Comparative Differential Scanning Calorimetric and FTIR and ^{31}P-NMR Spectroscopic Studies of the Effects of Cholesterol and Androstenol on the Thermotropic Phase Behavior and Organization of Phosphatidylcholine Bilayers

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ABSTRACT We have investigated the comparative effects of the incorporation of increasing quantities of androstenol and cholesterol on the thermotropic phase behavior of aqueous dispersions of members of a homologous series of linear saturated diacyl PCs[1] using high sensitivity DSC. We have also employed FTIR and ^{31}P-NMR spectroscopy to study the comparative effects of androstenol and cholesterol incorporation on the organization of the host PC bilayer in both the gel and liquid-crystalline states. The effects of androstenol and cholesterol incorporation on the thermotropic phase behavior of shorter chain PCs like 14:0 PC are generally similar but not identical. The incorporation of either sterol progressively decreases the temperature and enthalpy, but not the cooperativity, of the pretransition and completely abolishes it at sterol concentrations above 5 mol %. Moreover, at sterol concentrations of 1 to 20–25 mol %, both androstenol and cholesterol incorporation produce DSC endotherms consisting of superimposed sharp and broad components, the former due to the hydrocarbon chain melting of sterol-poor and the latter to the melting of sterol-rich 14:0 PC domains. The temperature and cooperativity of the sharp component are reduced slightly with increasing concentration of androstenol or cholesterol, and the enthalpy of the sharp component decreases progressively and becomes zero at 20–25 mol % sterol. As well, at cholesterol or androstenol concentrations above 20–25 mol %, the enthalpy of the broad component also decreases linearly with increasing sterol incorporation and becomes zero at sterol levels of about 50 mol %. However, whereas cholesterol incorporation progressively increases the temperature of the broad component of the DSC endotherm, androstenol incorporation decreases the temperature of this component. In contrast, the effects of androstenol and cholesterol incorporation on the thermotropic phase behavior of the intermediate and longer chain PCs studied here are considerably different. Although the incorporation of cholesterol increases the main phase transition temperature of 16:0 PC slightly and decreases the phase transition of 18:0 PC and 21:0 PC, androstenol incorporation decreases the main phase transition temperatures of all three PCs rather markedly. Moreover, androstenol is less effective in reducing the enthalpy and cooperativity of the broad component of the DSC endotherm of 16:0 PC and especially 18:0 PC bilayers in comparison to cholesterol. Androstenol incorporation (>5 mol %) also results in the appearance of a second, low temperature endotherm in the DSC traces of the intermediate and longer chain PC dispersions that is not observed in similar cholesterol/PC dispersions. FTIR and ^{31}P-NMR results suggest that this endotherm arises from a temperature-induced dissolution of androstenol in the gel phase PC bilayers. This second endotherm occurs at lower androstenol concentrations and increases in area at a given androstenol level as the chain length of the host PC bilayer increases. We ascribe the increasing immiscibility of androstenol in both the gel and liquid-crystalline states of PC bilayers of increasing thickness to an increasing degree of hydrophobic mismatch between the androstenol molecule and the host phospholipid bilayer.

INTRODUCTION

Cholesterol or a closely related sterol are major lipid components of the plasma membranes of most eukaryotic cells and are also found in lower concentrations in many intracellular membranes (Nes and McKean, 1977). Cholesterol is also known to be an essential cellular component inasmuch as cholesterol synthesis or availability are required for the growth and viability of a variety of eukaryotic cells (Dahl and

Dahl, 1988). Although cholesterol seems to have several different functions in eukaryotic cells, one of its primary roles is as a modulator of the physical properties of the plasma membrane phospholipid bilayer (Yeagle, 1988). Thus, a

Abbreviations used: PC, phosphatidylcholine (specific phosphatidylcholines are designated by the notation n:0 PC, where n is the number of carbon atoms per hydrocarbon chain and 0 indicates the absence of double bonds); DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; SOPC, 1-steroyl-2-oleoyl-sn-glycero-3-phosphocholine; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; ^{2}H-NMR, deuterium nuclear magnetic resonance; ^{31}P-NMR, phosphorus-31 nuclear magnetic resonance; TLC, thin-layer chromatography; T_m, gel to liquid-crystalline phase transition temperature; ΔH, enthalpy of the gel to liquid-crystalline phase transition; $\Delta T_{1/2}$, width of the gel to liquid-crystalline phase transition measured at DSC endotherm half-height (inversely related to the cooperativity of the phase transition); PPM, parts per million; $L_{\beta'}$, lamellar gel phase with tilted hydrocarbon chains; $P_{\beta'}$, rippled gel phase with tilted hydrocarbon chains; L_α, lamellar liquid-crystalline phase; L_c, lamellar crystalline phase; PE, phosphatidylethanolamine.

large number of studies of the effects of cholesterol incorporation on the properties of phospholipid monolayers and bilayers have been carried out using a wide variety of physical techniques (Demel and de Kruijff, 1976; Yeagle, 1985, 1988; Finean, 1990; Vist and Davis, 1990; McElhaney, 1992a). These studies have shown that cholesterol incorporation (i) broadens and eventually eliminates the cooperative gel to liquid-crystalline phase transition of phospholipid bilayers, (ii) decreases (increases) the area per molecule of liquid-crystalline (gel) state phospholipid monolayers, (iii) increases (decreases) the orientational order of the hydrocarbon chains of liquid-crystalline (gel) phospholipid bilayers, and iv) decreases (increases) the passive permeability of phospholipid bilayers above (below) their gel to liquid-crystalline phase transition temperatures. At relatively high concentration and higher temperatures, cholesterol seems to induce a "liquid-ordered" state in normally fluid phospholipid bilayers characterized by a mixture of gel and liquid-crystalline features. For example, in cholesterol-rich phospholipid bilayers the rates of phospholipid rotational (Sankaram and Thompson, 1990; Vist and Davis, 1990) and lateral (Alecio et al., 1982; Kuo and Wade, 1979; Lindblom et al., 1981; Rubenstein et al., 1979) diffusion are comparable to those observed in the fluid phase of pure phospholipid bilayers, phospholipid hydrocarbon chain orientational order is intermediate between gel and liquid-crystalline of the pure lipid phases (Ipsen et al., 1990; Vist and Davis, 1990), and the area compressibility modulus more closely resembles that of the gel phase of the pure phospholipid (Needham et al., 1988). The presence of cholesterol in biological membranes has also been shown to modulate a number of membrane functions, presumably via its effects on the properties of the phospholipid bilayer (Dahl and Dahl, 1988; Yeagle, 1988).

A number of researchers have investigated the effects of systematic variations in the structure and stereochemistry of the cholesterol molecule on the thermotropic phase behavior, organization, and passive permeability of phospholipid bilayers (Demel and de Kruijff, 1976; Yeagle, 1985, 1988; McElhaney 1992a). In general, most structural and stereochemical alterations result in some loss of the ability of the cholesterol molecule to produce its characteristic effects on phospholipid bilayers. In general, sterols must possess an equatorially oriented C3-hydroxy group, a rigid planar fused ring system, and a flexible hydrocarbon side chain at C17 for maximum effect, whereas the degree of unsaturation of the ring system and the size of the alkyl side chain are of less importance. Interestingly, exactly the same structural features are required for exogenous sterols to support the maximum growth of sterol-auxotropic mycoplasma, yeast, and mammalian cells (Dahl and Dahl, 1988; McElhaney, 1992b), confirming that one of the major roles of cholesterol in eukaryotic membranes is to regulate the physical properties of the lipid bilayer.

The cholesterol analogue androstenol (5-androsten-3-β-ol), also referred to as androsten, has proven to be very useful in studies of the effect of sterols on the physical properties

of phospholipid monolayers and bilayers and on the growth of sterol auxotropic cells. Androstenol has exactly the same structure and stereochemistry as the parent cholesterol molecule, except that it completely lacks the C17 alkyl side chain of the sterol nucleus. The incorporation of androstenol into phospholipid bilayers, however, seems to have little if any effect of the physical properties of model or biological membranes. Thus, low-sensitivity DSC studies of androstenol-containing egg PC (Ladbrooke and Chapman, 1969) or SOPC (de Kruyff et al., 1972) bilayers found only small effects of this sterol on the cooperativity and enthalpy of the gel to liquid-crystalline phase transition in comparison to cholesterol. Similarly, androstenol lacks the characteristic condensing effect exhibited by cholesterol in PC monolayers (Demel et al., 1972a). Moreover, fluorescence polarization (Vincent and Gallay, 1983) and FTIR (Senak et al., 1992) spectroscopic studies of hydrocarbon chain order in DPPC bilayers found that androstenol was much less effective than cholesterol in reducing conformational disorder in the liquid-crystalline state. As well, androstenol, unlike cholesterol, is unable to significantly reduce the Rb^+, glycerol, or glucose permeabilities of egg PC bilayers (Demel et al., 1972b) or the glycerol permeability of the human erythrocyte membrane (Bruckdorfer et al., 1969). Finally, androstenol is unable to support the growth of a number of cholesterol-auxotropic mycoplasma, yeast, and mammalian cells (Dahl and Dahl, 1988; McElhaney, 1992b). Thus the presence of an alkyl side chain at C17 seems to be a requirement for the cholesterol molecule to exert its characteristic effects in both model and biological membranes.

Singer and Finegold (1990a, b) recently carried out a low-sensitivity DSC study of the comparative effects of androstenol and cholesterol incorporation on the thermotropic phase behavior of a homologous series of PCs and PEs differing in the length of their hydrocarbon chains. Surprisingly, these workers report that the incorporation of increasing quantities of androstenol and cholesterol have virtually identical effects on the temperature, enthalpy, and cooperativity of the chain-melting phase transitions of PCs and PEs, and that the sterol concentration required to completely abolish the gel to liquid-crystalline phase transition increases linearly with the length of the phospholipid hydrocarbon chains. However, these results are at odds with those of earlier calorimetric and other studies of the comparative effects of androstenol and cholesterol incorporation on phospholipid thermotropic phase behavior and organization in monolayer and bilayer model membranes. In addition, a marked linear dependence of sterol/phospholipid interaction stoichiometry on phospholipid hydrocarbon chain length cannot be explained by any of the current models of cholesterol/phospholipid packing. Finally, the use of a low-sensitivity DSC instrument with constant and relatively small phospholipid samples can result in failure to detect the less energetic and less cooperative phase transitions characteristic particularly of shorter chain phospholipids and high sterol concentrations, as we have demonstrated previously

(McMullen et al., 1993). For these reasons we have reinvestigated the comparative effects of androstenol and cholesterol incorporation on the thermotropic phase behavior of several members of a homologous series of linear saturated diacyl PCs using high-sensitivity DSC and an experimental protocol insuring that broad, poorly energetic phase transitions can be accurately monitored. Indeed, using such an approach we have demonstrated that the effects of androstenol and cholesterol on PC thermotropic phase behavior are in some ways quite different. We have also used ^{31}P-NMR and FTIR spectroscopy to study the comparative effects of androstenol and cholesterol incorporation on the organization of the host PC bilayer in both the gel and liquid-crystalline states.

MATERIALS AND METHODS

The PCs used in these experiments were purchased from Avanti Polar Lipids (Alabaster, AL) and checked for purity by TLC using chloroform/methanol/ammonia (50:50:4, v/v) as the developing solvent followed by spraying with sulfuric acid. Each PC gave a single spot on the developed TLC plates. The chain perdeuterated 14:0 PC (14:0 PC-d_{54}) was synthesized and purified using the procedures outlined in Lewis et al., (1985) except that perdeuterated myristic acid replaced myristic acid. The cholesterol was purchased from Fisher Chemicals (Fairlawn, NJ) and recrystallized twice from ethanol before use. The 5-androsten-3-β-ol was purchased from Steraloids (Wilton, NH). To ensure purity both sterol stock batches were checked by TLC using hexane/ether (7:3 by volume) as the developing solvent. For each PC or sterol a known amount was then dissolved in chloroform to make stock solutions from which the PC/sterol dispersions would be quantitatively mixed. To ensure that the stock solutions gave homologous PC/sterol mixtures we evaluated the thermotropic behavior and quantitative proportions of PC/sterol mixtures prepared from stock solutions of chloroform, chloroform/methanol (2:1, by volume), chloroform/methanol (1:2, by volume), and methanol. We found that the overall thermotropic phase behavior, as well as the quantitative differences in the predicted amount of PC or sterol within the mixtures, did not vary significantly with the solvent system. PC/sterol mixtures were then dried under N_2 and evaporated to dryness under vacuum overnight.

For DSC experiments the dried PC/sterol mixtures were dispersed and resuspended with deionized water, heated to approximately 10–20°C above the phase transition of the mixture, and then vortexed to give a multilamellar suspension. The DSC thermograms for the PC/sterol suspensions were recorded with a Hart high-sensitivity differential scanning calorimeter. Initially the scan rates used were 5°C/h for PC/sterol mixtures with less than 10 mol % sterol, whereas increased amounts of sterol the scan rate was progressively increased to 20°C/h. In addition, the amount of PC used for DSC was progressively increased from 0.5 mg for pure PC bilayers to 5 mg for PC samples containing 45 or 50 mol % sterol. The Hart calorimeter has been calibrated using solid standards from Hart Scientific (Pleasant Groove, UT) as well as aqueous lipid samples synthetically prepared within this laboratory using methods previously shown to provide highly pure samples (Lewis and McElhaney, 1985). Sample runs were repeated at least three times to ensure reproducibility. After the DSC runs the samples were quantitated and checked for degradation using TLC with the developing solvent chloroform/methanol/ammonia (50:50:4, v/v) and sulfuric acid spray. No degradation was observed. 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. (1978) and Mabrey et al. (1978) for PC/sterol mixtures. The curve broadening is expressed in terms of the van't Hoff enthalpy, which is evaluated by the equation $\Delta H_{vH} = 4RT_m^2(c_{max}/\Delta q)$, where c_{max} is the excess specific heat capacity, and Δq is the area under the curve. Although these equations provide a convincing description of the overall endotherm curves and have become a standard part of PC/sterol bilayer melting component analysis, it is not the only possible interpretation of the melting behavior.

For FTIR analysis the dried PC and PC/sterol dispersions were resuspended in D_2O and prepared as described above for the DSC experiments. For the experiments involving perdeuterated 14:0 PC/sterol mixtures, the hydration step used Milli-Q deionized water. The samples were then placed between CaF$_2$ windows with a 25-μm spacer and mounted in a cell holder attached to a computer-controlled circulating water bath. FTIR spectra were recorded with a Digilab (Cambridge, MA) FTS-40 FTIR spectrometer. The data was processed using computer programs developed by the National Research Council of Canada.

Pure PC or PC/sterol mixtures for ^{31}P-NMR were prepared as outlined for the DSC experiments. Proton-decoupled ^{31}P-NMR spectra were acquired with a Varian 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. (1988). After completion of the NMR experiments the samples were checked for degradation by both TLC and DSC. No degradation or alterations in sample thermotropic behavior were observed.

RESULTS

Calorimetric studies of the thermotropic phase behavior of pure phosphatidylcholines

In this study the interactions of androstenol and cholesterol with a shorter chain, intermediate chain, and longer chain PC (14:0 PC, 16:0 PC, and 18:0 PC, respectively) were studied. High-sensitivity DSC heating scans of aqueous dispersions of these pure PCs are shown in Figs. 1, 2, and 3, respectively. In the absence of sterol, these PC bilayers exhibit two endotherms on heating, a lower temperature, lower enthalpy pretransition and a higher temperature, higher enthalpy main transition. (Because these PC dispersions were not annealed at low temperature before heating, the L_c phase does not form and no subtransition is observed.) The pretransition arises from the conversion of the $L_{\beta'}$ phase to the $P_{\beta'}$ phase and the main, or chain-melting, transition from a conversion of the $P_{\beta'}$ phase to the L_α phase. Very similar behavior is noted for 14:0 PC, 16:0 PC, and 18:0 PC, except that the temperature interval between these two transitions decreases as the length of the PC hydrocarbon chains increases. For a thorough description of the thermotropic phase behavior of these and other members of the homologous series of linear saturated PCs, the reader is referred to Lewis et al. (1987) and to the references cited therein.

Effect of androstenol and cholesterol on the pretransition

We have shown previously that the incorporation of low concentrations of cholesterol into PC bilayers progressively decreases the pretransition temperature and enthalpy in an approximately linear manner without significantly altering the cooperativity and that the pretransition becomes undetectable at cholesterol concentrations above 5 mol %, regardless of the hydrocarbon chain length of the PC molecule (McMullen et al., 1993). Essentially similar results were obtained with androstenol in the present study, indicating that
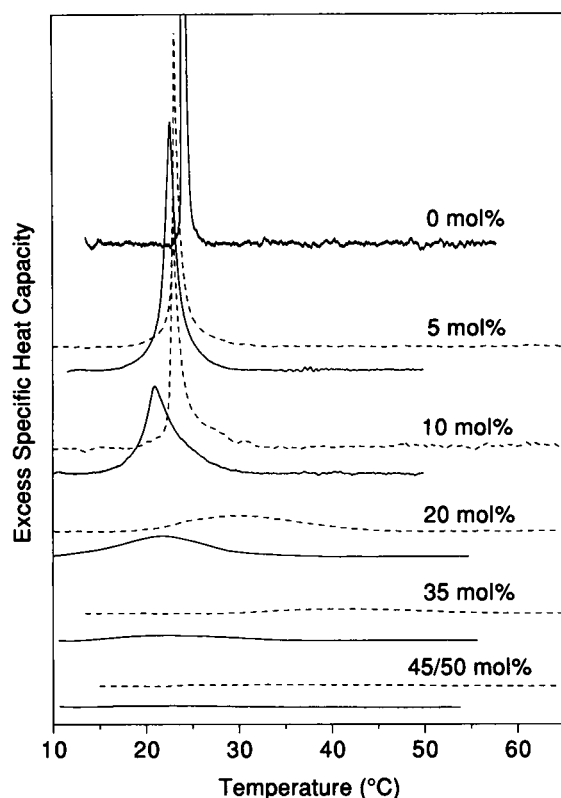
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FIGURE 1   Representative DSC scans of unannealed 14:0 PC bilayers with various concentrations of cholesterol or androstenol. 14:0 PC/cholesterol thermograms are represented by the dotted lines, whereas the 14:0 PC/androstenol traces are represented by the solid lines just below. The sterol concentrations represented by the DSC traces are shown in the figure. The highest androstenol concentration used was 45 mol %, whereas for cholesterol the highest concentration examined was 50 mol %. DSC scans containing 1, 2, 15, 25, 30, and 40 mol % cholesterol and 15, 25, and 30 mol % androstenol were also performed but not shown. Endotherms are not corrected for scan rate or mass of sample. (Comparative 14:0 PC/cholesterol data obtained from McMullen et al., 1993.)
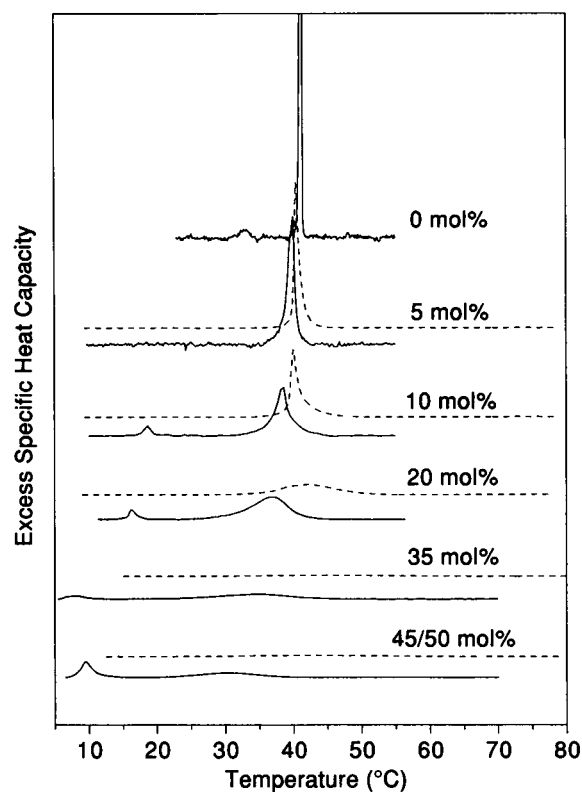
FIGURE 2   Representative DSC scans of unannealed 16:0 PC bilayers with various concentrations of cholesterol or androstenol. 16:0 PC/cholesterol thermograms are represented by the dotted lines, whereas the 16:0 PC/androstenol mixtures are represented by the solid lines just below. The sterol concentrations are shown in the figure for each set of DSC traces. The highest androstenol concentration used was 45 mol %, whereas for cholesterol the highest concentration examined was 50 mol %. DSC scans performed but not shown are 1, 2, 3, 4, 6, 15, 25, 30, and 40 mol % for 16:0 PC/cholesterol, and 15, 25, and 30 mol % for 16:0 PC/androstenol mixtures. Endotherms are not corrected for scan rate or mass of sample. Both endotherms are completely reversible. (16:0 PC/cholesterol traces obtained from McMullen et al., 1993.)

the alkyl side chain at C17 is not important for this particular sterol effect. However, because the incorporation of small quantities of free fatty acids and lysophospholipids (McElhaney, 1992a), or even transmembrane hydrophobic peptides (Zhang et al., 1992), also abolishes the pretransition of PCs, the significance of this result is unclear and will not be discussed further.

Effect of androstenol and cholesterol on the main transition

Representative high-sensitivity DSC heating scans for 14:0 PC dispersions containing increasing quantities of androstenol or cholesterol are presented in Fig. 1. In general, the incorporation of increasing quantities of androstenol and cholesterol have rather similar effects on the 14:0 PC endotherms. In both cases sterol incorporation decreases the enthalpy and cooperativity and the main phase transition of 14:0 PC to a comparable extent (Figs. 4 and 5, respectively) and abolishes cooperative chain melting at concentrations of

50 mol %. As well, the asymmetric DSC endotherms observed at androstenol concentrations of 1–20 mol % can be well fit by deconvolution into a sharp component, representing the melting of pure 14:0 domains, and a broad component, representing the melting of androstenol-containing 14:0 PC domains, just as previously demonstrated for 14:0 PC/cholesterol mixtures (McMullen et al., 1993 and references cited therein). These results indicate that the effective stoichiometry of cholesterol/phospholipid and androstenol/phospholipid interactions are similar in 14:0 PC bilayers. The one major difference between the effect of androstenol and cholesterol on 14:0 PC thermotropic phase behavior is that androstenol decreases the phase transition temperature of the broad component of the DSC endotherm, whereas cholesterol increases the phase transition temperature of this component (Fig. 6).

Representative high-sensitivity DSC heating scans of 16:0 PC and 18:0 PC dispersions containing increasing quantities of androstenol or cholesterol are presented in Figs. 2 and 3, respectively. In contrast to 14:0 PC bilayers, androstenol and
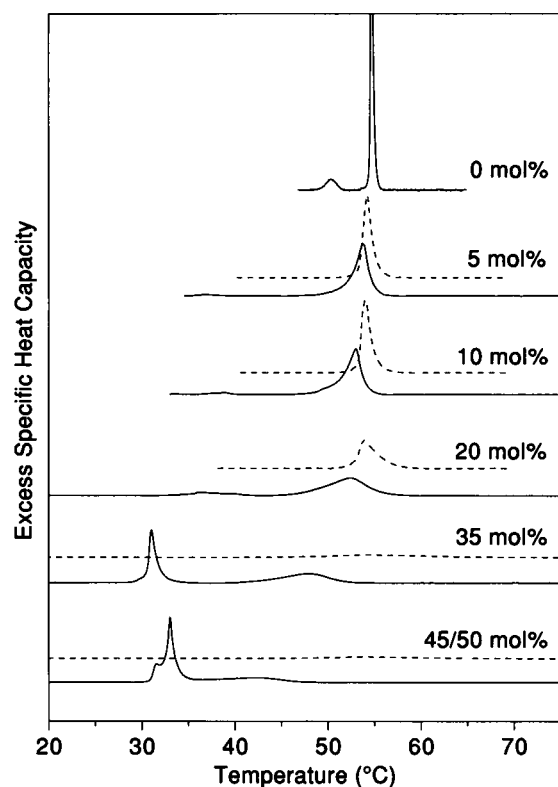
FIGURE 3 Representative DSC scans of unannealed 18:0 PC bilayers with various concentrations of cholesterol or androstenol. 18:0 PC/cholesterol thermograms are represented by the dotted lines, whereas the 18:0 PC/androstenol mixtures are represented by the solid lines just below. The sterol concentrations are shown in the figure for each set of DSC traces. The highest androstenol concentration used was 45 mol %, whereas for cholesterol the highest concentration examined was 50 mol %. DSC scans performed but not show are 1, 2, 15, 25, 30, and 40 mol % for 18:0 PC/cholesterol and 15, 25, and 30 mol % for 18:0 PC/androstenol mixtures. Endotherms are not corrected for scan rate or mass of sample. Both endotherms are completely reversible. (Comparative 18:0 PC/cholesterol data obtained from McMullen et al., 1993.)
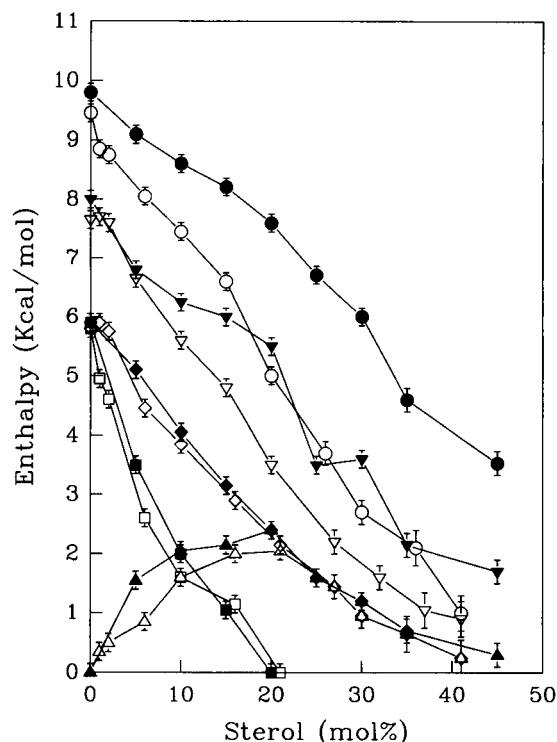

FIGURE 4 A comparison of the 14:0 PC, 16:0 PC, and 18:0 PC main transition enthalpies as a function of increasing sterol concentration. The legend for the figure is as follows: 14:0 PC/androstenol overall (◆), 14:0 PC/androstenol sharp melting component (■), 14:0 PC/androstenol broad melting component (▲), 14:0 PC/cholesterol overall (◇), 14:0 PC/cholesterol sharp melting component (□), 14:0 PC/cholesterol broad melting component, (△), 16:0 PC/androstenol (▼), 16:0 PC/cholesterol (▽), 18:0 PC/androstenol (●), and 18:0 PC/cholesterol (○), (CnPC/cholesterol data obtained from McMullen et al., 1993.)

cholesterol have rather different effects on the thermotropic phase behavior of these longer chain PCs. Specifically, at all but the lowest concentrations tested, the presence of androstenol produces a second, low-temperature DSC endotherm that is not observed in the cholesterol-containing dispersions. This low temperature endotherm is also seen on cooling scans and does not change in temperature or enthalpy over repeated scans. Moreover, the effects of androstenol and cholesterol on the higher temperature endotherm, which arises from the chain-melting transition of these longer chain PCs, are also quite different. Whereas increasing quantities of cholesterol progressively increase (decrease) the phase transition temperature of the broad component of the DSC endotherm of 16:0 PC (18:0 PC) bilayers only slightly, increasing quantities of androstenol decrease the phase transition temperature of this component markedly in both of these PC bilayers (Fig. 6). As well, androstenol is less effective in reducing the enthalpy and cooperativity of the broad component of 16:0 PC and especially 18:0 PC bilayers in comparison with cholesterol (see Figs. 4 and 5). Thus, a cooperative albeit broad

chain-melting phase transition is still observed in 16:0 PC and especially in 18:0 PC bilayers containing 50 mol % androstenol, whereas the presence of a comparable amount of cholesterol completely abolishes this phase transition. These results indicate that the effective stoichiometry of cholesterol/phospholipid and androstenol/phospholipid interactions are not the same in 16:0 PC and especially in 18:0 PC bilayers.

The DSC scans presented in Figs. 2 and 3 for 16:0 PC and 18:0 PC, respectively, illustrate qualitatively the overall effect of the incorporation of increasing quantities of androstenol on the development and behavior of the low temperature endotherm observed only in the intermediate and longer chain PC bilayers. However, the thermodynamic characteristics of this transition vary not only with the level of androstenol added but also with the chain length of the host PC bilayer. For example the T_m of the lower temperature transition varies in a complex manner with increasing androstenol concentration in the intermediate and longer chain PCs studied (Figs. 2 and 3). In addition, as shown in Table 1, at comparable androstenol concentrations the low temperature endotherm occurs at a higher temperature compared with the pure PC and becomes both more energetic and more cooperative as PC hydrocarbon chain length increases.
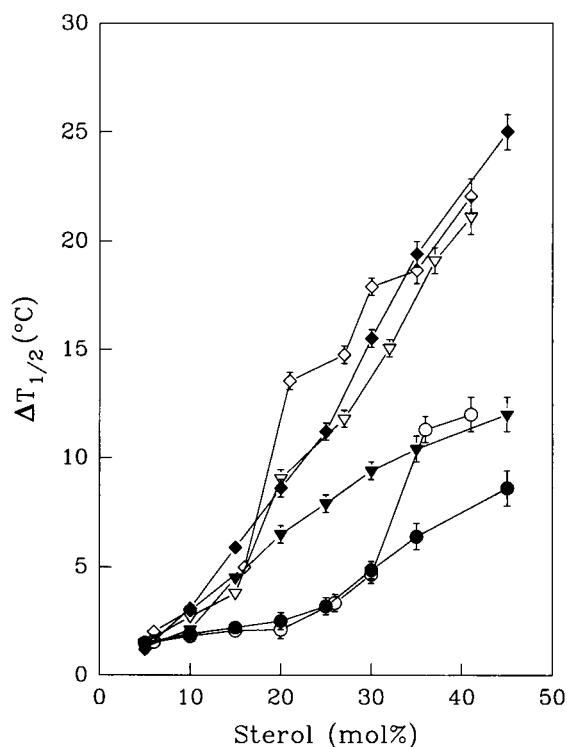
FIGURE 5 Dependence of the 14:0 PC/sterol, 16:0 PC/sterol, and 18:0 PC/sterol broad component transition $\Delta T_{1/2}$'s on increasing sterol concentration. Legend for the figure is as follows: 14:0 PC/androstenol (◆), 14:0 PC/cholesterol (◇), 16:0 PC/androstenol (▼), 16:0 PC/cholesterol (▽), 18:0 PC/androstenol (●), and 18:0 PC/cholesterol (○). (Data for CnPC/cholesterol mixtures from McMullen et al., 1993.)
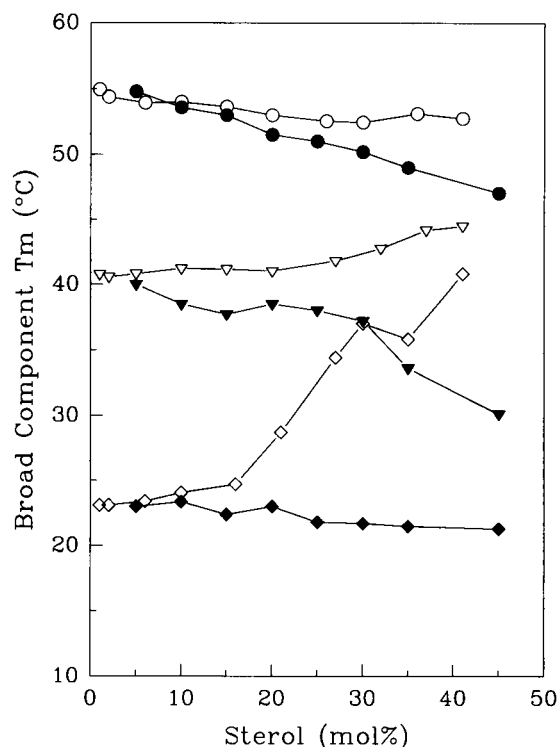
FIGURE 6 Plot of the broad component transition temperature shift as a function of increasing sterol concentration for short, intermediate, and long chain PCs. Legend for the figure is as follows: 14:0 PC/androstenol (◆), 14:0 PC/cholesterol (◇), 16:0 PC/androstenol (▼), 16:0 PC/cholesterol (▽), 18:0 PC/androstenol (●), 18:0 PC/cholesterol (○). Temperatures are corrected for scan rate. (Comparative CnPC/cholesterol data obtained from McMullen et al., 1993.)

FTIR studies of androstenol/PC and cholesterol/PC mixtures

In these studies FTIR was used to assess the comparative effects of androstenol and cholesterol incorporation on the organization of the host lipid bilayer as a function of temperature. Thus, temperature-induced shifts in the frequency of the CH_2 symmetric and asymmetric stretching bands near 2850 cm^{-1} and 2920 cm^{-1} were used to monitor changes in the conformational state of the PC hydrocarbon chains, while alterations in the frequency of the CH_2 scissoring band near 1468 cm^{-1} indicated changes in the solid-state PC hydrocarbon chain packing (Mendelsohn and Mantsch, 1986; Mantsch and McElhaney, 1991). Because there were no significant changes in the maximum absorption frequency or shape of the PC ester carbonyl band at 1735 cm^{-1} or the P-O asymmetric stretching band upon sterol incorporation (data not presented), we will not discuss these regions of the FTIR spectra further. However, the incorporation of androstenol and cholesterol does have qualitatively different effects on the organization of the hydrocarbon chains of the host PC bilayers at both high and low temperatures, as elaborated below.

Illustrated in Fig. 7 are the variations in the frequency of the CH_2 asymmetric stretching band of pure 14:0 PC, as well as 14:0 PC/androstenol and 14:0 PC/cholesterol binary mix-

TABLE 1 Chain length dependence of the CnPC/androstenol anomalous endotherm transition temperature and size

| PC	T_m (CM*)	T_m (LT‡)	ΔT_m (CM-LT)	Area LT/Area CM
16:0	37.2	11.0	26.2	0.07
18:0	49.0	30.2	18.8	0.40
21:0	63.3	53.1	10.3	0.80

All temperatures in °C and scan rate corrected. All CnPC bilayers with 35 mol% androstenol.
* Chain melting transition.
‡ Low temperature transition. The lower temperature transition is sized relative to the higher temperature chain melting transition because no stoichiometric constant is available to determine the number of moles of PC and androstenol participating in the energetics of mixing. Hence, no direct molar enthalpic comparison is permitted between the low temperature transition and the chain melting transition for a given PC.

tures containing 25 mol % sterol, as a function of temperature. The DSC endotherms of each sample are included to facilitate a comparison of the calorimetric and FTIR results. The concentration of 25 mol % sterol was chosen because at this level of incorporation domains of pure 14:0 PC are no longer present. Thus all of the PC hydrocarbon chains are presumably interacting with sterol molecules but sterol/sterol interactions are minimized. However, qualitatively similar results were obtained with androstenol/PC and

FIGURE 7   A comparison of FTIR and DSC data for: A, pure 14:0 PC; B, 14:0 PC/androstenol (25 mol %); and C, 14:0 PC/cholesterol (25 mol %) bilayers. Along the top are the plots of the CH₂ asymmetric stretching absorption maxima as a function of temperature. The corresponding DSC traces are shown for comparative purposes directly below. The CH₂ symmetric stretching spectra absorption maxima are not shown but gave qualitatively similar results. The endotherms are corrected for scan rate but are not corrected for sample mass.
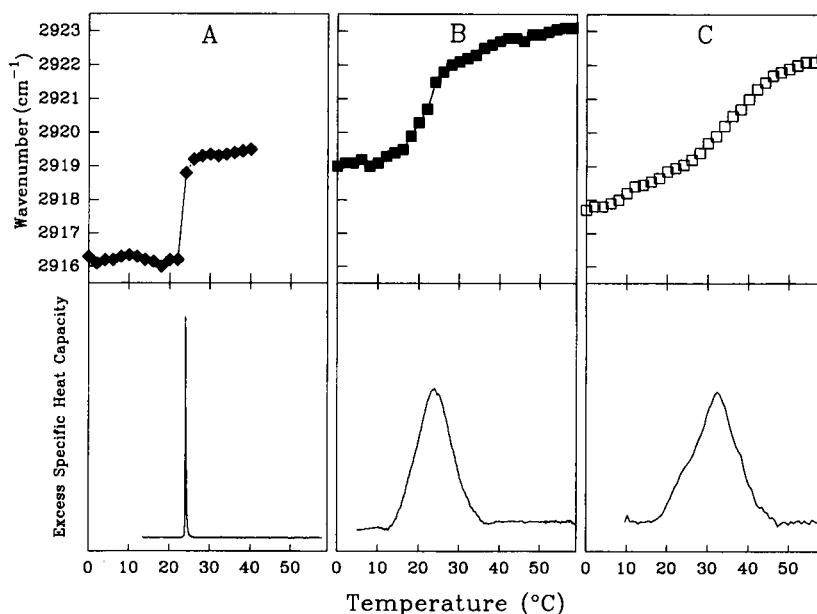
cholesterol/PC mixtures containing higher levels of sterol or when the CH₂ symmetric stretching band frequency was used to monitor hydrocarbon chain conformation (data not presented).

In the case of the pure 14:0 PC dispersion, a sharp increase in the CH₂ asymmetric stretching frequency of about 3 cm⁻¹ is observed at the temperature of the main phase transition as detected by DSC. Increases of this order of magnitude invariably accompany the melting of the hydrocarbon chains at the gel to liquid/crystalline phase transition of phospholipid bilayers (Mendelsohn and Mantsch, 1986; Mantsch and McElhaney, 1991). The 14:0 PC/androstenol and the 14:0 PC/cholesterol mixtures exhibit similar changes in CH₂

asymmetric frequency, but these both occur over a much broader range of temperatures than for the pure 14:0 PC dispersion. However, the temperature range over which most of the frequency change occurs in both systems again coincides fairly well with the DSC endotherms. This indicates that the DSC endotherms arise from the cooperative melting of the 14:0 PC hydrocarbon chains in all three systems.

Plots of the frequency of the CH₂ asymmetric stretching band as a function of temperature for pure 18:0 PC, and for 18:0 PC/androstenol and 18:0 PC/cholesterol mixtures containing 35 mol % sterol, are presented in Fig. 8. To facilitate comparison with the calorimetric data, the corresponding DSC endotherms for each of these three systems is also

FIGURE 8   A comparison of FTIR and DSC data for: A, pure 18:0 PC; B, 18:0 PC/androstenol (35 mol %); and C, 18:0 PC/cholesterol (35 mol %) bilayers. Along the top are plots of the CH₂ asymmetric stretching absorption maxima as a function of temperature. The corresponding DSC traces are shown for comparative purposes directly below. The CH₂ symmetric stretching spectra absorption maxima are not shown but gave qualitatively similar results. The endotherms are corrected for scan rate but are not corrected for sample mass.
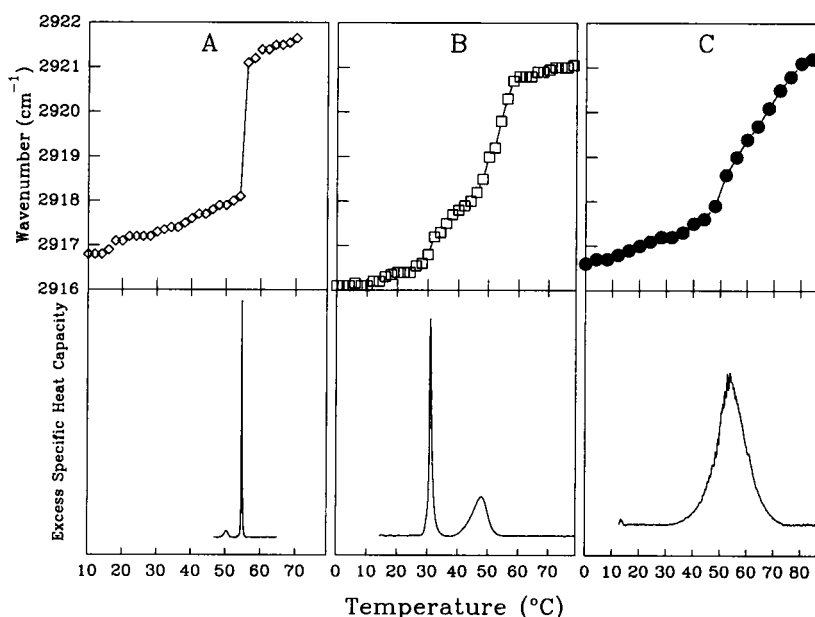
illustrated. For 18:0 PC alone, the CH_2 asymmetric stretching band frequency exhibits a large increase ($\sim$3.5 cm^{-1}) over a narrow range of temperatures centered near 55°C, which corresponds well to the midpoint temperature of the main phase transition detected by DSC. As mentioned above, frequency changes of this magnitude in the CH_2 symmetric and asymmetric absorption bands invariably accompany the gel to liquid-crystalline phase transitions of phospholipid bilayers. In the case of the 18:0 PC/cholesterol mixtures, the increase in the CH_2 asymmetric stretching frequency is of comparable magnitude but occurs over a much broader range of temperatures and is centered near 53°C, again in reasonable agreement with the DSC determination. However, the behavior of the 18:0 PC/androstenol system is more complex than that of the 18:0 PC and 18:0 PC/cholesterol systems. Although the magnitude of the shift in the frequency of the CH_2 asymmetric stretching band is comparable to the former two systems, this shift in frequency seems to take place in two stages upon heating. A relatively small increase ($\sim$0.6 cm^{-1}) in CH_2 asymmetric stretching band frequency occurs at temperatures near 30°C followed by a larger increase ($\sim$2.5 cm^{-1}) in band frequency near 50°C. Moreover, the range of temperature over which this two-component frequency shift is observed ($\sim$23°C) is somewhat less than that observed for the 18:0 PC/cholesterol system ($\sim$30–35°C). Again, the FTIR results for the 18:0 PC/androstenol mixture correspond well to the DSC endotherms. In particular, these FTIR results indicate that the sharp, low temperature DSC endothermic event is accompanied by the introduction of a small amount of conformational disorder into the 18:0 PC hydrocarbon chains, but also that the higher temperature endothermic event corresponds to the major chain-melting phase transition of the host 18:0 PC bilayer.

The absolute frequencies of the CH_2 symmetric and asymmetric stretching bands of any particular hydrocarbon chain are related to the degree of conformational disorder of that chain, with the introduction of increasing numbers of gauche conformers resulting in a progressively higher band frequency (Mendelsohn and Mantsch, 1986; Mantsch and McElhaney, 1991). Thus, in principle one can determine the relative effects of the incorporation of androstenol and cholesterol on the conformational disorder of the fatty acyl hydrocarbon chains of a phospholipid bilayer, in both the gel and liquid-crystalline states, by monitoring the CH_2 stretching band frequencies of sterol-containing phospholipid bilayers relative to those of the pure phospholipid. However, the data presented in Figs. 7 and 8 cannot be used for this purpose, inasmuch as the stretching bands of the CH_2 groups of the sterol ring system of androstenol and cholesterol, and of the CH_2 groups of the cholesterol alkyl side chain, overlap with those of the CH_2 groups of the phospholipid hydrocarbon chains. To overcome this problem, we monitored the CD_2 asymmetric stretching frequency of fully chain perdeuterated 14:0 PC alone and in the presence of either 25 mol % nonperdeuterated androstenol or cholesterol, and the results are presented in Fig. 9. Note that at temperatures above the upper boundary of the calorimetrically detected main
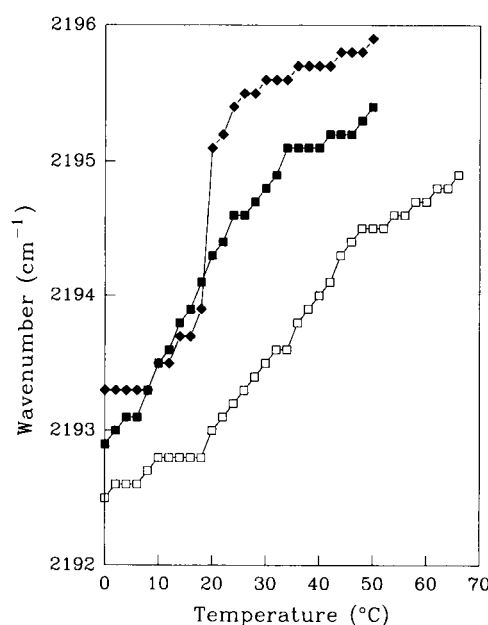

FIGURE 9   A comparison of the CD_2 asymmetric stretching absorption maxima as a function of temperature for 14:0 PC-d_{54} pure (♦), 14:0 PC-d_{54} with 25 mol % androstenol (■), and 14:0 PC-d_{54} with 25 mol % cholesterol (□). The CD_2 symmetric stretching spectra are not shown but gave qualitatively similar results.

phase transitions ($\sim$50°C), the frequency of the CH_2 asymmetric stretching band decreases somewhat upon the addition of androstenol to the 14:0 PC bilayers but decreases to a much greater extent upon the addition of comparable amounts of cholesterol. These results are compatible with those of previous fluorescence polarization (Vincent and Gallay, 1983) and FTIR (Senak et al., 1992) spectroscopic studies of hydrocarbon chain order in DPPC bilayers, which found that androstenol was much less effective than cholesterol in reducing hydrocarbon chain conformational disorder in the liquid-crystalline state. The incorporation of androstenol and to a greater extent cholesterol into gel-state 14:0 PC bilayers seems to increase 14:0 PC hydrocarbon chain conformational order at temperatures below the onset of the calorimetrically detected main phase transition temperature (<10°C). These findings are surprising in that previous monolayer film and ^{2}H-NMR studies of DPPC bilayers indicate that cholesterol disorders the hydrocarbon chains in the gel state. One would predict that both sterols, especially androstenol, would disorder the hydrocarbon chains of gel state 14:0 PC bilayers because of the mismatch in effective hydrophobic lengths of the longer all-*trans* phospholipid hydrocarbon chains and the shorter sterol molecules. At present we have no good explanations for these results. We note, however, that an earlier FTIR study analyzing the effect of cholesterol on the PC CH_2 stretching bands also reports that cholesterol seems to decrease hydrocarbon chain conformational disorder of gel-state DPPC bilayers (Senak et al., 1992).

Analysis of CH_2 bending absorption band of the PC hydrocarbon chains as a function of temperature for pure 18:0

PC, and for 18:0 PC/cholesterol and 18:0 PC/androstenol mixtures containing 35 mol % sterol, also demonstrated differences with respect to sterol incorporation (data not presented). For 18:0 PC alone, the CH_2 bending absorption band is split into two components at temperatures below the pretransition temperature because of the strong lateral interactions of the PC hydrocarbon chains in the $L_{\beta'}$ phase (see Lewis and McElhaney, 1990). Above the pretransition temperature these two components collapse into a single band because of the weaker lateral hydrocarbon chain interactions characteristic of the $P_{\beta'}$ and especially of the L_α phase. In the 18:0 PC/cholesterol system, no band splitting is observed even at low temperatures, presumably the results of a weakening of hydrophobic chain interactions because of the disordering effect of cholesterol on the gel phase PC bilayers. However, such band splitting is observed in the 18:0 PC/androstenol system, but only at temperatures below the low temperature endotherm observed by calorimetry. Significantly, the presence of comparable levels of androstenol in 14:0 PC dispersions does not produce splitting of the hydrocarbon chain CH_2 scissoring band, as expected from its disordering effect on the hydrophobic core of these bilayers (data not presented). Thus the presence of CH_2 bending band splitting at low temperatures in the 18:0 PC/androstenol mixtures indicates that domains of pure 18:0 PC $L_{\beta'}$ phase are present and thus that androstenol is largely immiscible in 18:0 PC bilayers at those temperatures. Similarly, the disappearance of this band splitting at temperatures above that of the low temperature DSC endotherm in the 18:0 PC/androstenol system indicates that domains of pure 18:0 PC $L_{\beta'}$ phase have largely disappeared, implying that androstenol is at least partially dispersed in the host PC bilayer at these higher temperatures even though the 18:0 PC hydrocarbon chains remain largely in their extended (unmelted) state.

^{31}P-NMR studies of androstenol/PC and cholesterol/PC mixtures

We employed ^{31}P-NMR spectroscopy to monitor the effect of androstenol and cholesterol on the mobility of the PC polar headgroup at the surface of the lipid bilayer as well as to confirm that the androstenol/PC binary mixtures remain lamellar over the range of sterol concentrations and temperatures examined (Seelig, 1978; Campbell et al., 1979). Illustrated in Fig. 10 are ^{31}P-NMR spectra of 14:0 PC alone, as well as 14:0 PC/androstenol and 14:0 PC/cholesterol at sterol concentrations of 25 mol %, at temperatures both above and below their respective phase transitions. At temperatures above the upper boundary of the DSC endotherm of the sterol/PC mixtures, the spectra of pure 14:0 PC and the 14:0 PC/sterol mixtures are virtually identical. Specifically, all three spectra exhibit the shape and basal linewidth ($\sim$55 ppm) characteristic of the fast, axially symmetric motion of the phosphate polar headgroup in a lamellar, and liquid-crystalline bilayer (Seelig, 1978; Campbell et al., 1979). Thus, although cholesterol, and to a lesser extent andro-
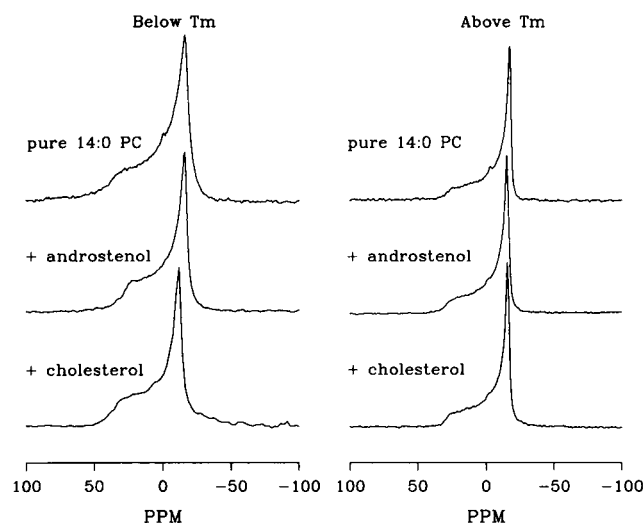

FIGURE 10   Comparative stacked plots of ^{31}P-NMR spectra for 14:0 PC pure, 14:0 PC with 25 mol % androstenol, and 14:0 PC with 25 mol % cholesterol at temperatures above and below their respective main transition temperatures. The temperatures for each of the spectra are as follows: below the Tm, 14:0 PC pure (5°C), 14:0 PC/androstenol (5°C), and 14:0 PC/cholesterol (1°C); above the Tm, 14:0 PC pure (40°C), 14:0 PC/androstenol (35°C), and 14:0 PC/cholesterol (50°C).

stenol, order the hydrocarbon chains of the liquid-crystalline 14:0 PC bilayer thereby decreasing the area occupied by the PC molecules, the incorporation of these sterols nevertheless actually increases the available surface area per PC headgroup in the bilayer. The net result is that the mobility of the PC polar headgroup, which is already high, remains so upon the incorporation of cholesterol or androstenol. In contrast, at temperatures below the lower boundary of the DSC endotherm of the 14:0 PC/sterol mixtures, both sterols decrease the basal linewidth of the pure 14:0 PC from $\sim$85 to $\sim$60 ppm, indicating that both cholesterol and androstenol increase the mobility of the polar headgroup of gel phase 14:0 PC to comparable extents. This effect is presumably because of both the disordering effect of both sterols on the 14:0 PC hydrocarbon chains at low temperatures observed by other techniques (see Introduction) and by the polar headgroup spacing effect just discussed. A key point here is that comparable amounts of cholesterol and androstenol are probably present in the 14:0 PC bilayer at low temperatures to produce spectral linewidths and shapes of comparable magnitude in the ^{31}P-NMR spectra.

The ^{31}P-NMR spectra of pure 18:0 PC, as well as 18:0 PC/cholesterol and 18:0 PC/androstenol mixtures containing about 35 mol % sterol, at three different temperatures are presented in Fig. 11. The ^{31}P spectra shown were taken at (A) 5°C (below the anomalous transition of 18:0 PC/androstenol), (B) 35°C (between the anomalous and chain melting transitions of 18:0 PC/androstenol), and (C) 60°C (above the 18:0 PC pure and 18:0 PC/androstenol chain melting transitions) and 70°C (above the 18:0 PC/cholesterol chain melting transition). At 5°C, both the ^{31}P power spectra for 18:0 PC/androstenol and 18:0 PC/cholesterol display a downfield
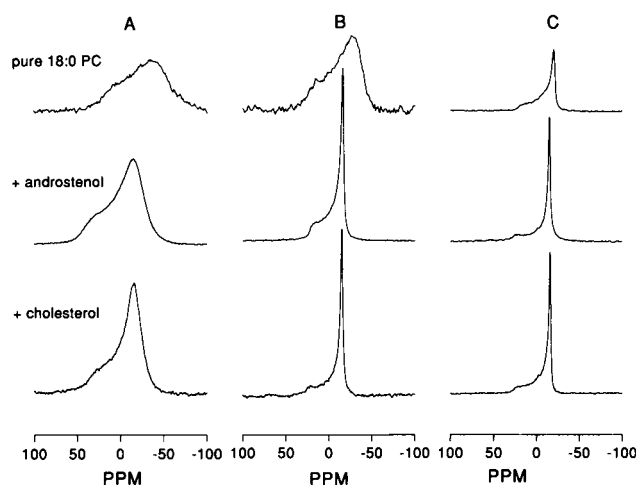
FIGURE 11 Comparative stacked plots of ^{31}P-NMR spectra for 18:0 PC pure, 18:0 PC with 35 mol % androstenol, and 18:0 PC with 35 mol % cholesterol. Plot A, at 5°C for all three spectra, compares spectral linewidths at temperatures below the anomalous transition of 18:0 PC/androstenol. Plot B, at 35°C for all three spectra, compares spectral linewidths at temperatures between the anomalous transition and the main transition of 18:0 PC/androstenol. Plot C, at 60°C for 18:0 PC pure and 18:0 PC/androstenol, and 70°C for 18:0 PC/cholesterol, compares spectral linewidths at temperatures above the main transition.

shoulder and upfield peak indicative of a lamellar phase, but the basal and peak linewidth shown for the 18:0 PC/androstenol bilayer is significantly larger (~25 ppm) and more like a gel-state pure PC bilayer than that shown for the 18:0 PC/cholesterol bilayer at the same temperature. At 35°C, both lamellar spectra display substantially decreased basal and peak linewidths, such that the spectra for 18:0 PC/cholesterol and 18:0 PC/androstenol are now virtually superimposable. At temperatures above the chain melting transitions of the 18:0 PC/androstenol and 18:0 PC/cholesterol mixtures, both bilayer dispersions exhibit only slight decreases in basal and peak linewidths, indicating that the chain melting of 18:0 PC/androstenol or 18:0 PC/cholesterol bilayers has not significantly altered the rotational freedom of the phosphate groups at the bilayer interface. Because in gel-phase 14:0 PC bilayers androstenol has a comparable effect to cholesterol in increasing polar headgroup mobility, it would seem that in 18:0 PC bilayers below the anomalous calorimetric phase transition relatively small amounts of this sterol are interacting with the 18:0 PC molecules. However, at temperatures above the anomalous transition, but below the chain-melting phase transition, considerably more androstenol interacts with the host 18:0 PC bilayer. These results, and the FTIR results just discussed, suggest that the low temperature phase transition observed by DSC results from a cooperative mutual solubilization of separate domains of pure androstenol and pure 18:0 PC.

DISCUSSION

We believe that many of the differences between the effects of cholesterol and androstenol on the thermotropic phase

behavior of PCs of different hydrocarbon chain lengths can be explained, at least qualitatively, by reference to the degree of hydrophobic mismatch between the effective lengths of these sterol molecules and the host PC hydrocarbon chains measured in a direction perpendicular to the bilayer plane (Mouritsen and Bloom, 1984, and references cited therein). At the gel/liquid-crystalline phase transition temperature, the hydrophobic thickness of PC bilayers decreases by approximately one-third because of the introductions of gauche conformers into the fully extended, all-trans hydrocarbon chains. Thus the presence of another type of amphiphilic molecule that does not undergo major temperature-induced changes in the length of its hydrophobic segment can differently affect the relative stabilities of the gel and liquid-crystalline phases according to whether its intrinsic hydrophobic length more closely resembles that of the solid or fluid PC bilayer. At least to a first approximation the relative stabilities of the gel and liquid-crystalline phases, and thus the chain-melting phase transition temperature, should be least affected when the hydrophobic length of the amphiphile equals the mean hydrophobic thickness of the bilayer (i.e., has an intrinsic hydrophobic length midway between that of the gel and liquid-crystalline phases). Amphiphiles with greater hydrophobic lengths would tend to differentially stabilize the gel phase of the host PC bilayer, thus increasing the gel to liquid-crystalline phase transition temperature, and vice versa. This hydrophobic mismatch effect has now been demonstrated to operate in synthetic peptide-PC (Zhang et al., 1992) and cholesterol-PC (McMullen et al., 1993) binary systems and in peptide-cholesterol-PC ternary systems (Nezil and Bloom, 1992).

We have presented evidence elsewhere that the effective hydrophobic length of the cholesterol molecule in PC bilayers is about 17.5 Å (McMullen et al., 1993), a value close to that of the mean hydrophobic thickness of a 17:0 PC bilayer. Thus the increase in the transition temperature of the PC/cholesterol broad component of the DSC endotherms observed for 14:0 PC and 16:0 PC bilayers, and the decrease observed for 18:0 PC bilayer, can be explained by the hydrophobic mismatch theory. Similarly, molecular modelling indicates that the length of the androstenol molecule, which lacks the cholesterol alkyl side chain, should be about 12.5 Å, 5 Å shorter than the cholesterol molecule. Because the hydrophobic length of the androstenol molecule is considerably shorter than the mean hydrophobic thicknesses of the hydrocarbon chains of all the PCs studied here, we predict and indeed observe that the incorporation of androstenol should decrease the transition temperature of the broad component of the DSC endotherm in all cases. In fact hydrophobic mismatch theory predicts that the best match of hydrophobic lengths should occur for androstenol in 12:0 PC bilayers, and indeed the temperatures of the sharp and the broad components of the DSC endotherms are very similar in androstenol/12:0 PC mixtures (data not presented).

The considerable and increasing degree of hydrophobic mismatch between the androstenol molecule and the gel phases of PCs of 16 carbons or more may also explain the

gel-phase immiscibility evident in these binary mixtures by DSC, and by FTIR and ^{31}P-NMR spectroscopy. It is certain that in these systems the hydrophobic thickness of each monolayer of the gel-state bilayer significantly and progressively exceeds the hydrophobic length of the androstenol molecule as PC chain length increases, even considering that this sterol molecule disorders, and thus shortens, the hydrocarbon chains of the PC molecules with which it is in contact. This increasing degree of hydrophobic mismatch, and thus gel phase immiscibility, is probably responsible for the low-temperature DSC endotherm, which become increasingly prominent as the amount of androstenol present in the phospholipid dispersion or as the length of the hydrocarbon chains of the host PC bilayer increases (Figs. 2 and 3). Although the FTIR results indicate that the process giving rise to this rather cooperative endotherm involves some disordering of the gel state hydrocarbon chains of the host PC bilayer, most of the hydrocarbon chain conformational disorder is introduced during the high temperature DSC endotherm that corresponds to the main phase transition (Fig. 8). Note that in the 18:0 PC/androstenol mixture containing 35 mol % sterol, the proportion of the total conformational disorder introduced during the lower temperature DSC endotherm (Fig. 8) seems to be much less than the fractional enthalpy of this endotherm (Table 1). We thus suggest that the chain disordering accompanying the apparent dissolution of androstenol into the gel phase 18:0 PC bilayers accounts for only a portion of the change in excess specific heat observed and that phospholipid-androstenol mixing itself accounts for the remainder.

Because the hydrophobic length of the 18:0 PC hydrocarbon chains in the gel state [~22.4 Å, see Zhang et al., 1992] greatly exceeds the hydrophobic length of the androstenol molecule (~12.5 Å), it is not surprising that the temperature-induced introduction of androstenol in the gel-state 18:0 PC bilayer would create some disorder in the phospholipid hydrocarbon chains. Moreover, even in the liquid-crystalline state of the pure 16:0 PC bilayer, the hydrophobic length of the melted hydrocarbon chain would still exceed that of the androstenol molecule by 0.5–1.0 Å, and this hydrophobic mismatch is probably exaggerated by the ordering effect of this sterol on the fluid 16:0 PC bilayer observed by FTIR. We therefore suggest that this increasing degree of hydrophobic mismatch between the liquid-crystalline PC bilayer and the androstenol molecule is responsible as well for the fluid phase immiscibility observed with these longer chain PC bilayers, which is manifested calorimetrically as the increasing large residual enthalpy observed at a sterol level of 50 mol % and as a smaller decrease in transition cooperativity relative to cholesterol. Conversely, the fact that the mean hydrophobic length of the androstenol molecule is intermediate between the hydrophobic length of gel and liquid-crystalline 14:0 PC monolayers probably explains the lack of evidence for gel or liquid-crystalline phase immiscibility in this or in shorter chain PC bilayers. Previous work on synthetic hydrophobic transmembrane α-helical peptide/PC (Zhang et al., 1992) and on natural membrane

protein/PC (Lewis and Engelman, 1983; Riegler and Möhwald, 1986) binary mixtures has shown that lipid/protein phase separation can occur in very long chain PC host bilayers.

It is clear from this and other recent studies that the interaction of sterols with phospholipid bilayers is a complex process that can be influenced by temperature, sterol concentration, and the structure of the phospholipids and sterols themselves. Clearly, additional studies, using modern structural, thermodynamic, and spectroscopic techniques and sterols and phospholipid molecules whose structures are systematically varied, will be required to fully understand these interactions. Such studies are currently underway in this laboratory.

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