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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 phosphatidylglycerol bilayer membranes

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ABSTRACT

We have examined the effects of cholesterol (Chol) on the thermotropic phase behavior and organization of aqueous dispersions of a homologous series of linear disaturated phosphatidylglycerols (PGs) by high-sensitivity differential scanning calorimetry and Fourier transform infrared and ^{31}P NMR spectroscopy. We find that the incorporation of increasing quantities of Chol alters the temperature and progressively reduces the enthalpy and cooperativity of the gel-to-liquid-crystalline phase transition of the host PG bilayer. With dimyristoyl-PG:Chol mixtures, cooperative chain-melting phase transitions are completely or almost completely abolished at Chol concentrations near 50 mol%, whereas with the dipalmitoyl- and distearoyl-PG:Chol mixtures, cooperative hydrocarbon chain-melting phase transitions are still discernable at Chol concentrations near 50 mol%. We are also unable to detect the presence of significant populations of separate domains of the anhydrous or monohydrate forms of Chol in our binary mixtures, in contrast to previous reports. We ascribe the previously reported large scale formation of Chol crystallites to the fractional crystallization of the Chol and phospholipid phases during the removal of organic solvent from the binary mixture before the hydration of the sample. We further show that the direction and magnitude of the change in the phase transition temperature induced by Chol addition is dependent on the hydrocarbon chain length of the PG studied. This finding agrees with our previous results with phosphatidylcholine bilayers, where we found that Chol increases or decreases the phase transition temperature in a hydrophobic mismatch-dependent manner (*Biochemistry* 1993, 32:516–522), but is in contrast to our previous results for phosphatidylethanolamine (*Biochim. Biophys. Acta* 1999, 1416:119–234) and phosphatidylserine (*Biophys. J.* 2000, 79:2056–2065) bilayers, where no such hydrophobic mismatch-dependent effects were observed. We also show that the addition of Chol facilitates the formation of the lamellar crystalline phase in PG bilayers, as it does in phosphatidylethanolamine and phosphatidylserine bilayers, whereas the formation of such phases in phosphatidylcholine bilayers is inhibited by the presence of Chol. Moreover, the formation of the lamellar crystalline phase in PG bilayers at lower temperatures excludes Chol, resulting in an apparent Chol immiscibility in gel-state PG bilayers. We suggest that the magnitude of the effect of Chol on the thermotropic phase behavior of the host phospholipid bilayer, and its miscibility in phospholipids dispersions generally, depend on the strength of the attractive interactions between the polar headgroups and the hydrocarbon chains of the phospholipid molecule, and not on the charge of the polar headgroups *per se*.

1. Introduction

Cholesterol (Chol), or a closely related sterol, is an essential constituent of the plasma membrane of virtually all eukaryotic cells [1,2]. Chol has a profound effect on the physical properties of the host phospholipid membrane and Chol–phospholipid interactions have been the focus of a large number of studies using a wide range of different physical techniques [for reviews, see 2–4]. While the number of studies of Chol–phospholipid interactions is large, the majority of prior studies of Chol–phospholipid interactions involve

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; ^{31}P, phosphorus-31; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; PPM, parts per million; L_c, lamellar crystalline phase; L_{β}' and L_{β}, lamellar gel phase with tilted or untilted hydrocarbon chains; P_{β}', rippled gel phase, L_{α}, lamellar liquid-crystalline phase

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only two phospholipids, dimyristoylphosphatidylcholine (DMPC) and DPPC. Based primarily on the extensive studies of DMPC: and DPPC:Chol mixtures, the major effects of Chol on phospholipid bilayers can be summarized as follows: (i) Chol broadens and eventually eliminates the cooperative gel to liquid-crystalline phase transition of phospholipid bilayers; (ii) Chol decreases (increases) the area per molecule of the liquid-crystalline (gel) state monolayers; (iii) Chol increases (decreases) the orientational order of the hydrocarbon chains of liquid-crystalline (gel) bilayers; and (iv) Chol decreases (increases) the passive permeability of phospholipid bilayers above (below) their gel to liquid-crystalline phase transition temperatures. Thus, Chol-rich phospholipid domains are characterized by phospholipid intra- and intermolecular motional rates which are modestly reduced compared to those of a Chol-free fluid phospholipid bilayer, but with hydrocarbon chain orientational order and phospholipid area compressibility values which more closely resemble those of the gel phase of a pure phospholipid. The unique properties of the Chol-rich phospholipid phase, termed the liquid-ordered phase, are postulated to represent the phase state of eukaryotic cell plasma membranes [5,6]. However, whether or not a single thermodynamically discrete liquid-ordered state actually exists in Chol-containing phospholipids bilayers is controversial [see 4,7,8]. Alternatively, Chol- and sphingolipid-enriched liquid-ordered domains have also been postulated to form the molecular basis for the putative existence of lipid rafts in eukaryotic cell membranes [9,10], although the existence of such domains in biological membranes has recently been questioned [11–13].

In addition to the zwitterionic phospholipid PC, virtually all eukaryotic plasma membranes are composed of a substantial fraction of the zwitterionic phospholipid PE and several anionic phospholipids, particularly PS [14]. However, prior reports examining the effect of Chol on the phase behavior and organization of PEs or negatively charged phospholipids are limited in scope and number [15–22]. Moreover, the results of prior studies are often incompatible, and thus we do not have a consistent picture of the effect of phospholipid molecular structure on Chol–phospholipid interactions. For example, initial low-sensitivity DSC studies on the effect of Chol on the chain melting transitions of monotectic binary phospholipid mixtures indicated that Chol exhibits a decreasing affinity for various phospholipids in the order PG ~ PS > PC >> PE [15,16,23]. However, Chol does not appear to exhibit a preferential affinity for PCs or PEs in non-monotectic PC/PE mixtures, and the effect of Chol on the thermotropic phase behavior of PE bilayers was reported to be qualitatively similar to that documented for PC:Chol mixtures [24–26]. Moreover, studies of Chol-containing saturated PS and PG bilayers concluded that Chol has relatively limited effects on the chain-melting transition temperature and enthalpy of these anionic phospholipid bilayers, even at 50 mol% Chol [17,18,20,27]. These same studies also reported that saturated PS:Chol and PG:Chol mixtures both exhibit phase separation above 25 to 35 and 30 to 40 mol% Chol, in both the gel and liquid-crystalline states, respectively, resulting in the presence of solid-phase Chol crystallites and Chol-poor phospholipid domains in these mixtures, thus accounting for their purportedly limited effects on the gel/liquid-crystalline phase transition at higher Chol concentrations. Moreover, Chol crystallites were not detected in a 1-palmitoyl, 2-oleoyl-PG:cholsterol system until Chol concentrations approached values near or above 45 mol%, suggesting that Chol is more miscible with unsaturated phospholipids, when the opposite is usually the case [2–4]. Paradoxically, however, mixtures of Chol with various unsaturated PEs were also reported to exhibit Chol phase separation, but Chol levels of 40 mol% or more were nevertheless enough to completely abolish the phase transition of the host unsaturated PE bilayer [28,29].

We suspect that the varied results and conclusions of prior studies regarding the influence of phospholipid headgroup structure on phospholipid–Chol interactions may be due primarily to the varied composition of the phospholipid hydrocarbon chains that were utilized in these studies. Recently, we investigated the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated PC, PE and PS bilayers with linear hydrocarbon chains varying from 14 to 20 carbon atoms [21,22,30]. We found that the effect of Chol on the thermotropic phase behavior of PC, PE and PS bilayers varies markedly with changes in both phospholipid headgroup structure and hydrocarbon chain length. Furthermore, we also found that the thermotropic phase behavior of PS:Chol and PE:Chol mixtures depended on the thermal history of the sample. Generally, the differences in the effect of Chol on the thermotropic phase behavior and organization of the host bilayer were found to be due primarily to changes in the apparent miscibility of Chol in the gel-state phospholipid bilayer due to the Chol-induced formation of lamellar crystalline (L_c) phases and the resulting changes in the effective stoichiometry of Chol–phospholipid interactions. If L_c phase formation was avoided, then Chol was found to be fully miscible in both the L_β and L_α phases of PC, PE and PS bilayers up to at least 50 mol%, with the exception of phospholipid molecular species with very long or very short hydrocarbon chains. The results of these and other studies indicate that the relative contributions of phospholipid headgroup electrostatic and hydrogen bonding interactions and hydrocarbon chain van der Waals and hydrophobic forces to Chol–phospholipid interactions are complex and require further study [2,4,31]. As part of our continued efforts to systematically characterize the effects of variations in phospholipid headgroup structure and hydrocarbon chain length and structure and on Chol–phospholipid interactions, we present here the results of our investigation of the effect of the incorporation of various quantities of Chol on the thermotropic behavior and organization of a series of linear saturated PGs using high-sensitivity DSC and FTIR and ^{31}P-NMR spectroscopy.

2. Materials and methods

The PGs and Chol used in these experiments were obtained from Avanti Polar Lipids (Alabaster, AB) and were used as supplied. The PG:Chol mixtures used in these studies were prepared from stock solutions in chloroform/methanol (2:1, vol/vol). Appropriate volumes of the stock solutions of PG and Chol were thoroughly mixed, heated to temperatures 10–20 °C above the expected hydrocarbon chain-melting phase transition of the mixture, and the solvent removed with a stream of nitrogen. The mixture obtained was then dried *in vacuo* overnight. Subsequently, the dried PG:Chol mixture was heated to temperatures approximately 10–20 °C above the expected phase transition of the PG:Chol mixture and hydrated with a pre-warmed buffer solution by vigorous vortex mixing at elevated temperatures. We note here that because of the seemingly low miscibility of Chol in gel-state PG bilayers, one needs to be very careful to avoid the phase separation and possible fractional crystallization of Chol when preparing PG:Chol mixtures in general, and Chol-rich PG mixtures in particular. To this end, methanol-rich solvent mixtures should be avoided because Chol readily crystallizes from methanol-rich solvents at moderate temperatures, and the organic solvents should be removed at temperatures above the expected hydrocarbon chain-melting phase transition temperatures of the mixtures. Moreover, because of the differential affinities of PG and Chol for water, and the apparently low-miscibility of Chol with gel-state PG bilayers, hydration/dispersal of the dry PG:Chol films should be performed with pre-warmed buffers at elevated temperatures to avoid Chol-PG phase separation during the hydration process. In our hands the procedure described above produces well-mixed PG:Chol films from which fully reproducible DSC thermograms were obtained.

For the DSC experiments, the dried PG:Chol mixtures were dispersed by vigorous vortex mixing in a buffer containing 100 mM Tris, 100 mM NaCl and 10 mM EDTA, pH 7.4, at temperatures 10–20 °C

above the phase transition of the PG:Chol mixture. The multilamellar dispersions obtained were first heated and cooled between 0 and 60 °C at 10 °C/h, and the data acquisition runs were done immediately afterwards (i.e., without explicit low-temperature annealing of the samples). Data acquisition runs involved at least three heating/cooling cycles obtained with a Calorimetry Sciences Corporation high-sensitivity multicell differential scanning calorimeter (Lindon, UT) operating at heating and cooling rates near 10 °C/h. The amount of PG used was progressively increased from 0.5 mg for pure PG bilayers to 20 mg for PG samples containing 45 or 50 mol% Chol. We have shown previously that this protocol is required to accurately monitor the broad, low-enthalpy phase transitions observed at higher Chol concentrations [30]. The calorimeter was calibrated using solid standards from Calorimetry Sciences Corporation, as well as aqueous lipid samples synthetically prepared and purified within this laboratory, using methods previously shown to provide highly pure samples [32]. Sample runs were repeated at least three times to ensure reproducibility. At the end of each DSC experiment, the phospholipid–Chol mixture was examined by TLC and there was no discernable evidence of sample degradation under our conditions. The data obtained were analyzed and plotted with the Origin software package (OriginLab Corporation, Northampton, MA). In cases where the DSC thermograms appeared to be a summation of overlapping components, the midpoint temperatures, areas and widths of the components were estimated with the aid of Origin's non-linear least squares curve- and peak-fitting procedures and a custom-coded function, based on the assumption that the observed thermogram was a linear combination of components, each of which could be approximated by a reversible, two-state transition at thermodynamic equilibrium.

For the FTIR spectroscopic analyses, the PG:Chol dispersions were prepared and dispersed in D_2O-based buffer containing 100 mM PO_4, 100 mM NaCl and 10 mM EDTA, pH 7.4, using methods similar to that used for the DSC samples. The dispersion was squeezed between the CaF_2 windows of a heatable, demountable liquid cell (NSG Precision Cells, Farmingdale, NY) equipped with a 25 μM teflon spacer. Once mounted in the sample holder of the spectrometer, the sample could be heated between −20 °C and 90 °C by an external, computer-controlled water bath. FTIR spectra were recorded in both heating and cooling runs with a Digilab (Cambridge, MA) FTS-40 Fourier transform infrared spectrometer. As with the DSC experiments, data acquisition was initiated immediately after the samples were cooled from high temperatures. The experiment involved a sequential series of 2 °C temperature ramps with a 10-min inter-ramp delay for thermal equilibration, and was equivalent to a scanning rate of ~8 °C/h. The data obtained were analyzed and plotted using the Origin software package and other computer programs developed by the National Research Council of Canada. The data obtained were analyzed using computer programs obtained from the instrument manufacturer and from the National Research Council of Canada, and plotted with the Origin software package. In cases where absorption bands appeared to be a summation of components, a combination of Fourier deconvolution and curve-fitting procedures was used to obtain estimates of the position of the component bands and to reconstruct the contours of the original band envelope.

PG and PG:Chol mixtures for ^{31}P-NMR spectroscopy were prepared as outlined for the DSC experiments, except that the amount of PG in the sample was typically 13–30 mg. Proton-decoupled ^{31}P-NMR spectra were acquired between 0 °C and 70 °C with a Varian (Palo Alto, CA) Unity-300 spectrometer operating at 121.42 MHz for ^{31}P. The data acquisition and data processing protocols were the same as the single-pulse, direct-excitation techniques described by Lewis et al. [33]. After completion of the NMR experiments, the samples were checked for degradation by both TLC and DSC. No degradation or alteration in sample thermotropic behavior was observed.

3. Results

3.1. Thermotropic phase behavior of pure phosphatidylglycerol bilayers

Zhang et al. [34] have examined the thermotropic phase behavior and organization of a homologous series of linear, saturated PGs varied with respect to the lengths of their hydrocarbon chains, using DSC, FTIR and ^{31}P-NMR. A brief summary of the thermotropic phase behavior of these lipids is as follows. In the absence of Chol and under physiologically relevant conditions (7.4 pH and 0.1–0.2 M salt), fully hydrated PGs which have not been annealed at low temperatures exhibit only a low temperature, low enthalpy L_{β}'/P_{β}' pretransition and a gel to liquid-crystalline (P_{β}'/L_{α}) chain-melting phase transition. However, when incubated at low temperatures, the L_{β}' phase transforms into one or more lamellar crystalline (L_c) phases in which the PGs are highly ordered and partially dehydrated. At shorter chain lengths (≤16:0), the crystal-like phase converts upon heating into the liquid-crystalline state directly (L_c/L_{α} phase transition), but with longer chain compounds (>16:0), the Lc phases transform first to the gel state (L_c/L_{β} phase transition) and then from the gel to the liquid-crystalline state (L_{β}/L_{α} phase transition) with increased temperature. Also, as the hydrocarbon chain length of the PG bilayer increases, progressively longer low-temperature annealing times are required to form the L_c phases. An understanding of the inherent complexity of the thermotropic phase behavior of PG itself is essential for the accurate interpretation of the complex phase behavior exhibited by Chol:PG mixtures (see below). We believe that this may have not been fully appreciated in many of the studies of PG:Chol interactions published so far.

3.2. Effect of cholesterol on the thermotropic phase behavior and organization of PG bilayers: differential scanning calorimetric studies

The overall effect of Chol on the thermotropic phase behavior of DMPG, DPPG and DSPG bilayers is summarized in Figs. 1–5. As noted in the Materials and methods, the DSC data shown were acquired

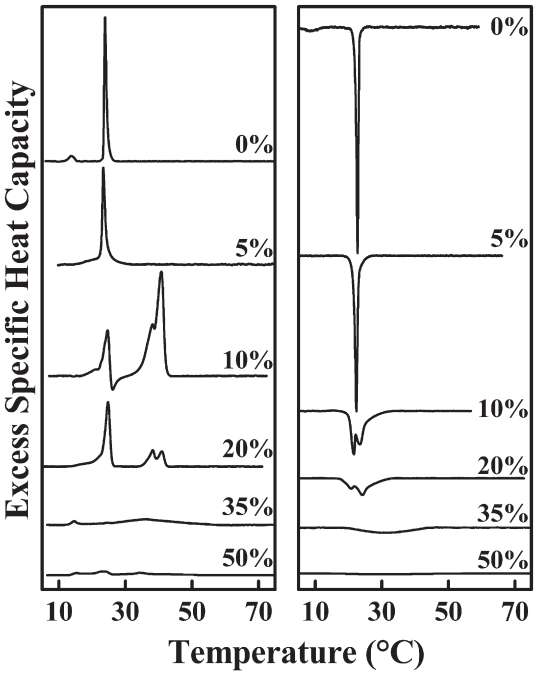

Fig. 1. Representative heating (left panel) and cooling (right panel) thermograms of DMPG bilayers containing progressively increasing levels of cholesterol as indicated in the figure. The thermograms presented were obtained at a scan rate of 10 °C/h and have been normalized for phospholipid sample mass.

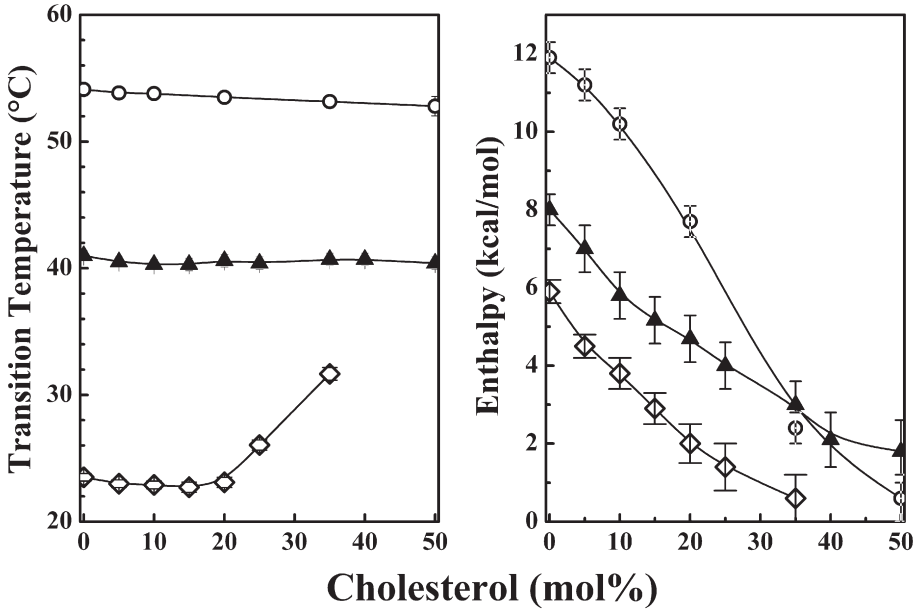

Fig. 2. Summary of the effect of cholesterol on the transition temperatures (left panel) and enthalpy changes (right panel) of the gel/liquid-crystalline (L_β/L_α) phase transitions exhibited by cholesterol-containing PG bilayers. Because of interference by the L_c/L_α phase transitions exhibited by many of the cholesterol-containing mixtures, the results shown were all obtained from analyses of cooling thermograms. Also, because of the multi-component nature of the thermograms exhibited by most mixtures, the transition temperatures reported are those at which the enthalpy change is 50% of the total. Data are presented for mixtures of cholesterol with DMPG (—◇—), DPPG (—▲—) and DSPG (—○—).

without explicit low temperature incubation of the samples. These PG–Chol binary mixtures exhibit a complex pattern of thermotropic phase behavior which varies with the amount of Chol incorporated and with the length of the PG hydrocarbon chain, and may also differ markedly upon heating and cooling. These overall effects contrast sharply from the behavior observed with PC:Chol mixtures, for which the heating and cooling curves are similar [30], but shows some general similarities to the behavior observed with bilayers composed of PE:Chol and PS:Chol mixtures [21,22]. A more detailed examination of the effect of Chol on the thermotropic phase behavior of each PG is presented below.

Illustrated in Fig. 1 are the heating (left panel) and cooling (right panel) DSC thermograms exhibited by Chol-free and Chol-containing DMPG bilayers. As expected, the Chol-free DMPG bilayers exhibit the lower temperature, low enthalpy L_β'/P_β' pretransition at approximately 16 °C and the main chain-melting P_β'/L_α transition at temperatures near 23 °C [41]. Moreover, these processes are essentially freely reversible upon cooling, with the pretransition exhibiting a modest cooling hysteresis [34]. This general pattern of thermotropic phase behavior is also observed with DMPG:Chol mixtures which contain small (≤5 mol%) amounts of sterol. However, the presence of Chol increases the width and decreases the enthalpy of both transitions. The magnitude of these effects increases as the Chol content of the mixtures increases, but is more pronounced with the pretransition, which becomes calorimetrically undetectable when the Chol content of the mixture exceeds 5 mol%. The Chol-induced peak broadening and decrease in the transition enthalpy are much less marked for the main or gel/liquid-crystalline phase transition and, at low sterol concentrations, relatively sharp heating and cooling thermograms can still be resolved (see Fig. 1). However, at higher Chol concentrations, the DSC peak arising from the gel/liquid-crystalline phase transition consists of overlapping sharp and broad components (see Fig. 5), with the relative intensity of the sharp component progressively diminishing and that of the broad component increasing as the Chol content increases. This general pattern of thermotropic phase behavior has also been observed in other phospholipid–Chol systems and has been ascribed to the melting of sterol-poor and sterol-rich lipid domains [21,22,30].

The DMPG:Chol mixtures containing ~5–20 mol% sterol exhibit a more complex pattern of thermotropic phase behavior and there are marked differences between the heating and cooling thermograms observed (see Fig. 1). Specifically, the heating endotherms observed over this range of Chol concentration contain fairly enthalpic thermotropic events centered near 23 °C and 40 °C, whereas the cooling exotherms seem to be composed of multi-component exotherms centered near 23 °C (see Fig. 1). We also note that the

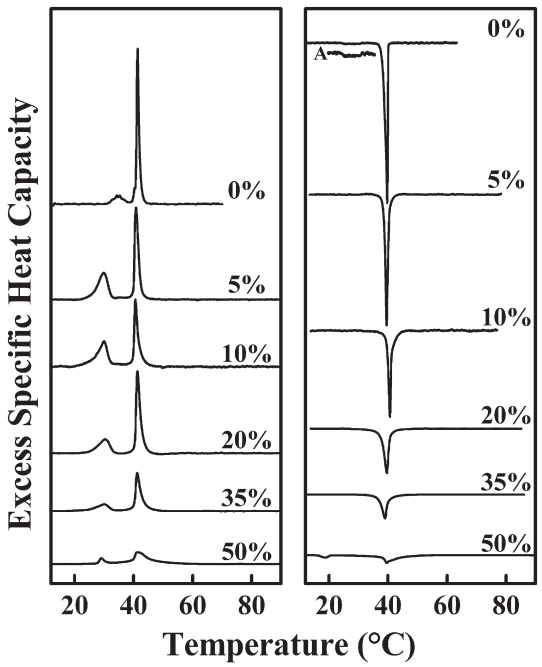

Fig. 3. Representative heating (left panel) and cooling (right panel) thermograms of DPPG bilayers containing progressively increasing levels of cholesterol as indicated in the figure. The DSC thermograms presented were obtained at a scan rate of 10 °C/h and have been normalized for phospholipid sample mass. The cooling exotherm labeled A shows the pretransition exotherms on a 3-fold expanded scale.

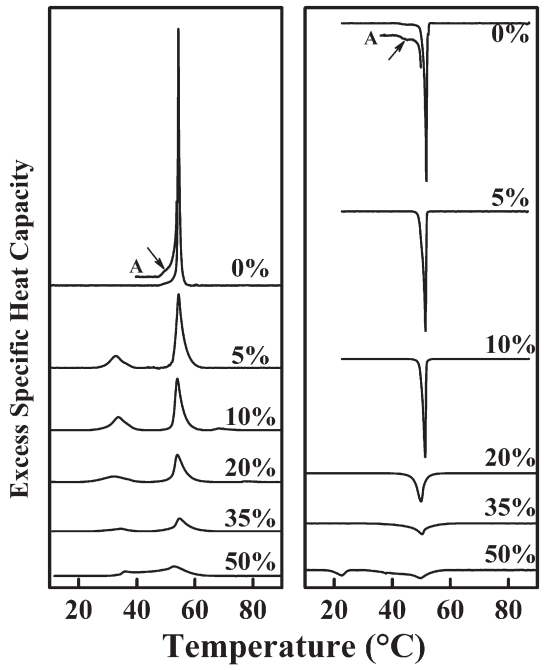

Fig. 4. Representative heating (left panel) and cooling (right panel) thermograms of DSPG bilayers containing progressively increasing levels of cholesterol as indicated in the figure. The thermograms presented were obtained at a scan rate of 10 °C/h and have been normalized for phospholipid sample mass. The thermograms labeled A show the pretransitions (indicated by the arrows) on a 3-fold expanded scale.

temperature ranges over which the heating endotherms occur (~23 °C and ~40 °C) are comparable to those at which unannealed aqueous DMPG bilayers exhibit their $P_{\beta'}/L_{\alpha}$ phase transition and low-temperature annealed DMPG bilayers exhibit their L_c/L_{α} phase transition, respectively [34]. The latter observation, and the absence of the higher-temperature thermotropic events from the cooling scans, suggest that DMPG L_c phases were being formed in the DMPG:Chol mixtures over the time scale of the DSC cooling scans, in marked contrast to the 3–5 days of low-temperature incubation that is normally required to induce L_c phase formation in pure DMPG bilayers [34]. Thus, the presence of modest amounts Chol is actually markedly facilitating L_c phase formation when such samples are briefly cooled to low temperatures, as confirmed by our FTIR and ^{31}P-NMR spectroscopic experiments (see below). However, the fact that endothermic peaks corresponding to the $L_{\beta'}/L_{\alpha}$ and L_c/L_{α} phase transitions of DMPG are observed in the heating thermograms of DMPG:Chol mixtures containing 5–20 mol% Chol suggests that L_c phase formation does not proceed to completion during our DSC cooling scans and, as a result, there is a coexistence of $L_{\beta'}$ and L_c phases under such conditions. These suggestions have interesting implications as regards the apparent miscibility of Chol with DMPG (and the other linear saturated PGs) at low temperatures which will be explored in the Discussion. However, the cooling exotherms exhibited by the DMPG bilayers containing ~5–20 mol% Chol are all broader than those observed at the lower range of Chol concentrations (see Fig. 1) and seem to be a summation of sharp and broad components. Moreover, the temperature range of the sharp components is consistently lower than that of the broad component, the relative intensity of the sharp component progressively diminishes as the Chol content increases (see Fig. 1), and the enthalpy changes of these thermotropic events progressively diminish as the Chol content of the mixtures increases (Fig. 2). This aspect of the behavior of these DMPG:Chol mixtures is consistent with that observed at the lower range of Chol concentrations examined.

Fig. 1 also shows that the dominant feature of the DSC thermograms exhibited by DMPG:Chol mixtures with a sterol content that significantly exceeds ~25 mol% is the presence of a broad, relatively energetic endotherm centered near 32 °C. This endotherm seems to be composed of a single component, suggesting that the sharp component of the main phase transition observed at lower sterol concentrations has been abolished in these Chol-rich mixtures. In addition, two other weakly energetic endotherms occur in the temperature range 15–40 °C on heating. These weaker thermotropic events are not observed on cooling, suggesting that they may be attributed to the low temperature-induced formation of crystalline forms of the host phospholipid or possibly Chol itself. However, since these two weakly energetic heating endotherms comprise a very small fraction of the observed total enthalpy change, it seems unlikely that extensive formation of crystalline material was occurring under our conditions at these high Chol levels.

Illustrated in Fig. 2 is a summary of the effect of Chol on the temperature and enthalpy of the gel/liquid-crystalline phase transitions of the three PGs studied. With many of the DMPG:Chol mixtures studied, we could not extract meaningful thermodynamic parameters from the heating thermograms because of the fact that a large fraction of the DMPG present exists in the L_c phase in the presence of Chol and undergoes a separate conversion to the L_a phase at a temperature well

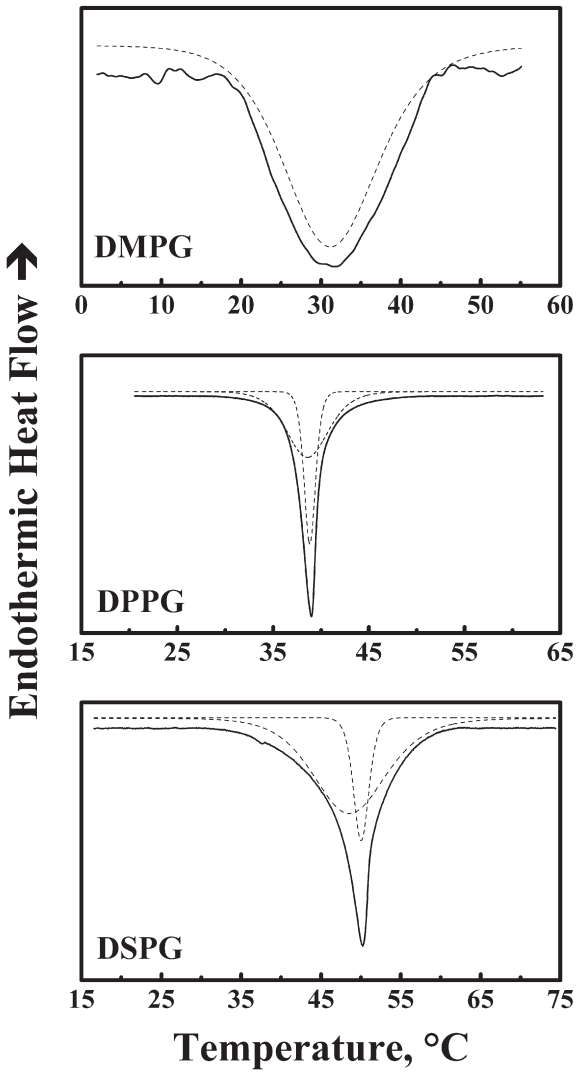

Fig. 5. Comparison of the multi-component structure of DSC thermograms exhibited by cholesterol-rich PG bilayers. The data presented illustrate a subcomponent analysis of cooling exotherms exhibited by cholesterol-rich (35 mol%) PG bilayers, with the solid line representing the observed cooling exotherms and the dashed line representing our estimates of the subcomponents. Curves illustrating the subcomponents have been shifted from the baseline for clarity.

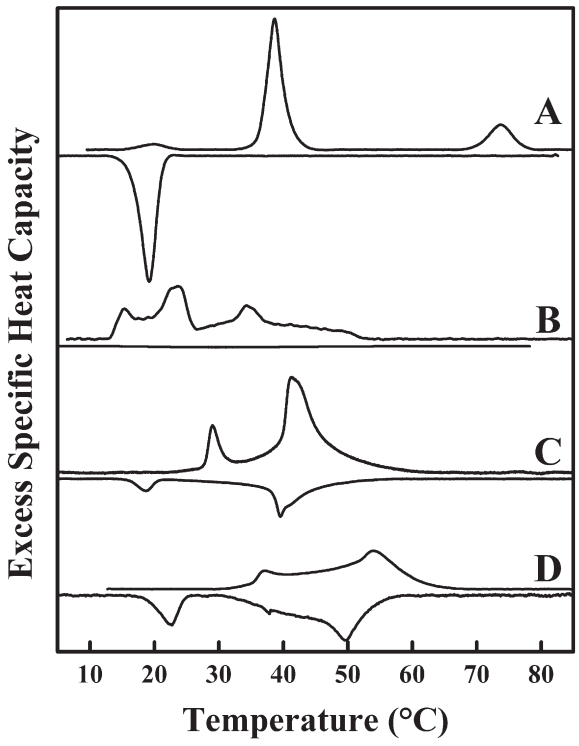

Fig. 6. Comparative plots of the phase transitions of anhydrous cholesterol suspended in water (A) and 50 mol% cholesterol in DMPG (B), DPPG (C) and DSPG (D) bilayers, respectively. The excess specific heat capacity is not to scale and is optimized for comparative purposes.

above the gel/liquid-crystalline phase transition temperature of pure DMPG (see Fig. 1 and Ref. 34). Therefore, an accurate enthalpy value for the gel/liquid-crystalline phase transition cannot be determined from the heating thermograms because the relative amounts of DMPG existing in the gel and L_c phases are not known. However, accurate enthalpy values could be determined from the appropriate cooling scans, as significant quantities of the L_c phase do not form at temperatures above the liquid-crystalline/gel phase transition temperature upon cooling and thus all of the DMPG is participating in this L_α/L_β phase transition. The results presented in Fig. 2 show that for mixtures of low to modest Chol content, the area-averaged midpoint of the gel/liquid-crystalline phase transition was comparable to that of pure DMPG (~23 °C), and that it increased progressively to values well above 30 °C with the Chol-rich samples. This upward shift in temperature occurs because the higher-temperature broad component becomes progressively dominant in the higher range of Chol concentrations examined. Fig. 2 also shows that there is a progressive decline in the total enthalpy of these thermotropic events and that the enthalpy approaches zero at Chol contents near 50 mol%. These aspects of the behavior of these DMPG:Chol mixtures are remarkably similar to those exhibited by Chol-containing DMPC bilayers [30].

Figs. 3 and 4 are high-sensitivity DSC heating and cooling thermograms which illustrate the thermotropic phase behavior exhibited by DPPG:Chol and DSPG:Chol mixtures with Chol contents ranging from 0–50 mol%, respectively. The DSC thermograms shown were also obtained with samples that have not been explicitly incubated at low temperatures. Bilayers composed of DPPG:Chol and DSPG Chol mixtures of low (<5 mol%) Chol content all exhibit a low enthalpy phase transition near 35 °C and 51 °C, respectively, and a sharp, reversible higher enthalpy phase transition near 41 °C and 54 °C, respectively. These events are analogous to the well characterized the L_β'/P_β' and P_β'/L_α phase transitions normally exhibited by DPPG and DSPG bilayers, respectively [34]. Over this range of Chol content, the effects of Chol are generally manifest by considerable broadening and eventual elimination of the pretransition, but by only a modest increase in the width and a modest decrease in the overall enthalpy of the gel/liquid-crystalline phase transition. These effects are quite similar to those exhibited by the corresponding Chol-containing DMPG mixtures (see Fig. 1).

At modest (~5–35 mol%) sterol concentrations, Chol-containing DPPG and DSPG bilayers exhibit DSC heating thermograms which all contain a broad, fairly energetic endotherm centered at temperatures well below the onset of the gel/liquid-crystalline phase transitions of these lipid:sterol mixtures (Figs. 3 and 4, left panel). The temperature ranges spanned by the broad, lower temperature thermotropic events (~30 °C for DPPG:Chol and ~36 °C for DSPG:Chol mixtures, respectively) are comparable to that spanned by the L_c/L_β phase transitions observed upon initial heating of samples of the sterol-free PG bilayers after prolonged incubation at low temperatures [34]. However, the reversal of these thermotropic events was not detected in the corresponding cooling exotherms (see Figs. 3 and 4, right panel) and, if the cooling scans were terminated at temperatures near 15–20 °C, these transitions did not appear in the following heating scan (data not shown). Indeed, these broad endothermic events were observed only if samples were slowly (≤10 °C/h) cooled to temperatures near 0 °C, and their intensities were markedly reduced when DSC thermograms were recorded at significantly faster scan rates (data not shown). These observations suggest that the lower-temperature endotherms are L_c/L_β phase transitions, as confirmed by our FTIR and ^{31}P-NMR spectroscopic data (see below). Moreover, since the L_c phases were formed without explicit low-temperature incubation of the samples, we can also conclude that, as observed with the corresponding DMPG:Chol mixtures, the presence of Chol in DPPG and DSPG bilayers markedly facilitates the formation of the L_c phases of these lipids. With these longer-chain PGs, however, Chol-induced L_c phase formation was even observed at Chol concentrations near 35–40 mol% (see Figs. 3 and 4), a range significantly higher than that observed with the corresponding DMPG:Chol mixtures, suggesting that there may also be a hydrocarbon chain length-dependent component to this phenomenon. The possible basis of this and other aspects of the Chol-induced facilitation of L_c phase formation in these PG bilayers will be explored in the Discussion.

As expected, the presence of Chol also affects the properties of the gel/liquid-crystalline phase transitions exhibited by these longer-chain PG:Chol mixtures and, as observed with the DMPG system, these effects are manifest primarily by increases in overall transition width and by decreases in overall transition enthalpy (Figs. 2–4). With the longer chain PG:Chol mixtures, sterol concentration-induced broadening of the phase transition seems to be relatively less pronounced than observed with the DMPG:Chol mixtures, and DSC thermograms arising from the gel/liquid-crystalline phase transitions of such mixtures are still resolvable at Chol concentrations near 50 mol% (Figs. 3 and 4). This result differs markedly from the DMPG:Chol system, for which thermograms arising from the gel-liquid-crystalline phase transition are not detectable when the Chol concentration approaches 50 mol% (see Fig. 1), as reflected in the effects of Chol concentration on the overall enthalpy changes measured (Fig. 2, right panel). We also find that at all sterol concentrations examined, the heating and cooling thermograms arising from the gel/liquid-crystalline phase transitions of Chol-containing DPPG and DSPG bilayers appear to be summation of overlapping sharp and broad components (for examples, see Fig. 5). Also, as observed with the DMPG:Chol mixtures, the relative intensity of the sharp component decreases as the Chol content of the mixture increases. However, unlike the DMPG:Chol mixtures, the sharp components of the DSC thermograms exhibited by these longer chain PG:Chol mixtures persist at Chol contents above 25 mol% and can still be resolved at Chol contents near 50 mol% (Figs. 3–5). Moreover, with these longer chain PG:Chol mixtures, the midpoint temperatures of the broad component either remains similar to that of

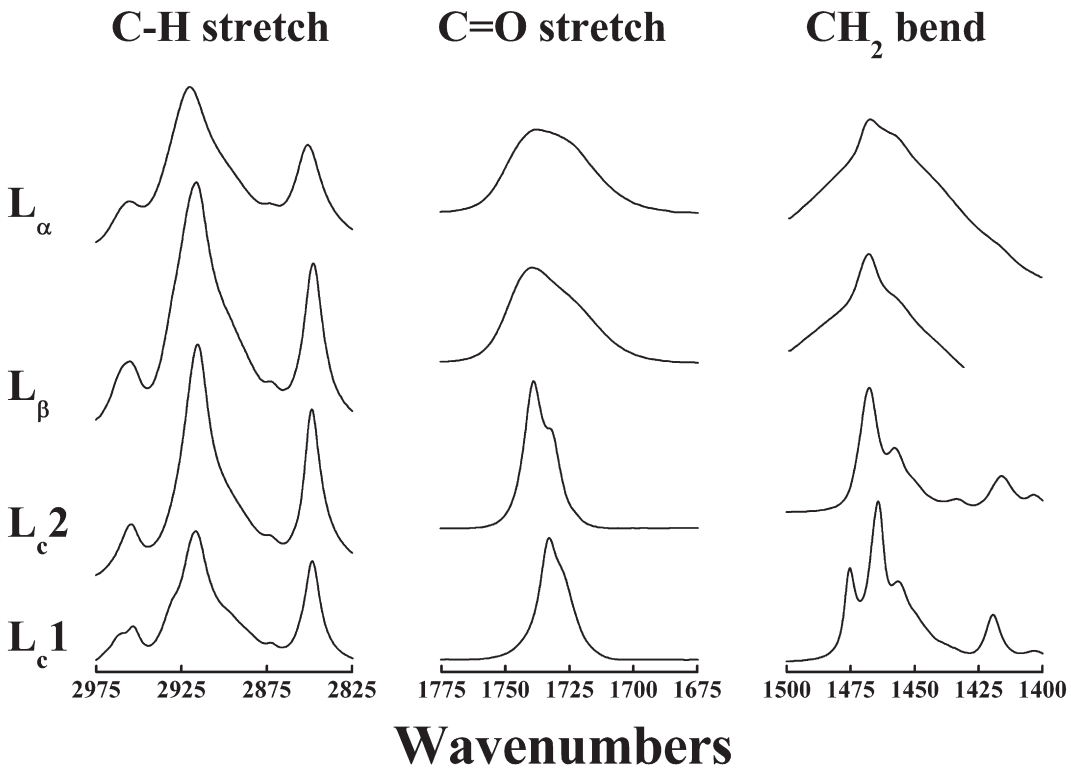

Fig. 7. Representative FTIR spectra illustrating the contours of the C–H stretching, C=O stretching and CH_2 scissoring absorption bands for the L_α, L_β, L_c1 and L_c2 phases formed by fully hydrated n-saturated diacyl PG bilayers. Spectra were obtained Zhang et al. [34].

the sharp component (DPPG) or decreases slightly relative to that the sharp component (DSPG) as the Chol content of the mixtures increases, whereas the midpoint temperature of the broad component in DMPG bilayers increases relative to that of the sharp component as the sterol content of those bilayers increase (see Fig. 1). Thus, the direction of the shift in the gel/liquid-crystalline phase transition temperature of the broad component depends on the hydrocarbon chain length of the host PG bilayer. However, the fact that a weak, two-component gel/liquid-crystalline phase transition persists in cholesterol-rich DPPG and DSPG mixtures suggests a reduced miscibility of Chol with these longer chain host PG bilayers.

Previous studies have suggested that the thermotropic phase behavior exhibited by Chol-rich PG preparations can be significantly affected by the formation of Chol crystallites [20]. Thus, in order to determine whether our experimental results were significantly affected by such phenomena, the thermotropic phase behavior of aqueous dispersions of pure Chol was examined and compared with that exhibited by some of the Chol-rich PG preparations studied here. We chose to study PG-Chol mixtures containing the highest amounts of Chol investigated (50 mol%) in order to maximize the degree of potential Chol crystallite formation and to repress L_c phase formation, thus minimizing possible DSC peak overlap between the thermotropic events arising from the Chol and the PG component present in each sample. Note also that the absolute amounts of Chol present as free Chol or as Chol incorporated as equimolar mixtures with the three PGs studied are comparable, thus permitting a determination of the relative quantities of Chol crystallites present in the four samples, if any, via a direct comparison of the observed Chol phase transition enthalpies. As illustrated in Fig. 6A, aqueous dispersions of Chol exhibit two distinctive thermotropic transitions on heating. These events are observed at temperatures near 38 °C and 76 °C, and have been assigned to a solid-phase packing rearrangement of anhydrous Chol and to a monohydrate/anhydrous Chol phase transition, respectively, while the phase transition observed at 18 °C on cooling has been assigned to a reversal of the anhydrous Chol phase transition seen at 38 °C on heating [35]. As illustrated in Fig. 6B, C and D, the DSC thermograms obtained from Chol-rich preparations of DMPG, DPPG and DSPG (~50 mol% Chol) not annealed at low temperatures exhibit one or more weak thermotropic events attributable to the transitions involving small amounts of the L_c and L_β phases which persist at these high Chol concentrations (i.e., L_c/L_α, L_c/L_β and L_β/L_α transitions). These weakly energetic residual thermotropic events are generally clearly discernable from the phase transitions of anhydrous Chol or Chol monohydrate, especially if both the heating and cooling thermograms of each sample are compared. Specifically, note that no endotherm located near 78 °C corresponding to the Chol monohydrate-anhydrous Chol transition is observed in any of these PG–Chol mixtures, indicating that no Chol monohydrate is present in any of these samples. Similarly, there is no endotherm observed at 38 °C on heating and no exotherm is observed at 18 °C on cooling in the DMPG–Chol sample. Although one could argue that the endotherm seen at 33 °C on heating could potentially be a down-shifted anhydrous Chol phase transition, the absence of any exotherm on cooling rules out this possibility. As well, although one could argue that the very small exotherm observed near 20 °C on cooling of the DPPG–Chol sample could represent an anhydrous Chol phase transition, the absence of an endotherm at 38 °C on heating does not support this interpretation. Finally, although the small peak at 38 °C observed on heating of the DSPG–Chol sample could be due to an anhydrous Chol phase transition, the absence of an exotherm near 18 °C on cooling makes this possibility unlikely. We also note that even if any of these thermal events do arise from the presence of Chol crystallites, a comparison of the underlying broad peak-corrected enthalpies of these thermal events with those of the pure Chol sample indicates that only very small amounts (<5%) of the Chol present in the PG–Chol mixtures would exist in the crystallite form, an amount far less than that reported for the DMPG–Chol preparations reported previously [20]. It is thus clear that even at the highest Chol concentrations studied (~50 mol%), significant crystallization of Chol from PG bilayers is not occurring. However, we find that PG:Chol mixtures containing

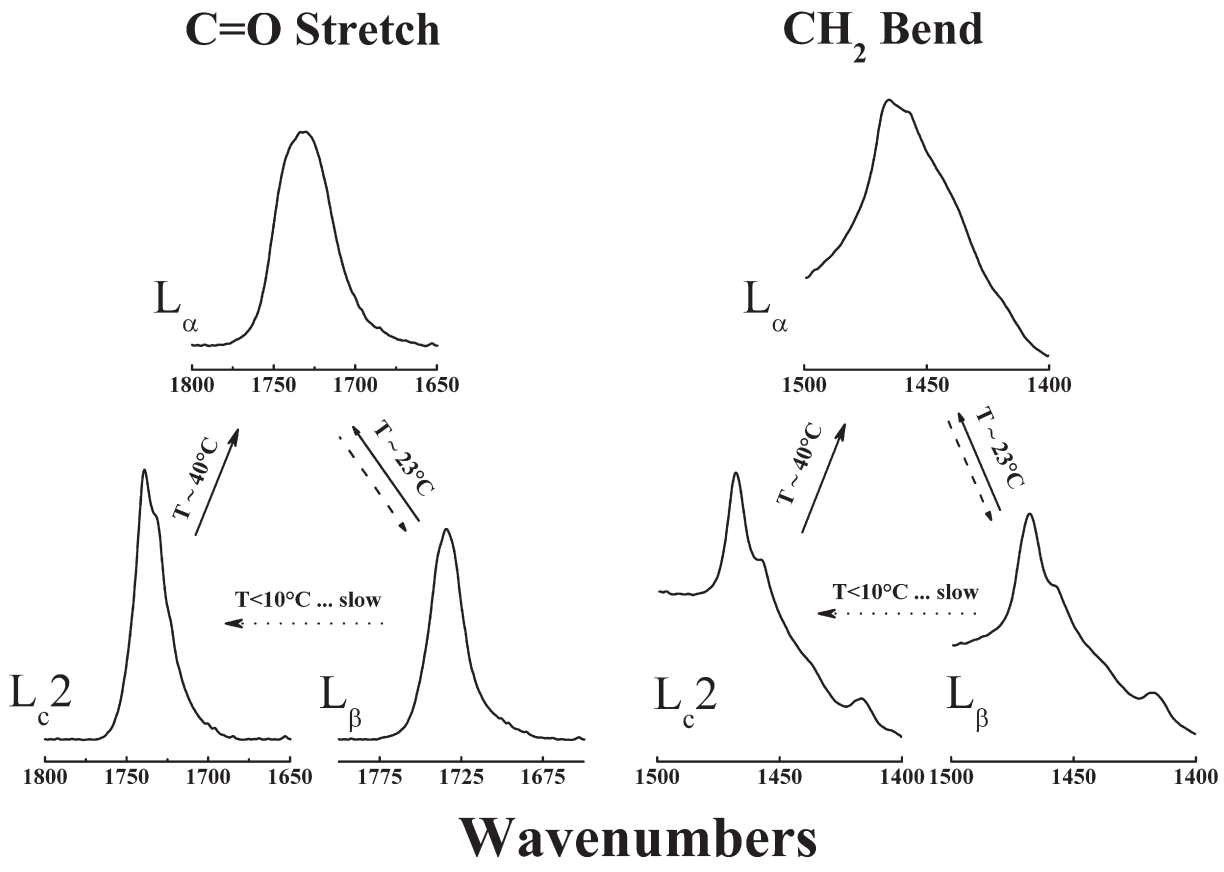

Fig. 8. Representative FTIR spectra illustrating the general contours of the C=O stretching and CH_2 scissoring absorption bands exhibited by DMPG bilayers containing 5–30 mol% cholesterol. The spectra shown were obtained with a DMPG:mixtures containing 25 mol% cholesterol. The types and approximate temperature ranges of the thermotropic phase transitions observed upon heating (solid arrows) and cooling (dashed arrows) of such mixtures are as indicated. The conversion of the L_β phase to the L_c phase (see doted arrow), which occurs at low temperature, is normally too slow to be resolved by DSC.

considerably higher Chol concentrations (~70 mol% or more), do exhibit thermotropic events near 38 °C and 76 °C on heating and near 18 °C on cooling, consistent with the formation of Chol crystallites from those mixtures (data not shown). We therefore conclude that the formation of Chol crystallites is not a significant contributor to the thermotropic phase behavior exhibited by the PG:Chol mixtures prepared under our conditions.

3.3. Effect of cholesterol on the thermotropic phase behavior and organization of DMPG, DPPG and DSPG bilayers: FTIR spectroscopic studies

FTIR spectroscopic experiments were performed to determine the effect of Chol on the structure and organization of PG:Chol bilayers as a function of temperature and to correlate this information with the observed calorimetric transitions. A detailed FTIR spectroscopic characterization of the thermotropic phase behavior and organization of bilayers composed of a homologous series of n-saturated 1,2-diacyl PGs has been presented elsewhere [34] and will not be discussed here. However, the distinguishing features of the FTIR spectra exhibited by the various polymorphic phases formed by aqueous dispersions of the PGs studied here are shown in Fig. 7 in order to facilitate the interpretation of the FTIR spectra of the various PG:Chol mixtures examined. Note that the infrared spectra exhibited by the various polymorphic forms of these PGs are quite distinct, enabling straightforward assignments of the PG phases present in these PG:Chol mixtures, and therefore of the nature of the various thermotropic phase transitions detected by DSC.

At temperatures below 23 °C, DMPG bilayers which contain up to 5 mol% Chol and have not been not incubated at low temperatures exhibit FTIR spectra which closely resemble those presented in Fig. 7 for the L_β phase of pure PG bilayers. Upon heating to temperatures above 23 °C, the IR absorption bands all broaden and adopt features comparable to those shown in Fig. 7 for the L_α phase of pure PG bilayers, thus providing strong evidence that the main thermotropic phase transition exhibited by these Chol-poor DMPG bilayers is a simple L_β–L_α type of hydrocarbon chain-melting phase transition. In contrast, when DMPG bilayers containing modest (5–30 mol%) amounts of Chol are cooled to low temperatures, they exhibit FTIR spectra containing features comparable to the L_c2 phase of pure PG bilayers samples (see Fig. 8 and compare with Fig. 7). However, the spectroscopic signatures observed are not identical to those of the L_c2 phase of the pure lipid, presumably because significant populations of PG molecules in the L_β phase persist under these conditions, suggesting the coexistence of L_c and L_β phases. Upon heating, these spectroscopic features persist to temperatures near 40 °C and then convert directly to those typical of the L_α phase (Figs. 7 and 8). Upon cooling, the spectroscopic features of the L_α phase persist to temperatures near 25 °C, at which point conversion to those typical of the L_β phase begins. This process continues with further decreases in sample temperature and features characteristic of L_c2 phase formation gradually begin to emerge as sample temperatures begin to approach 0 °C. We therefore conclude that the heating endotherms observed in our DSC experiments are the result of differential melting of L_β (~23 °C) and L_c (~40 °C) phases which coexist after these Chol-containing DMPG samples have been slowly cooled to lower temperatures. Comparable patterns of behavior have also been observed with Chol-containing DMPE and DMPS bilayers [21,22], but not with the corresponding PC bilayers [30]. Finally, with the Chol-rich (>30 mol%) DMPG bilayers, the FTIR spectra observed at low

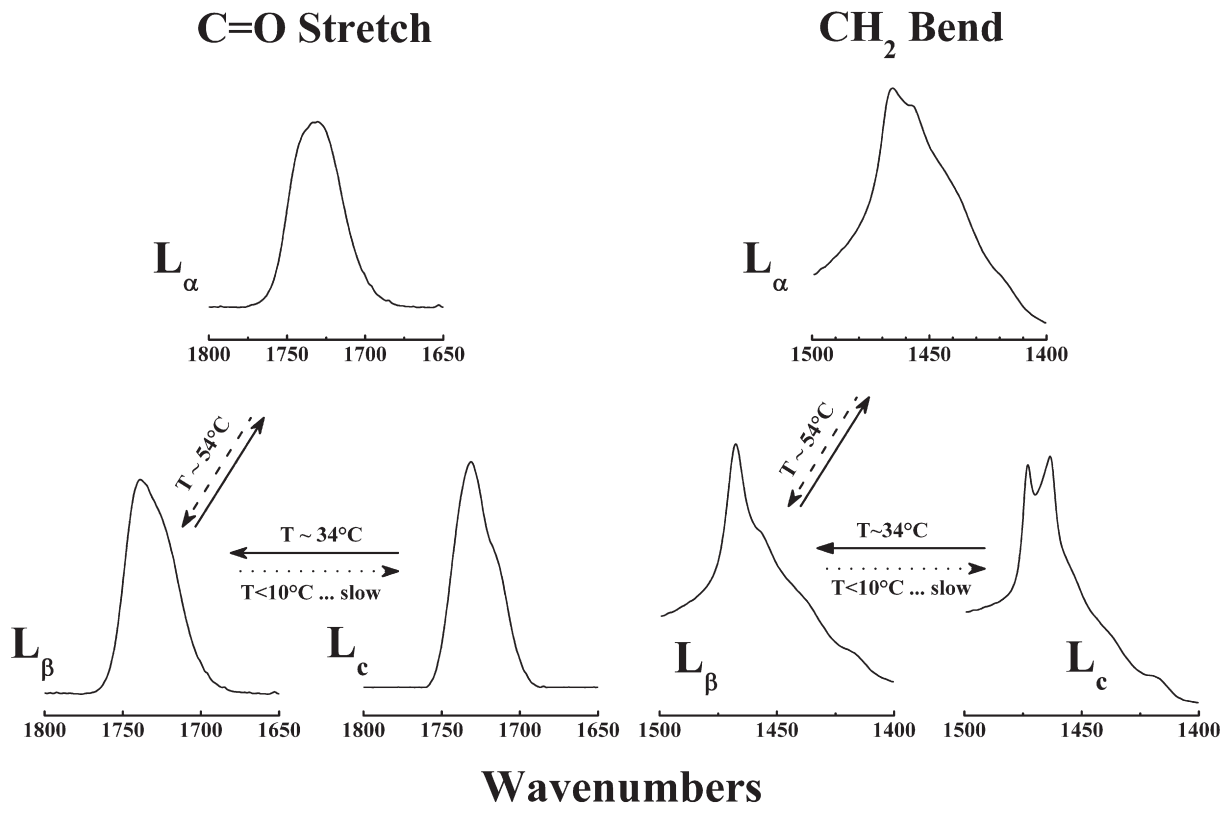

Fig. 9. Representative FTIR spectra illustrating the general contours of the C=O stretching and CH_2 scissoring absorption bands exhibited by DPPG and DSPG bilayers containing 5–30 mol% cholesterol. The spectra shown were obtained with a DSPG:mixture containing 25 mol% cholesterol. The types and approximate temperature ranges of the thermotropic phase transitions observed upon heating (solid arrows) and cooling (dashed arrows) such mixtures are as indicated. The conversion of the L_{β} phase to the L_c phase (see dotted arrow) which occurs at low temperature, is normally too slow to be resolved by DSC.

temperatures closely resembles that of the L_{β} phase of pure PG bilayers and, when heated to temperatures well above the range of the broad thermograms resolved by DSC, the absorption bands all broaden and adopt the features of the L_{α} phases of pure PG bilayers (Fig. 7). We therefore conclude that the broad thermotropic events exhibited by these Chol-rich DMPG bilayers are simple hydrocarbon chain-melting phase transitions whose thermodynamic properties have been altered by the presence of large amounts of Chol. Note that the FTIR spectroscopic data obtained are not sufficiently resolved to enable a determination of the nature of the weak thermotropic events that are superimposed on the broad endotherm observed upon heating, except to indicate that the proportion of PG molecules involved in these events is very small.

The FTIR spectroscopic changes observed upon heating and cooling Chol-poor (≤5 mol%), DPPG and DSPG bilayers are very similar to those described above for the corresponding Chol-poor DMPG bilayers and differ only with respect to temperature ranges over which the main thermotropic events are observed (i.e. ~23 °C for DMPG, ~41 °C for DPPG and ~54 °C for DSPG). We therefore conclude that, as observed with the Chol-poor DMPG bilayers, the predominant structural changes occurring with the corresponding Chol-containing DPPG and DSPG bilayers are also hydrocarbon chain-melting phase transitions similar in nature to those which occur in the pure PG bilayers. We also find that when DPPG and DSPG bilayers containing modest (~5–30 mol%) amounts of Chol are cooled to temperatures near 0 °C under conditions comparable to those used in our DSC experiments, FTIR spectroscopic features characteristic of the L_c phases of pure PG are also observed. However, as illustrated in Fig. 9, these FTIR spectroscopic features more closely resemble those of the L_c1 phase of pure PGs (Fig. 7), and not L_c2 phase as occurs with the corresponding DMPG bilayers (see Fig. 8). Also, as observed with the corresponding Chol-containing DMPG mixtures, the spectroscopic features observed are not identical to those of the L_c phase of the pure lipid, probably because of the presence of significant amounts of the lipid in the L_{β} phase. Upon heating, these spectroscopic features persist to temperatures just below the onset of the lower-temperature endotherms resolved in the DSC heating thermograms of the Chol-containing DPPG and DSPG mixtures (i.e. ~25 °C for DPPG and ~32 °C for DSPG). Moreover, with further heating, these features slowly convert to those typical of the L_{β} phase of the pure lipid (see Fig. 9). This conversion typically occurs over a temperature range of some 10 °C and is essentially complete before the onset of the higher-temperature calorimetrically resolved phase transition. We therefore conclude that the structural changes underlying the lower-temperature endotherms observed upon heating DPPG and DSPG bilayers containing modest (5–30 mol%) amounts of Chol are actually L_c/L_{β} type of transitions, analogous the so-called subtransitions exhibited by the pure lipid species. We also observe a marked splitting of the CH_2 scissoring band in these Chol-containing DPPG and DSPG mixtures, when the samples are cooled to low temperature (see Fig. 9). This phenomenon, the so-called correlation field splitting of the CH_2 scissoring band, occurs only in the L_c phase, when large domains of rotationally hindered, all-*trans* polymethylene chains, packed in an orthorhombic perpendicular subcells with the zigzag planes of the hydrocarbon chains perpendicular to each other, are formed [36,37]. Thus, at low temperatures, domains of virtually pure PG molecules in the L_c phase must be formed even in the presence of relatively high (~35 mol%) amounts of Chol. Upon heating to temperatures above the lower temperature phase transition temperature reported by DSC, the CH_2 scissoring band splitting collapses and the spectra initially adopts the contours typical of the L_{β} phase and, with further heating, convert to those typical of the L_{α} phase (see Fig. 9). On cooling, the spectroscopic features of the L_{α}-like phase initially convert to those typical of a L_{β}-like phase, at temperatures comparable to those

resolved calorimetrically, and eventually adopt characteristics consistent with significant L_c phase content when cooled to low temperatures.

Finally, with the Chol-rich (>35 mol%) DPPG and DSPG bilayers, the FTIR spectra observed at low temperatures closely resembles those shown in Fig. 9 for the $L_{\beta'}$ phase of pure PG bilayers, as also observed with the corresponding DMPG bilayers. Also, upon heating to temperatures well above the range of the broad calorimetrically resolved phase transition, all infrared absorption bands broaden and adopt the features similar to those illustrated in Fig. 7 for the L_α phases of pure PG bilayers. Thus, as described for the Chol-rich DMPG bilayers, the structural changes underlying the broad thermotropic transition exhibited by Chol-rich DPPG and DSPG bilayers are predominantly simple hydrocarbon chain-melting phase transitions that have been altered by the presence of large amount of Chol. Moreover, as was the case with the Chol-rich DMPG bilayers, the spectroscopic data obtained is not sufficiently resolved to enable the characterization of the weak thermotropic events that are superimposed on the broad heating endotherm observed, presumably because the population of the lipid molecules involved comprises a small fraction of the lipid molecules present in the mixture.

3.4. Effect of cholesterol on the thermotropic phase behavior and organization of DMPG, DPPG and DSPG bilayers: ^{31}P-NMR spectroscopic studies

^{31}P-NMR spectroscopic measurements were performed primarily to determine whether the lamellar/nonlamellar phase preferences of these PG bilayers were altered by Chol incorporation. Prior to low-temperature annealing, all of the PG and PG:Chol mixtures examined exhibit ^{31}P-NMR powder patterns consistent with the existence of lamellar phospholipid assemblies in which phosphate headgroup motions are axially symmetric about the bilayer normal through the temperature range examined (0–90 °C, spectra not shown). In particular, there was no evidence of a sharp isotropic peak near ~2 ppm downfield, or of the sharp downfield shoulder component of an H_{ii} phase (~9 ppm downfield), even after prolonged incubation (~1–2 h) at temperatures near 90 °C. These observations thus eliminate the possibility of either native or Chol-induced nonlamellar phase formation in these PG bilayers under our experimental conditions. However, we also observed a considerable but reversible loss of NMR signal intensity when samples were annealed under conditions favorable to the formation of L_c phases. Similar behavior has been observed when the $L_{\beta'}$ phases of PE and PG convert to the L_c phases [34,38,39] and when PE L_c phases are formed from PE:Chol mixtures [21]. The loss of ^{31}P-NMR signal intensity under such conditions has been ascribed to a change in the relaxation behavior of the ^{31}P nucleus coincident with the formation of an L_c phase having immobilized phosphate headgroups and long-lived, hydrogen-bonding interactions with their H-bonding donors [39 and references cited therein]. Our observations are thus consistent with the formation of L_c phases, as indicated by the DSC and FTIR spectroscopic data presented above.

4. Discussion

These studies clearly show that the incorporation of modest amounts of Chol into n-saturated diacyl PG bilayers facilitates the formation lamellar crystalline domains of PG at temperatures well below the chain melting phase transition temperature at significantly faster rates than occurs with the pure lipid. Similar results have been obtained in comparable studies of the effect of Chol on the structure and organization of PE and PS bilayers [21,22], but Chol incorporation inhibits L_c phase formation in PC bilayers [8]. We have shown previously that the incorporation of α-helical transmembrane peptides into PE but not PC bilayers also facilitates L_c phase formation [40], so this effect is not unique to Chol. Thus, it appears that the presence of some sterol or peptide inclusions in PG and some other phospholipid bilayers nucleates L_c phase formation at faster rates than occurs in the pure lipid alone. Clearly, additional work will be required to elucidate the molecular mechanism by which Chol and other agents facilitate L_c phase formation in some phospholipid bilayers. However, whatever its molecular mechanism, the possibility of sterol- or peptide-induced facilitation of L_c phases in phospholipid bilayer membranes should always be considered in studies of the interactions of such agents with model phospholipid membranes, to ensure that the data acquired in such studies can be appropriately interpreted. Moreover, the fact that the sterols, peptides and other agents that nucleate L_c phase formation in some phospholipid bilayers are usually excluded from the lamellar-crystalline domains that are formed, should also be considered in the interpretation of the data obtained. In the specific case of the linear saturated PGs studied here, Chol-induced L_c phase formation seems to be largely responsible for the seemingly low miscibility of Chol in the gel states of these PG bilayers.

This study also suggests that there is a hydrocarbon chain length-dependent component to the miscibility of Chol in linear saturated PG bilayers. In particular, we note that sterol-induced increases in the widths and decreases in the enthalpy of the PG gel/liquid-crystalline phase transition are more pronounced with the DMPG:Chol mixtures, for which the gel/liquid-crystalline phase transition is completely abolished at sterol concentration near 50 mol%, whereas with the longer chain PGs, hydrocarbon chain-melting phase transitions are still discernable at Chol concentrations near 50 ml%. Moreover, with the DMPG:Chol mixtures, sharp components are not detected in the cooling exotherms of the liquid-crystalline/gel phase transitions when the Chol content of the mixture significantly exceeds 25 mol%, consistent with a phospholipid:Chol interaction stoichiometry of 3.5:1, as predicted theoretically from the geometry of the molecules [41] and inferred from experiments with PC:Chol mixtures [30]. With bilayers composed of mixtures of Chol with the longer chain PGs, relatively weakly cooperative hydrocarbon chain-melting phase transitions are still discernable in mixtures containing ~50 mol% Chol and, even in these mixtures, there are significant contributions from sharp components arising from relatively Chol-poor lipid domains. Together, these observations suggest a modestly reduced Chol miscibility with the longer chain PGs in the gel state. This aspect of the behavior of Chol-containing PG bilayers differs markedly from that of the corresponding Chol-containing PC bilayers, which do not exhibit chain length-dependent reductions in enthalpy or cooperativity with increasing levels of Chol due to differences in the miscibility of Chol for the PC gel or liquid-crystalline states, except for cases of extreme hydrophobic mismatch [33], but is similar to our previously published results on Chol-containing PS and PE bilayers [21,22].

It is instructive to compare the effects of Chol on the chain-melting phase transition temperatures of zwitterionic PC [30] and PE [21] bilayers with that of anionic PS [22] and PG (present study) bilayers. These studies reveal that Chol affects the transition temperature of PC and PG bilayers in a manner dependent on the hydrophobic mismatch between the Chol molecule and the host bilayers. Specifically, when the mean hydrophobic thickness of the bilayer is less than the Chol hydrophobic length, the sterol-associated lipid melts at higher temperatures than does the Chol-poor lipid and *vice versa*. In contrast, the incorporation of Chol into PE and PS bilayers results in a progressive and more significant reduction in the phase transition temperature in a manner independent of the hydrocarbon mismatch between the phospholipids and the sterol, but with a larger reduction in temperature occurring in PE as opposed to PS bilayers. The fact that Chol has different effects on the gel phase stabilities of these various phospholipid bilayers, even with comparable hydrocarbon chain lengths, indicates that the Chol–phospholipid interactions can be influenced not only by hydrophobic mismatch between Chol and the

host lipid bilayers, but by the differential electrostatic and H-bonding interactions in the polar headgroup regions of different phospholipid bilayers.

Previous studies have shown that the phase transition temperatures of saturated PCs are almost equal to those of saturated PG bilayers, both of which are about 20–25 °C and 9–12 °C lower than that of saturated PE and PS bilayers of comparable hydrocarbon chain length, respectively. This has been explained by the fact that gel state PE bilayers have stronger electrostatic and H-bonding attractive interactions in the polar headgroup region than do gel state PS or especially PC and PG bilayers [42]. The polar headgroup region of zwitterionic PE bilayers contains several H-bond donors and acceptors, such as the amino and phosphate groups, that can form strong intermolecular H-bonds. In addition, the positively charged amino groups and the negatively charged phosphate moieties can also undergo electrostatic attractions with each other. These two types of attractive interactions reinforce one another, leading to very stable gel state bilayers. The polar headgroups of anionic PS bilayers can also form a number of intermolecular H-bonds between the amino, phosphate and carboxyl moieties of the polar headgroup. However, the net electrostatic repulsions between adjacent polar headgroups reduce gel state bilayer stability somewhat. Similarly, the polar headgroup region of PG bilayers also contains a number of H-bond donors and acceptors, especially the glycerol hydroxyl groups, that can form strong intermolecular H-bond interactions. However, the overall intermolecular attractive force in PG bilayers is weakened by the electrostatic repulsive interactions between the negatively charged phosphate moieties. Finally, the polar headgroup region of PC bilayers also contain a partially shielded positively charged choline and the negatively charged phosphate groups, which can form only weak electrostatic attractive interactions with each other. However, the polar headgroup of the PC molecule does not contain a H-bond donor and thus has weaker intermolecular H-bond interactions than PE, PS and PG. Thus, the intermolecular attractive forces that favor the gel phase over the liquid-crystalline phase decreases in the order PE>PS>PG=PC. We suggest that the incorporation of Chol will increase the distance between the polar headgroups in the bilayer, reducing the potential of adjacent phospholipid molecules for intermolecular hydrogen-bonding, thus weakening the hydrogen-bonding network, particularly in PE and PS bilayers. Moreover, the presence of Chol molecules will weaken the electrostatic attractions between lipid polar headgroups in zwitterionic PC and PE bilayers, while alleviating the electrostatic repulsions between the negatively-charged phosphate moieties in PS and PG bilayers. Thus, the incorporation of Chol would be expected to disrupt the overall intermolecular attractive forces in lipid bilayers to a greater degree in PE and PS than in PG and PC bilayers, leading to the observed larger decrease in the chain-melting phase transition temperature of PE and PS as compared to PG and PC bilayers. Moreover, given the importance of the H-bonding interactions and the electrostatic attractions to the stability of PE and PS bilayers, incorporated Chol molecules may disrupt the attractive interactions in PE and PS bilayers to such an extent as to effectively mask effects attributable to the mismatch of Chol hydrophobic length and bilayer hydrophobic thickness, which is manifest in PC and PG bilayers.

The results of our present high-sensitivity DSC and FTIR and ^{31}P-NMR spectroscopic study of the thermotropic phase behavior of aqueous dispersions of binary mixtures of saturated PG with Chol do not agree with some respects with the results of a prior low-sensitivity DSC and X-ray diffraction study of saturated PG:Chol binary mixtures [18,20]. Although both of these studies report that the addition of Chol broadens and shifts the temperature of the gel-to-liquid crystalline phase transition and reduces its enthalpy, there are quantitative differences in the results obtained. For example, Borochov et al. [20] report that the addition of Chol shifts the phase transition temperature of PG bilayers only slightly, whereas we find an appreciable hydrocarbon chain length-dependent shift in this parameter. They also reported that a cooperative gel-to-liquid-crystalline phase transition is still prominent in DMPG:Chol bilayers containing ~50 mol% Chol, whereas our results indicate that this phase transition is abolished (DMPG) or almost abolished (DPPG and DSPG) when the Chol content of such mixtures approaches ~50 mol%. Finally, a major qualitative difference in these studies is that Borochov et al. report significant phase separation of crystalline Chol in both the gel and liquid-crystalline PG:Chol mixtures at Chol concentrations above about 30 mol%, whereas we find no calorimetric or spectroscopic evidence for the existence of a separate Chol phase at sterol concentrations of up to 50 mol%. We also note that similar discrepancies in the results were obtained by the two research groups in studies of binary mixtures of Chol and linear saturated PS [17,19]. We believe that the discrepancies in experimental results summarized above could arise from differences in the preparation and treatment of the PG:Chol and PS:Chol mixtures utilized in this and in the previous studies. We find, for example, that if the temperature of the binary mixture is not maintained above the transition temperature of the phospholipid component when the organic solvents are evaporated, then Chol appears to be much less miscible in PG and PS bilayers and DSC endotherms corresponding to the melting of Chol monohydrate crystals are observed (data not shown). Moreover, Huang et al. [43] have recently examined the maximum solubility limit of Chol using an X-ray diffraction technique that is very sensitive to the formation of Chol monohydrate crystals. These workers found that the maximum solubility limit of Chol in four different PCs of different hydrocarbon chain length and degree of unsaturation falls near 67 mol% sterol, whereas that for 1-palmitoyl-2-oleoyl PE falls near 50 mol%. Moreover, these workers also showed that the previous reports of the existence of Chol crystals at lower Chol concentrations in these systems were due to the artifactual demixing of Chol that can occur during conventional sample preparation, particularly when the Chol/phospholipid mixtures pass through an intermediary solid state. Moreover, neither rehydration, heating, nor mechanical agitation can bring about the complete remixing of this demixed Chol once crystals of Chol are formed. In addition, the results of recent X-ray diffraction studies indicate that Chol is soluble in liquid-crystalline l-palmitoyl, 2-oleoyl PG levels to levels of about 67 mol% sterol (G.W. Feigenson, personal communication), as is the case for the corresponding PC bilayer. This latter result, and the absence of calorimetric and spectroscopic evidence for a separate Chol phase in our present experiments with various PG bilayers, strongly suggest that an artifactual demixing of Chol occurred in these earlier studies of PS:Chol [17–19,27] and PG:Chol [20] systems.

In all aspects of their thermotropic phase behavior, these four classes of phospholipids form a graded series from PC to PG to PS to PE. The fact that the behavior of mixtures of the two zwitterionic phospholipids, PC and PE, with Chol are very different, and that the anionic lipid PG:Chol and PS:Chol mixtures occupy an intermediate position in this series, argues against a unique general role for polar headgroup charge *per se* in determining the strength and nature of phospholipid–Chol interactions. This conclusion is supported by earlier work on the effect of limiting amounts of Chol on the thermotropic behavior of binary phospholipids mixtures, which indicate that the anionic phospholipids PS and PG exhibit greater affinity for Chol than do the zwitterionic phospholipids PC and PE, respectively [15], and by our recent calorimetric study, which showed that Chol exhibits greater miscibility in anionic PG than in uncharged glycolipid bilayers [45]. Although more subtle and specific Chol–phospholipid interactions may occur in particular binary systems, it seems that, in general, the relative miscibility of Chol in the gel phase of various phospholipid bilayers is determined primarily by the strength of phospholipid–phospholipid interactions in most phospholipid bilayer systems. Thus, phospholipids with strong intermolecular interactions in both the polar headgroup and hydrocarbon chain regions tend to exclude Chol from the bilayer

above certain critical concentrations. This finding seems also to hold for the monoglucosyl diacylglycerol, diglucosyldiacylglycerol, and PG components of the *Acholeplasma laidlawii* membrane, where the higher-melting neutral glycolipids exhibit a more limited ability to mix with Chol than the anionic phospholipid PG [44]. Conversely, the tendency for phospholipid–Chol lateral phase separation can be reduced by decreasing the strength of the intermolecular phospholipid–phospholipid interactions. This can be accomplished by decreasing the phospholipid phase transition temperature by changes in polar headgroup structure, by introducing hydrocarbon chain unsaturation, by decreasing hydrocarbon chain length, or by increasing the temperature of the system.

The interaction of Chol with phospholipids have been reported to vary with phospholipid headgroup size [17,18], charge [18,20], and hydrogen bonding potential [25–26], as well as with hydrocarbon chain length [this work, 20] and degree of unsaturation [28,29,44–48]. Based on our studies of the effect of Chol on the saturated PC, PE, PS and PG bilayers varied with respect to the length of their hydrocarbon chains, we find that the miscibility of Chol in the gel states of the host bilayer is governed primarily by Chol/host bilayer mean hydrophobic mismatch and the strength of phospholipid interheadgroup electrostatic and hydrogen bonding interactions, and by the strength of van der Waal's and hydrophobic interactions between the phospholipid hydrocarbon chains. Differences in the miscibility of Chol in the gel states of different phospholipid bilayers also account for most of the differences in the thermotropic behavior observed and specific Chol–phospholipid interactions *per se* are not required to explain the observed behavior. In particular, we find that the miscibility of Chol in the gel-state host phospholipid bilayer decreases in roughly the same order as their increasing gel/liquid-crystalline phase transition temperatures at comparable hydrocarbon chain lengths, i.e., PC ~ PG < PS < PE [42], and is not determined by polar headgroup charge *per se*. Moreover, provided extreme hydrophobic mismatch or degrees of hydrocarbon chain unsaturation are avoided, all phospholipid systems examined to date seem to exhibit a high miscibility of Chol and phospholipid in the biologically relevant liquid-crystalline state at all physiologically relevant Chol levels (~30–40 mol% sterol).

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