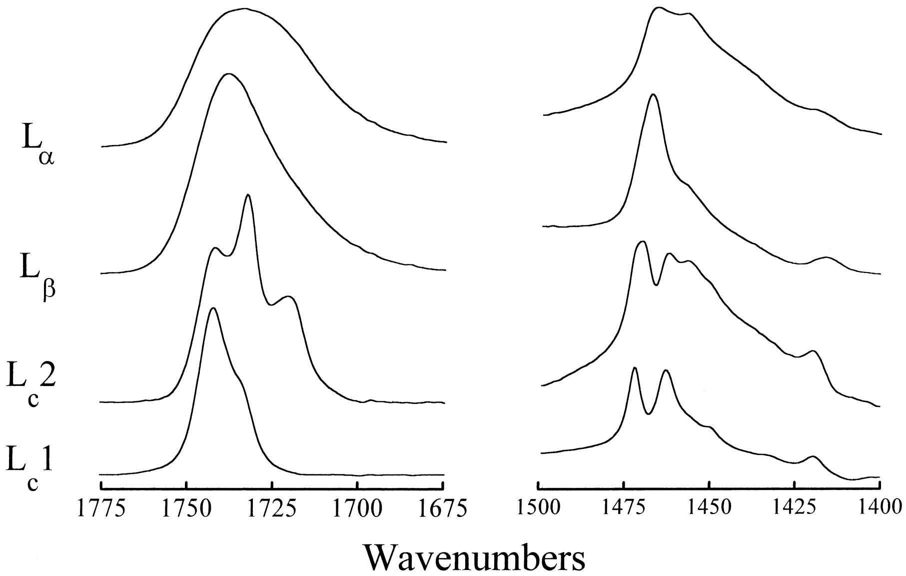

Fig. 6. FTIR spectra showing the C = O stretching and CH_2 scissoring bands of the L_α, L_β, L_c1 and L_c2 phases of the *n*-saturated 1,2-diacyl PEs (from Lewis and McElhaney [41]).

of 10 mol%, additional transitions are apparent in the heating thermograms (see Fig. 3). These components are relatively minor at 10–15 mol% cholesterol but they become progressively more pronounced as the cholesterol content increases and are the predominant thermotropic events observed at cholesterol contents near 50 mol%. The appearance of these thermal events coincides with the discontinuous changes in the enthalpy and temperature values measured from the heating thermograms (see Fig. 5). Upon cooling, these low temperature events are also observed, but exhibit substantially lower enthalpy values compared to corresponding events observed on heating. These results suggest that the low temperature transitions observed in cholesterol/DSPE mixtures with more than 15 mol% cholesterol are solid-phase events which do not involve the melting of the lipid hydrocarbon chains. These findings are consistent with the formation of the L_c phase in DSPE bilayers, and with its conversion to the L_β rather than the L_α phase upon heating, as documented by Lewis and McElhaney [41], and are supported by the results of our spectroscopic studies (see below). Moreover, based on these findings and our results for the cholesterol/DMPE and cholesterol/DPPE mixtures, it is clear that the formation of highly ordered crystalline arrays of PEs induced by the presence of cholesterol is accelerated with increasing PE hydrocarbon chain length. The significance of these observations will be examined later.

3.2. *FTIR spectroscopic studies*

FTIR spectroscopy was employed to examine the effects of cholesterol on the phase state and conformational order of cholesterol/PE bilayers as a function of temperature, as well as to examine and characterize the physical basis of the various thermotropic events observed by DSC. The contours of the CH_2 stretching, C = O stretching and CH_2 scissoring bands of the lamellar gel (L_β), lamellar liquid-crystalline (L_α) and lamellar crystalline (L_c1 and L_c2) phases formed by the *n*-saturated 1,2-diacyl PEs in the absence of cholesterol are shown in Fig. 6. These are the main spectroscopic signatures of the various lamellar phases formed by the *n*-saturated 1,2-diacyl PEs, and are interpretable in terms of changes in hydrocarbon chain conformational and orientational order, hydrocarbon chain-packing motif, as well as differences in headgroup and interfacial hydration [41]. The spectroscopic characterization of *n*-saturated 1,2-diacyl PEs and their structural interpretation have been fully described previously (see [41]) and will not be discussed again in detail here. The spectroscopic data shown in Fig. 6 for the PEs alone are rather presented as a reference to assist in

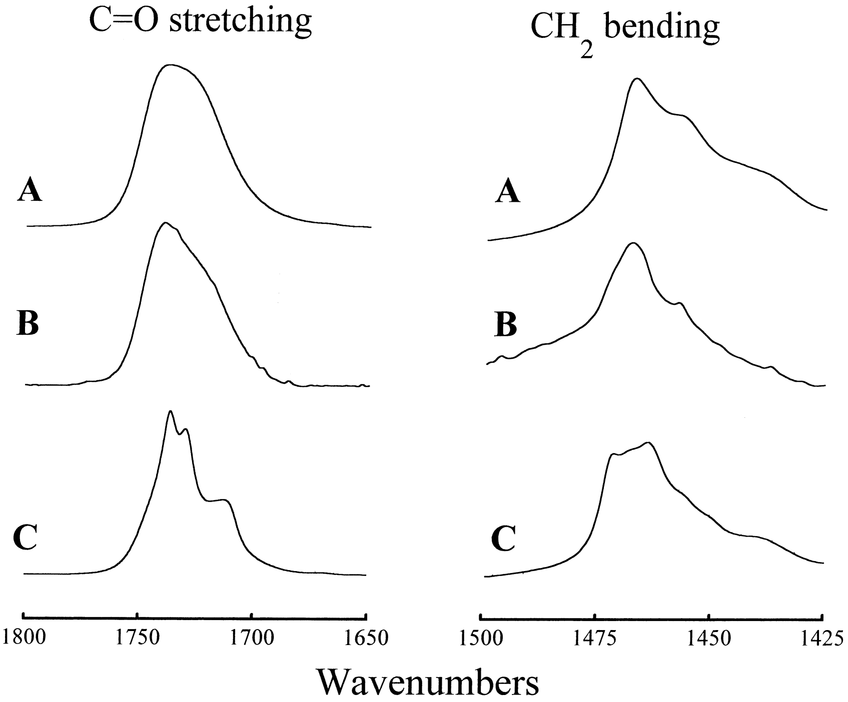

Fig. 7. FTIR spectra showing the C = O stretching and CH_2 scissoring bands observed with cholesterol-containing DMPE and DPPE bilayers. The spectra shown were acquired with cholesterol-containing (40 mol%) DMPE bilayers under the following conditions: (A) sample heated to a temperature above the L_β/L_α phase transition temperature (60°C); (B) sample cooled to a temperature just below the L_β/L_α phase transition (30°C); and (C) sample cooled to a temperature well below the L_β/L_α phase transition (2°C).

the interpretation of the spectroscopic data obtained with the cholesterol/PE mixtures.

Illustrated in Fig. 7 are the C = O stretching and CH_2 scissoring regions of representative FTIR spectra obtained with cholesterol-containing DMPE and DPPE bilayers. At low temperatures and with cholesterol levels up to 30 mol%, cholesterol/DMPE and cholesterol/DPPE mixtures exhibit spectroscopic features which are essentially identical to those of spectra 7B. At temperatures above the calorimetrically observed phase transitions of these same mixtures, the spectroscopic features are essentially identical to those of spectra 7A. These results are obtained whether the samples were heated or cooled. Thus the thermotropic events observed upon heating and cooling of cholesterol/DMPE and cholesterol/DPPE mixtures of low cholesterol content (< 20–30 mol% cholesterol) are comparable to the L_β/L_α phase transitions of the pure PEs. However, at and above 20–30 mol% cholesterol, the L_β phase of these mixtures is not stable at any temperature and the spectra convert from the L_β phase (Fig. 7B) to a form similar to that labeled C in Fig. 7. A comparison of these latter spectroscopic features with the reference data shown in Fig. 6 indicates these features are virtually identical to those exhibited by the L_c2 crystalline PE phase described by Lewis and McElhaney [41]. Upon heating, these spectroscopic features convert directly to the form characteristic of the PE liquid-crystalline phase (see spectra labeled A in Fig. 7) at temperatures which coincide with the calorimetrically determined phase transition temperatures. Thus the higher temperature calorimetric events observed upon heating cholesterol/DMPE and cholesterol/DPPE mixtures of high cholesterol content are structurally analogous to the phase transitions from lamellar crystalline to lamellar liquid-crystalline (i.e., L_c/L_α) in pure DMPE and DPPE bilayers. Moreover, the considerably accelerated formation of the L_c phase in cholesterol/PE bilayers compared to that normally observed with the pure PEs (see [41]) indicates that cholesterol potentiates the formation of the L_c phases of these PEs. Since the formation of PE L_c phases requires specific and very tight intermolecular interactions between extended domains of PE molecules (see [40]), we can further conclude that choles-

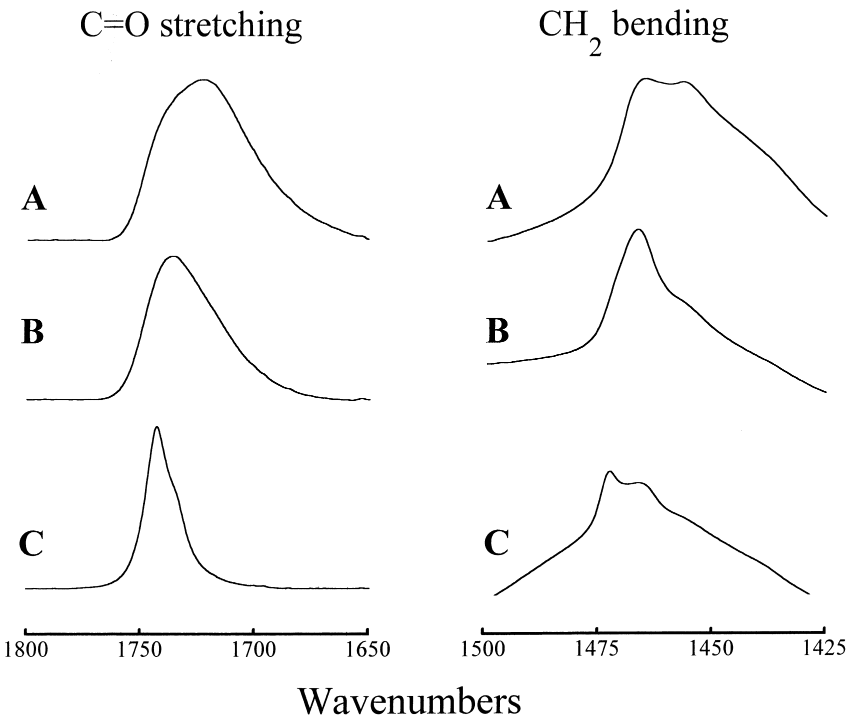

Fig. 8. FTIR spectra showing the C = O stretching and CH_2 scissoring bands observed with cholesterol-containing (25 mol%) DSPE bilayers. The spectra shown were acquired under the following conditions: (A) sample heated to temperatures above the L_α/L_β phase transition (82°C); (B) sample cooled to temperatures just below the L_β/L_α phase transition (46°C); and (C) sample cooled to temperatures well below the L_β/L_α phase transition (2°C).

terol is essentially excluded from these domains. The poor miscibility of cholesterol in PE bilayers due to the formation of cholesterol-poor L_c phases can explain the significant increase in transition temperature and enthalpy observed in cholesterol/DMPE and cholesterol/DPPE mixtures with high levels of cholesterol (see Figs. 4 and 5), since the L_c phase melts at a higher temperature and with a greater enthalpy than does the L_β phase.

Illustrated in Fig. 8 are examples of the C = O stretching and CH_2 scissoring bands of FTIR spectra exhibited by the various cholesterol/DSPE mixtures studied. At temperatures above and below the calorimetrically detected phase transition, DSPE bilayers containing less than 10–15 mol% cholesterol exhibit spectra similar to those shown in A and B, respectively (Fig. 8). These spectroscopic features resemble those exhibited by the L_α and L_β phases of the pure PEs and support the conclusion that the thermotropic events observed in these mixtures are the gel/liquid-crystalline L_β/L_α phase transitions. Similar spectroscopic features are also found with mixtures of higher cholesterol content ($>$20 mol% cholesterol); however, such changes coincide only with the high-temperature component of the DSC thermograms of DSPE/cholesterol mixtures observed upon heating and cooling (see Fig. 3). Thus, we conclude that the high temperature transition in cholesterol/DSPE mixtures is structurally analogous to the gel/liquid-crystalline (L_β/L_α) phase transition observed with the pure DSPE bilayers. In addition to the presence of the L_β phase at lower temperatures, cholesterol/DSPE mixtures of higher cholesterol content ($>$15 mol% cholesterol) also exhibit spectroscopic features which closely resemble the L_c1 quasi-crystalline phase of the pure PEs (see reference spectra in Fig. 6). Upon heating, these particular features disappear at temperatures which coincide with the major lower temperature component of the DSC thermogram and are replaced by spectroscopic signatures typified by those labeled B in Fig. 8. Thus the lower temperature thermotropic events exhibited by cholesterol/DSPE mixtures ($>$15 mol% cholesterol) represent lamellar crystalline to lamellar gel (i.e., L_c/L_β)

phase transitions which are followed by L_β/L_α transitions at higher temperatures. This assignment is further supported by the observation that discontinuous increases in the frequency of the CH_2 symmetric stretching band (a diagnostic of hydrocarbon chain-melting processes, see [41] and references cited therein) are not observed over the course of the lower temperature transitions (data not shown), but are observed over the course of the high-temperature phase transitions shown by DSC.

3.3. ^{31}P-NMR spectroscopic studies

^{31}P-NMR spectroscopy was used to monitor the effect of cholesterol on the mobility of PE headgroups and to aid in the structural assignments of the thermotropic events observed in our DSC experiments. The ^{31}P-NMR spectra shown in Fig. 9 were obtained with DMPE alone and are shown as a reference. These spectra were recorded at temperatures above and below the lipid phase transition temperature (Fig. 9, spectra A and B, respectively), and after low-temperature incubation under conditions favoring the formation of the lamellar crystalline phases of this lipid (spectrum C). At temperatures bracketing the normal L_β/L_α phase transition of this lipid, so-called axially symmetric ^{31}P-NMR powder patterns, with an estimated chemical shift anisotropy of 38–40 ppm, are obtained (Fig. 9, spectra A and B). These spectra are consistent with axially symmetric motion of the PE phosphodiester headgroup on the surface of a gel or liquid-crystalline phospholipid bilayer, respectively. The spectrum recorded at low temperature, favoring the formation of lamellar crystalline PE phases, also exhibits features expected of lamellar phospholipids undergoing axially symmetric motion (Fig. 9, spectrum C), but the intensity of the recorded signal is markedly diminished when compared to those shown in spectra A and B. The loss of ^{31}P-NMR signal intensity coincident with the formation of lamellar crystalline phospholipids has been observed in previous studies of PEs [41,42] and has recently been reported in studies of lamellar crystalline phosphatidylglycerols (see [43]). This phenomenon has been attributed to substantial increases in the longitudinal relaxation times of the population of ^{31}P nuclei involved in the L_c phases of these lipids [41–43]. Consequently, the L_c phase does not contribute significantly to the observed NMR signal and the residual domains of L_β phase which coexist with the L_c phase of DMPE are the dominant contributors to the observed spectrum (for a more detail discussion of this phenomenon, see [41]). An understanding of this aspect of the ^{31}P-NMR spectroscopic data is crucial to the correct interpretation of the data presented below.

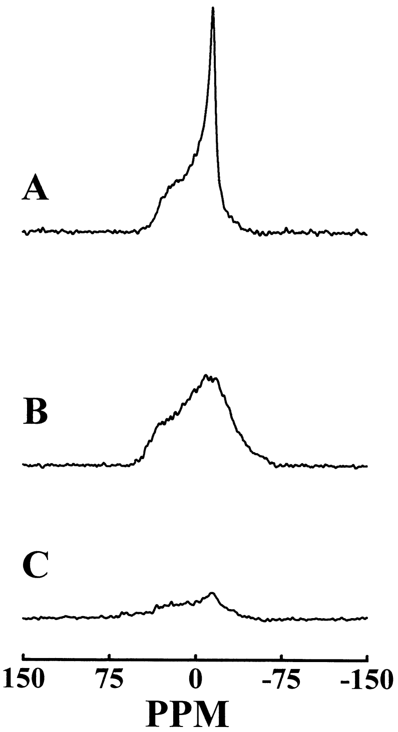

Fig. 9. Proton decoupled ^{31}P-NMR spectra which typify those obtained with the *n*-saturated 1,2-diacyl PEs. The spectra shown were obtained with a sample of fully hydrated DMPE and were obtained by processing the same number of transients (10 000). Data acquisition conditions were: (A) sample heated to a temperature above the L_β/L_α phase transition (60°C); (B) sample cooled to a temperature just below the L_β/L_α phase transition (30°C); and (C) sample incubated under conditions favorable to the formation of the L_c phase (2°C).

Illustrated in Fig. 10 are ^{31}P-NMR powder patterns that exemplify the types of spectra obtained for all of the cholesterol/PE mixtures examined. For DMPE, DPPE and DSPE bilayers with 5–50 mol% cholesterol, spectra A and B in Fig. 10 typify those obtained when samples are cooled from high temperatures (A) to temperatures just below the phase transition temperature obtained by calorimetry (B). These spectra are essentially identical to those obtained in the L_α and L_β phases of PEs and other

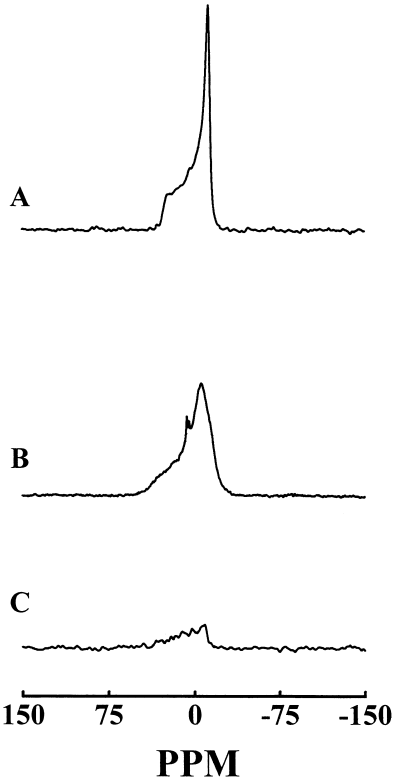

Fig. 10. Proton-decoupled ^{31}P-NMR spectra which typify those obtained with cholesterol-containing samples of the *n*-saturated 1,2-diacyl PEs. The spectra shown were obtained with a sample of a fully hydrated DMPE containing 40 mol% cholesterol and were obtained by processing the same number of transients. Data acquisition conditions were: (A) sample heated to a temperature above the L_β/L_α phase transition (60°C); (B) sample cooled to temperatures just below the L_β/L_α phase transition (30°C); and (C) sample cooled to temperatures well below the L_β/L_α phase transition (4°C).

phospholipids and their appearance is therefore consistent with the assignment of these thermotropic events as processes analogous to the L_β/L_α phase transitions of the pure lipids. However, when cholesterol-containing DMPE, DPPE and DSPE bilayers are cooled to temperatures well below the phase transition temperature of the respective pure phospholipid, powder patterns analogous to spectrum C are observed. This result clearly indicates that at low temperatures cholesterol/PE mixtures convert from the lamellar gel phase to the quasi-crystalline L_c phase. Moreover, in all of the PEs examined, increasing levels of cholesterol facilitate formation of the L_c phase. Upon subsequent heating, the spectra of cholesterol/DMPE and cholesterol/DPPE mixtures (spectrum C) convert directly to spectrum A. This correlates with our DSC and FTIR data on the corresponding mixtures, which indicate that these systems convert directly from the L_c to the lamellar liquid-crystalline L_α phase on heating. In cholesterol/DSPE mixtures, the low temperature spectrum (Fig. 10, spectrum C) first converts to a form exemplified by spectrum B and, upon further heating, converts to the form exemplified by spectrum C in Fig. 10. Thus in cholesterol/DSPE mixtures, the L_c phase first converts to a L_β-like phase upon heating, and with further heating, conversion to the L_α-phase is observed. These spectroscopic changes all coincide with the thermotropic events observed by DSC and are consistent with the results and interpretation of the FTIR spectroscopic data presented above.

4. Discussion

We reported that the incorporation of increasing amounts of cholesterol into PC bilayers with hydrocarbon chain lengths of 13:0 to 21:0 carbons progressively decreases the cooperativity and enthalpy of the chain-melting transition of the host PC bilayer, in both the heating and cooling modes, such that at 50 mol% cholesterol the chain-melting phase transition was completely abolished [30]. Thus, cholesterol appears to be fully miscible in both the gel and liquid-crystalline states of the host PC bilayer, regardless of PC hydrocarbon chain length or degree of unsaturation (see also [19,31–34,37,38]). The only chain length-dependent effect observed in cholesterol/PC mixtures is the relative destabilization (stabilization) of the PC gel state upon cholesterol incorporation into PC bilayers with hydrocarbon chains longer (shorter) than 17:0 PC [30,44]. These results were attributed to the hydrophobic mismatch effect first described by Mouritsen and Bloom [45] for transmembrane peptides in PC bilayers of varying hydrophobic thickness. However, in the present study, we find that cholesterol always decreases the L_β/L_α phase transition temperature, regardless of PE hydrocarbon chain length. Moreover, the effect of cholesterol on the thermotropic phase behavior and organization of cholesterol/PE mixtures of varying hydrocarbon chain length and cholesterol concentrations differs markedly from that reported for cholesterol/PC mixtures, as well as from prior studies of cholesterol/PE mixtures [22,23,26,29]. We find that upon heating, the effect of increasing levels of cholesterol in DMPE and DPPE bilayers is to progressively decrease both the enthalpy and temperature of the chain-melting phase transition, but only up to levels of 30–20 mol% cholesterol, respectively. At higher levels of cholesterol, the temperature and total enthalpy of the chain-melting phase transitions of these cholesterol/PE mixtures actually *increase* dramatically, due to the chain melting of the highly ordered crystalline phase of pure PEs induced by cholesterol. This behavior appears to be due both to the limited miscibility of cholesterol in gel state PE bilayers and to the propensity of cholesterol to facilitate the formation of a cholesterol-free lamellar crystalline phase in these systems. Only on subsequent cooling scans do the temperature and enthalpy of the chain-melting transition of these mixtures decrease in a monotonic manner, because cholesterol is fully miscible in liquid-crystalline phases of DMPE and DPPE bilayers and because the formation of L_c phases is not observed during the course of the DSC cooling run. As the hydrocarbon chain length of the PEs increases to 18 carbons (DSPE), both heating and cooling scans exhibit cooperative and relatively energetic transitions even at 50 mol% cholesterol. FTIR and ^{31}P-NMR spectroscopy also reveal the coexistence of cholesterol-rich and cholesterol-poor PE domains in cholesterol/DSPE mixtures from 5 mol% up to 50 mol% cholesterol, fully consistent with our DSC findings. Generally, we find that increasing hydrocarbon chain length and cholesterol levels, as well as decreasing temperature, favors the formation of cholesterol-poor PE domains over cholesterol-rich domains. Thus, contrary to the usually accepted role of cholesterol in biological membranes based on work on cholesterol/PC systems, our results indicate that increasing levels of cholesterol may actually facilitate lateral phase separation in PE bilayers, and that this effect becomes more pronounced with increasing PE hydrocarbon chain length and decreasing temperature. Only at very low cholesterol levels, short hydrocarbon chain lengths and high temperatures do the phase state and organization of cholesterol/PE mixtures resemble that documented for the more laterally homogeneous cholesterol/PC mixtures.

The limited lateral miscibility of cholesterol in PE relative to PC bilayers is probably due to the relatively strong inter-headgroup hydrogen bonding and electrostatic interactions of the former phospholipid, which favors phospholipid-phospholipid contacts over cholesterol-phospholipid interactions [23,41,46]. Thus, at low temperatures, where the tight packing of neighboring PE molecules permits extensive non-polar and polar intermolecular interactions, the intercalation of cholesterol between neighboring PEs is unfavorable, and becomes more so with increasing cholesterol levels. As a result, the number of cholesterol-PE contacts, and thus the effective cholesterol-PE interaction stoichiometry, is considerably lower than in corresponding cholesterol/PC mixtures. This allows for the formation of extended arrays of ordered, crystalline PE domains in cholesterol/PE mixtures from which cholesterol must be completely excluded [41]. The magnitude, and the rate, of the lateral phase separation of cholesterol-rich and cholesterol-poor cholesterol/PE mixtures also increases with increasing PE hydrocarbon chain length. This is probably due to the increased van der Waals forces between the hydrocarbon chains of neighboring PE molecules, which further favors phospholipid-phospholipid contacts over cholesterol-phospholipid interactions. However, as the temperature increases and progresses beyond both the L_c/L_α or L_β/L_α chain-melting phase transition temperature and the strength of the PE attractive intermolecular forces decreases, the lateral exclusion of cholesterol becomes less favorable and larger amounts of cholesterol become miscible in the host PE bilayer. This accounts for the substantially enhanced effect of cholesterol on the enthalpy, cooperativity and temperature of the PE chain-melting phase transition observed upon subsequent cooling of a given cholesterol/PE sample, such that the thermotropic phase behavior of the cholesterol/PE system more closely resembles corresponding cholesterol/PC mixtures. However, our calorimetric and spectroscopic studies of cholesterol/DSPE mixtures reveal that cholesterol levels above 50 mol% are not sufficient to abolish the chain-melting transition on either heating or cooling. Thus, in contrast to the shorter chain cholesterol/PE mixtures or corresponding longer chain cholesterol/PC mixtures, the apparent stoichiometry of cholesterol-PE interactions is limited even in the relatively disordered state of liquid-crystalline DSPE bilayers. In fact, when we attempted to study cholesterol/PE mixtures where the hydrocarbon chain length of the PE was 20 carbons, we found it extremely difficult to form stable mixtures due to the very low miscibility of cholesterol in both gel and liquid-crystalline state diarachadonylphosphatidylethanolamine bilayers. Clearly both PE-cholesterol hydrophobic mismatch and the strong PE headgroup interactions contribute to the relative immiscibility of cholesterol in PE as compared to PC bilayers. Interestingly, the effect of synthetic α-helical transmembrane peptides on the thermotropic phase behavior of PC and PE bilayers is qualitatively similar to that produced by cholesterol [47,48]. Our conclusion that the miscibility of cholesterol in PE bilayers, and thus the effective stoichiometry of cholesterol-PE interactions, varies with hydrocarbon chain length, temperature and cholesterol concentration is in marked contrast to the interpretation utilized in prior studies of cholesterol/PE mixtures, which assume constant and cholesterol concentration-independent cholesterol-phospholipid interaction stoichiometries to account for the effect of cholesterol on the thermotropic phase behavior and organization of the host bilayer [22–29]. Moreover, our studies of the interaction of cholesterol with different synthetic phospholipids [34] and with the phospho- and glycolipids of the *Acholeplasma laidlawii* membrane [49], also indicate that the lateral miscibility of this sterol can vary markedly with lipid polar headgroup structure and fatty acid unsaturation. In this regard, we note that a recent ^{2}H-NMR and FTIR spectroscopic study suggests that cholesterol is more miscible with POPE bilayers [50] than with the DPPE or especially the DSPE bilayers studied here.

In order to determine the 'affinity' of cholesterol for different phospholipid species, in particular for PCs and PEs, prior DSC studies used monotectic mixtures invariably containing a combination of saturated and unsaturated phospholipids with varying hydrocarbon chain lengths [22,26,51,52]. However, it is now clear that the hydrocarbon chain composition, the level of cholesterol and even the scan rate all have a considerable influence on the thermotropic phase behavior and organization of the cholesterol/PE mixtures, in marked contrast to the largely chain length-independent lateral miscibility of cholesterol in corresponding PC bilayers [30–34]. Moreover, the distribution of cholesterol in higher order cholesterol/phospholipid mixtures may also depend on whether or not the presence of cholesterol will inhibit (as in the case of the PCs) or promote (as in the case of the PEs) the ability of the phospholipid species to form crystalline phases. These facts may account for the discrepant results of prior cholesterol/DMPE and cholesterol/DPPE DSC and cholesterol/DPPE ^{13}C- and ^{2}H-NMR studies [23,29]. The NMR spectroscopic experiments involved holding the cholesterol/DPPE mixtures at a given temperature for considerable periods of time, while the corresponding cholesterol/DMPE DSC experiments involved scan rates of no less than 60°C/h. As a result, the NMR experiments favor the formation of laterally phase separated cholesterol-rich L_β and cholesterol-poor L_c phases in cholesterol-DPPE mixtures, especially at low temperatures and high cholesterol levels. In fact, the authors observe metastability in that region of their temperature/composition plot, while the DSC experiments identified only the L_β and L_α phases of the shorter chain cholesterol/DMPE mixtures. Thus, the present work demonstrates that the simultaneous use of several different physical techniques is required to rigorously characterize the thermotropic phase behavior and organization of binary and higher order cholesterol-containing phospholipid mixtures.

The differential affinity of cholesterol for, or miscibility of cholesterol with, different phospholipids may account at least in part for the formation of domains of phospholipids selectively enriched (PC) or depleted (PE) in cholesterol in model and perhaps even in biological membranes. We note that differences in the size and morphology of cholesterol-rich and cholesterol-poor domains as a function of temperature and phospholipid composition has been directly observed in cholesterol/SPM and cholesterol/PC mixtures of varying hydrocarbon chain lengths [53–56]. We predict that investigations using magic angle spinning-NMR or fluorescent spectroscopy will demonstrate that the lateral organization of cholesterol in PE bilayers also differs significantly from cholesterol/PC (or cholesterol/SPM) mixtures and will also vary with temperature and the level of cholesterol present (see [53–57]). Moreover, the homogeneous and temperature-independent distribution of cholesterol within the bilayer of eukaryotic cell plasma membranes sometimes assumed, based on the properties of the so-called liquid-ordered phase of PC bilayers containing high levels of cholesterol [19,58,59], may be an oversimplification, since we have shown that this is clearly not the case for cholesterol/PE mixtures. Moreover, differential interactions of cholesterol with various phospho-, sphingo- and glycolipids could have a considerable impact on the lateral organization of the lipid phase of biological membranes and may be partly responsible for the formation of lipid domains in these systems [14,16,49,59–61].

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