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The effect of side-chain analogues of cholesterol on the thermotropic phase behavior of 1-stearoyl-2-oleoylphosphatidylcholine bilayers: a differential scanning calorimetric study

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Abstract

In this study we have examined the effects of analogues of cholesterol differing with respect to alkyl side-chain length and structure on the thermotropic phase behavior of bilayers formed from 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), an important subclass of naturally occurring phosphatidylcholines (PCs). The synthetic sterols we studied contained either a terminally unbranched (n-series) or a single methyl-branched (iso-series) side chain of 3 to 10 carbon atoms. The phase transition behavior was examined by high-sensitivity differential scanning calorimetry (DSC). The main phase transition endotherm of SOPC/sterol bilayers consists of superimposed sharp and broad components, which represent the hydrocarbon chain melting of sterol-poor and sterol-rich phospholipid domains, respectively. The transition temperature and the cooperativity of the sharp component are moderately reduced upon sterol incorporation and the enthalpy decreases to zero when sterol levels of 20–30 mol% are reached. The enthalpy of the broad component transition initially increases to a maximum around 25 or 25–30 mol% sterol and thereafter decreases with further increases in sterol concentration. However, the broad transition of SOPC bilayers containing both short (C-22, i-C5 and n-C3) and long (i-C9 and i-C10) side-chain sterols still persists at levels of 50 mol% sterol. Thus the effective stoichiometry of SOPC–sterol interactions varies with changes in sterol alkyl side-chain length. The incorporation of short linear or branched side-chain sterols (C-22, n-C3, n-C4, i-C5) causes the broad component transition temperature and cooperativity to decrease dramatically, whereas the incorporation of medium- and long-chain sterols in both the n- and iso-series has less effect on the transition temperature and cooperativity of the broad component. Overall, no significant differences were found between the n- and iso-series sterols for a given side-chain length. A comparison of the phase behavior of dipalmitoylphosphatidylcholine (DPPC)/sterol (McMullen et al. (1995) Biophys. J. 69, 169–176) and SOPC/sterol mixtures indicates that the primary factor responsible for changes in the thermotropic phase behavior of these systems is the extent of the hydrophobic mismatch between the sterol and the host lipid bilayer. However, sterol miscibility in PC bilayers, and thus the stoichiometry of lipid–sterol interactions, also appears to depend on the degree of unsaturation of the host lipid bilayer.

Keywords: Cholesterol analog; Thermotropic phase behavior; Phase behavior; DSC; 1-Stearoyl-2-oleoylphosphatidylcholine bilayer

1. Introduction

The occurrence of high concentrations of cholesterol in the plasma membrane of higher organisms has prompted numerous investigations into its role in the structure and function of cell membranes [1,2]. One of the primary roles of cholesterol in eukaryotic cells is to modulate the physical properties of the plasma membrane phospholipid bilayer [2]. 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

Abbreviations: SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; DSC, differential scanning calorimetry; n(iso)-C#, C17 side chain where n specifies unbranched and iso specifies terminal methyl branch, and # specifies number of carbon atoms; T_m, gel to liquid-crystalline transition temperature; $\Delta T_{1/2}$, width of the gel to liquid-crystalline phase transition measured at DSC endotherm half-height (inversely related to the cooperativity of the phase transition); FTIR, Fourier transform infrared.

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wide variety of physical techniques [2–7]. These studies have shown that cholesterol decreases the area per molecule of liquid-crystalline phospholipid monolayers. Moreover, cholesterol increases the orientational order of the hydrocarbon chains of liquid-crystalline phospholipid bilayers and eventually eliminates their cooperative gel to liquid-crystalline phase transition. Lastly, cholesterol incorporation also decreases the passive permeability of phospholipid bilayers above their gel to liquid-crystalline phase transition temperatures.

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 [2–4,7]. For example 5-androsten-3β-ol, an analogue of cholesterol that lacks the C17 isooctyl side chain, is incapable of condensing phosphatidylcholine (PC) monolayers [8,9] and is less effective than cholesterol in reducing the solute permeability of egg PC [8] and erythrocyte bilayers [8,10,11], or in decreasing the conformational disorder of PC fatty acyl chains [12–18]. Androstenol is also less effective than cholesterol in decreasing the main phase transition enthalpy and cooperativity of egg PC [19], 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) [20], or saturated PCs with chain lengths longer than myristoyl [18]. Moreover, sterols that have either a shorter or longer side-chain than cholesterol are not as effective as cholesterol in increasing the hydrocarbon chain order of liquid-crystalline PC bilayers [21–23]. The binding of polyene antibiotics to sterols in bilayers is also dependent on sterol side-chain structure [24], as is the rate of transbilayer movement of sterols across the membrane of growing mycoplasma cells [25] and the rate of spontaneous exchange of sterols between vesicles [26,27] and lysophospholipid dispersions [28]. Thus, most structural and stereochemical alterations result in some loss of the ability of the cholesterol molecule to produce its characteristic effects on phospholipid bilayers. The same is true for the ability of exogenous sterols to support the maximum growth of sterol-auxotrophic mycoplasma, yeast and mammalian cells [1,29,30], confirming that one of the major roles of cholesterol in eukaryotic membranes is to regulate the physical properties of the lipid bilayer.

The vast majority of previous DSC studies of cholesterol–phospholipid interactions have used PCs bearing two identical saturated fatty acyl chains. However, except in lung surfactant and nerve endings where high levels of DPPC are found, naturally occurring PCs rarely contain identical fatty acyl chains [31,32]. Instead, PCs that contain a saturated chain at the *sn*-1 position and an unsaturated chain at the *sn*-2 position are most abundant in biological membranes [33]. Prior low-sensitivity DSC studies of the effect of cholesterol on the thermotropic behavior of mixed-chain PCs revealed that cholesterol interacts differentially with *sn*-1 and *sn*-2 positional isomers [34–36]. In addition, PC bilayers with various levels of unsaturation exhibit significantly different thermotropic responses to the incorporation of cholesterol [35]. In order to provide additional insights into the effects of sterol alkyl side-chain length and branching on sterol interactions with mixed-chain saturated-unsaturated PC bilayers, we have undertaken the present high-sensitivity differential scanning calorimetric (DSC) study of SOPC/sterol mixtures (see Fig. 1 for the structures of the cholesterol analogues used). We find that variations in the length, but not the structure, of the sterol side chain produce significant changes in the thermotropic phase behavior of the host SOPC bilayer. Although the effects of these cholesterol side-chain analogues on the thermotropic behavior of SOPC and DPPC [37] bilayers are qualitatively similar, significant quantitative differences are also observed. We ascribe these differences in the thermotropic behavior of SOPC/sterol and DPPC/sterol mixtures primarily to differences in the degree of hydrophobic mismatch between the sterol and host bilayer and secondarily to differences in the interaction of these sterols with saturated and unsaturated *sn*-2 hydrocarbon chains in the gel-state [38–41].

2. Materials and methods

2.1. Materials

The SOPC used in these experiments was purchased from Avanti Polar Lipids (Alabaster, AL). The purity was checked using thin layer chromatography with a chloroform/methanol/water (65:25:4) solvent system. Both moderately and heavily loaded plates showed only a single spot at the expected position upon visualization. The cholesterol side-chain analogues used in this study were synthesized as described previously [17]. These cholesterol analogues were purified by flash chromatography on silica gel 60 (230-400 ASTM mesh) and then recrystallized from methanol. Their structures were confirmed by NMR spectroscopy [17].

2.2. High-sensitivity DSC measurements

The SOPC/sterol binary mixtures were prepared from SOPC and sterol stock solutions in chloroform. The SOPC/sterol mixtures were dried under N_2 and evaporated to dryness in a vacuum overnight. The dried lipid mixture was then suspended in deionized water containing 50 mM KCl, 1 mM Na_2EDTA, and 0.05% NaN_3 and heated approx. 20°C above the phase transition temperature with vortexing to give a multilamellar suspension. These aqueous dispersions were stored overnight under N_2 at 4°C. A Hart Scientific high-sensitivity differential scanning calorimeter (Pleasant Grove, UT) was used to collect the DSC thermograms. Samples were incubated in the calorimeter at −10°C for at least 60 min prior to the start of the DSC heating scans to ensure an initial state of

equilibrium. Each sample was scanned at least three times. The DSC scan rates were increased with increasing sterol concentration, from 5 to 30°C/h, and the amount of SOPC in each sample was increased from 2 to 10 mg. This experimental protocol ensured that the broad, low-enthalpy endotherms observed at high sterol concentrations are accurately recorded [38].

The analysis and decomposition of the DSC endotherms was done using Microcal (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 previously [38,42–44]. 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 our experimental DSC endotherms. Although other methods of estimating the temperature, enthalpy and cooperativity of the components of these DSC endotherms were also employed, these analyses yielded qualitatively similar results.

3. Results

High-sensitivity DSC thermograms of SOPC in the absence of sterol are presented at the top of Fig. 2; only the bottom half of these very sharp endotherms could be accommodated in this figure without a drastic reduction of the excess specific heat scale. Pure SOPC exhibits a single, fully reversible gel to liquid-crystalline phase transition over the temperature range examined. The transition temperature is 6.0°C, the transition enthalpy 5.9 kcal/mol, and the apparent $\Delta T_{1/2}$ (a measure of the cooperativity of this chain-melting phase transition) is about 0.75°C. The transition temperature and enthalpy values almost exactly match the average values for SOPC reported in previous low- and high-sensitivity DSC studies but the $\Delta T_{1/2}$ values we report here are much smaller (the SOPC phase transition is considerably more cooperative). These sharp DSC endotherms confirm that the sample of SOPC used in these experiments is of high purity.

Shown in Fig. 2A and B are DSC endotherms of the main phase transition of SOPC bilayers as a function of

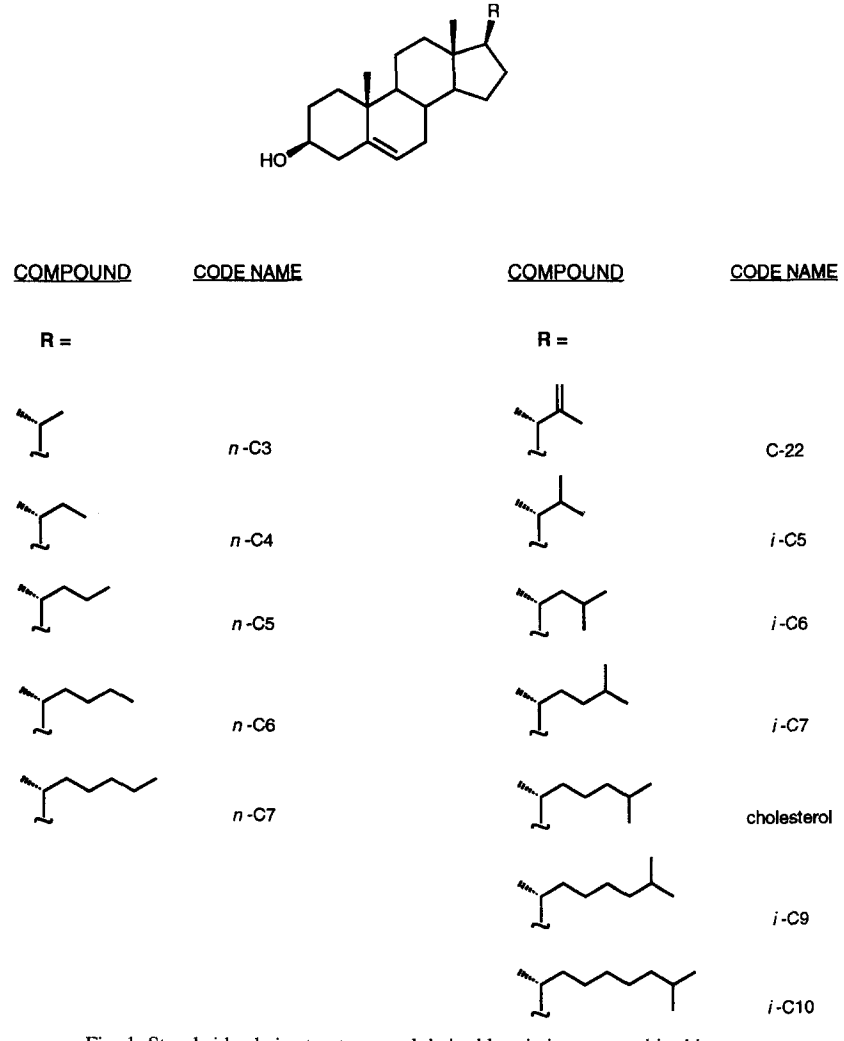

Fig. 1. Sterol side chain structures and their abbreviations as used in this paper.

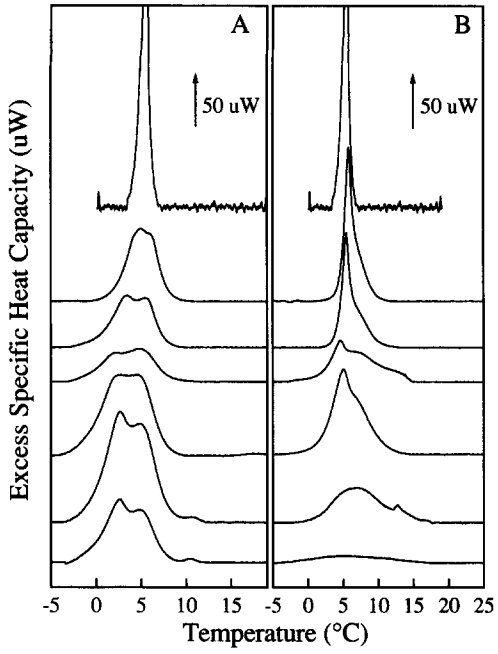

Fig. 2. Representative DSC thermograms of SOPC bilayers containing increasing amounts of (A) C-22 and (B) i-C10. Sterol concentrations, shown in descending order, are 0, 3, 6, 9, 12, 20, and 40 mol%. Sterol concentrations done but not shown are 15, 25 and 30 mol%.

increasing sterol concentration for the branched short side-chain sterol C-22 and branched long side-chain sterol i-C10, respectively. It is clear in both mixtures that the addition of both cholesterol analogues dramatically alters the enthalpy, transition temperature, and cooperativity of the overall SOPC gel to liquid-crystalline phase transition. Moreover, the overall endotherm for each SOPC/sterol mixture is clearly a composite of two overlapping components. This multicomponent melting behavior is exhibited by all of the SOPC/sterol mixtures examined. However, the shifts in enthalpy, transition temperature, and cooperativity of the overall endotherm vary significantly depending on the sterol. The detailed data obtained with each sterol are described in the following paragraphs.

The overall enthalpy of the SOPC main phase transition as a function of increasing sterol concentration is shown in Fig. 3A for the n-series and in Fig. 3B for the iso-series of cholesterol side-chain analogues. For the n-series sterols, the overall chain-melting phase transition enthalpy of SOPC decreases progressively towards zero by 50 mol% sterol, with the exception of n-C3, where residual enthalpy persists at high sterol levels. (We did not study n-series sterols with longer side-chains than cholesterol.) We observed a similar progressive decrease in enthalpy for SOPC bilayers containing the iso-series sterols; however, the overall chain-melting transition of SOPC persisted at sterol levels of 50 mol% with the shorter side-chain sterols C-22 and i-C5 and with the longer side-chain sterols and i-C9 and i-C10. This effect is much more pronounced for the shorter chain sterols, as indicated by the larger residual enthalpy at 50 mol% sterol. The effects of cholesterol analogues containing either linear or branched side-chains on the overall enthalpy of the gel to liquid-crystalline phase transition of the host SOPC bilayer were the same at comparable sterol concentrations.

Since the chain-melting phase transition of SOPC clearly consists of both a sharp (sterol-poor) and a broad (sterol-rich) melting component at lower sterol concentrations, we decomposed the overall endotherm to detail the sterol-dependent behavior of each component [34,38,42–44]. The variation in the enthalpy of the sharp component with sterol concentration for the n-series of cholesterol analogues is shown in Fig. 4A. With the exception of n-C7,

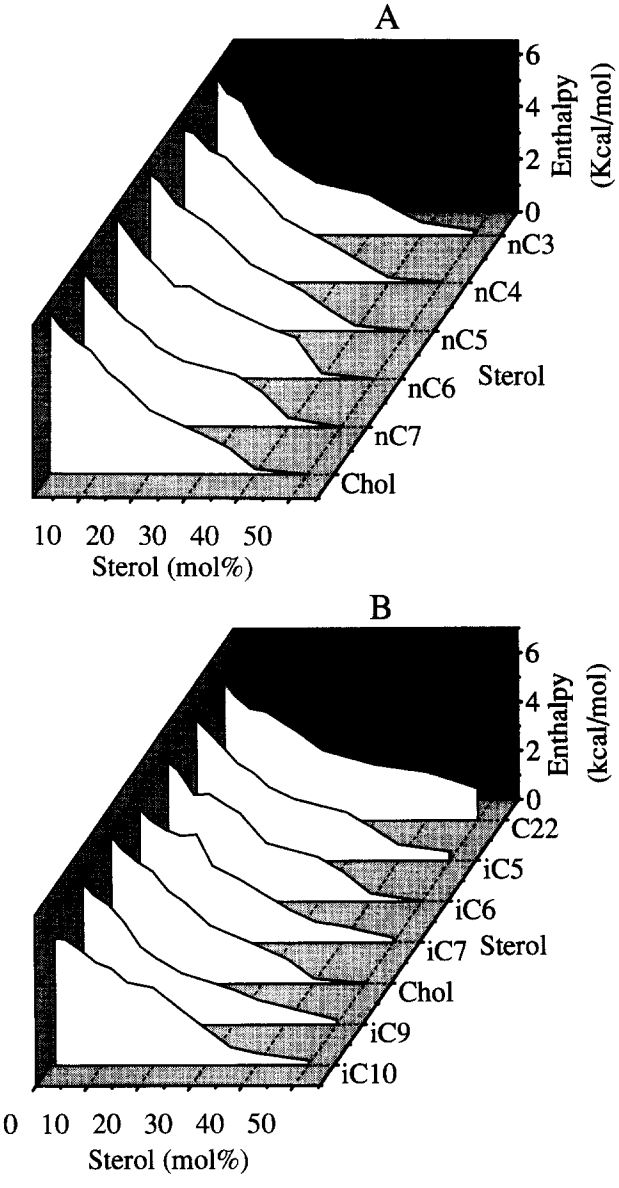

Fig. 3. Three-dimensional plot of the effect of increasing sterol concentration on the SOPC main transition overall enthalpy for the (A) n- and (B) iso-series of sterols. Sterols and their respective concentrations and are shown on the figure.

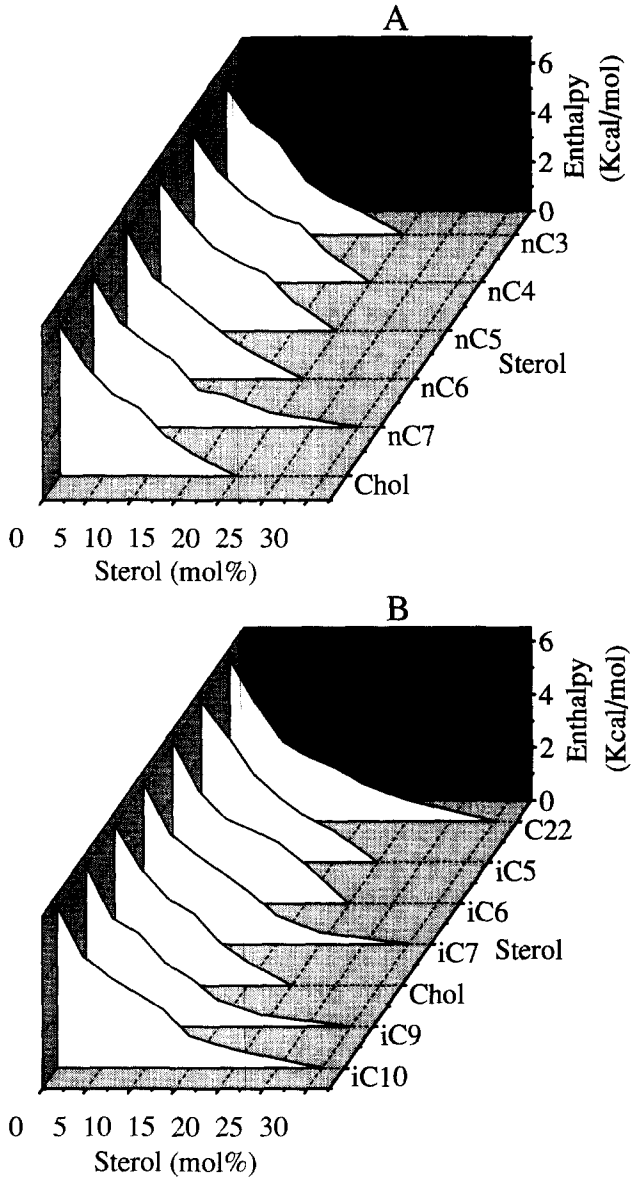

Fig. 4. Sharp component enthalpy as a function of increasing sterol concentration for the (A) n- and (B) iso-series sterols. Sterols and their respective concentrations are shown on the figure.

the enthalpy of the sharp component of each mixture decreases linearly toward zero by approx. 20 mol% sterol. The iso-series sterols, shown in Fig. 4B, generally exhibit a similar behavior; however, the enthalpy of the sharp component of SOPC bilayers containing the shortest side-chain sterol (C-22) or the longer side-chain sterols (i-C7, i-C9 and i-C10) does not approach zero until approximately 30 mol% sterol. From 1 to 25–30 mol% sterol, the enthalpy of the broad component of all of the SOPC/sterol mixtures increases in conjunction with decreasing sharp component enthalpy, thereafter decreasing toward zero at 50 mol% sterol. However, at 50 mol% sterol the broad component of the SOPC phase transition persists for the short side-chain sterols C-22, i-C5 and n-C3, and to a lesser degree the longest side-chain sterols i-C9 and i-C10.

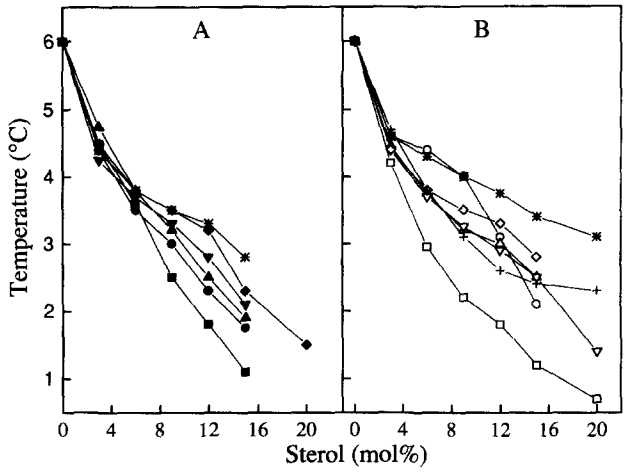

Fig. 5. Sharp component transition temperature as a function of increasing sterol concentration: (A) n-C3 (■); n-C4 (●); n-C5 (▲); n-C6 (▼); n-C7 (◆); Chol (*). (B) C-22 (□); i-C5 (○); i-C6 (△); i-C7(▽); Chol (◇); i-C9 (+); i-C10 (*). All values ± 0.30°C.

The variations in the transition temperatures of the sharp component of the SOPC chain-melting phase transition with sterol concentration are shown for both the n- and iso-series in Fig. 5A and B, respectively. In both the n- and iso-series the transition temperature of the sharp component decreases progressively as a function of increasing sterol concentration. However, the extent of the decrease depends on sterol side-chain length, with the shorter chain sterols inducing more dramatic decreases in the transition temperature. The transition temperature of the broad component of the SOPC phase transition for both the n- and iso-series exhibited similar sterol-dependent transition temperature shifts, as shown in Fig. 6A and B, respectively. The incorporation of the shorter side-chain sterols n-C3, n-C4, C-22, and i-C5 induced significant decreases in the transition temperature of the broad component, while in-

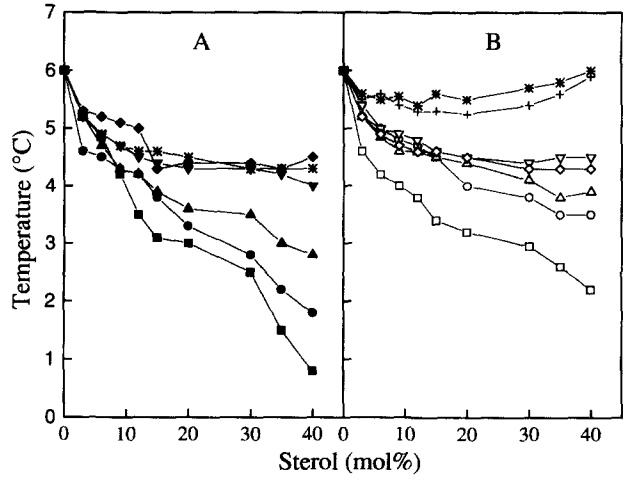

Fig. 6. Broad component transition temperature as a function of increasing sterol concentration: (A) n-C3 (■); n-C4 (●); n-C5 (▲); n-C6 (▼); n-C7 (◆); Chol (*). (B) C-22 (□); i-C5 (○); i-C6 (△); i-C7(▽); Chol (◇); i-C9 (+); i-C10 (*). All values ± 0.40°C.

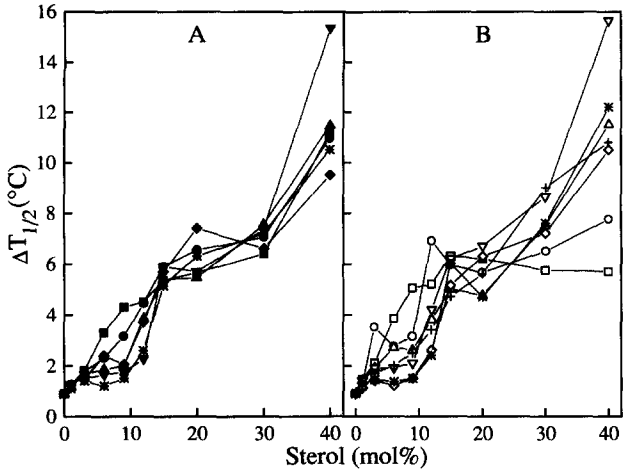

Fig. 7. Overall SOPC endotherm $\Delta T_{1/2}$ as a function of increasing sterol concentration: (A) n-C3 (■); n-C4 (●); n-C5 (▲); n-C6 (▼); n-C7 (◆); Chol (*). (B) C-22 (□); i-C5 (○); i-C6 (△); i-C7(▽); Chol (◇); i-C9 (+); i-C10 (*). Up to 30 mol%, $\Delta T_{1/2}$ values ± 0.40°C; for 40 mol% ± 1.0°C.

creased incorporation of sterols i-C9 and i-C10 slightly increased the transition temperature of the broad component. Clearly, there is no significant difference between the temperature shifts induced by the n- and iso-series sterols for a given sterol side-chain length and sterol concentration.

Lastly, the effect of sterol incorporation on the cooperativity of the overall transition of SOPC is shown in Fig. 7A (n-series) and B (iso-series). Increases in sterol incorporation dramatically increase the $\Delta T_{1/2}$ of the overall chain-melting phase transition of SOPC, and this increase in $\Delta T_{1/2}$ is independent of the iso- or n-structure. However, SOPC bilayers containing longer side-chain sterols initially exhibited relatively small decreases in cooperativity compared to shorter-chain sterols. This situation reverses with progressive increases in sterol concentration, such that by 40 mol% sterol the longer side-chain sterols exhibit $\Delta T_{1/2}$ values as much as twice that observed in short-chain sterols.

4. Discussion

The overall chain-melting phase transition of SOPC clearly exhibits multiple melting components upon the addition of each sterol, indicative of the formation of sterol-poor (sharp transition) and sterol-rich (broad transition) phospholipid regions similar to that observed previously by DSC in PC/cholesterol and DPPC/sterol mixtures [34,38,42–44]. Alterations in the length of the sterol side-chain have marked effects on the thermotropic phase behavior of each of these components. Both the sharp and broad components exhibited sterol-specific variations in enthalpy, transition temperature and cooperativity. However, while the DSC endotherms of SOPC/sterol bilayers respond to alterations in the length of the sterol side-chain, we did not observe significant differences between terminally branched (iso) and unbranched (n) side chains of a given length. Our results correlate well with those of parallel FTIR [17] and sterol oxidation studies [9], as well as prior DSC studies in PC/cholesterol and other DPPC/sterol mixtures [37,38]. In conjunction with our earlier study, we also demonstrate that SOPC–sterol interactions depend primarily on the degree of hydrophobic mismatch. The formation of a crankshaft-kink in the SOPC *sn*-2 acyl-chains (see Li et al. [40] and Wang et al. [41]), which only moderately shortens the average thickness of the SOPC bilayer, appears to permit more favorable sterol/SOPC contacts in the gel-state than would be predicted for the much shorter, boomerang-like, *sn*-2 chain with a single C9–10 *cis*-kink [39,41].

Both the sharp and broad components of the gel to liquid-crystalline phase transition of SOPC bilayers exhibit dramatic sterol-dependent shifts in transition temperature. The hydrophobic mismatch effect clearly accounts for the direction of these shifts in temperature. Briefly, hydrophobic mismatch represents the difference in mean hydrophobic length between the sterol and host phospholipid bilayer [38]. The mean hydrophobic thickness of the host PC bilayer is defined as the mean hydrophobic length of the gel and liquid-crystalline phases while the hydrophobic thickness of the sterol molecule extends roughly from the C3 of the ring system to the tip of the alkyl side-chain. Sterols with hydrophobic lengths greater than the mean hydrophobic length of the SOPC molecule will preferentially stabilize the gel state of SOPC bilayers, while sterols with short alkyl side-chains will preferentially destabilize the gel state. McMullen et al. [37,38] have shown previously that differences as small as 1.25 Å, or one CH_2 group, in hydrophobic length between the sterol and host lipid molecules produce significant shifts in the phase transition temperature of the broad component in DPPC bilayers. In DPPC bilayers, sterols containing alkyl side-chains of seven carbon atoms or more produce increases in the temperature of the broad component of the chain-melting phase transition, while sterols containing 5 carbon atoms or less produce decreases in this temperature [37]. This result indicates that the mean hydrophobic length of the DPPC molecule in the bilayer is roughly equivalent to the hydrophobic length of a sterol having an alkyl side-chain containing about six carbon atoms. In contrast, a significant decrease in the temperature of the broad component phase transition of SOPC bilayers is induced by all of the sterols studied here, except those with the longest alkyl chains (see Fig. 8). This finding confirms that the mean hydrophobic thickness of SOPC bilayers must be greater than that of DPPC bilayers. In fact, the mean hydrophobic thickness of DPPC bilayers has been determined to be 32.9 Å [37,38] while that of SOPC bilayers has been estimated at about 36 Å, assuming that the oleoyl chain assumes predominately the crankshaft-kink confor-

mation [40,41]. Thus, our experimental results conform to the predictions of the hydrophobic mismatch model of sterol–phospholipid interactions, at least qualitatively. Moreover, the fact that SOPC bilayers in this study behave as if they are significantly thicker than DPPC bilayers lends some support to the postulate that the oleoyl chains of SOPC do adapt a more extended crankshaft-kink conformation in the gel-state, as postulated earlier [40,41].

Interestingly, the effective stoichiometry of SOPC–sterol interactions also varies from that observed for DPPC/sterol systems, especially when the sterol side-chain is significantly shorter than the isooctyl chain of cholesterol. We believe that the persistence of the sharp component of the chain-melting transition of SOPC to sterol levels beyond 20 mol%, and the persistence of the broad component to sterol levels beyond 50 mol%, indicates that these sterols do not mix ideally in the SOPC bilayer. The non-homogeneous dispersion of these sterols in the gel-state SOPC bilayer subsequently lowers the effective stoichiometry of sterol–SOPC interactions. Fig. 9 clearly shows that the short side-chain sterol C-22 is less effective in reducing the enthalpy of the main transition of longer SOPC bilayers than that of DPPC bilayers. The significant hydrophobic mismatch between short side-chain sterols and the host SOPC bilayer creates an unfavorable free volume in the bilayer core which limits the miscibility of short side-chain sterols in gel-state SOPC bilayers. Non-ideal mixing of SOPC and short side-chain sterols also explains the relatively small increases in $\Delta T_{1/2}$ observed even with large increases in sterol concentration. The segregation of short side-chain sterols into sterol-rich regions lowers the number of effective SOPC/sterol contacts, permitting a relatively more cooperative chain-melting phase transition than seen for SOPC bilayers with long side-chain sterols.

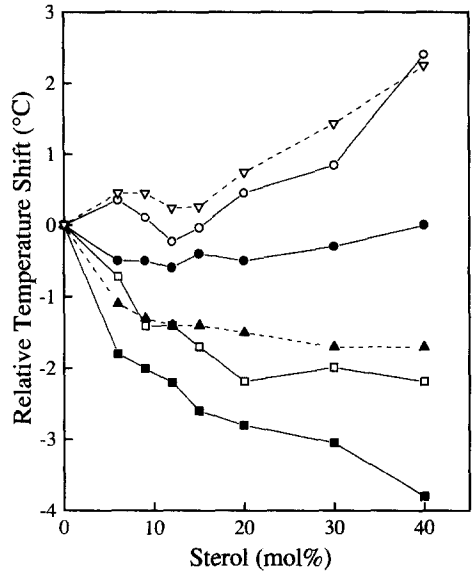

Fig. 8. Broad component transition temperature shift for cholesterol, C-22 and i-C10 sterols in SOPC and DPPC bilayers: SOPC/cholesterol (▲); SOPC/C-22 (■); SOPC/i-C10 (●); DPPC/cholesterol (▽); DPPC/C-22 (□); DPPC/i-C10 (○). All values ± 0.40°C.

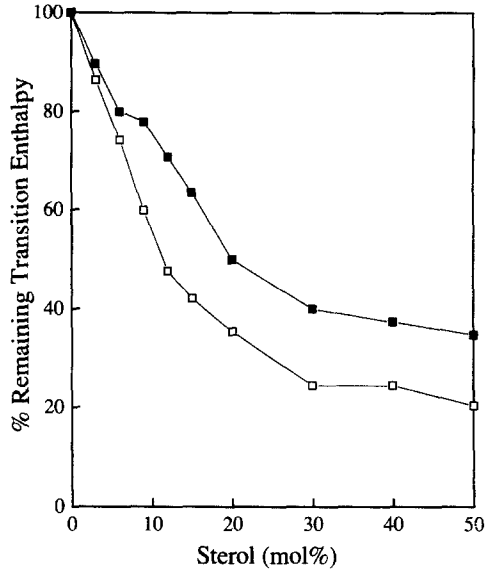

Fig. 9. Plots of the % remaining enthalpy of the overall chain-melting transition for SOPC and DPPC bilayers as a function of increasing C-22 sterol concentration: SOPC/C-22 (■); DPPC/C-22 (□). All values ± 5.0%.

In summary, we have demonstrated that the thermotropic behavior and organization of sterol-containing SOPC mixtures depends primarily on the degree of hydrophobic mismatch between the sterol and the host PC bilayer. The degree of sterol-bilayer hydrophobic mismatch in turn clearly depends on the relative lengths (number of carbon atoms) of the alkyl chain of the sterol and the fatty acid chains of the phospholipid. Although this study provides no support for preferential interactions of cholesterol or its side-chain analogues with the *cis*-monounsaturated PC hydrocarbon chains as proposed previously [39], the presence of unsaturation may be important in that it appears to determine the effective length of the PC bilayer, at least in the gel state. In particular, the crankshaft-kink conformation of the SOPC *sn*-2 acyl chain, which seems to be the most stable conformation within the bilayer, decreases the hydrophobic length of the SOPC molecule only slightly compared to its saturated analogue. Thus, any change in hydrocarbon chain structure which alters the hydrophobic thickness of the bilayer will have dramatic effects on the thermotropic behavior and organization of sterol/PC mixtures.

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