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A comparative calorimetric study of the effects of cholesterol and the plant sterols campesterol and brassicasterol on the thermotropic phase behavior of dipalmitoylphosphatidylcholine bilayer membranes

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ABSTRACT

We present a comparative differential scanning calorimetric study of the effects of the animal sterol cholesterol (Chol) and the plant sterols campesterol (Camp) and brassicasterol (Bras) on the thermotropic phase behavior of dipalmitoylphosphatidylcholine (DPPC) bilayers. Camp and Bras differ from Chol in having a C24 methyl group and, additionally for Bras, a C22 trans-double bond. Camp and especially Bras decrease the temperature, cooperativity and enthalpy of the DPPC pretransition more than Chol, although these effects are attenuated at higher sterol levels. This indicates that they destabilize gel-state DPPC bilayers to a greater extent, but are less soluble, than Chol. Not surprisingly, all three sterols have similar effects on the sterol-poor sharp component of the DPPC main phase transition. However, Camp and especially Bras less effectively increase the temperature and decrease the cooperativity and enthalpy of the broad component of the main transition than Chol. This indicates that at higher sterol concentrations, Camp and Bras are less miscible and less effective than Chol at ordering the hydrocarbon chains of the sterol-enriched fluid DPPC bilayers. Overall, these alkyl side chain modifications generally reduce the ability of Chol to produce its characteristic effects on DPPC bilayer physical properties. These differences are likely due to the less extended and more bent conformations of the alkyl side chains of Camp and Bras, producing sterols with a greater effective cross-sectional area and reduced length than Chol. Hence, the structure of Chol is likely optimized for maximum solubility in, as opposed to maximum ordering of, phospholipid bilayers.

1. Introduction

Cholesterol (Chol) is a major and essential lipid component of the plasma membranes of the cells of higher animals and is also found in lower concentrations in certain intracellular membranes in vesicular communication with the plasma membrane [1–3]. Certainly one of the primary roles of Chol is as a modulator of the physical properties and lateral organization of the plasma membrane lipid bilayer, although Chol plays other important regulatory and signaling roles in animal cells. The interaction of Chol with phospholipid monolayer and bilayer model membranes has been extensively studied with a wide range of different physical techniques [2,4–7]. The majority of those studies have utilized symmetrical chain, linear saturated PCs, although other desaturated or mixed-chain glycerophospholipids and sphingomyelin (SpM) have also been studied. This work has shown that one of the major effects of Chol incorporation on phospholipid monolayer and bilayer model membranes is a broadening and eventual elimination of the cooperative gel/liquid-crystalline phase transition and the progressive replacement of both the gel and liquid-crystalline states by a state with an intermediate degree of organization. Thus, the presence of Chol significantly increases the orientational order of the phospholipid hydrocarbon chains and decreases the cross-sectional area occupied by the phospholipid molecules, while only moderately restricting the rates of phospholipid lateral diffusion or hydrocarbon chain motion, compared to the liquid-crystalline state that would otherwise exist in animal cell membranes in the absence of Chol. The presence of Chol also increases both the thickness and mechanical strength and decreases the permeability of the phospholipid bilayer in the

Abbreviations: Chol, cholesterol (cholest-5-en-3β-ol); Camp, campesterol (cholest-5-en-24α-methyl-3β-ol); Bras, brassicasterol (cholest-5,22-dien-24β-methyl-3β-ol); Sito, sitosterol (cholest-5-en-24α-ethyl-3β-ol); Stig, stigmasterol (cholest-5,22-dien-24α-ethyl-3β-ol); Ergo, ergosterol (cholest-5,7,22-trien-24β-methyl-3β-ol); PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; POPC, 1-palmitoyl, 2-oleoylphosphatidylcholine; SpM, sphingomyelin; DSC, differential scanning calorimetry; ^{2}H NMR, deuterium nuclear magnetic resonance; $L_{\beta'}$ and L_{β}, lamellar gel phases with tilted and untilted hydrocarbon chains, respectively; $P_{\beta'}$, rippled gel phase with tilted hydrocarbon chains; L_{α}, lamellar liquid-crystalline phase; L_o, lamellar liquid-ordered phase; L_d, lamellar liquid-disordered phase; T_p/T_m, the pretransition/main phase transition temperature maximum, respectively; $\Delta T_{1/2(p)}/\Delta T_{1/2(m)}$, the width of the pretransition/main phase transition at half height, inversely related to the cooperativity of the phase transition, respectively; $\Delta H_p/\Delta H_m$, enthalpy of the pretransition/main phase transition, respectively. The superscripts "shp" and "brd" appended to these thermodynamic parameters refer to the sharp and broad components of the main phase transition of sterol- or steroid-containing DPPC bilayers, respectively.

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physiologically relevant liquid-crystalline phase. The relatively high rates of intramolecular and intermolecular motions characteristic of phospholipid model membranes in the presence of high levels of Chol, coupled with an increased hydrocarbon chain order and a decreased area compressibility, have prompted several workers to postulate the existence of a discrete lamellar liquid-ordered (L_o) phase in binary phospholipid/Chol model membranes [5,8,9]. However, as many of the physical properties of model membranes composed of Chol and a single phospholipid change smoothly and monotonically with progressive increases in Chol concentration up to 50 mol% [2,4–7], the existence of thermodynamically discrete, macroscopic lamellar L_o and liquid-disordered (Ld) phases in such binary systems has been questioned [7,10,11]. In particular, a study of thermally-induced volume changes in binary Chol/1-palmitoyl, 2-oleoylphosphatidylcholine (POPC) mixtures indicates that this system shows intermediate behavior between that expected for lateral phase separation and random mixing within one phase, due to submicroscopic demixing effects [12]. Other work has suggested that the behavior of phospholipid/Chol binary systems can be explained by the formation of various super-lattices [13] or molecular complexes [14], although evidence for these postulates is unconvincing. However, in ternary model membranes composed of Chol, unsaturated phosphatidylcholines (PCs) and natural SpMs, the specificity of the interaction of Chol for SpM can result in the formation of macroscopic L_o and L_d domains enriched in Chol and SpMs and depleted in unsaturated PCs [15], and such domains are thought to form the molecular basis for the possible existence of detergent-insoluble, Chol- and SpM-enriched lipid rafts in biological membranes [16–21]. However, co-existing L_o and L_d domains can also form in ternary Chol/unsaturated PC/saturated PC mixtures, indicating that specific Chol-SpM interactions are not required for their formation [16–21]. Even in such ternary systems, however, the existence of thermodynamically discrete L_d and L_o systems has not been detected by differential scanning calorimetry (DSC) or X-ray diffraction [22], although other biophysical techniques have demonstrated their existence. Although co-existing L_d and L_o domains can form in lipid model membranes with specific compositions, several investigators have found the evidence for the existence of relatively large, long-lived lipid rafts in biological membranes equivocal at best [23–25]. However, it is clear that the presence of Chol in biological membranes does modulate a number of different membrane functions, either directly or via its effects on the properties and lateral organization of the phospholipid bilayer [2,26–28].

Although Chol and ergosterol (Ergo) are by far the dominant sterols in animals and yeast, respectively, the membranes of photosynthetic microorganisms and plants usually contain a mixture of sterols, the majority of which have an alkyl group at C24 and some of which also have a *trans*-double bond at C22 of the alkyl side chain [1]. The structures of the two sterols studied here, campesterol (Camp) and brassicasterol (Bras), differ from that of Chol in having either a C24R methyl group (Camp) or a C24S methyl group and a *trans*-double bond at C22 (Bras) (Fig. 1). Camp is a minor sterol found in a wide variety of plants, while Bras occurs in a more restricted group of plants and in algae [1] and is used as a biomarker for marine phytoplankton [29]. As is the case for animal and yeast sterols, plant sterols have signaling and regulatory roles in addition to their structural role in maintaining membrane structure and function, and are the biosynthetic precursors of a wide variety of secondary metabolites and of the steroid hormones which regulate plant development and homeostasis [30]. In this regard, Camp is the biosynthetic precursor to the brassinosteroids, an important group of plant hormones [31].

In contrast to the relatively well studied major C24-ethyl plant sterols β-sitosterol (Sito) and stigmasterol (Stig), studies of the effects of Camp and Bras on the thermotropic phase behavior and organization of lipid bilayer model membranes are limited, particularly for the latter sterol. Moreover, the results of such studies are not always concordant. The first comparative low sensitivity DSC and X-ray diffraction study of the effects of Chol and Camp on dipalmitoylphosphatidylcholine (DPPC) bilayers indicated that the effects of both sterols on the phase behavior of this phospholipid were essentially identical, in that the incorporation

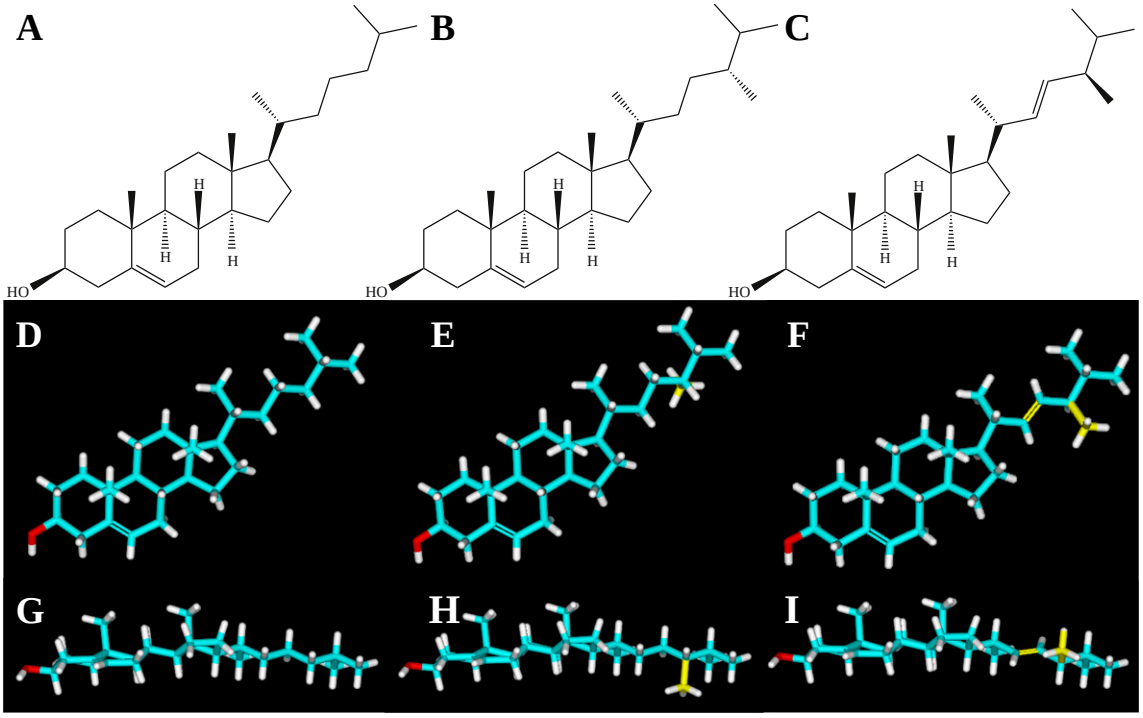

Fig. 1. *Molecular models for cholesterol, campesterol and brassicasterol.* The top figure in each panel shows views normal to the plane of the sterol ring to highlight differences between the structures of cholesterol (A), campesterol (B) and brassicasterol (C). The middle row shows views normal to the plane of the sterol ring for cholesterol (D), campesterol (E) and brassicasterol (F). The bottom row shows views parallel to the plane of the sterol ring for cholesterol (G), campesterol (H) and brassicasterol (I). The functional group at C3 is red, and the C24R methyl group in campesterol and C24S methyl group and trans-double bond at C22 for brassicasterol are in yellow. The molecules were minimized using Ds Viewer Pro 5.0 (Accelrys Software Inc., San Diego, CA).

of both sterols broadened and eventually eliminated the pretransition and main transition at 5 and 33 mol% sterol, respectively, and otherwise had similar effects on the phase transition temperatures, enthalpies and cooperativities of both phase transitions [32]. Moreover, the X-ray diffraction results demonstrated that at 33 mol%, both sterols induced a state of organization intermediate between the gel and liquid-crystalline state of DPPC alone. However, at sterol concentrations above 33 mol%, Camp formed pure crystallites in the DPPC bilayer whereas Chol did not, indicating that the solubility of Camp in this saturated phospholipid bilayer system was less than that of Chol. However, a subsequent high sensitivity DSC study of Camp and Chol incorporation into DPPC (and N-palmitoyl SpM) vesicles found that incorporation of Camp decreased the temperature of the pretransition and increased the temperature of the main phase transition to a greater and smaller extent, respectively, than Chol, indicating that Camp was slightly less effective than Chol in stabilizing the gel states of DPPC bilayers [33]. Similarly, one study using fluorescence polarization spectroscopy with a diphenyl hexatriene probe reported that Camp ordered bilayers of dimyristoylphosphatidylcholine (DMPC) and its sulfonium analog about twice as effectively as Chol [34], whereas another study using soybean PC bilayers reported that Camp was only slightly more effective than Chol in this regard [35]. The former result is surprising in that the Chol molecule is considered to be evolutionarily optimized for providing the maximum degree of order in model and animal cell membranes [36]. Moreover, a subsequent deuterium nuclear magnetic resonance (^{2}H NMR) study using small amounts of fatty acyl chain perdeuterated DMPC as a probe in soybean PC vesicles found that Camp was considerably less effective than Chol at increasing the orientational order of the DMPC hydrocarbon chains [37]. Finally, a study of POPC vesicles using resonance energy transfer and detergent solubility reported that Camp was not quite as effective as Chol in inducing the formation of ordered domains in this mixed-chain phospholipid [33]. The only biophysical study to our knowledge of Bras-phospholipid interactions is the ^{2}H NMR study discussed above, which indicated that Bras was somewhat less effective than Camp, and much less effective than Chol, in ordering the hydrocarbon chains of perdeuterated DMPC dispersed in soybean PC bilayers [37].

In the present study, we again use high sensitivity DSC to investigate the comparative effects of Camp and Chol on the thermotropic phase behavior of DPPC bilayer membranes. However, we utilize here much smaller increments in sterol concentration to more carefully investigate the effects of Camp on all of the thermodynamic parameters of the DPPC pretransition, and higher sterol concentrations to more carefully explore the effects of this sterol on the thermodynamic parameters of main phase transition. These higher sterol concentrations also allow us to determine the maximum solubility of this sterol in DPPC vesicles. Moreover, we employ a DSC protocol which allows us to detect and accurately measure the thermodynamic parameters of the broad, low enthalpy phase transitions observed in phospholipid vesicles containing high concentrations of sterol [11,38]. As well, we study for the first time the effects of Bras incorporation of the thermotropic phase behavior of DPPC bilayers.

2. Materials and methods

DPPC and Chol were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and were >99% pure, whereas Camp and Bras were supplied by Tama Biochemical Company Ltd. (Kanagawa-ken, Japan) and were >97% pure. All organic solvents were of at least analytical grade quality and were redistilled before use. Samples for hydration were prepared exactly as described previously [39]. The sterol/DPPC films were subsequently dispersed in an appropriate volume of deionized water by vigorous vortex mixing at temperatures near 55–60 °C. This procedure avoids any fractional crystallization of the sterol during sample preparation.

The samples used for the DSC experiments were prepared by dispersing appropriate amounts of the dried sterol/lipid mixture in 1 ml of deionized water. The dispersion was then degassed and either 900 μl (for studying the main phase transition) or 324 μl aliquots (for studying the pretransition) were withdrawn for DSC analyses. To ensure better resolution of the broad low-enthalpy thermotropic transitions exhibited by sterol-rich mixtures, the amount of lipid used for DSC measurements was progressively increased with the sterol content of the mixture [11,38]. Typically, samples containing 1–3 mg phospholipid were used at sterol concentrations below 5 mol%, 5–8 mg phospholipid at sterol concentrations between 5–15 mol%, and 25 mg of phospholipid at all higher sterol concentrations. DSC heating and cooling thermograms were obtained at a scan rate of 10 °C/h using either a Hart multi-cell high-sensitivity DSC instrument for the main phase transition measurements or a high-sensitivity Nano II DSC instrument for the pretransition measurements (both instruments were supplied by Calorimetry Sciences Corporation, Lindon, UT). The data acquired were analyzed and plotted with the Origin Pro 7.5 software package (OriginLab Corporation, Northampton, MA). In cases where the DSC thermograms were clearly composed of a summation of overlapping components, the midpoint temperatures, areas and widths of the components were estimated with the aid of the Origin non-linear least squares curve- and peak-fitting procedures and a custom-coded function, based on the assumption that the observed thermogram was a linear combination of components, each of which could be approximated by a reversible two-state transition at thermal equilibrium [40].

3. Results

3.1. Differential scanning calorimetry measurements of the thermotropic phase behavior of Chol/, Camp/and Bras/DPPC mixtures

In the present study, we use our previously published measurements of Chol/DPPC binary mixtures [39] as reference data for a comparison with our new DSC studies of Camp/DPPC and Bras/DPPC mixtures. Fig. 2 shows DSC heating scans of DPPC dispersions containing differing concentrations of all three sterols. The overall pattern of thermotropic phase behavior seen on heating is broadly similar to that reported previously for Chol/DPPC [11,38], for other sterol/DPPC binary mixtures [39,41–49], and for mixtures of Chol with other phospholipids [50–55], which we have studied previously. Pure DPPC heating scans show two sharp endothermic peaks centered at 35 °C and 41.7 °C, which correspond to the pretransition ($L_{\beta'}/P_{\beta'}$) and main ($P_{\beta'}/L_{\alpha}$) phase transition, respectively. Increasing the sterol concentration gradually broadens the pretransition and reduces its temperature, enthalpy and cooperativity in both cases. Similarly, in the case of the main phase transition, increasing the sterol concentration initially produces a multicomponent DSC endotherm, consisting of a sharp component that is progressively reduced in temperature, enthalpy and cooperativity, and a broad component that increases in both temperature and enthalpy, but decreases in cooperativity. Thus, with increasing sterol concentrations, the sharp component disappears as the broad component grows. However, there are subtle but significant differences in the pattern of thermal events observed in the Chol/DPPC (Fig. 2A), and in the Camp/DPPC (Fig. 2B) and Bras/DPPC (Fig. 2C) samples, which indicate that the behaviour of the latter two sterols is quantitatively different from the former and from each other, despite their broadly similar chemical structures. We will first focus on the effect of both sterols on the pretransition and then on the two deconvolved components of the main phase transition of DPPC.

3.2. Effects of Chol, Camp and Bras on the pretransition of DPPC

In order to study the disappearance of the DPPC pretransition in greater detail, we utilized sterol/DPPC mixtures in which sterol concentrations were increased in increments of 1 mol% from 0–10 mol%. Some representative DSC heating thermograms illustrating the effects of these incremental increases in Chol, Camp and Bras concentrations are

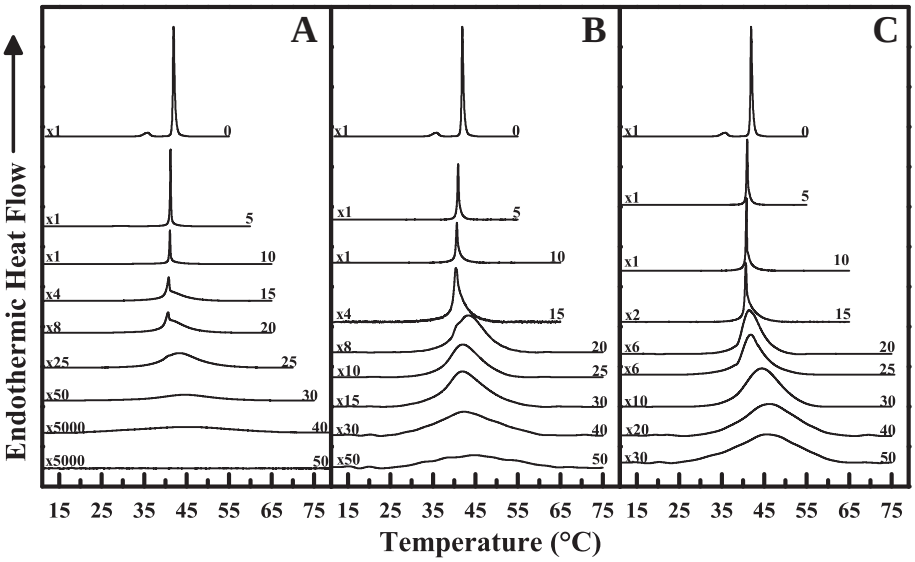

Fig. 2. *DSC thermograms illustrating the effect of cholesterol (A), campesterol (B) and brassicasterol (C) on the gel/liquid-crystalline phase transition of DPPC.* The thermograms shown were acquired at the sterol concentrations (mol%) indicated on the right hand side at a scan rate of 10 °C/h and have all been normalized against the mass of DPPC used. Y-axis scaling factors are indicated on the left hand side of each thermogram.

presented in Fig. 3A, B and C, respectively, and the thermodynamic parameters derived from these three sets of calorimetric measurements are presented in Fig. 4A, B and C. As reported previously [11,38], incremental increases in Chol concentration results in a monotonic and approximately linear decrease in pretransition temperature maximum (T_p) and pretransition enthalpy (ΔH_p), and an increase in width of the phase transition at half height ($\Delta T_{1/2(p)}$) (decrease in cooperativity). In this regard, note that the pretransition persists until Chol concentrations exceed 10 mol%. In contrast, the effects of incremental increase in Camp and Bras concentrations on these thermodynamic parameters are usually nonlinear and often biphasic. For example, at lower concentrations, increases in Camp and Bras incorporation result in larger decreases in T_p than is the case for Chol, but at higher sterols concentrations, T_p's are decreased to a smaller degree, although at the highest sterol levels, increasing Camp but not Bras incorporation results in a reduction of T_p more marked than observed at lower sterol concentrations. Similarly, although incremental increases in the concentrations of all three sterols initially result in comparable increases in $\Delta T_{1/2(p)}$, this effect levels off for Camp and is attenuated for Bras at higher sterol concentrations. Finally, increases in sterol concentration initially result in somewhat and markedly greater reductions in ΔH_p for Camp and Bras, respectively, compared to Chol, However, this reduction in ΔH_p plateaus

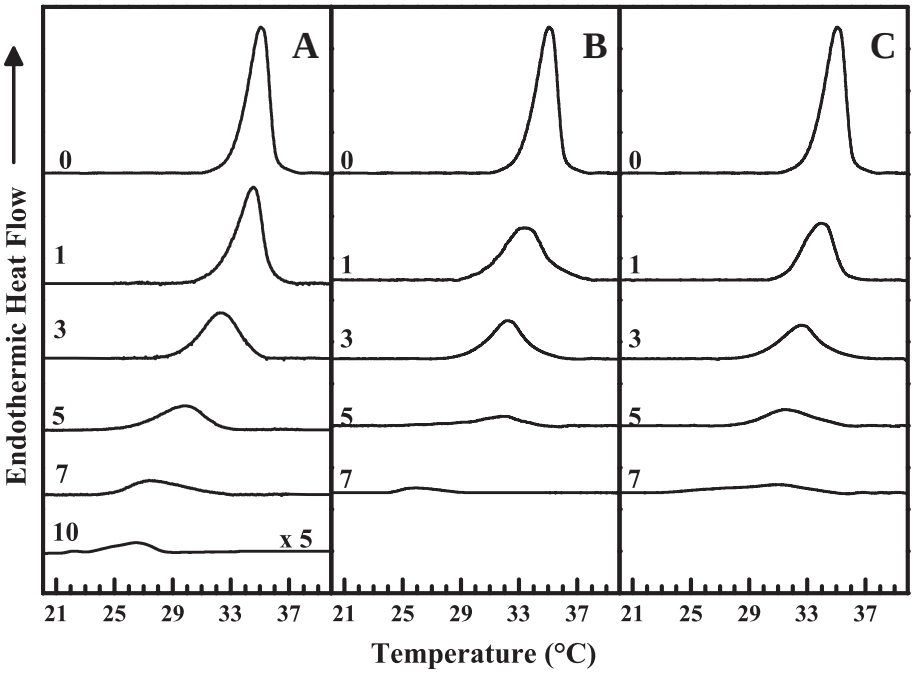

Fig. 3. *DSC thermograms illustrating the effect of cholesterol (A), campesterol (B) and brassicasterol (C) on the pretransition of DPPC.* The thermograms shown were acquired at the sterol concentrations (mol%) indicated on the left hand side at a scan rate of 10 °C/h and have all been normalized against the mass of DPPC used. Y-axis scaling factors where magnified are indicated on the right hand side of each thermogram.

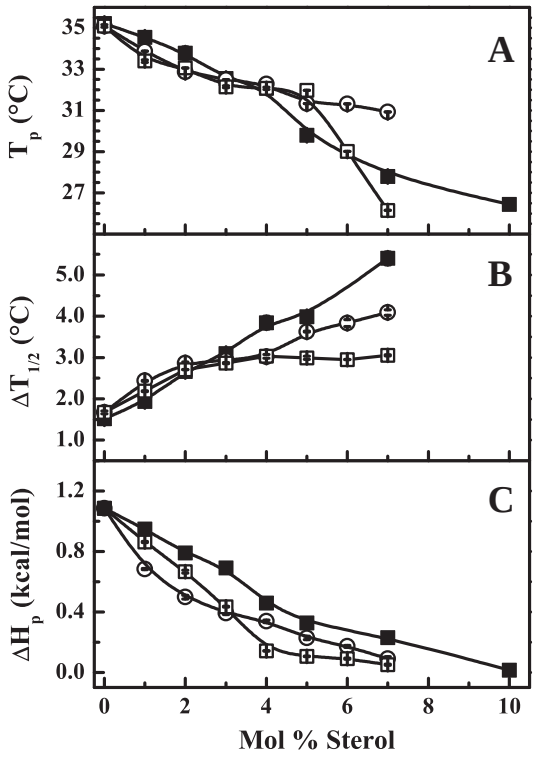

Fig. 4. *Effect of increases in sterol concentration on the T_p, $\Delta T_{½(p)}$, ΔH_p of the pretransition of DPPC:* cholesterol/DPPC (■), campesterol/DPPC (□) and brassicasterol/DPPC (○) mixtures. The error bars were typically equal to, or smaller than, the size of the symbols.

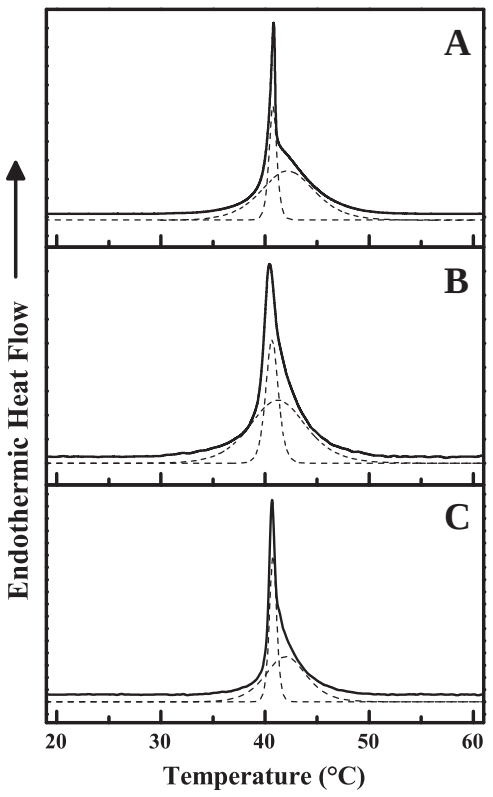

Fig. 5. *Illustration of the results typically obtained in our peak-fitting deconvolution analyses of the DSC thermograms exhibited by cholesterol-containing (A), campesterol-containing (B) and brassicasterol-containing (C) DPPC bilayers.* All thermograms are from samples containing 15 mol% sterol and acquired at a scan rate of 10 °C/h. To facilitate visibility, the fitted curves are slightly displaced along the y-axis.

at higher concentrations of Camp and is reduced at higher concentrations of Bras. Note as well that the DPPC pretransition is abolished above sterol levels above 7 mol% for both plant sterols. These results suggest that although all three sterols generally reduce the thermal stability of the gel states of DPPC and decrease the energetics and cooperativity of the transition between these gel states, the molecular mechanisms by which effects are produced must differ between each sterol, and that these mechanisms change with sterol concentration for Camp and Bras.

3.3. Effects of Chol, Camp and Bras on the main phase transition of DPPC

Our previous work on mixtures of Chol [38] and other sterols with DPPC [39,41–49] and of Chol with other phospholipids [50–55], indicates that at lower sterol concentrations, the main phase transition of DPPC consists of overlapping sharp and broad components, due to DPPC hydrocarbon chain-melting phase transitions arising from coexisting sterol-poor and sterol-rich domains, respectively. As illustrated in Fig. 5A, B and C, this is also true of the Camp/DPPC and Bras/DPPC mixtures studied here. We present below an analysis of the thermodynamic parameters associated with the sharp and broad components of the DPPC main phase transition.

3.4. Effects of Chol, Camp and Bras on the sharp component of the main phase transition of DPPC

The main transition temperature maximum (T_m), $\Delta T_{1/2(m)}$ and ΔH_m values of the sharp component of the main phase transition for mixtures of Chol/DPPC, Camp/DPPC and Bras/DPPC, as a function of sterol concentration, are shown in Fig. 6A, B and C, respectively. The T_m for all three sterol/DPPC mixtures initially decreases more rapidly with increasing sterol concentrations at lower sterol levels and less rapidly at higher sterol concentrations, where it tends to level off. Although Chol incorporation produces a slightly smaller decrease in T_m than do Camp and Bras, this decrease is small in each case (<1 °C). This result indicates that the incorporation of each sterol decreases the thermal stability of the gel state of the sterol-poor DPPC domains only slightly. In contrast, the $\Delta T_{1/2(m)}$ values of the sharp component change little at low sterol concentrations but increase markedly at higher sterol levels, particularly for Bras. Thus all three sterols have little effect on the cooperativity of the sharp component of the main phase transition of DPPC at low sterol concentrations but decrease it substantially at higher sterol levels. Finally, the ΔH_m values of the sharp component decrease more markedly at lower sterol concentrations and somewhat less markedly at higher sterol levels, and in all three cases the sharp component disappears entirely at sterol concentrations above 20 mol%. Overall, these results indicate that the incorporation of all three sterols have similar effects on the thermodynamic parameters associated the sharp component of the main phase transition of DPPC.

3.5. Effects of Chol, Camp and Bras on the broad component of the main phase transition of DPPC

The T_m, $\Delta T_{1/2(m)}$ and ΔH_m values associated with the broad component of the DPPC main phase transition, as a function of increasing Chol, Camp and Bras concentrations, are shown in Fig. 6D, E and F, respectively. In contrast to the similar effects of the incorporation of these three sterols on the sharp component of the main phase transition, the effects of each sterol on the thermodynamic parameters of the broad component of the main phase transition of DPPC differ significantly. In particular, while the incorporation of higher levels of Chol and Camp increases the T_m of the broad component significantly (~5 °C), the effect of Bras incorporation on T_m is considerably smaller, particularly at intermediate sterol levels. This result indicates that Chol and Camp are somewhat more effective at increasing the thermal stability of the gel phase in the sterol-rich DPPC domains than is Bras. Moreover, although the

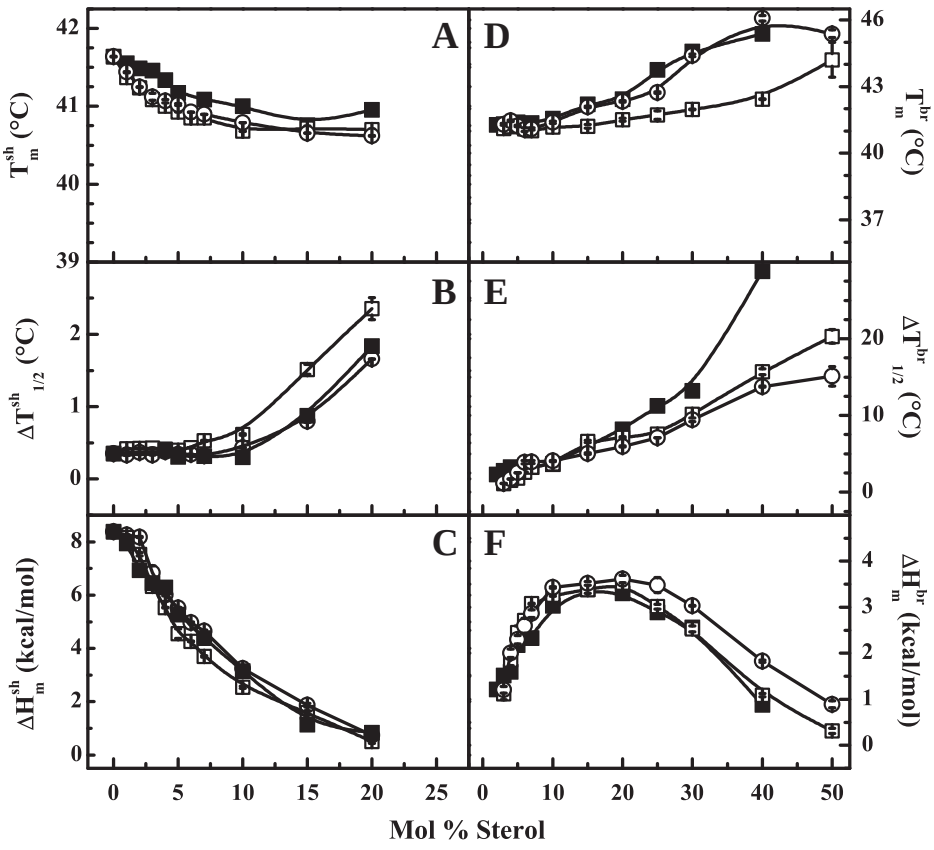

Fig. 6. *Thermodynamic parameters (T_m, $\Delta T_{½(m)}$, ΔH_m) for the deconvolved sharp (sh) (A–C) and broad (br) (D–F) components obtained from the DSC heating thermograms of sterol/DPPC mixtures:* cholesterol/DPPC (■), campesterol/DPPC (□) and brassicasterol/DPPC (○) mixtures as a function of sterol concentration, acquired at scan rate of 10 °C/h. The error bars were typically equal to, or smaller than, the size of the symbols.

incorporation of all three sterols initially increases the $\Delta T_{1/2(m)}$ values comparably at lower sterol concentrations, at higher sterol levels Chol is increasingly more effective than Camp and especially Bras in decreasing the cooperativity of the broad component of the DPPC main phase transition. Finally, at lower sterol concentrations, the incorporation of all three sterols initially causes an increase in the ΔH_m values of the broad component of the DPPC main phase transition, followed by a decrease at higher sterol levels. However, Chol is more effective in reducing the ΔH_m values of the broad component than are Camp and particularly Bras at all sterol levels. Note that the broad component of the DPPC main phase transition disappears at 50 mol% Chol incorporation, while the broad component persists at sterol concentrations of 50 mol% in the case of Camp and especially of Bras, which exhibit residual ΔH_m values of 0.3 and 0.9 kcal/mol, respectively, at the highest sterol levels tested. These $\Delta T_{1/2(m)}$ and ΔH_m results indicate that Camp and particularly Bras are less miscible in DPPC bilayers at high sterol levels than is Chol.

4. Discussion

Our comparative high-sensitivity DSC study of the effects of the progressive incorporation of Chol and the two plant sterols Camp and Bras on the pretransition of DPPC vesicles indicates that although each of these sterols produces qualitatively similar effects, these effects exhibit significant quantitative differences. In particular, although the incorporation of all three sterols progressively decreases the pretransition temperature, the presence of Chol reduces the pretransition temperature to a smaller extent than the presence of Camp and Bras, particularly at lower sterol concentrations. This result indicates that the presence of Camp and especially Bras decreases the overall thermal stability of the two low temperature gel phases of DPPC to a greater extent than does Chol. This result agrees with the findings of a previous high- sensitivity DSC investigation of the effects of the incorporation of Chol and Camp into DPPC and N-palmitoyl SpM vesicles [33]. Similarly, although the presence of all three sterols produces roughly comparable decreases in the cooperativity of the pretransition at lower sterol concentrations, Chol produces much greater decreases in cooperativity than Camp and Bras at higher sterol levels. As well, at higher sterol concentrations, the presence of Chol produces smaller decreases in the enthalpy of the pretransition compared to Camp and Bras, such that the pretransition persists to a Chol concentration of 10 mol%, whereas the pretransition persists only to sterol concentrations of 7 mol% in the case of Camp and Bras incorporation. These latter two results indicate that Camp and Bras are less miscible in gel state DPPC bilayers than is Chol. This finding may appear to differ from the conclusions reached in previous low-sensitivity and high-sensitivity DSC studies, where it was reported that both Chol and Camp abolished the pretransition of DPPC vesicles at sterol concentrations above 5 mol% [32,33]. However, in the former low-sensitivity study, the DSC instrument was incapable of detecting broad, poorly energetic phase transitions, and in the high-sensitivity DSC study, increments in sterol concentrations of 5 mol% were utilized, so that the exact sterol concentration at which the pretransition disappears could not be determined. Thus, these previous results are not really in conflict with the present findings.

An earlier X-ray diffraction study also reported that the pretransition of DPPC bilayers was abolished above a sterol concentration of 10 mol% in Chol/DPPC binary mixtures [56], in agreement with the present DSC study. This X-ray diffraction study also found that the abolition of the pretransition of DPPC was due to the progressive replacement of both the $L_{\beta'}$ and $P_{\beta'}$ phases, in which the all-trans hydrocarbon chains are tilted with respect to the bilayer plane, with a slightly disordered L_{β}-like phase in which the DPPC hydrocarbon chains are not tilted. This shift in

gel phases occurs because the progressive insertion of Chol molecules, with their small polar headgroups but large steroid nucleus, into the bilayer increases the space available to the relatively larger polar headgroups of DPPC and their smaller all-trans hydrocarbon chains, thus relieving the intrinsic mismatch in cross-sectional areas which is ultimately responsible for hydrocarbon chains tilting in the gel state. This effect is augmented by the small disordering effect of Chol incorporation on the all-trans hydrocarbon chains of the adjacent DPPC molecules in the bilayer. As in our previous work with other sterol/DPPC binary mixtures [57], we ascribe the greater decrease in the thermal stability of the gel phases of DPPC resulting from Camp and Bras incorporation, and their enhanced ability to abolish the pretransition of DPPC in comparison to Chol, to their greater disordering of the hydrocarbon chains of adjacent DPPC molecules and to their larger intrinsic cross-sectional areas [33,37].

Our comparative DSC results indicate that incorporation of each of the three sterols studied here reduces the T_m, $\Delta T_{1/2(m)}$ and the ΔH_m of the sharp component of the DPPC main phase transition to a very similar degree. In this regard, we have also reported comparable results for mixtures of DPPC with a wide variety of other sterols [57]. Since the sharp component of the main phase transition is due to the hydrocarbon chain melting of sterol-poor domains of DPPC, the relative insensitivity of this component of the main phase transition to the small changes in the chemical structure of the sterol molecule is not surprising. Only when larger changes in the structure of the sterol molecule are made are greater decreases in T_m, increases in $\Delta T_{1/2(m)}$ and decreases in ΔH_m observed than is the case for mixtures of Chol, Camp and Bras with DPPC [57].

In contrast, our DSC results indicate that the progressive incorporation of each of these three sterols produces somewhat different effects on the broad component of the main phase transition of DPPC. This general result is not unexpected, as the broad component of the main phase transition of DPPC arises from the hydrocarbon chain melting of sterol-rich DPPC domains, and has been observed in all of our sterol/DPPC studies to date [57]. Specifically, both Chol and Camp incorporation progressively increase the T_m of this component to a greater extent than Bras, indicating that the former two sterols are more effective at increasing the thermal stability of the disordered gel phase of the DPPC bilayer than is the latter sterol. This result is compatible with the fact that the incorporation of Chol and Camp are more effective than ordering fluid DPPC bilayers than is Bras, as shown in the ^2H NMR study discussed earlier [37]. Also, at higher sterol concentrations, Chol incorporation results in a larger increase in $\Delta T_{1/2(m)}$ and a greater decrease in ΔH_m than does the incorporation of Camp and especially Bras. In particular, the presence of 50 mol% Chol completely abolishes the main phase transition of DPPC, while the incorporation of 50 mol% Camp or Bras results in a relatively smaller or larger residual phase transition, respectively. These latter results indicate that the lateral miscibility of Chol in DPPC bilayers is slightly greater than that of Camp and especially of Bras, respectively. This result does not agree with the DPH fluorescence polarization studies discussed earlier [34,35], where Camp solubility in DPPC bilayers was reported to be greater then Chol, but does agree with the earlier X-ray diffraction study, in which the solubility of Chol was reported to be greater than that of Camp [32]. We believe that our present DSC and others previous X-ray results are more reliable, as these thermodynamic and structural techniques, respectively, are more direct and do not rely on the introduction of an extrinsic fluorescent probe, which has a chemical structure and physical properties which are very different from the lipid molecules being studied and which are known to perturb the DPPC bilayer (see [27] and references therein).

The fact that Camp is both more effective than Bras in ordering both gel and fluid state DPPC bilayers and is more soluble in such bilayers, is compatible with a number of comparative biophysical studies of the C24 ethyl plant sterols Sito and Stig. As with the C24 methyl plant sterols Bras and Camp, Stig differs from Sito only in the presence of a trans-double bond at C22. The presence of this C22 trans-double bond has also been reported to reduce the ability of Stig relative to Sito to order fluid DPPC bilayers [35,37], to induce larger, more thermally stable and less detergent-soluble ordered domains in POPC bilayers [33], and to decrease sterol solubility in soy PC model membranes [35]. Thus, the introduction of a trans-double bond at C22 generally reduces the ability of C24 alkyl-substituted plant and algal sterols to produce Chol-like effects in phospholipid bilayer membranes. However, whether the C24 methyl or the ethyl substitution is more disruptive of sterol-phospholipid interactions is not clear at present. For example, Camp has been reported to order the hydrocarbon chains of fluid PC bilayers to a greater [34] or to a lesser [36,37] extent than Sito, to be either more [35] or less [32] laterally miscible in such bilayers, and to be either more or less effective in inducing ordered domains in POPC bilayers, depending on the criteria for domain formation being employed (domain size, thermal stability, or detergent insolubility) [33]. However, the smaller size of the methyl as compared to the ethyl substitution at C24 might suggest that Camp would generally have effects on phospholipid bilayers more similar to Chol than would Sito. Although as noted above, the various comparative biophysical studies of the effects of Camp and Sito on phospholipid bilayers are not in agreement, it is interesting to note that in sterol auxotrophic mammalian cells, Camp is able to partially replace the growth requirement for Chol whereas Sito cannot, and Bras or Stig are also ineffective [58,59]. These results suggest both that the C24 methyl substitution is less disruptive of the presumably optimal Chol-phospholipid bilayer interactions than is the C24 ethyl substitution, and that the additional presence of the C22 trans-double bond further reduces the magnitude of these interactions. However, the introduction of a trans- (but not a cis-) double bond at C22 or C24 of the isooctyl side chain of Chol does not reduce its ability to substitute for Chol itself in supporting the growth of sterol-auxotrophic mammalian cells [58,60], indicating that a trans-double bond in the alkyl side chain only reduces cell growth in the presence of an alkyl substitution at C24. Although the growth-promoting activities of these sterols seem to correlate generally with their biophysical effects on phospholipid bilayers, one should keep in mind that as discussed earlier, animal cell growth can be influenced by the biochemical as well as by the biophysical properties of these sterols.

A comparison of the present DSC results for Bras/DPPC binary mixtures with those that we previously reported for Ergo/DPPC mixtures [45] may be instructive, Ergo being the predominant sterol in yeast and fungi [1]. Ergo, like Bras, contains a methyl group at C24 and a trans-double bond at C22, but unlike Bras, also contains an additional double bond at C7 of ring B. The presence of the additional conjugated double bond in ring B would be expected to produce a more planar B ring and a generally flatter conformation of both rings B and C (see [45] and references therein). However, the conformations of the chemically identical alkyl side chains should be at least generally similar in both sterols. In general, the overall effects of Bras and Ergo on the pretransition of DPPC are almost identical, with both sterols progressively decreasing T_p, increasing $\Delta T_{1/2(p)}$ and reducing ΔH_p in a similar fashion, and in both cases the pretransition is abolished above 7 mol% sterol. The only significant difference between the two sterol/DPPC binary mixtures is that Ergo incorporation results in a significantly greater reduction of T_p than Bras, indicating that it is more disruptive to packing in the DPPC gel states. Erg and Bras also have very similar effects on the T_m and $\Delta T_{1/2(m)}$ of the sharp component of the DPPC main phase transition, but Ergo decreases ΔH_m to a greater extent, such that the sharp component of the main phase transition is abolished above an Ergo concentration of only 15 mol%, whereas it persists to above 22 mol% in Bras (and Camp and Chol)/DPPC mixtures. Similarly, Ergo and Bras incorporation produces very similar increases in the T_m and $\Delta T_{1/2(m)}$ and very similar decreases in the ΔH_m of the broad component of the DPPC main phase transition, except that Ergo is less soluble in the DPPC bilayer than Bras. In our original comparative DSC and FTIR spectroscopic of Chol and Ergo, we rationalized the increased ability of Ergo in

comparison to Chol to abolish the pretransition of DPPC and its reduced miscibility in fluid DPPC bilayers to its greater intrinsic cross-sectional area and reduced effective length. More specifically, we suggested that this increase in cross-sectional area and decrease in length of the Ergo molecule in comparison to Chol is due to a difference in the dominant conformers of these two sterols (see [45] and references therein). Specifically, Chol has a single dominant conformer in which the more flexible saturated isooctyl side chain assumes a fully extended, all-trans conformation, such that the steroid ring system and the alkyl side chain are essentially coplanar. This conformation provides Chol with its smallest cross-sectional area and maximum molecular length. In contrast, due to the lack of free rotation about the trans-double bond at C22 and the presence of an additional methyl group at C24, Ergo appears to exist in two major conformers. In one, the alkyl side chain is largely extended but is slightly bent relative to the plane of the steroid ring system, while in the other the alkyl side chain is less extended and forms a sharper angle with the steroid ring system. Thus, the effective area of Ergo would be greater and its effective length would be less than in the case for Chol. It would seem that similar arguments should apply to the preferred conformations of Bras, although this should be confirmed by experimental and molecular modeling studies. It would also be of interest to extend these studies to Camp, which contains a methyl group at C24 but lacks the trans-double bond at C22, although it seems that Camp would likely assume a more Chol-like conformation, which would explain most of the present results. Presumably, the small differences in the effects of Ergo and Bras on the thermotropic phase behavior of DPPC bilayers can be ascribed to the presence of the additional double bond in ring B in the former.

Finally, we point out again that every sterol and steroid we have examined thus far, now including the plant and algal sterols Camp and Bras, exhibits a reduced lateral miscibility in DPPC bilayer membranes as compared to Chol. This finding indicates that the structure of Chol may actually be optimized for maximal solubility, at least in saturated phospholipids, and not necessarily for maximum hydrocarbon chain ordering or maximum ordered domain formation, since a few other natural sterols are actually slightly more effective than Chol, although Chol is certainly more potent in these regards than most sterols or Chol analogs (see [45,61,62] and references therein). Nevertheless, since plant cell membrane phospholipids and particularly glycolipids are typically enriched in polyunsaturated fatty acids, it is possible that plant sterols might have a greater effect on the lipid bilayers of their host cell membranes than Chol would have. This possibility should be investigated in future studies.

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