Biochimica et Biophysica Acta 1848 (2015) 1629–1638

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

A comparative calorimetric and spectroscopic study of the effects of cholesterol and of the plant sterols β-sitosterol and stigmasterol on the thermotropic phase behavior and organization of dipalmitoylphosphatidylcholine bilayer membranes

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ARTICLE INFO

Article history:
Received 5 January 2015
Received in revised form 8 April 2015
Accepted 15 April 2015
Available online 22 April 2015

Keywords:
Cholesterol
β-sitosterol
Stigmasterol
Brassicasterol
Campesterol
Sterol-phospholipid interactions

ABSTRACT

We performed comparative DSC and FTIR spectroscopic measurements of the effects of β-sitosterol (Sito) and stigmasterol (Stig) on the thermotropic phase behavior and organization of DPPC bilayers. Sito and Stig are the major sterols in the biological membranes of higher plants, whereas cholesterol (Chol) is the major sterol in mammalian membranes. Sito differs in structure from Chol in having an ethyl group at C24 of the alkyl side-chain, and Stig in having both the C24 ethyl group and *trans*-double bond at C22. Our DSC studies indicate that the progressive incorporation of Sito and Stig decrease the temperature and cooperativity of the pretransition of DPPC to a slightly lesser and greater extent than Chol, respectively, but the pretransition persists to 10 mol % sterol concentration in all cases. All three sterols produce essentially identical effects on the thermodynamic parameters of the sharp component of the DPPC main phase transition. However, the ability to increase the temperature and decrease the cooperativity and enthalpy of the broad component decreases in the order Chol > Sito > Stig. Nevertheless, at higher Sito/Stig concentrations, there is no evidence of sterol crystallites. Our FTIR spectroscopic studies demonstrate that Sito and especially Stig incorporation produces a smaller ordering of the hydrocarbon chains of fluid DPPC bilayers than does Chol. In general, the presence of a C24 ethyl group in the alkyl side-chain reduces the characteristic effects of Chol on the thermotropic phase behavior and organization of DPPC bilayer membranes, and a *trans*-double bond at C22 magnifies this effect.

1. Introduction

Animal and fungal cell membranes normally contain only a single major class of sterol, in these cases cholesterol (Chol) and ergosterol (Ergo), respectively [1]. However, plant cell membranes typically contain a complex mixture of sterols, of which β-sitosterol (Sito) and stigmasterol (Stig) are usually the most abundant and widespread [1]. Sito differs in structure from Chol only in having an ethyl group at C24 of the alkyl side chain, and Stig differs from Chol in having both the ethyl group at C24 and a *trans*-double bond at C22, thus having an alkyl side chain similar in structure to that of Ergo, except that a methyl rather than an ethyl group is substituted at C24 (Fig. 1). These and other plant sterols, however, are also found in mammalian tissues, since they are often the major dietary sterols [2]. In this regard, Sito, alone or when combined with other plants sterols such as Stig, is known to reduce blood Chol levels, most likely by blocking Chol absorption [3,4] and thus is anti-atherogenic [5]. Moreover, plant sterols may be important for the proper functioning of the mammalian immune system [6] and as cancer preventative or anti-tumor agents, and Sito in particular has been reported to have anti-inflammatory and antimicrobial activities [7–9]. Thus, the study of plant sterols is important, both from the

Abbreviations: Chol, cholesterol (cholest-5-en-3β–ol); Sito, β-sitosterol (cholest-5-en-24α-ethyl-3β-ol); Stig, stigmasterol (cholest-5,22-dien-24α-ethyl-3β–ol); Camp, campesterol (cholest-5-en-24α–methyl-3β–ol); Bras, brassicasterol (cholest-5,22-dien-24β–methyl-3β–ol); Ergo, ergosterol (cholest-5,7,22-trien-24β-methyl-3β-ol); PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; SpM, sphingomyelin; N-PSpM, N-palmitoylsphingomyelin; DPPE, dipalmitoylphosphtidylethanolamine; ESR, electron spin resonance; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared; DSC, differential scanning calorimetry. $L_{\beta'}$ 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-containing DPPC bilayers, respectively

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http://dx.doi.org/10.1016/j.bbamem.2015.04.009
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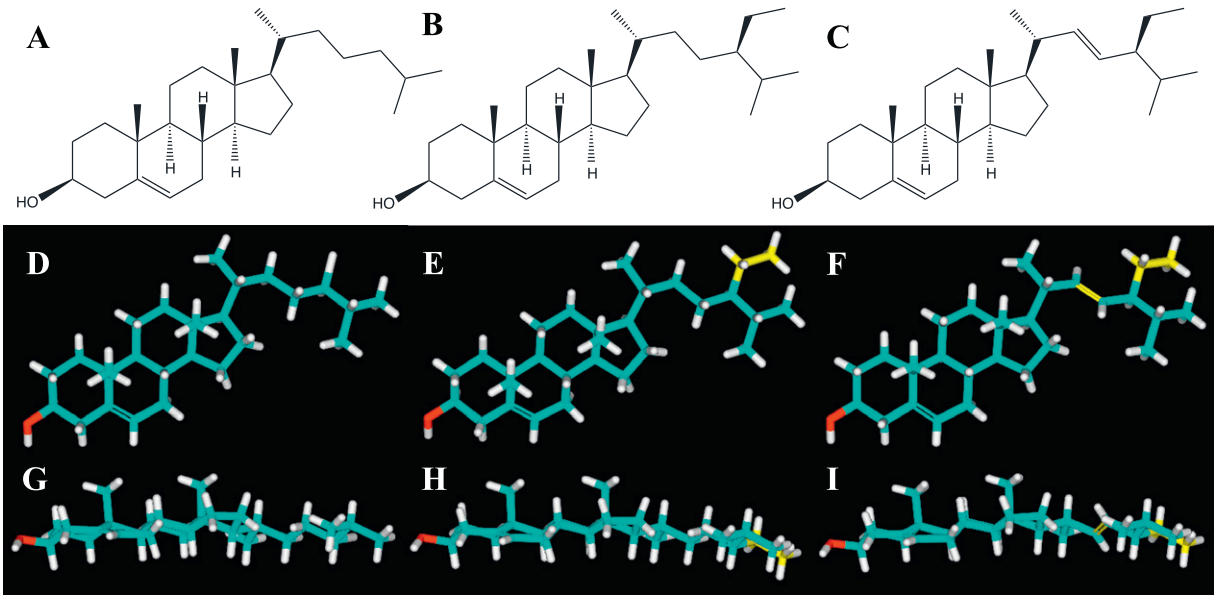

Fig. 1. Molecular models for cholesterol, β-sitosterol and stigmasterol. The top figure in each panel shows views normal to the plane of the sterol ring to highlight differences between the structures of cholesterol (A), β-sitosterol (B) and stigmasterol (C). The middle row shows views normal to the of the sterol ring for cholesterol (D), β-sitosterol (E) and stigmasterol (F). The bottom row shows views parallel to the plane of the sterol ring for cholesterol (G), β-sitosterol (H) and stigmasterol (I). The functional group at C3 is red, and the C24 ethyl group in β-sitosterol and C24 ethyl group and *trans*-double bond at C22 for stigmasterol are in yellow. The molecules were minimized using DsViewer Pro 5.0 (Accelyrs, Software Inc., San Diego, CA).

standpoint of understanding the specific structural features of sterols required for the stabilization of the structure and organization of the lipid bilayers in biological membranes, and also the metabolic and regulatory functions of sterols in eukaryotic cells.

In general, the plant sterols have not been nearly as intensively studied as Chol, but there is nonetheless a fairly substantial collection of previous papers, particularly for Sito and Stig. Thus, in lipid monolayer films, Sito and Stig have been reported to have slightly larger areas/molecule than Chol at the collapse pressure, which in turn is modestly reduced for these phytosterols relative to Chol [10–14]. Moreover, the magnitude of the monolayer condensation of sterol/POPC or sterol/DPPC binary mixtures by these three sterols was reported to decrease in the order Chol > Sito > Stig [10–16].

A number of comparative spectroscopic, structural and mechanical studies of the effects of Chol, Sito and Stig on various phospholipid bilayers have been carried out. An early ^{2}H NMR study of bilayers composed of soybean PC and low sterol levels found that the unsaturated alkyl side chain of ^{2}H-labeled Stig was more ordered than that of the saturated alkyl side chain of Sito [17], which seems counterintuitive, since Stig produces less ordered soybean PC bilayers than Sito, as determined by fluorescence anisotropy [18] and ^{2}H NMR [19,20]. Similarly, a neutron diffraction study of the effects of these two plant sterols on soybean PC bilayers showed that Sito incorporation leads to a progressive increase in bilayer hydrophobic thickness whereas the incorporation of Stig did not, and that Sito and Stig were soluble to levels of only 16 and 30 mol %, respectively [21]. Unfortunately, in none of these studies on soybean PC bilayers was the effect of Chol itself determined, precluding a direct comparison of the primary animal and plant sterols. However, a subsequent study of DMPC bilayers by small angle X-ray scattering and dilatometry and of POPC bilayers by ultrasound velocity [22], as well as steady state fluorescence anisotropy studies of DPPC bilayers [23,24], revealed that the ordering of the PC hydrocarbon chains by various sterols, and modulation of bilayer thickness and elasticity, decrease in the order Chol > Sito > Stig. Moreover, in the two former studies [22,23], a more limited solubility of Sito and especially Stig in DMPC bilayers was reported. However, a previous ^{2}H and ^{31}P NMR study of Chol and stigmastanol (the fully saturated analog of Stig) in POPC and DOPC bilayers found both sterols to be freely miscible up to 50 mol % sterol [25], suggesting that the presence of a double bond in the steroid nucleus and alkyl side chain may reduce Stig solubility in phospholipid bilayers. Moreover, although Chol restricted the flexing motions of the fatty acyl chains to a greater extent than stigmastanol, neither sterol affected the conformation or dynamics of the polar headgroup [25]. In contrast, a small angle X-ray diffraction and Fourier transform infrared (FTIR) spectroscopic study of a series of dimonounsaturated PCs of various chain lengths found no differences in the effect of Chol and Sito on increasing either bilayer thickness or the number of additional water molecules located in the polar headgroup after sterol addition, although the ability of various sterols to order these model membranes did decrease in the order Chol > Sito > Stig [26]. In contrast, Chol, Sito and Stig were reported to have similar effects on the polarity and molecular mobility of the hydrophilic/hydrophobic interfacial (glycerol backbone) region of various PC molecular species as probed by fluorescence spectroscopy [24,27]. Thus, the above studies show that the potency of these sterols in increasing phospholipid fatty acyl chain order, increasing bilayer thickness and decreasing bilayer mechanical flexibility decrease in the order Chol > Sito > Stig, although any differential effects of these three sterols on the glycerol backbone and polar headgroup regions of the phospholipid molecule seem to be at least markedly attenuated.

A number of studies of the effects of Chol and plant sterol incorporation on the passive permeability of phospholipid bilayer membranes to water, nonelectrolytes and ions have also been carried out. The reduction in the permeability of egg PC vesicles to glycerol, glucose and Rb^{+} was originally reported to be greatest for Chol and less in the plant sterols tested [28], and similar results were reported for water permeability in various synthetic and natural PCs, with Chol being much more effective in reducing water permeability than Sito, which was in turn slightly more effective than Stig [29]. Similar results were reported for the rates of glucose leakage from DPPC vesicles [28]. In contrast, the water permeability of soybean PC vesicles was reported to be more strongly reduced by Sito than by Chol, with Stig having essentially no effect, leading to the suggestion that Sito is more effective than Chol in the highly unsaturated phospholipids of plant cell membranes [29]. Finally, it has been reported that the spontaneous rate of exchange of Sito between various phospholipid vesicles is much lower than that of

Chol, a result the authors ascribe to the stronger van der Waals forces between the larger Sito molecule and the phospholipid hydrocarbon chains, and to the reduced aqueous solubility of Sito compared to Chol [30].

The relative abilities of Chol and the major plant sterols to induce the formation of the lamellar liquid-ordered phase (L_o) phase ("lipid rafts") in ternary mixtures of unsaturated phospholipid/sphingolipid/sterols have been investigated by a number of groups using several different techniques. The original fluorescence quenching studies indicated that the L_o phase formed by Chol was more thermally stable than those formed by Sito or Stig [31]. However, Sito and Stig appeared to promote domain formation to a slightly greater extent than Chol, and the L_o phase formed by the plant sterols were more resistant to detergent solubilization than that formed by Chol [31]. However, a subsequent study utilizing DSC, fluorescence energy transfer and detergent solubility assays in DPPC/SpM/sterol mixtures demonstrated that Sito and Stig formed less extensive and less stable L_o phase domains than Chol, and that the L_o domains formed in POPC/sterol binary mixtures were more easily solubilized by Triton X-100, indicating that they interact less favorably with this phospholipid [32]. A later study, utilizing pulsed field gradient NMR spectroscopy to determine lipid lateral diffusion coefficients in bilayers composed of DOPC/egg SpM/sterol, also indicated that the L_o domains formed by Chol were more stable than those formed by Sito, whereas Stig did not induce the formation of an L_o phase [33]. Lastly, it was shown by ^{2}H-NMR than Chol induced less stable L_o phases in animal "lipid raft mixtures" consisting of DPPC/SpM/Chol ternary mixtures, than Sito and Stig did in plant "lipid raft mixtures" consisting of DPPC/glucosylcerebroside/sterol ternary mixtures [34]. These authors thus concluded that these two plant sterols are more effective in forming lipid rafts in plant cell membranes than Chol is in animal cell membranes. However, the plant sterols were not tested in the animal cell plasma membrane raft mixture and Chol was not tested in the plant cell plasma membrane lipid raft mixture. Moreover, as expected on first principles and as demonstrated experimentally [31], the presence of the glucosylceremide in the plant lipid raft mixture, which has a much higher gel/liquid-crystalline phase transition temperature than the SpM component of the animal lipid raft mixture, would itself produce a much more stable L_o phase than would plant SpM in the presence of a sterol molecule. Therefore, the suggestion that the plant sterols interact more strongly with plant membrane lipids than Chol remains to be confirmed.

High-sensitivity differential scanning calorimetry (DSC) is a powerful and nonperturbing thermodynamic technique which has proven of great value in studies of the effects of Chol and other sterols on the thermotropic phase behavior of glycerophospholipid and phosphosphingolipid bilayer membranes [35–38]. There have been several comparative DSC studies of the effects of Chol and the major plant sterols on the thermotropic phase behavior of PC and SpM model membranes. An early low-sensitivity DSC and X-ray diffraction study of the effects of sterol incorporation on DPPC bilayer indicated that all three sterols eliminated the cooperative gel/liquid-crystalline phase transition at 33 mol % [39], a value doubtlessly too low due to the limited sensitivity of the calorimeter employed [38]. Moreover, the X-ray diffraction data revealed that the Sito and Stig formed a separate crystalline phase at 50 mol % sterol, whereas Chol did not, indicating a reduced miscibility of the plant sterols at higher concentrations [40]. A later high sensitivity DSC study of DPPC vesicles containing up to 25 mol % sterol demonstrated that the two plant sterols produced a greater decrease in the temperatures of the sharp and broad components of the DSC endotherms, representing the hydrocarbon chain-melting transition of sterol-poor and sterol-rich DPPC domains, respectively, than did Chol [32]. A subsequent low sensitivity DSC study of the effect of lower levels of Sito on DMPC vesicles confirmed that this sterol decreased the overall gel/liquid-crystalline phase transition temperature and cooperativity, and also reduced the phase transition enthalpy by over 40% at 15 mol % sterol [41]. Finally, a high-sensitivity DSC study of sterol/egg SpM binary mixtures indicated that at sterol concentrations of 17 and 30 mol %, the magnitude of the increase in the gel/liquid-crystalline phase transition temperature, and the decrease in the phase transition enthalpy, was greater for Chol than for the two plant sterols, and slightly greater for Sito than for Stig [42]. Although these previous DSC studies are of considerable value, none have provided a full and complete comparative thermodynamic analysis for all three sterols over a full range of sterol concentrations from 0-50 mol %. We have thus reinvestigated the effects of Chol, Sito and Stig on the thermotropic phase behavior of the intensively studied DPPC bilayers, using a high sensitivity calorimeter and an experimental protocol ensuring that the broad, lower enthalpy phase transitions occurring at higher sterol levels are accurately monitored [39] and employing high purity sterol samples. Moreover, we also investigated the comparative effects of these three sterols on the organization of DPPC bilayers by FTIR spectroscopy. Overall, our results indicate that the effects of Chol and the two plant sterols on the theromotropic phase behavior and organization of DPPC membranes are somewhat different, as are the effects of Sito and Stig themselves. Finally, we have also studied the effects of the plant sterols campesterol (Camp) and brassicasterol (Bras) on the organization of DPPC bilayers by FTIR spectroscopy, in order to complement our earlier DSC studies on the compounds [43].

2. Materials and methods

The DPPC and Chol were both were >99% purity and were obtained from Avanti Polar Lipids Inc. (Alabaster, AL), whereas the Sito and Stig (>95%) were supplied by Sigma-Aldrich (St Louis, MO) and Steraloids Inc. (Newport, RI), respectively. All organic solvents were of at least analytical grade quality and were redistilled before use. Samples for hydration were prepared exactly as described previously [44]. The DPPC/sterol 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 sterol during sample preparation.

The samples used for the DSC experiments were prepared by dispersing appropriate amounts of the dried lipid:sterol mixture in 1 ml of deionized water. The dispersion was then degassed and 324 μl aliquots 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 [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 and 15 mol %, and 10–15 mg of phospholipid at all higher sterol concentrations. DSC heating and cooling thermograms were recorded with a high-sensitivity Nano II DSC (Calorimetry Sciences Corporation, Lindon, UT) operating at a scan rate of 10 °C/hr. The data acquired were analyzed and plotted with the Origin software package (OriginLab Corporation, Northampton, MA). In cases where the DSC thermograms appeared to be a summation of overlapping components, the midpoint temperatures, areas and widths of the components were estimated with the aid of 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 thermodynamic equilibrium [45].

Samples used for FTIR spectroscopic experiments were prepared by dispersing dried sterol/DPPC mixtures containing 2–3 mg of phospholipid in 50 μl of D_2O at temperatures near 55–60 °C. The paste so formed was then sealed as a thin film between the CaF_2 windows of a heatable, demountable liquid cell equipped with a 25 μm Teflon spacer. Once mounted in the sample holder of the instrument, sample temperature could be controlled between 0 and 60 °C by means of an external computer-controlled water bath. FTIR spectra were acquired with a Digilab FTS-40 spectrometer (Biorad, Digilab Division, Cambridge, MA)

or a Nicolet Magna 750 spectrometer (Thermo Scientific, Waltham MA) using data acquisition and data processing protocols, all as previously described [46].

3. Results

3.1. Differential scanning calorimetry studies of the thermotropic phase behavior of sterol/DPPC mixtures

In this study, we compare our previously published DSC studies of Chol/DPPC mixtures [38,47] with our new calorimetric results on mixtures of the plant sterols Sito and Stig with DPPC. Fig. 2 shows DSC heating scans of DPPC dispersions containing differing concentrations of Chol and of both phytosterols. The overall pattern of thermotropic phase behavior seen on heating these mixtures is broadly similar to that previously observed for a mixtures of a variety of other Chol precursors, metabolites or analogs [44,48–58], and for the fungal sterol Ergo [59] and plant sterols Camp and Bras [43] with DPPC. Pure DPPC heating scans show two sharp endothermic peaks centered at 34 °C and 41.2 °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 and enthalpy in all cases. Similarly, for 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 initially increases in both temperature and enthalpy, but decreases in cooperativity. Thus, with increasing sterol concentrations, the sharp component disappears as the broad component grows. At higher sterol levels, the enthalpy and cooperativity of the broad component decrease. However, there are distinct differences in the pattern of thermal events observed in the Chol/DPPC (Fig. 2A), Sito/DPPC (Fig. 2B) and Stig/DPPC (Fig. 2C) samples, which indicate that the behavior of the plant sterol-containing mixtures are different and more complex than that of Chol-containing mixtures. We will first focus on the effects of the incorporation of these three sterols on the pretransition and then on the two components of main phase transition of DPPC.

3.2. The effects of Chol, Sito and Stig incorporation on the pretransition of DPPC

In order to investigate the disappearance of the pretransition in greater detail, we prepared sterol/DPPC samples with a narrower range of more closely spaced sterol concentrations. DSC heating thermograms obtained from multilamellar DPPC vesicles containing increasing quantities of Chol, Sito and Stig are shown in Fig. 3A–C, respectively, and the thermodynamic parameters associated with the pretransition for Chol/DPPC, Sito/DPPC and Stig/DPPC mixtures as a function of increasing sterol concentration are presented in Fig. 4. In all cases, the progressive incorporation of all three sterols results in a monotonic and approximately linear decrease in the temperature, cooperativity and enthalpy of the pretransition of DPPC. Moreover, the rates of reduction in the pretransition temperature maximum (T_p) are almost comparable in the Chol/DPPC and Stig/DPPC mixtures, which are in turn slightly greater than that observed for the Sito/DPPC mixture (Fig. 4A). At the maximum sterol concentration at which the DPPC pretransition is still observed (10 mol %), the reduction in the T_p is about 8.5, 8.0 and 6.5 °C for Chol, Stig and Sito, respectively. Similarly, incremental increases in sterol concentration result in progressive increases in the width of the pretransition at half-height ($\Delta T_{1/2}$), although in this case the cooperativity of the pretransition decreases slightly more rapidly with increasing sterol concentration for Stig/DPPC than for Chol/DPPC and Sito/DPPC mixtures (Fig. 4B). Finally, progressive increases in sterol concentration result in comparable reductions in the pretransition enthalpy (ΔH_p) for all three mixtures and in each case, the pretransition of DPPC is abolished above a sterol concentration of 10 mol % (Fig. 4C). Thus, the incorporation of all three sterols reduces the thermal stability of the gel states of DPPC, which exist at lower temperatures, and the energetics and cooperativity of the conversion between these gel states, to a generally similar if not identical degree.

3.3. The effects of sterol concentration on the main phase transition of DPPC.

The DSC data shown in Fig. 2 indicate that at low to moderate sterol concentrations, all three sterol/DPPC mixtures exhibit asymmetric heating thermograms which consist of two overlapping thermal events.

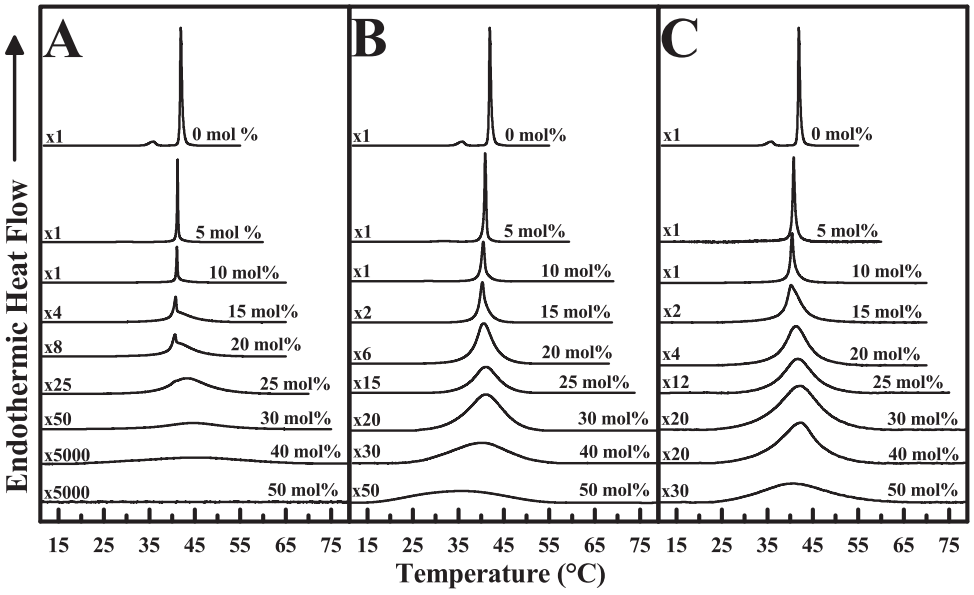

Fig. 2. DSC thermograms illustrating the effect of cholesterol (A), β-sitosterol (B) and stigmasterol (C) on the gel/liquid-crystalline phase transition of DPPC. The thermograms shown were acquired at the sterol concentrations (mol %) indicated at a scan rate of 10 °C/h and have all been normalized against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

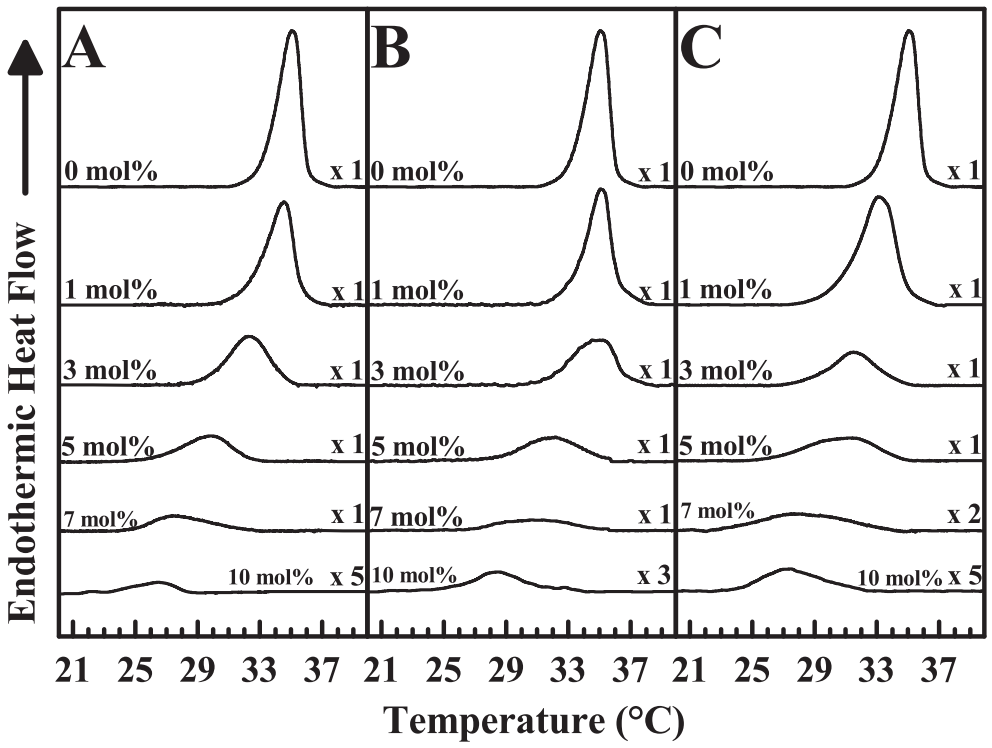

Fig. 3. DSC thermograms illustrating the effect of cholesterol (A), β-sitosterol (B) and stigmasterol (C) on the pretransition of DPPC. The thermograms shown were acquired at the sterol concentrations (mol %) indicated at a scan rate of 10 °C/h and have all been normalized against the mass of DPPC used.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

One of these components is considerably sharper than the other, its peak temperature and cooperativity decrease slightly, but its enthalpy decreases markedly with increasing sterol content. The other component is considerably broader, its midpoint temperature exhibits a more complex dependence on sterol content, and it is the only component persisting at higher sterol concentrations. This pattern of sterol concentration-dependent behavior has been observed previously in mixtures of Chol and other sterols with DPPC, and the resolved sharp and broad components have been ascribed to the differential melting of sterol-poor and sterol-rich lipid domains, respectively [38,48]. As illustrated in Fig. 5B and C, this is also the case for the Sito/DPPC and Stig/DPPC mixtures studied here. However, there are significant differences between the sterol concentration-dependent behaviors exhibited by the Chol-, Sito- and Stig-containing DPPC mixtures, especially with regard to the quantitative aspects of the sterol concentration dependence of the thermodynamic parameters associated with the underlying sharp and broad peaks (Fig. 5). We present below a detailed analysis of the effects of variations in the Chol, Sito and Stig content on the sharp and broad components seen in our DSC thermograms.

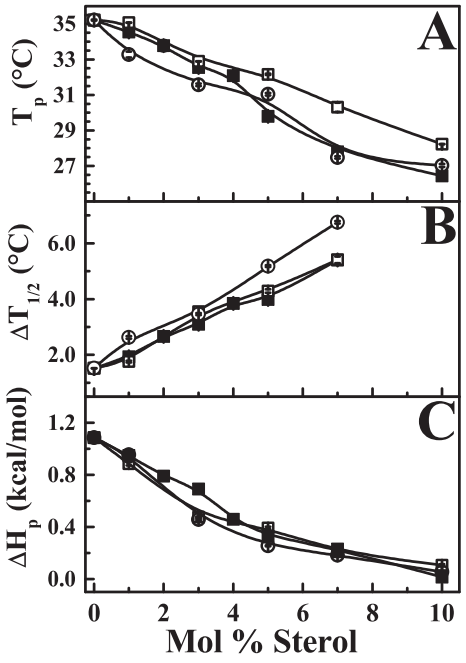

Fig. 4. Effect of increases in sterol concentration on the T_p, $\Delta T_{½}$, and ΔH_p of the pretransition of DPPC: cholesterol/DPPC (■), β-sitosterol/DPPC (□) and stigmasterol/DPPC (○) mixtures. The error bars were typically equal to, or smaller than, the size of the symbols.

3.4. The effects of Chol, Sito and Stig on the sharp component of the DPPC main phase transition

The thermodynamic parameters associated with the sharp component of the main phase transition for Chol/DPPC, Sito/DPPC and Stig/DPPC mixtures, as a function of sterol concentration, are presented in Fig. 6A, B and C, respectively. The T_m^{shp} values for all three mixtures initially decrease more rapidly with increasing sterol concentration at lower sterol levels before decreasing less rapidly at higher sterol concentrations, where they level off. However, the progressive incorporation of Chol produces a slightly smaller decrease in T_m^{shp} than does Sito, which in turn produces a slightly smaller decrease than Stig. This result indicates that the incorporation of each of these sterols reduces the thermal stability of the gel states of the sterol-poor DPPC domains slightly, with Stig producing the largest effect, Sito producing an intermediate effect, and Chol producing the smallest effect. Similarly, the progressive incorporation of all three sterols increases the $\Delta T_{1/2}^{shp}$ values modestly at low sterols concentrations, but to a much greater degree at higher sterol levels. Thus, all three sterols decrease the cooperativity of the sharp component of the main phase transition slightly at low sterol concentrations but more markedly at higher sterol levels. However, Stig

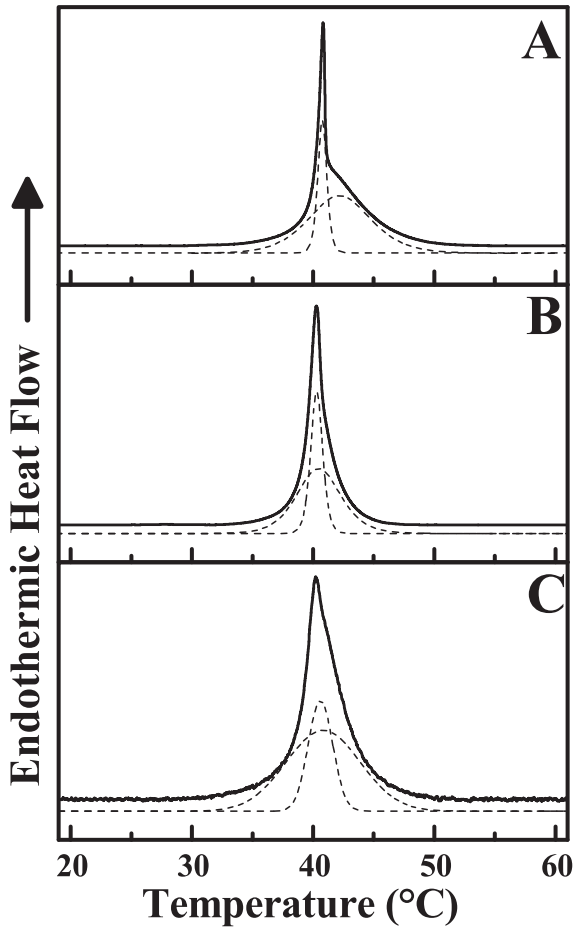

Fig. 5. Illustration of the results typically obtained in our peak-fitting deconvolution analyses of the DSC thermograms exhibited by cholesterol-containing (A), β-sitosterol-containing (B) and stigmasterol-containing (C) DPPC bilayers. All thermograms are from samples containing 15 mol % sterol and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

again has the greatest effect in this regard, Sito is intermediate, and Chol has the smallest effect. Finally, the progressive incorporation of all three sterols lowers the ΔH_m^{shp} values of the sharp component of the main phase transition of DPPC more markedly at lower sterol concentrations and less markedly at higher sterol levels. However, in this case the magnitude of this effect is similar for all three sterols, and in each case the sharp component of the main phase transition disappears entirely at sterol concentrations above 20 mol %.

3.5. The effects of Chol, Sito and Stig on the broad component of the DPPC main phase transition

The thermodynamic parameters associated with the broad component of the main phase transition in Chol/DPPC, Sito/DPPC and Stig/DPPC mixtures, plotted as a function of sterol concentration, are presented 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 of DPPC, the effects of each sterol on the thermodynamic parameters of the broad component of this phase transition differ significantly. In particular, the progressive incorporation of Chol into DPPC bilayers results in a significant and monotonic increase in T_m^{brd} values. In contrast, the incorporation of Sito and Stig initially have no effect on the T_m^{brd} values, although at higher sterol concentrations, Stig produces first an increase and then a decrease in the T_m^{brd}, while Sito produces a large decrease in the T_m^{brd} at the highest sterol concentration examined. These results indicate that Chol incorporation

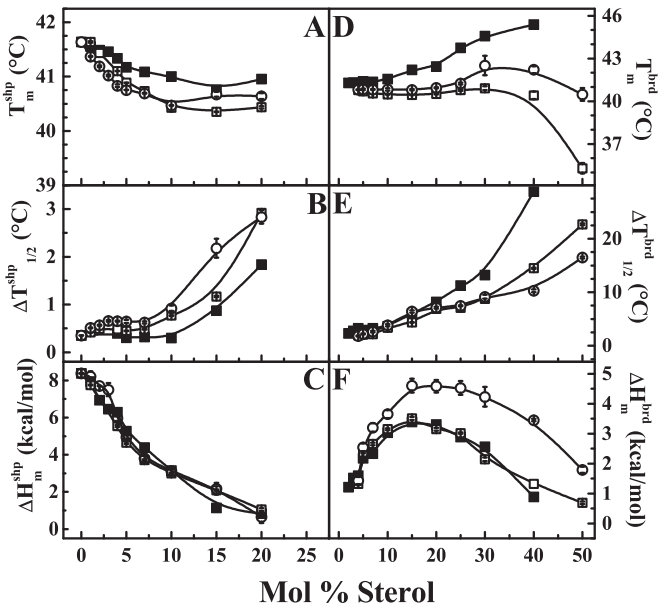

Fig. 6. Thermodynamic parameters (T_m, $\Delta T_{½}$, ΔH_m) for the deconvolved sharp (shp) (A-C) and broad (brd) (D-F) components obtained from the DSC heating thermograms of sterol/DPPC mixtures: cholesterol/DPPC (■), β-sitosterol/DPPC (□) and stigmasterol/DPPC (○) mixtures as a function of sterol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

increases the thermal stability of the slightly disordered gel-like phases present in the sterol-rich DPPC domains, while Sito and Stig are initially without effect. Similarly, although the incorporation of all three sterols progressively increases the $\Delta T_{1/2}^{brd}$ values, at higher sterol concentrations, Chol is more effective in this regard than Sito, which is in turn more effective than Stig in decreasing the cooperativity of the broad component of the DPPC main phase transition. Finally, at lower sterol concentrations, the progressive incorporation of all three sterols initially produces an increase in the ΔH_m^{brd} values of the broad component of the main phase transition of DPPC, followed by a decrease at higher sterol levels. However, Stig incorporation produces larger increases in the ΔH_m^{brd} values at lower sterol concentrations and smaller decreases in the ΔH_m^{brd} values at higher sterol concentrations than do Chol and Sito. As well, at the two highest sterol concentrations tested, Chol incorporations produces somewhat larger decreases in the ΔH_m^{brd} values than does Sito. Note that at a nominal sterol concentration of 50 mol %, the broad component of the main phase transition of DPPC disappears entirely (residual $\Delta H_m^{brd} = 0$ kcal/mol) in the Chol/DPPC mixture, whereas the broad component persists in the Sito/DPPC and Stig/DPPC mixtures, which exhibit residual ΔH_m^{brd} values of about 0.7 and 1.8 kcal/mol, respectively. Taken together, the different variations in both the $\Delta T_{1/2}^{brd}$ and the ΔH_m^{brd} values at higher sterol concentrations indicate that Sito and particularly Stig are less laterally miscible in fluid DPPC bilayers than is Chol.

Finally, we present a plot of the total enthalpy of the main phase transition of DPPC (sum of the enthalpies of the sharp and broad components) as a function of sterol concentration in Fig. 7. This plot reveals that although the ΔH_m^{shp} values decrease rapidly at low sterol concentrations before the sharp component disappears entirely, and the ΔH_m^{brd} values initially increase rapidly at low sterols concentrations before decreasing less rapidly at higher sterol levels (Fig. 6C and F), the net effect of these two changes nevertheless produces a monotonic and nearly linear decrease in the total enthalpy of the main phase transition of DPPC with increasing sterol levels. However, it is clear that the rate at which the total enthalpy decreases, and thus the magnitude of the residual enthalpy remaining at nominal sterol concentrations 50 mol %, varies with the structure of the sterol being studied. Specifically, although the initial rates of decrease in the total enthalpy values at the

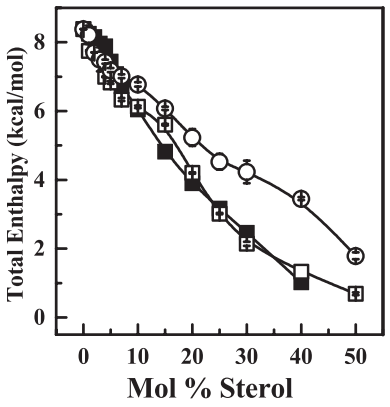

Fig. 7. Overall enthalpy values for cholesterol/DPPC (■), β-sitosterol/DPPC (□) and stigmasterol/DPPC (○) mixtures.

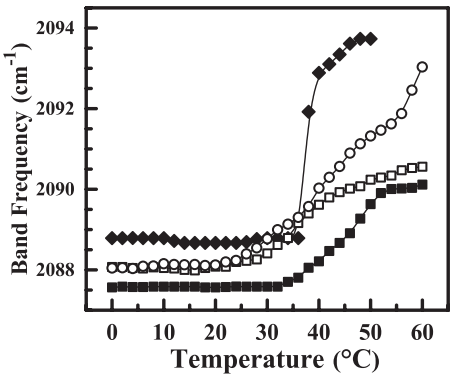

Fig. 8. Temperature-dependent changes of the properties of the C-D stretching band exhibited by sterol/DPPC-d_{62} bilayers. C–D stretching of sterol-free for DPPC-d_{62} bilayers (◆), DPPC-d_{62} bilayers containing ~30 mol % cholesterol (■), DPPC-d_{62} bilayers containing ~30 mol % β-sitosterol (□) and DPPC-d_{62} bilayers containing ~30 mol % stigmasterol (○). The data shown were obtained by analyses of temperature-induced changes in the band maxima spectra acquired in the heating mode.

lowest sterol concentrations are roughly comparable for all three sterols, the rate of decrease becomes significantly less for Stig than for Chol and Sito as sterol concentration increases further. A similar effect in also noted for Sito, although in this case a differential effect in the rate at which the total enthalpy decreases is only observed at the highest sterol levels. These results, as well as the differences in the magnitudes of the residual enthalpy of the DPPC main phase noted above, indicate that Chol is somewhat more laterally miscible in fluid DPPC bilayers than is Sito at the highest sterol levels examined, and that Stig is much less miscible still, even at much lower sterol concentrations. We include this additional figure here because it more clearly illustrates these effects than do the separate plots of the variations of the ΔH_m^{shp} and ΔH_m^{brd} values as a function of sterol concentration presented earlier.

3.6. FTIR spectroscopic studies of the thermotropic phase behavior and organization of sterol/DPPC mixtures

We have studied the temperature-induced changes in the FTIR spectra exhibited by DPPC alone and by mixtures of DPPC and 30 mol % of each of the three sterols studied here over the temperature range from 0-60 °C (full spectra not presented). We summarize below the results obtained for the asymmertric C-D stretching bands and present results for the carbonyl stretching bands and CH_2 scissoring absorption bands in Supplementary Material.

One of the most important attributes of the Chol molecule is its ability to markedly order the hydrocarbon chains of liquid-crystalline phospholipid bilayers into which it is incorporated. This reduction in the rotational conformational disorder of the phospholipid hydrocarbon chains in manifested most clearly in FTIR spectroscopy as a decrease in the frequency and an increase in the width of the asymmetric C-H (or C-D) stretching band of the DPPC hydrocarbon chains observed at higher temperatures in Chol-containing DPPC membranes [46,60,61]. We have therefore compared the magnitudes of the reduction in the asymmetric C-D stretching frequencies of chain-perdeuterated DPPC bilayers upon the incorporation of 30 mol % of each of the three sterols studied at higher temperatures and the results of such studies are presented in Fig. 8. An examination of the respective C-D frequency versus temperature plots reveals that the incorporation of each of these three sterols reduces the cooperativity of the DPPC phase transition and the progressive decrease in the C-D stretching frequency and increase in band width over the same temperature range observed by DSC indicates that the broad component of the main phase transition of DPPC is indeed a hydrocarbon chain-melting phase transition. The plots presented in this figure also show that the incorporation of these sterols results in a decrease of the C-D stretching frequency, indicating that the presence of Chol, Sito and Stig all reduce the ratio of *gauche* to *trans* rotational conformers in fluid DPPC hydrocarbon chains compared to DPPC alone at a comparable higher temperature. However, the magnitude of the reduction in the C-D frequency varies markedly with the structure of the sterol incorporated. In particular, the DPPC hydrocarbon chain ordering effect of Chol is much greater than that of Sito, which in turn is greater than that of Stig. These results indicate that the introduction of an ethyl group at C24 of the alkyl side chain of Chol dramatically reduces its ability to order the hydrocarbon chains of fluid DPPC bilayers, and that the additional presence of a *trans* double bond at C22 in the alkyl side chain further reduces its hydrocarbon chain ordering ability.

4. Discussion

Our comparative high-sensitivity DSC study of the effects of the progressive incorporation of the animal sterol Chol and the plant sterols Sito and Stig on the pretransition of DPPC bilayers indicate that these sterols all have rather similar effects, although there are small quantitative differences. In particular, the incorporation of Sito produces a smaller decrease in the pretransition temperature than Chol or Stig, indicating a smaller reduction in the overall thermal stability of the two low-temperature gel phases of DPPC. This result agrees with the findings of a previous high-sensitivity DSC investigation of the effects of Chol, Sito and Stig on DPPC and N-palmitoyl SpM bilayers [32]. Similarly, although the progressive incorporation of all three sterols produce roughly linear decreases in the cooperativity of the DPPC pretransition, the rate at which the cooperativity decreases is slightly greater for Stig than for Chol or Sito. Nevertheless, the rates of decrease in the enthalpy of the pretransition of DPPC are essentially the same for all three sterols and in each case the pretransition persists until sterol concentrations of 10 mol % are reached. This latter result is unique, in that all of the natural sterols and sterol analogs which we have studied to date (other than Chol itself) abolish the DPPC pretransition at sterol concentrations well below 10 mol %, including the closely related C24-methyl plant sterols Camp and Bras [43].

An earlier X-ray diffraction study also reported that the pretransition of DPPC bilayers was abolished above a Chol concentration of 10 mol % [62], in agreement with the present DSC study. This and subsequent studies have found that the gradual abolition of the DPPC pretransition by Chol is due to the progressive replacement the $L_{\beta'}$ and $P_{\beta'}$ phases, both of which are characterized by all-*trans* hydrocarbon chains which are tilted with respect to the bilayer plane, by a slightly disordered L_β-like phase in which the hydrocarbon chains contain a small number of *gauche* rotational conformers but are oriented perpendicular to the bilayer plane. This phase replacement in turn occurs because the Chol molecule, with its very small polar headgroup but large steroid nucleus, pushes adjacent DPPC molecules apart, making more space

available to the polar headgroups of DPPC, whose intrinsic cross-sectional area is greater than that of the two all-*trans* hydrocarbon chains. This in turn allows the now slightly disordered but still largely all-*trans* hydrocarbon chains of DPPC to assume a perpendicular orientation, since chain tilting is no longer required to relieve the intrinsic mismatch in cross-sectional areas between the larger polar headgroup and smaller hydrocarbon chains in the gel state bilayer. Moreover, as previously, we ascribe the decreased thermal stability of the gel phases of DPPC accompanying Chol incorporation to its hydrocarbon chain disordering effect. Thus, in principle, the effectiveness of any sterol in abolishing the pretransition of DPPC should increase as a function of both the cross-sectional area of the sterol, which is related to its ability to increase the space between adjacent phospholipid molecules in the gel state bilayer, and its ability to disorder the hydrocarbon chains of adjacent phospholipid molecules in the bilayer, thus further decreasing the intrinsic mismatch in the sizes of the relatively larger polar headgroup and the smaller all-*trans* hydrocarbon chains of DPPC by increasing the cross-sectional area of the latter. We thus ascribe the similar behavior of Chol and the plant sterols Sito and Stig to the similar albeit slightly larger sizes (cross-sectional areas) of the two plant sterols [10–14] and perhaps also to their greater abilities to disorder the all-*trans* hydrocarbon chains of adjacent DPPC molecules, although the abilities of Sito and Stig to disorder the hydrocarbon chains of gel state DPPC bilayers has not actually been studied.

Our comparative DSC studies also show that the progressive incorporation of Chol, Sito and Stig into DPPC bilayers results in similar if not identical effects on the thermodynamic parameters associated with the sharp component of the main phase transition of DPPC. In this regard, we have previously presented similar results for a wide variety of Chol precursors, metabolites and synthetic analogs [44,48–58], as well as the fungal sterol Ergo [59] and the plant sterols Camp and Bras [43]. Since the sharp component of the main phase transition is due the hydrocarbon chain melting of the sterol-poor domains in Sterol/DPPC mixtures, the relative insensitivity of this component of the main phase transition to relatively small changes in sterol structure, such as altering the number and position of double bonds in the steroid ring system [49–58] or the length and structure of the alkyl side-chain [43,44,47,49,59], is not unexpected. However, larger changes in the structure of the Chol molecule, such as changes in the stereochemistry or structure of the polar headgroup [52–54,56–58], or introducing additional methyl substitutions into the steroid ring system [51] or changing its all-*trans* conformation [54], can produce greater decreases in the temperature, cooperativity and enthalpy of the sharp component of the main phase transition of DPPC than are observed with the three sterols studied here.

In contrast, our DSC results indicate that the progressive incorporation of each of these sterols into DPPC bilayers produces somewhat different effects on the broad component of the main phase transition of DPPC. This result is not surprising, since the broad component of the main phase transition is due to the hydrocarbon chain melting of the sterol-rich domains of sterol/DPPC mixtures, so greater effects of variations in sterol structure on the thermodynamic parameters associated with this component of the main phase transition of DPPC would be expected and have been observed in our previous studies of a number of sterols and steroids [43,44,47,49–59]. In particular, the ability to decrease the cooperativity and reduce the enthalpy of the broad component of the main phase transition decrease in the order Chol > Sito > Stig, but the ability to increase the phase transition temperature decreases in the order Chol > Stig > Sito. The greater ability of Chol compared to the plant sterols to thermally stabilize the sterol-rich domains of DPPC bilayers is compatible with its relatively greater ability to increase the order of the hydrocarbon chains and to condense various phospholipid and SpM bilayers (see the Introduction), although Sito is generally found to be more effective than Stig in this regard. Moreover, the greater ability of Chol as compared to Sito and especially Stig to decrease the cooperativity and enthalpy of the broad component of the DPPC main phase transition is compatible with the reduced lateral miscibility of Sito and particularly Stig in various phospholipid bilayers reported elsewhere [21–23].

It is instructive to compare our present DSC results on the effects of the C24 ethyl-substituted plant sterols Sito and Stig on the main phase transition of DPPC with our previously published results for their C24 methyl-substituted analogs Camp and Bras [43]. The progressive incorporation all four sterols had very similar effects on the thermodynamic parameters associated with the sharp component of the main phase transition, due to the hydrocarbon chain melting of DPPC in the sterol-poor domains, as expected for the reasons discussed above. However, the progressive incorporation of Camp and Bras both increased the temperature of the broad component to a slightly greater extent than for Sito and Stig, and the incorporation of the former pair of sterols decreased the cooperativity of the broad component to a slightly smaller extent than the latter pair. Moreover, the presence of the *trans*-double bond at C22 in both sterol pairs increased the magnitude of these characteristic effects. Moreover, the sterol-induced decrease in the enthalpy of the broad component of the main phase transition was greater for Camp and Bras than for Sito and Stig. Specifically, the residual enthalpies at nominal sterol concentrations of 50 mol % were Camp (0.3 kcal/mol) < Sito (0.7 kcal/mol) < Bras (0.9 kcal/mol) < Stig (1.8 kcal/mol). These results indicate that sterols having a methyl rather than an ethyl group at C24, and sterols lacking the *trans*-double bond at C22, are more effective at thermally stabilizing the sterol-rich domains in DPPC bilayers and are also more laterally miscible in fluid DPPC bilayers. This latter finding also agrees with the results of the previous studies of the relative solubility of these plant sterols in various other phospholipid bilayers [21–23].

Our FTIR results indicate that at nominal sterol concentrations of 30 mol %, Chol has a much greater ordering effect on fluid DPPC bilayers than does Sito and especially Stig, as measured by the ratio of *gauche* to *trans* rotational conformers present in their hydrocarbon chains. The finding that the animal sterol Chol is more effective in this regard than the plant sterols Sito and especially Stig is in agreement with most of the studies reviewed in the Introduction, which indicate that Chol is more effective at condensing phospholipid monolayers [10–16] and in ordering phospholipid bilayers [18–26] than are these two plant sterols, and that Sito is more effective than Stig in this regard. The FTIR results (Suppl. Fig. S1) presented in the supplementary materials also indicate that the incorporation of the plant sterols Camp and Bras are more effective than Sito and Stig, respectively, in ordering the hydrocarbon chains of fluid DPPC bilayers, and that Camp is likely slightly more effective in this regard than is Bras. Taken together, these results indicate that the presence of a methyl group at C24 of the alkyl side chain diminishes the ability of Chol to order DPPC bilayers to a somewhat lesser extent than the presence of an ethyl group at this position, and that the presence of a *trans*-double bond at C24 of the alkyl side chain results in a further weakening of its ordering ability in both cases. However, we note that a fluorescence anisotropy study using soybean PC vesicles reported that Sito and Camp were essentially equally effective in their bilayer ordering abilities [22], while a ^{2}H NMR study of soybean PC vesicles containing a dueterated DMPC probe found that Sito was more effective than was Camp at ordering the DMPC hydrocarbon chains [24]. Whether this difference in results is due to the different PC systems studied, to differences in the techniques employed, or both, remain to be determined.

In closing, we point out again that every sterol and steroid which we have studied to date, now including the major plant sterols Sito and Stig, exhibit a reduced maximum lateral miscibility in DPPC bilayer membranes as compared to Chol. This observation suggests that the structure of Chol may be evolutionarily optimized for maximum miscibility in phospholipid bilayer membranes, as well as for a high but not necessarily optimal degree of hydrocarbon chain ordering ability. This conclusion follows from the fact that several natural sterols, including the fungal sterol Ergo [59] and the saturated Chol analog cholestanol [58],

actually order DPPC bilayer membranes to a higher or to a similar extent, respectively, at sterol concentrations below their solubility limits, yet have lower maximum lateral miscibilities in DPPC bilayers. Nevertheless, Chol does have a greater ability to order the hydrocarbon chains of DPPC bilayer membranes than do most sterols, certainly including the major plant sterols Sito and Stig studied here. However, since plant cell membranes are typically enriched in phospholipids and particularly in glycolipids containing high concentrations of polyunsaturated fatty acids, it is possible that plant sterols containing an alkyl group at C22 and sometimes also a *trans*-double bond at C24 may be relatively more effective at ordering and condensing the lipid bilayers of such membranes than is Chol.

Conflict of interest statement

The authors disclose no conflicts of interest.

Acknowledgments

This work was supported by operating and major equipment grants from the Canadian Institutes of Health Research and by major equipment grants from the Alberta Heritage Fund for Medical Research. M.G.K.B. was supported by Undergraduate Summer Student Research Awards from the Natural Sciences and Engineering Research Council of Canada and the Alberta Heritage Fund for Medical Research. We thank Mr. Wayne Moffat, Manager of the Analytical and Instrumental Laboratory, Department of Chemistry, University of Alberta, for assistance with FTIR spectroscopy acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.04.009.

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