Differential Scanning Calorimetric Study of the Effect of Sterol Side Chain Length and Structure on Dipalmitoylphosphatidylcholine Thermotropic Phase Behavior

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ABSTRACT We have investigated the thermotropic phase behavior of dipalmitoylphosphatidylcholine (DPPC) bilayers containing a series of cholesterol analogues varying in the length and structure of their alkyl side chains. We find that upon the incorporation of up to ~25 mol % of any of the side chain analogues, the DPPC main transition endotherm consists of superimposed sharp and broad components representing the hydrocarbon chain melting of sterol-poor and sterol-rich phospholipid domains, respectively. Moreover, the behavior of these components is dependent on sterol side chain length. Specifically, for all sterol/DPPC mixtures, the sharp component enthalpy decreases linearly to zero by 25 mol % sterol while the cooperativity is only moderately reduced from that observed in the pure phospholipid. In addition, the sharp component transition temperature decreases for all sterol/DPPC mixtures; however, the magnitude of the decrease is dependent on the sterol side chain length. With respect to the broad component, the enthalpy initially increases to a maximum around 25 mol % sterol, thereafter decreasing toward zero by 50 mol % sterol with the exception of the sterols with very short alkyl side chains. Both the transition temperature and cooperativity of the broad component clearly exhibit alkyl chain length-dependent effects, with both the transition temperature and cooperativity decreasing more dramatically for sterols with progressively shorter side chains. We ascribe the chain length-dependent effects on transition temperature and cooperativity to the hydrophobic mismatch between the sterol and the host DPPC bilayer (see McMullen, T. P. W., Lewis, R. N. A. H., and McElhaney, R. N. (1993) *Biochemistry* 32:516–522). Moreover, the effective stoichiometry of sterol/DPPC interactions is altered by a significantly large degree of hydrophobic mismatch between the sterol and the DPPC bilayer. Thus the short chain sterols appear to exhibit considerable immiscibility in gel state DPPC bilayers, effectively limiting their interaction with adjacent phospholipid molecules.

INTRODUCTION

Cholesterol (or a closely related sterol) is a major lipid component of the plasma membranes of most eukaryotic cells and is also found in lower concentrations in many intracellular membranes (Nes and McKean, 1977). Although cholesterol appears to have several different functions in eukaryotic cells, one of its primary roles is to modulate the physical properties of the plasma membrane phospholipid bilayer (Yeagle, 1988). Thus a large number of studies of the effects of cholesterol incorporation on the properties of phospholipid monolayers and bilayers have been carried out utilizing a wide variety of physical techniques (see Demel and de Kruyff, 1976; Bittman et al., 1981; Yeagle, 1985, 1988; Finean, 1990; Vist and Davis, 1990; McElhaney, 1992a). These studies have shown that cholesterol incorporation 1) broadens and eventually eliminates the cooperative gel to liquid-crystalline phase transition of phospholipid bilayers; 2) decreases (increases) the area per molecule of liquid-crystalline (gel) state phospholipid monolayers; 3) increases (decreases) the orientational order of the hydrocarbon chains of liquid-crystalline (gel) phospholipid bilayers; and 4) decreases (increases) the passive permeability of phospholipid bilayers above (below) their gel to liquid-crystalline phase transition temperatures. 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 workers have investigated the effects of systematic variations in the structure of the cholesterol molecule on the thermotropic phase behavior, organization, and passive permeability of phospholipid bilayers (Demel and de Kruyff, 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. Thus, sterols must possess an equatorially oriented C3-hydroxy group, a rigid planar fused ring system, and a flexible hydrocarbon side chain at C17 for

Received for publication 17 January 1995 and in final form 6 April 1995.
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Abbreviations used in this article: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; ESR, electron spin resonance; FTIR, Fourier transform infrared; *n(iso)*-C#, C17 side chain in which *n* specifies unbranched and *iso* specifies terminal methyl branch, and # specifies number of carbon atoms; $\Delta T_{1/2}$, width of the gel to liquid-crystalline phase transition measured at DSC endotherm half-height (inversely related to the cooperativity of the phase transition); $L_{\beta'}$, lamellar gel phase with tilted hydrocarbon chains; $P_{\beta'}$, rippled gel phase with tilted hydrocarbon chains; L_{α}, lamellar liquid-crystalline phase.

0006-3495/95/07/169/08 $2.00

maximum effect, while the degree of unsaturation of the ring system and the size of the alkyl side chain are of less importance. The presence of additional methyl groups on the steroid ring system or of polar functions on the alkyl side chain also markedly reduces the effectiveness of the sterol in condensing and ordering liquid-crystalline phospholipid bilayers. Interestingly, similar structural features are required for exogenous sterols to support the maximum growth of sterol-auxotrophic mycoplasma, yeast, and mammalian cells (Dahl and Dahl, 1988; McElhaney, 1992b; Bittman, 1988), 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) and its ring-saturated counterpart androstanol have 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. When androstenol, which lacks the C17 alkyl side chain, is incorporated into phospholipid bilayers, it has little effect on the physical properties of model or biological membranes. Low-sensitivity DSC studies revealed that androstenol had only small effects on the cooperativity and enthalpy of the gel to liquid-crystalline phase transition of egg PC (Ladbrooke and Chapman, 1969) or SOPC (de Kruyff et al., 1972) bilayers in comparison with cholesterol. McMullen et al. (1994), using high sensitivity DSC, demonstrated that the thermotropic behavior of androstenol-containing 14:0 PC bilayers is similar to cholesterol, but with increases in PC chain length androstenol is less effective at decreasing main transition cooperativity and enthalpy due to hydrophobic mismatch-induced androstenol-PC immiscibility. Androstenol also lacks the characteristic condensing effect exhibited by cholesterol in PC monolayers (Demel et al., 1972a). Moreover, ESR (Butler et al., 1970; Hsia et al., 1972), fluorescence polarization (Vincent and Galley, 1983), and FTIR spectroscopic (Senak et al., 1992; McMullen et al., 1994) studies of hydrocarbon chain order in synthetic and natural phospholipid bilayers found that androstenol was much less effective than cholesterol in reducing conformational disorder in the liquid-crystalline state. In addition, 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.

Fewer studies of the effect of sterol side chain structure on sterol-phospholipid interactions have been carried out to date. ESR spectroscopic studies (Suckling and Boyd, 1976; Craig et al., 1978; Suckling et al., 1979) indicate that sterols containing side chains that are either shorter or longer than the isooctyl side chain of cholesterol are less effective in increasing the order of liquid-crystalline PC bilayers than is cholesterol. Moreover, the binding of the polyene antibiotics to sterols in bilayers is also dependent on sterol side chain structure (Clejan and Bittman, 1985). The rate of transbilayer movement of sterols across the membrane of growing mycoplasma cells (Clejan and Bittman, 1984), and the rate of spontaneous exchange of sterols between vesicles (Kan and Bittman, 1990; Kan et al. 1992) and lysophospholipid dispersions (Kan and Bittman, 1991), also depend on sterol side chain structure. However, the ability of sterols to condense DPPC/sterol monolayers is not dependent on the length or structure of the sterol side chain (Suckling et al., 1979; Slotte et al., 1994), nor is the degree of the reduction of the permeability of PC liposomes (Nakamura et al., 1980). Similarly, while the longer and more highly branched plant sterols, such as ergosterol and sitosterol, have been reported to be as effective as cholesterol in condensing PC monolayers (Demel et al., 1972a) or in ordering PC bilayers (Butler et al., 1970; Vincent and Galley, 1983), other studies have found these sterols to be less effective in reducing liposomal permeability (Demel et al., 1972b) or in increasing phospholipid bilayer order (Butler et al., 1970; Hsia et al., 1972). Clearly, additional work is required to resolve some of the apparent inconsistencies in the previous literature and to provide insight into the effect of sterol side chain structure on sterol-phospholipid interactions.

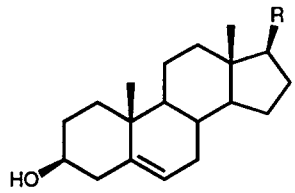
The use of high sensitivity calorimetry has provided investigators with vital information about the physical chemistry of phospholipid/cholesterol mixtures. At cholesterol concentrations of 1–25 mol %, the DSC endotherms of DPPC/cholesterol mixtures consist of superimposed sharp and broad components, the former due to the melting of cholesterol-poor and the latter to the melting of cholesterol-rich DPPC domains (Estep et al., 1978; Mabrey et al., 1978; Vist and Davis, 1990; McMullen et al., 1993, 1995). The sharp component exhibits a phase transition temperature and cooperativity only slightly reduced from those observed in the pure phospholipid and the enthalpy decreases to zero by ~20–25 mol % cholesterol. Conversely, increased levels of cholesterol decrease the broad component cooperativity significantly, while the enthalpy initially increases to a maximum at 25 mol % cholesterol, thereafter decreasing to zero by 50 mol %. Moreover, both the broad component cooperativity and transition temperature exhibit chain length-dependent behavior. Cholesterol incorporation progressively increases the transition temperature of the broad component in PCs having hydrocarbon chains of 16 or fewer carbons, while decreasing the transition temperature of PCs with hydrocarbon chains longer than 17 carbons. The broad component cooperativity also decreases more rapidly for shorter chain PCs upon cholesterol incorporation. These results were ascribed to a hydrophobic mismatch between the sterol molecule and the PC hydrocarbon chains by McMullen et al. (1993; see also Mouritsen and Bloom, 1984; Chia et al., 1993).

In the present work we have used high sensitivity DSC to study the effect of a series of cholesterol analogues, varying in the length and structure of the alkyl side chain, on the thermotropic phase behavior of DPPC bilayers. The sterol analogues employed (see Fig. 1) have either terminally unbranched or terminally branched alkyl side chains varying in length from C3 to C7 and from C5 to C10, respectively. In addition, a sterol with an exo-methylene group in the side chain was examined (C-22). We find that variations in the length of the sterol side chain produce significant changes in the thermotropic phase behavior of the host DPPC bilayer relative to cholesterol. These changes can be largely explained as a result of the varying degrees of hydrophobic mismatch between the sterol and host DPPC bilayer (see also McMullen et al., 1993; Chia et al., 1993; Slotte et al., 1994).

MATERIALS AND METHODS

The DPPC used in these experiments was purchased from Avanti Polar Lipids (Alabaster, AL). The purity was checked using TLC with a chloroform/methanol/water (65:25:4) solvent system. In addition we carried out comparative high sensitivity DSC runs with DPPC prepared in this laboratory by methods previously shown to ensure the highest purity (Lewis and McElhaney, 1985). The results were virtually identical. The sterols were synthesized using the methods described previously by Chia et al. (1993) and Slotte et al. (1994); see Fig. 1 for structures. The sterols were purified by flash chromatography on silica gel 60 (230–400 ASTM mesh) and then recrystallized from methanol. The structures were confirmed by nuclear magnetic resonance spectroscopy.

For the high sensitivity DSC experiments, DPPC and sterol stock solutions were prepared in chloroform from which the DPPC/sterol mixtures would be prepared. The mixtures were dried under N_2 and evaporated to dryness in a vacuum overnight. Final sample preparation involved dispersion of dried DPPC/sterol mixtures in deionized water with 50 mM KCl, 1 mM Na_2EDTA, and 0.05% NaN_3, heating to ~20°C above the main phase transition temperature of DPPC, and then vortexing to give a multilamellar suspension. Samples were incubated in a cold room overnight before calorimetric analysis. A Hart Scientific (Pleasant Grove, UT) high sensitivity differential scanning calorimeter was used to collect the DSC thermograms. The scan rate was increased from 5 to 30°C/h and the amount of DPPC in each sample was increased from 1 to 9 mg with increasing sterol concentration to accurately monitor the broad endotherms of DPPC bilayers containing high levels of cholesterol (McMullen et al., 1993).



COMPOUND	CODE NAME	COMPOUND	CODE NAME
R =		R =	
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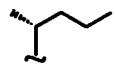
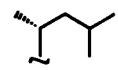
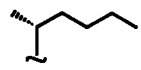
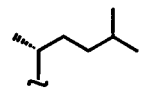
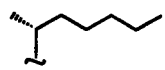
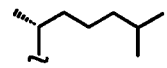
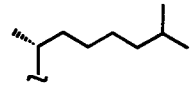
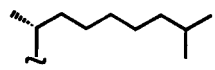
	*n*-C4		*i*-C5
	*n*-C5		*i*-C6
	*n*-C6		*i*-C7
	*n*-C7		cholesterol
			*i*-C9
			*i*-C10

FIGURE 1 Sterol side chain structures and their abbreviations as used in this paper.

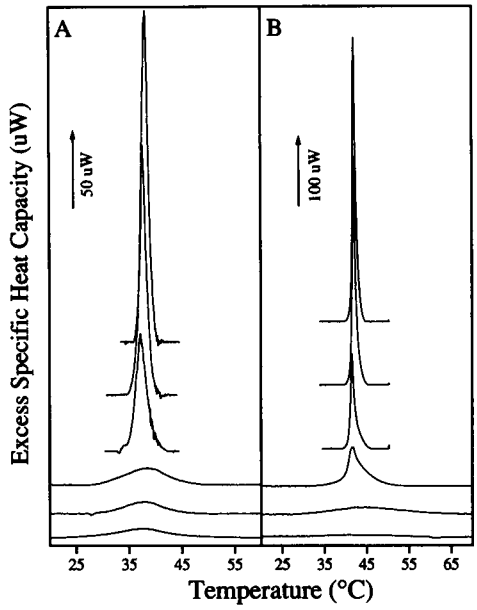


FIGURE 2 Representative plots of DPPC bilayer endotherms as a function of increasing C-22 (*A*) and *i*-C10 (*B*) sterol concentration. Sterol concentrations, shown in descending order, are 3, 6, 9, 12, 20, and 40 mol %. Sterol concentrations done but not shown are 0, 15, 25, and 30 mol %.

The analysis and decomposition of the DSC endotherms was done using Microcal's (Northampton, MA) Origin and DA-2 software. This procedure approximates each component of the endotherm as a combination of independent, two-state transitions as shown in Estep et al. (1978), Mabrey et al. (1978), McMullen et al. (1993), and McMullen and McElhaney (1995). The curve broadening is expressed in terms of the van't Hoff enthalpy, which is evaluated by the equation $\Delta H = 4RT^2(c_{max}/\Delta_q)$, where c_{max} is the excess specific heat capacity and Δ_q is the area under the curve. This protocol accurately reproduces the experimental DSC endotherms. Although other methods of estimating the temperature, enthalpy, and cooperativity of the components of these DSC endotherms were employed, these yielded qualitatively similar results.

RESULTS

In the absence of sterol, unannealed DPPC bilayers exhibit two transitions, a lower temperature and lower enthalpy pretransition (conversion of the L_β' to the P_β' phase), and a higher temperature and higher enthalpy main transition (conversion of the P_β' to the L_α phase). For all of the sterols examined, incorporation of >5 mol % was sufficient to abolish the pretransition. This confirms our earlier report that the C17 side chain is not important for this particular sterol effect (McMullen et al., 1994). Henceforth, our results focus on the changes observed in the main or chain-melting transition of DPPC upon the addition of different sterols.

Raw DSC thermograms for a select number of sterol concentrations are shown for DPPC bilayers containing increasing amounts of C-22 and *i*-C10, respectively, in Fig. 2, *a* and *b*. All DPPC/sterol mixtures exhibit two common features as a function of increasing sterol concentration, 1) a decrease in the overall enthalpy of the DPPC chain-melting endotherm, and 2) two partially superimposed and symmetrical components within the overall asymmetrical endotherm. However, at a given sterol concentration, variations in side chain length induce markedly different thermotropic behavior in the host DPPC bilayer, including dramatic differences in enthalpy, transition temperature, and cooperativity. The overall enthalpy of the DPPC chain-melting transition as a function of sterol concentration for the *n*- and *iso*-sterol series is shown in Fig. 3, *a* and *b*, respectively. Generally, with sterol side chains longer than five carbons and regardless of *n*- or *iso*- structure, the DPPC main transition enthalpy is abolished by 50 mol % sterol. In addition, the DPPC phase transition enthalpy decreases in an approximately linear manner with increases in sterol concentration. However, for the shorter chain C-22, *n*-C3, and *i*-C5 sterols, the DPPC gel to liquid-crystalline phase transition is still observable at 50 mol % sterol. The residual enthalpy of the transition at 50 mol % sterol increases with reductions in sterol side chain length. These results indicate that the effective stoichiometry of DPPC/sterol interactions depends on sterol alkyl side chain length but not structure.

Decomposition of the sharp and broad components of the DPPC main phase transition reveals the complex thermotropic behavior of these mixtures, which appears to be governed primarily by the degree of DPPC-sterol lateral phase separation. Shown in Fig. 4, *a* and *b* are the enthalpies

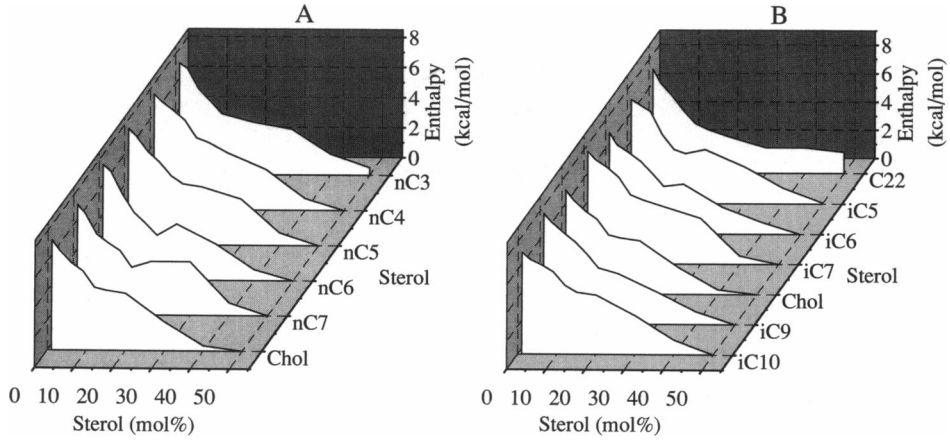


FIGURE 3 Three-dimensional plot of the effect of increasing sterol concentrations on the DPPC main transition overall enthalpy for the (*A*) *n*- and (*B*) *iso*- series of sterols. Sterols and their respective concentrations and are shown in the figure.

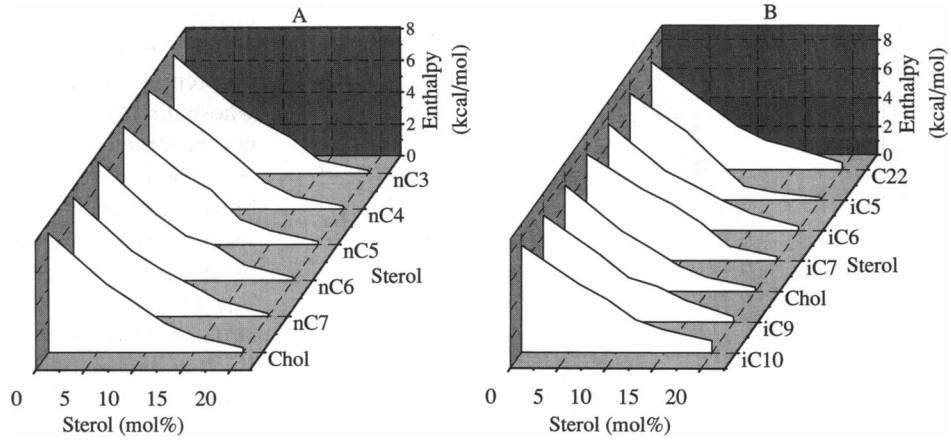


FIGURE 4 Sharp component enthalpy as a function of increasing sterol concentration for (*A*) the *n*- and (*B*) *iso*- series sterols. Sterols and their respective concentrations are shown in the figure.

of the sharp (sterol-poor phospholipid) components of the DSC endotherms as a function of sterol concentration for both sterol series. The sharp component endotherm presumably arises from an L_β to L_α phase transition and the broad component endotherm from a liquid-ordered to liquid-disordered phase transition (Vist and Davis, 1990; Huang et al., 1993). In most mixtures the sharp component persists from 0 mol % to ~20–25 mol % sterol, with an approximately linear decline in enthalpy as a function of increasing sterol level. With C-22, *i*-C9, and *i*-C10, however, the sharp component displays significantly higher enthalpies at 15 and 20 mol % than seen with other sterols. Nevertheless, for every DPPC/sterol mixture the sharp component is abolished by ~30 mol % sterol. For all of the sterols examined, the enthalpy of the broad component initially increases to a maximum at ~20–30 mol % sterol and subsequently decreases toward 0 by 50 mol % sterol, with the exception of the shortest side chains as noted above (data not shown).

The transition temperatures of the sharp and broad components of the DPPC chain-melting endotherm are shown in Fig. 5 and 6, respectively. The transition temperatures of the sharp component decrease in a sterol-dependent manner. For DPPC bilayers with the *iso*-branched sterols (Fig. 5 *b*), C-22 and *i*-C5 sterols exhibit the most significant decreases, while the rest of the *iso*- series are clustered in a temperature range only slightly reduced from the pure phospholipid. The *n*-sterol series, shown in Fig. 5 *a*, exhibit a similarly ordered distribution in transition temperature shifts with the shortest side chain analogues exhibiting the largest decreases. A sterol-specific distribution of transition temperature shifts was also observed in the DPPC broad component upon the addition of the *n*- or *iso*- series sterols, as shown in Fig. 6, *a* and *b*. However, the transition temperature shifts of the broad component are significantly larger than that seen with the sharp component. Longer chain sterols induce progressive increases in the transition temperature of the broad component, while shorter chain sterols induce progressive decreases in the transition temperature of the broad component. Clearly both the sharp and broad components of the DSC endotherms of DPPC/sterol mixtures are sensitive to alterations in sterol side chain length. However, no signif-

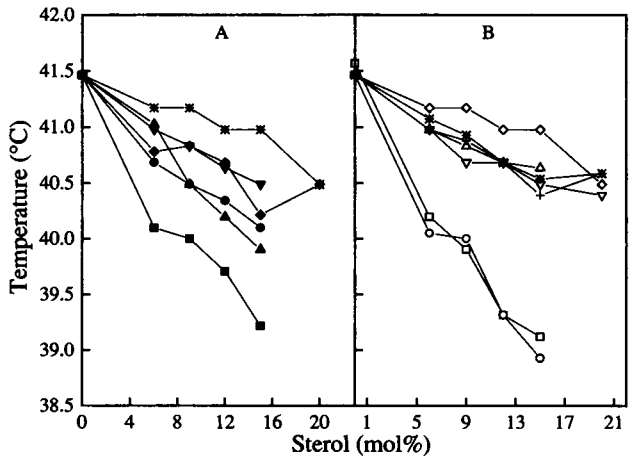


FIGURE 5 Sharp component transition temperature as a function of increasing sterol concentration. (*A*) *n*-C3, ■; *n*-C4, ●; *n*-C5, ▲ *n*-C6, ▼; *n*-C7, ◆; cholesterol, *. (*B*) C-22, □; *i*-C5, ○; *i*-C6, Δ; *i*-C7, ▽; cholesterol, ◇; *i*-C9, +; *i*-C10, *.

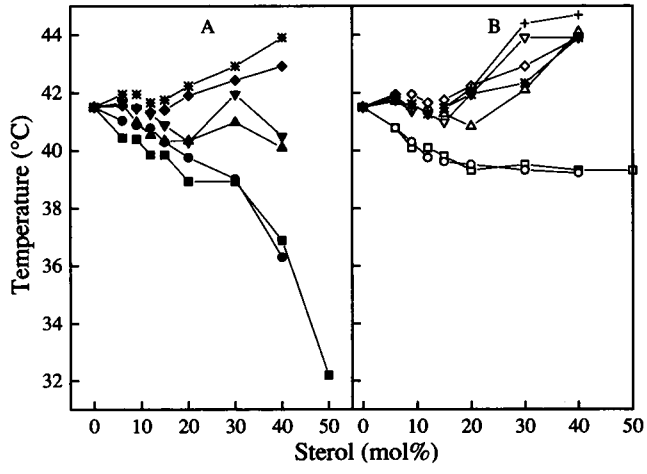


FIGURE 6 Broad component transition temperature as a function of increasing sterol concentration. (*A*) *n*-C3, ■; *n*-C4, ●; *n*-C5, ▲ *n*-C6, ▼; *n*-C7, ◆; cholesterol, *. (*B*) C-22, □; *i*-C5, ○; *i*-C6, Δ; *i*-C7, ▼; cholesterol, ◇; *i*-C9, +; *i*-C10, *.

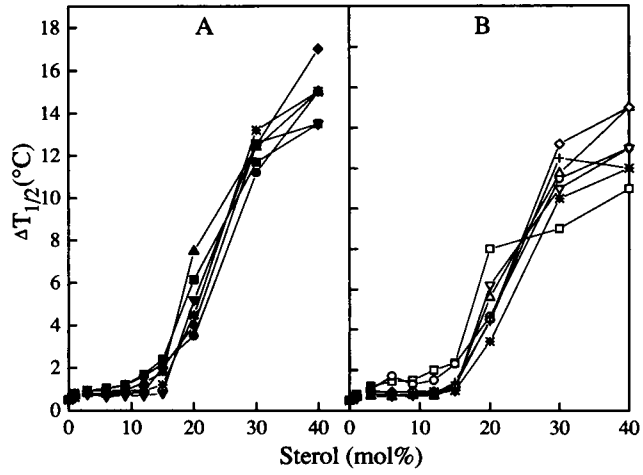


FIGURE 7 Overall DPPC endotherm $\Delta T_{1/2}$ as a function of increasing sterol concentration. (*A*) *n*-C3, ■; *n*-C4, ●; *n*-C5, ▲ *n*-C6, ▼; *n*-C7, ◆; cholesterol, *. (*B*) C-22, □; *i*-C5, ○; *i*-C6, Δ *i*-C7, ▽; cholesterol, ◇; *i*-C9, +; *i*-C10, *.

icant difference is apparent in the thermotropic behavior of DPPC bilayers containing *iso*-branched or unbranched side chains of comparable length.

Finally, the $\Delta T_{1/2}$ of the overall DPPC endotherm also varies depending on sterol side chain structure (Fig. 7, *a* and *b*). DPPC bilayers with shorter chain sterols generally exhibit significantly increased endotherm $\Delta T_{1/2}$ values (lower cooperativity) as compared to longer chain sterols at comparable levels of sterol incorporation. By 20 mol % sterol the shorter chain sterols exhibit $\Delta T_{1/2}$ values twice as large as the longer side chain sterols. This holds true for both the *n*- and *iso*- series of sterols. While this general trend is clearly observable in DPPC bilayers containing lower sterol levels (<25 mol %), increases in sterol incorporation toward 40–50 mol % obscure this relationship because of the increased immiscibility of the shorter chain sterols in the gel state DPPC bilayers (see McMullen et al., 1994).

DISCUSSION

Our results indicate that even small changes in the length of the C17 side chain of cholesterol will alter the thermotropic phase behavior of the host DPPC bilayer. Each sterol induces significantly different shifts in the transition temperature, enthalpy, and cooperativity of the sharp and broad components of DPPC/sterol mixtures. However, our observations, as well as those of Chia et al. (1993), indicate that differences in the structure (*n*- versus *iso*-) of the sterol side chain do not significantly alter the thermotropic behavior of DPPC bilayers. Thus the observed results conform well to those predicted by the hydrophobic mismatch effect as outlined for PC/sterol systems by McMullen et al. (1993, 1994) and by Chia et al. (1993).

In all of the DPPC/sterol mixtures the sharp, sterol-poor, phospholipid chain-melting endotherm exhibited only relatively small changes in transition temperature and cooperativity. However, the broad, sterol-rich, phospholipid chain-melting endotherm exhibited dramatic and progressive differences in transition temperature and cooperativity whose direction and magnitude depended on the length but not the structure of the alkyl chain of the sterol. The differences in the transition temperature and cooperativity of the broad component of the different DPPC/sterol mixtures seem to arise primarily from varying degrees of hydrophobic mismatch between the sterol and the host DPPC bilayer. The hydrophobic region of the bilayer is defined as the region between the *sn*-1 carbonyl groups of DPPC molecules in opposing monolayers. At the gel to liquid-crystalline phase transition the hydrophobic thickness of the PC bilayers decreases by approximately one-third. The presence of another amphiphile in the bilayer that does not undergo a similar change in hydrophobic thickness will differentially affect gel and liquid-crystalline bilayer stability unless its length is approximately equal to the mean thickness of the host bilayer (i.e., the sterol has a hydrophobic length midway between that of the gel and liquid-crystalline DPPC phases). Thus sterols with lengths greater than the mean thickness of the host PC bilayer will preferentially stabilize the gel phase of a PC bilayer (increase transition temperature), while short side chain sterols will destabilize the gel state (decrease transition temperature) (Mouritsen and Bloom, 1984). We previously demonstrated that the effective length of the cholesterol molecule is approximately 17.5 Å, close to the mean hydrophobic length of di-17:0 PC (McMullen et al., 1993). The mean hydrophobic thickness of one of the DPPC monolayers is ~16.5 Å; thus we predict and observe a slight increase in the transition temperature of the cholesterol-rich broad component of the DPPC endotherm. With changes in the sterol side chain length from ~19.5 Å for *i*-C10 to 13.0 Å for C-22 or *i*-C5, we see progressive increases and decreases, respectively, in the transition temperature of the broad component of the DPPC chain-melting endotherm. For intermediate length sterols we observe a general progression of transition temperatures between the two extremes C-22 or *i*-C5 and *i*-C10. These results complement the CH_2 wagging progression analysis of DPPC bilayers containing a similar series sterols by Chia et al. (1993). These workers determined that sterols with shorter side chains (<C6) induce a significantly higher degree of conformational disorder in the DPPC hydrocarbon chains than cholesterol for a given temperature. Conversely, liquid-crystalline DPPC bilayers containing longer chain sterols (>C8) exhibit a greater degree of conformational order relative to cholesterol. Moreover, CH_2 wagging intensity profiles as a function of temperature indicate that the chain melting of DPPC bilayers containing shorter chain sterols occurs at significantly lower temperatures than that seen for cholesterol and vice versa. These spectroscopic results clearly corroborate our DSC results and the hydrophobic mismatch model of PC/sterol interactions.

With most of the sterols studied here, the decreases in the DPPC chain-melting transition enthalpy as a function of

increasing sterol concentration are similar, with an approximately linear decrease to zero by 50 mol % sterol. Thus the effective stoichiometry of DPPC/sterol interactions is not dependent on the length or structure of the alkyl side chain of the sterol. However, for the shorter chain sterols, a residual DPPC chain-melting phase transition can be detected at sterol concentrations above 50 mol %. We suggest that as the degree of hydrophobic mismatch between these sterols and the host DPPC bilayer increases, these sterols may no longer be completely miscible in the gel state bilayer. As a result the effective stoichiometry of DPPC/sterol interactions is altered such that, on average, each DPPC molecule is in contact with fewer sterol molecules. This is seen with androstenol in DPPC and DSPC bilayers, where a significant chain-melting transition is apparent even at androstenol levels of 50 mol % (McMullen et al., 1994). Also, the appearance of a new, low temperature endotherm was noted in these systems at andostenol concentrations as low as 5 mol %. The low temperature endotherm was shown to be the result of temperature-induced dissolution of phase-separated androstenol in these gel state PC bilayers. Clearly with C-22, and to a lesser degree *i*-C3 and *i*-C5, the chain-melting transition observable at 50 mol % sterol is indicative of partial gel state sterol immiscibility due to DPPC/sterol hydrophobic mismatch. However, the presence of an alkyl side chain, even as short as three carbons, is apparently sufficient to prevent a lateral segregation of sterol molecules in gel state DPPC bilayers as is observed with androstenol.

The hydrophobic mismatch effect also accounts for the variations in the main transition cooperativity of the various DPPC/sterol mixtures. Due to the significant disordering of the DPPC gel state bilayer by the incorporation of sterols such as C-22, *i*-C5 and *n*-C3, the temperature required to induce increased gauche conformer formation decreases. Decreased conformational order in short chain sterol-containing DPPC bilayers compared with cholesterol-containing DPPC bilayers was documented by Chia et al. (1993). The result is a broader, lower temperature endotherm as seen by DSC. The converse is true with DPPC bilayers with longer chain sterols. Increased steric restriction of the PC hydrocarbon chain by the sterol side chain, although to a lesser degree than that induced by the sterol ring system, significantly increases the conformational order in the DPPC hydrocarbon chains compared with a sterol lacking a side chain. The result is a higher temperature and more cooperative phase transition. Note that under conditions of gel state immiscibility, such as with C-22, the decrease in transition cooperativity as a function of increasing sterol concentration is significantly reduced. This is due to the segregation of short-chain sterols into phase-separated sterol-rich domains, thus lowering the number of effective sterol/DPPC contacts and reducing the influence of the sterol on DPPC thermotropic behavior.

The complex and sometimes conflicting results observed in prior calorimetric and spectroscopic studies of cholesterol side chain analogues in phospholipid bilayers may be related in part to the complex thermotropic behavior of these systems. This study, as well as those by Chia et al. (1993) and Slotte et al. (1994), clearly demonstrate that the thermotropic behavior and organization of sterol-containing DPPC mixtures depend on sterol side chain length. Depending on the sterol and the host phospholipid bilayer, the degree of hydrophobic mismatch may lower or raise the temperature required for the gel to liquid-crystalline phase transition. Moreover, the phospholipid conformational order as a function of temperature would be dramatically different than that observed for cholesterol-containing bilayers. Thus the conflicting observations of studies examining the physical properties of various sterols (see Introduction) on phospholipid bilayers may be due in part to different sterol-induced shifts in the gel to liquid-crystalline phase transition of the host phospholipid bilayer as well as differences in sterol gel state miscibility. The results of a parallel calorimetric study of these same side chain analogues in SOPC bilayers support this conclusion. We believe that comparative studies of the effect of sterols on phospholipid thermotropic phase behavior, hydrocarbon chain conformational order, and permeability should be carried out on the same host phospholipid bilayer, and at comparable reduced temperature.

Finally, this study demonstrates, in conjunction with the studies of McMullen et al. (1993, 1994) and the spectroscopic data of Senak et al. (1992) and Chia et al. (1993), that the complex DPPC/sterol endothermic curves observed by DSC represent the behavior of superimposed but independent symmetrical endotherms. This determination was made possible by the sterol-specific differential temperature shifts exhibited by the sharp and broad components of the DPPC endotherm. Moreover, the temperature and width of the broad component of the DSC endotherm correlate well with the temperature-dependent changes observed in acyl chain conformational order of cholesterol-containing DPPC bilayers as observed by Chia et al. (1993) using FTIR spectroscopy. Thus the DSC endotherm curves accurately represent the chain melting of both the cholesterol-rich and cholesterol-poor DPPC domains, and the decomposition of the overall endotherm enables us to determine the relative contributions of each of the domains.

These results confirm that the evolution of the structure of cholesterol is based in part on regulation of the physical properties of the phospholipid bilayer. The complexities of the thermotropic behavior and organization of PC/sterol mixtures are only now becoming apparent with application of high sensitivity DSC and various spectroscopic techniques to these systems. Further examination of phospholipid/sterol mixtures in which both components are systematically varied may reveal additional features of these systems not yet identified.

This work was supported by operating and major equipment grants from the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research, respectively, to R.N.M, and grant HL-16660 from the National Institutes of Health to R.B. T.P.W.M is a Teagle Scholar

and the recipient of a studentship from the Alberta Heritage Foundation for Medical Research.

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