

Effects of Natural and Enantiomeric Cholesterol on the Thermotropic Phase Behavior and Structure of Egg Sphingomyelin Bilayer Membranes

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ABSTRACT Phospholipids, sphingolipids, and sterols are the major lipid components of the plasma membranes of eukaryotic cells. Because these three lipid classes occur naturally as enantiomerically pure compounds, enantiospecific lipid-lipid and lipid-sterol interactions could in principle occur in the lipid bilayers of eukaryotic plasma membranes. Although previous biophysical studies of phospholipid and phospholipid-sterol model membrane systems have consistently failed to observe such enantiomerically selective interactions, a recent monolayer study of the interactions of natural and enantiomeric cholesterol with egg sphingomyelin has apparently revealed the existence of enantiospecific sterol-sphingolipid interactions. To determine whether enantiospecific sterol-sphingolipid interactions also occur in more biologically relevant lipid bilayer systems, differential scanning calorimetric, x-ray diffraction, and neutral buoyant-density measurements were utilized to study the effects of natural and enantiomeric cholesterol on the thermotropic phase behavior and structure of egg sphingomyelin bilayers. The calorimetry experiments show that natural and enantiomeric cholesterol have essentially identical effects on the temperature, enthalpy, and cooperativity of the gel/liquid-crystalline phase transition of egg sphingomyelin bilayers within the limits of experimental error. As well, the x-ray diffraction and neutral buoyancy experiments indicate that bilayers formed from mixtures of natural or enantiomeric cholesterol and egg sphingomyelin have, within experimental uncertainty, the same structure and mass density. We thus conclude that significant enantioselective cholesterol-sphingolipid interactions do not occur in this lipid bilayer model membrane system.

INTRODUCTION

Cholesterol is a major and essential lipid component of the plasma membranes of cells of higher animals and is also found in lower concentrations in certain intracellular membranes in vesicular communication with the plasma membrane (Nes and McKean, 1977; Yeagle, 1988; Liscum and Munn, 1999). Although cholesterol has a number of different functions in animal cells, one of its primary roles is as a modulator of the physical properties of the plasma membrane lipid bilayer. Thus, many studies of the interaction of cholesterol with phospholipid monolayer and bilayer model membranes have been performed, utilizing a wide range of physical techniques (Demel and de Kruijff, 1976; Yeagle, 1988; Vist and Davis, 1990; McMullen and McElhaney, 1996; McIntosh et al., 1989; Needham and Nunn, 1990). These studies, most of which have utilized symmetrical chain linear saturated phosphatidylcholines (PCs), have established that one of the major effects of cholesterol incorporation on phospholipid monolayer and bilayer model membranes is a broadening and eventual elimination of the cooperative gel-to-liquid-crystalline phase transition and its replacement by a phase with an intermediate degree of or-

ganization. Thus, in the liquid-crystalline phase that would exist at physiological temperatures in the absence of sterols, the presence of cholesterol significantly increases the orientational order of the phospholipid hydrocarbon chains and decreases the cross-sectional area occupied by the phospholipid molecule without significantly restricting the rates of phospholipid lateral diffusion or hydrocarbon chain motion. As well, the presence of cholesterol increases both the thickness and mechanical strength and decreases the permeability of the phospholipid bilayer in the physiologically relevant liquid-crystalline phase. The relatively high rates of intramolecular and intermolecular motion that are characteristic of phospholipid model membranes in the presence of high levels of cholesterol, coupled with an increased hydrocarbon-chain order and a decreased area compressibility, have prompted several workers to postulate the existence of a discrete liquid-ordered state in model and biological membranes with cholesterol levels $> \sim 25$ mol % (Ipsen et al., 1987; Vist and Davis, 1990; Thewalt and Bloom, 1992). Moreover, the preferential interactions of cholesterol with natural sphingomyelins (SpMs) as opposed to the natural phospholipids appears to be particularly important in the formation of these putative liquid-ordered states in biological and model membranes (Ahmed et al., 1997; Brown and London, 2000; Dietrich et al., 2001; Ikonen, 2001; Simons and Ikonen, 2000).

Because the phospholipids and sterols found in cellular membranes contain one or more chiral carbon atoms and occur in an enantiomerically pure form, enantiospecific inter-

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actions can in principle occur in the lipid bilayers of such membranes. However, previous studies of phospholipid-phospholipid (van Deenen et al., 1962; Rainier et al., 1979; Arnett et al., 1988; Rose et al., 1993) and sterol-phospholipid (Ghosh et al., 1971; Arnett and Gold, 1982; Hermetter and Paltauf, 1982; Guyer and Block, 1983; Agarwal et al., 1986a,b) interactions utilizing dipalmitoylphosphatidylcholine (DPPC) or close structural analogs have not provided evidence for significant enantioselectivity for either type of interaction. Recently, however, a report has appeared in which enantioselective interactions between natural cholesterol or enantiomeric cholesterol and egg SpM in monolayer films have apparently been detected (Lalitha et al., 2001). In particular, it has been reported that at 24°C and at a lateral pressure of 30 mN/m (a value near the bilayer-equivalent lateral pressure for DPPC (Marsh, 1996)), enantiomeric cholesterol is approximately twice as effective as natural cholesterol in condensing egg SpM monolayers at sterol concentrations of 30 mol %. If shown also in lipid bilayer systems, such strongly enantiospecific interactions between cholesterol and naturally occurring SpMs could be potentially important, because it is well established that cholesterol preferentially interacts with SpM in both model and biological membrane systems (Demel and de Kruijff, 1976; Yeagle, 1988; McMullen and McElhaney, 1996), perhaps forming the molecular basis for the existence of detergent-insoluble, cholesterol- and SpM-enriched rafts in mammalian plasma membranes (Brown, 1998; Kinnunen, 1991).

Although the function of cholesterol as a modulator of the physical properties of the lipid bilayer of plasma membranes is the best known and most extensively studied, cholesterol has been shown to perform other essential roles in living organisms. For example, even in the sterol-requiring cell wall-less bacteria *Mycoplasma capricolum*, cholesterol has been shown to stimulate the biosynthesis of phospholipids that contain unsaturated fatty acids, which in turn act as fatty acyl group donors for the biosynthesis of fatty acylated membrane proteins. Furthermore, cholesterol is required for macromolecular synthesis and cell growth as well as for membrane biogenesis in this microorganism in addition to its role of regulating membrane lipid phase state and fluidity (Dahl and Dahl, 1988). Similarly, in the yeast *Saccharomyces cerevisiae*, ergosterol has been shown to play specific roles in cell budding, phospholipid biosynthesis, phosphoinositide signaling, and protein kinase activity modulation (Parks et al., 1995, 1999), and cholesterol has been shown to be involved in an even wider range of functions in mammalian cells (Dahl and Dahl, 1988). It is noteworthy that these alternate functions of sterols are generally characterized by the requirement for much lower levels of sterol but by a much higher stereospecificity than for the so-called bulk or lipid bilayer-modulation sterol function (Dahl and Dahl, 1988; Parks et al., 1995, 1999).

In this article, we present the results of differential scanning calorimetry (DSC), x-ray diffraction, and neutral

buoyant-density experiments, which address the question of the existence of enantioselective interactions between cholesterol and egg SpM in lipid bilayer model membranes. The results of this study are of special interest, because enantioselectivity has hitherto been assumed to be characteristic only of the interactions of sterols with membrane proteins and not of sterols with membrane lipids.

MATERIALS AND METHODS

The SpM and the natural cholesterol were both obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and were used without further purification, whereas the cholesterol enantiomer was synthesized using a procedure described previously (Jiang and Covey, 2002). The egg SpM utilized is highly enriched in long-chain saturated fatty acids, particularly in 16:0 (83.9%), and contained only small amounts of 24:1 (2–3%).

We utilized the DSC protocol described previously, which involved increasing total sample size with increasing sterol concentrations to insure that the broad, low-enthalpy phase transitions occurring under these circumstances could be accurately monitored (McMullen et al., 1993). However, in this study, DSC was performed using a Calorimetry Sciences Corporation 6100 NanoDSC II (American Fork, UT) with deionized water as a reference. The appropriate amounts of SpM and either cholesterol or its enantiomer were dissolved in chloroform containing a minimum amount of methanol and were mixed from stock solutions to give the appropriate sterol and SpM concentrations. These mixtures were then dried at 50–60°C under a stream of N₂, and traces of solvent were removed by placing the samples in vacuo overnight. The dried mixtures were then resuspended in deionized water at 50–60°C and vortexed to give multilamellar suspensions. Samples were heated and cooled at a rate of 10°C/h starting at a minimum of 5–10°C and finishing at 70–95°C depending on the sample composition. Thermograms were obtained with the cells pressurized to 3 atm to suppress bubble formation as recommended by the manufacturer. Each sample was subjected to at least three heating-and-cooling cycles. Data were collected using the N-DSC II control program and imported into Origin 7.0 (Originlab Corp., Northampton, MA). A typical analysis consisted of subtraction of a fast Fourier transform-smoothed reference water baseline from the sample thermogram. Correction for baseline curvature was achieved by subtraction of a computer-generated baseline of 100–200 points over the temperature range of the scan. The lower and upper intersections of the baseline with the DSC trace were estimated by eye and were reproducible in both heating and cooling runs. At lower sterol concentrations, where there are both sharp and broad phase transitions present, peak fitting was performed using the Origin peak-fitting module to resolve the individual components.

Two types of systems were studied by x-ray diffraction: unoriented dispersions and oriented multilayers, both of which are composed of binary mixtures of either natural or enantiomeric cholesterol and egg SpM at a molar ratio of 3:7. This sterol/egg SpM ratio was chosen because it exhibited the greatest degree of enantioselectivity in the previous monolayer experiments (Lalitha et al., 2001). To prepare both types of model membrane systems, multilamellar vesicles (MLVs) were first prepared by codissolving the lipids in chloroform, removing the solvent by rotary evaporation, and hydrating the lipids in excess water. To ensure proper hydration, the lipid-water suspensions were heated to 60°C and extensively vortexed. To study the diffraction of unoriented specimens, the MLVs were centrifuged with a bench centrifuge, sealed in a thin-walled glass capillary tube, and mounted in a point-collimation x-ray camera. Oriented lipid multilayers were prepared by placing a drop of the MLV suspension onto a curved glass substrate and drying it under a gentle stream of nitrogen. The lipid multilayers oriented on the glass substrate were mounted in a temperature-controlled constant-humidity sample chamber on a line-focus (single-mirror) x-ray camera (McIntosh et al., 1987, 1989, 1992a,b). Relative humidities were set by incubation with saturated salt solutions (McIntosh et al., 1987, 1989). All

x-ray patterns were recorded on Kodak DEF-5 x-ray film at 20°C to facilitate a comparison with the previously published monolayer data, which was collected at a similar temperature (Lalitha et al., 2001). To obtain electron-density profiles across the bilayer, a Fourier analysis of the x-ray diffraction patterns was performed. Integrated intensities were obtained for each diffraction order by measuring the area under each diffraction peak, and structure amplitudes were obtained by applying standard correction factors for either oriented or unoriented specimens (McIntosh et al., 1987; McIntosh and Simon, 1986). As described in detail previously (McIntosh and Simon, 1986; McIntosh et al., 1989, 1992a,b; Gandhavadi et al., 2002), phase angles were determined by using osmotic stress experiments to trace out the continuous transform of the bilayer. For each bilayer system, continuous transforms were calculated by use of the sampling theorem (Shannon, 1949) for one data set for each possible phase combination. The phase combination that gave the best match to the other structure factors was selected (McIntosh et al., 1984, 1987; McIntosh and Holloway, 1987). Electron-density profiles across the bilayer were calculated from Fourier reconstructions using the x-ray structure factors

$$\rho(x) = (2/d) \sum \exp(i\phi(h)) \cdot F(h) \cdot \cos(2\pi xh/d), \quad (1)$$

where $F(h)$ is the x-ray structure amplitude, x is the distance from the center of the bilayer, d is the lamellar repeat period, $\phi(h)$ is the phase angle of order h (either 0 or 180° for these centrosymmetric systems), and the sum is over h .

Measurements of the density of bilayers composed of either natural or enantiomeric cholesterol and egg SpM at a molar ratio of 3:7 were made using the neutral density procedure of Wiener et al. (1988). MLVs were prepared in precisely measured volumes of H₂O, and successive volumes of D₂O were added, vortexed, and equilibrated for 30 min to ensure complete transfer of water across the multilayers (Wiener et al., 1988). After each addition of D₂O, the MLV suspensions were centrifuged using an Eppendorf bench centrifuge to determine whether the MLVs pelleted or floated. Again, these buoyant-density measurements were carried out at 20°C to facilitate comparison with the previously published monolayer experiments (Lalitha et al., 2001).

RESULTS

Differential scanning calorimetry studies

High-sensitivity DSC heating and cooling scans for egg SpM alone are presented in Fig. 1. In the absence of sterol, large

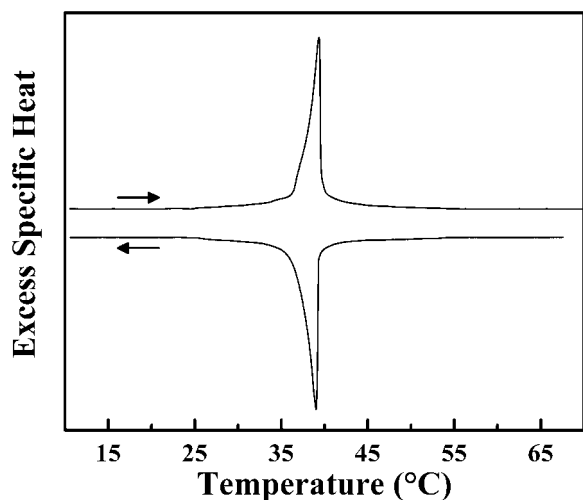


FIGURE 1 Representative DSC heating and cooling scans of large MLVs of egg SpM dispersed in excess water.

MLVs of egg SpM dispersed in water exhibit a single, fairly cooperative but slightly asymmetric multicomponent endotherm centered at 39.2°C on heating and a similar exotherm centered at 39.0°C on cooling. The calorimetric enthalpy of the heating endotherm and cooling exotherm are 5.9 and 6.0 kcal/mol, respectively. Based on previous studies of the thermotropic phase behavior of synthetic and natural SpMs (Marsh, 1999), we assigned the single multicomponent heating endotherm and cooling exotherm observed to a fully reversible gel/liquid-crystalline phase transition of egg SpM bilayers. The unusually cooperative phase transition exhibited by this particular natural SpM doubtlessly arises from the fact that it contains primarily even-chain saturated fatty acids, with 16:0 making up nearly 84% by weight of the total fatty acyl chains (see Materials and Methods). Thus the major sharp component of the egg SpM heating exotherm and cooling exotherm must be due to the chain-melting phase transition of *N*-palmitoyl-SpM.

High-sensitivity DSC heating scans for MLVs composed of SpM alone and of binary mixtures of natural or enantiomeric cholesterol with SpM are presented in Fig. 2. In

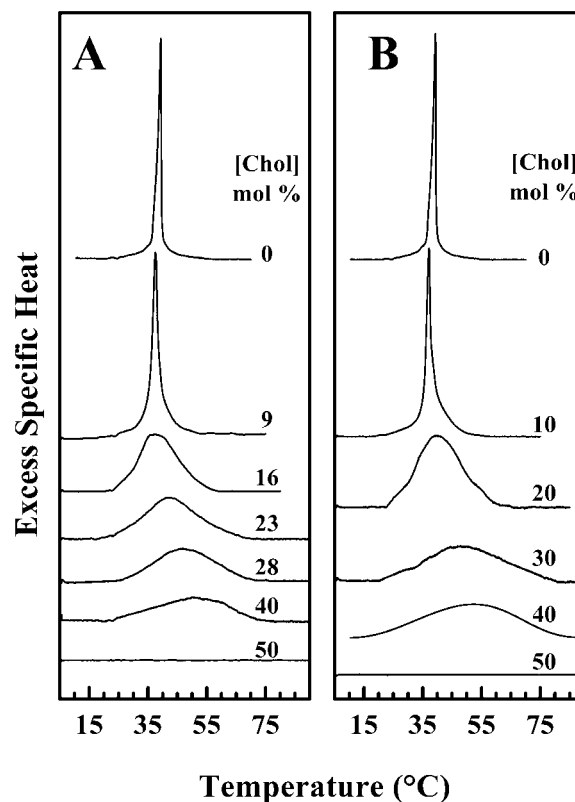


FIGURE 2 Representative DSC heating scans of large MLVs generated from binary mixtures of (A) natural cholesterol or (B) enantiomeric cholesterol with egg SpM that contain the sterol concentrations ([Chol]) indicated. Note that the various endotherms are not drawn to the same scale, to make the broader and less-enthalpic endotherms that occur at the higher sterol concentrations more visible to the reader, and that the sterol concentrations utilized are not all identical in the natural and enantiomeric cholesterol series.

both cases, the overall effect of increasing sterol concentration is to increase the temperature of the gel/liquid-crystalline phase transition of egg SpM slightly and to decrease its cooperativity and enthalpy markedly. In fact, at sterol concentrations of 50 mol %, the cooperative gel/liquid-crystalline phase transition of egg SpM large MLVs is abolished entirely. These results are very similar to those reported earlier for DPPC (Vist and Davis, 1990; McMullen et al., 1993).

The markedly asymmetric DSC endotherms observed in sterol/SpM binary mixtures containing up to 20 mol % sterol can be deconvolved into two major components, as was observed previously for binary mixtures of natural cholesterol with linear saturated PCs (Estep et al., 1978; Mabrey et al., 1978; Genz et al., 1986; Vist and Davis, 1990; McMullen et al., 1993), phosphatidylethanolamines (PEs; McMullen et al., 1999), and phosphatidylserines (PSs; McMullen et al., 2000). The phase transition temperature of the sharp component of the DSC endotherm decreases slightly with increases in sterol concentration, whereas its enthalpy decreases markedly and its cooperativity decreases slightly. In fact, the sharp component of the DSC endotherm is absent entirely at higher sterol concentrations. In contrast, the phase transition temperature of the broad component of the DSC endotherm increases with increasing sterol concentration, and its cooperativity decreases markedly. The enthalpy of the broad component initially increases with increasing sterol concentration but then decreases and approaches zero as sterol levels approach 50 mol %. In accordance with previous work on cholesterol/phospholipid systems (Estep et al., 1978; Mabrey et al., 1978; Genz et al., 1986; McMullen et al., 1993, 1999, 2000), the sharp component of the DSC endotherm is assigned to the relatively unperturbed gel/liquid-crystalline phase transition characteristic of domains of SpM alone, and the broad component is assigned to SpM domains increasingly enriched in sterol. However, because we could not deconvolve the DSC endotherms of binary mixtures of natural or enantiomeric cholesterol and egg SpM that contain low levels of sterol accurately due to the multicomponent nature of the egg SpM endotherm itself, we could not make quantitative comparisons between the effects of these cholesterol enantiomers on the sharp and broad components of the DSC endotherms, although these effects were certainly qualitatively very similar. Instead, we compare here only the overall effects of these two sterols on egg SpM thermotropic phase behavior and not their effects on both the sharp and broad components of the DSC endotherms, which are present in these mixtures at lower sterol levels.

The effects of increasing concentrations of natural and enantiomeric cholesterol on the apparent peak temperature of the DSC heating endotherm of egg SpM bilayers is presented in Fig. 3. At low sterol concentrations, the peak temperature decreases slightly and then increases modestly at higher sterol concentrations in both cases. As discussed above, this apparent reversal of peak temperature is actually an artifact

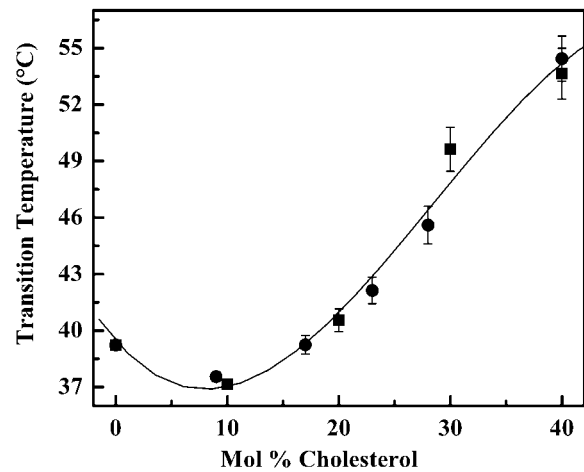


FIGURE 3 Plots of the peak temperatures of the DSC heating endotherms of large MLVs generated from binary mixtures of natural (●) or enantiomeric (■) cholesterol and egg SpM as a function of sterol concentration.

of sorts due to the superposition of the sharper component arising from the gel/liquid-crystalline phase-transition temperature of domains of SpM alone, which slightly but progressively decreases in temperature before disappearing entirely, and the broad component arising from the chain-melting phase transition of egg SpM domains enriched in cholesterol, which progressively increases in temperature with increasing sterol concentration. The key result, however, is that both natural and enantiomeric cholesterol have identical effects on the apparent peak temperature of egg SpM MLVs within experimental error.

The effect of increasing concentrations of natural and enantiomeric cholesterol on the total enthalpy of the gel/liquid-crystalline phase transition of egg SpM bilayers is illustrated in Fig. 4. In both cases, the overall phase-transition enthalpy decreases monotonically with increasing

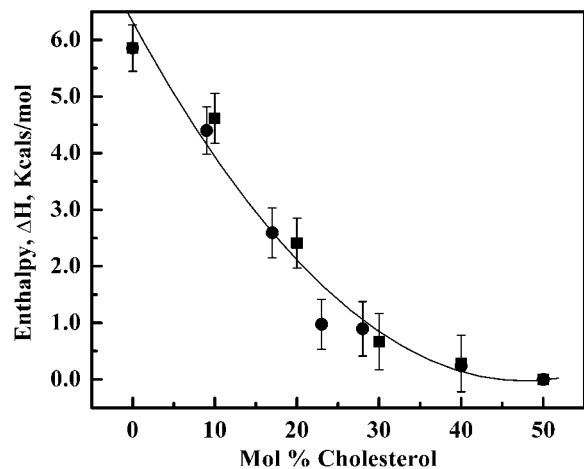


FIGURE 4 Plots of the total enthalpy of the DSC heating endotherms of large MLVs generated from binary mixtures of natural (●) or enantiomeric (■) cholesterol and egg SpM as a function of sterol concentration.

sterol incorporation and approaches zero at 50 mol % sterol, although this simple behavior masks the more complex behavior of the sharp and broad components that are present at lower sterol levels. Again, within experimental error, the effects of the two cholesterol enantiomers on the overall phase-transition enthalpy of egg SpM large MLVs are identical.

Finally, the effect of increasing concentrations of natural and enantiomeric cholesterol on the apparent cooperativity of the gel/liquid-crystalline phase transition of egg SpM bilayers is presented in Fig. 5. As before, these alterations in the apparent $\Delta T_{1/2}$ of the overall thermogram are due to a superposition of the differential qualitative and quantitative effects of both of these sterols on the sharp and broad components of the DSC endotherms present at lower sterol levels. Nevertheless, the effects of natural and enantiomeric cholesterol on the apparent cooperativity of the chain-melting phase transition of egg SpM large MLVs are again identical within experimental error.

X-ray diffraction studies

For all samples studied, each x-ray diffraction pattern consists of a broad wide-angle band centered at 4.4 Å and 5–7 sharp, low-angle reflections that index as orders of a lamellar repeat period. Such patterns are expected from stacks of liquid-crystalline bilayers (Tardieu et al., 1973). Table 1 shows the lamellar repeat periods (d) for bilayers formed from natural or enantiomeric cholesterol and egg SpM (molar ratio, 3:7) in excess water and at various relative humidities. As previously found for a variety of bilayer systems (Lis et al., 1982; McIntosh et al., 1987, 1989, 1992a,b), the repeat period was largest in excess water and

TABLE 1 X-ray diffraction values for bilayers composed of natural and enantiomeric cholesterol and egg sphingomyelin at different levels of hydration

Lipid system	Preparation*	Repeat period (Å) [†]	dpp (Å) [†]
Natural cholesterol/egg SpM (molar ratio, 3:7)	Excess water	67.0	47.0
	98% RH	60.0	45.0
	86% RH	59.5	45.0
Enantiomeric cholesterol/egg SpM (molar ratio, 3:7)	66% RH	55.8	44.6
	Excess water	67.2	45.6
	Excess water	67.0	47.0
	66% RH	55.6	45.0

*RH, relative humidity.

[†]Standard deviations of these measurements, ± 1.0 Å.

decreased as the relative humidity decreased. For either excess water or 66% relative humidity, the repeat periods were very similar for bilayers formed from either natural or enantiomeric cholesterol and egg SpM.

The repeat periods in Table 1 correspond to the width of a unit cell, which contains the bilayer and the fluid spacing between adjacent bilayers. To determine the relative widths of bilayers of natural or enantiomeric cholesterol and egg SpM (molar ratio, 3:7), a Fourier analysis of the x-ray data was used (McIntosh and Simon, 1986; McIntosh and Holloway, 1987). As a first step, the structure factors for all of the x-ray data were plotted versus reciprocal spacing (Fig. 6). The solid line corresponds to the continuous Fourier transform calculated for natural cholesterol/egg SpM bilayers by use of the sampling theorem (Shannon, 1949). It can be seen that for each of these lipid systems, the data points for both natural or enantiomeric cholesterol/egg SpM bilayers fall quite close to this continuous transform, indicating that: 1) the structure of these two bilayers does not appreciably change as water is removed from either system (McIntosh and Simon, 1986; McIntosh et al., 1987); and 2) the structure of these two bilayers is very similar.

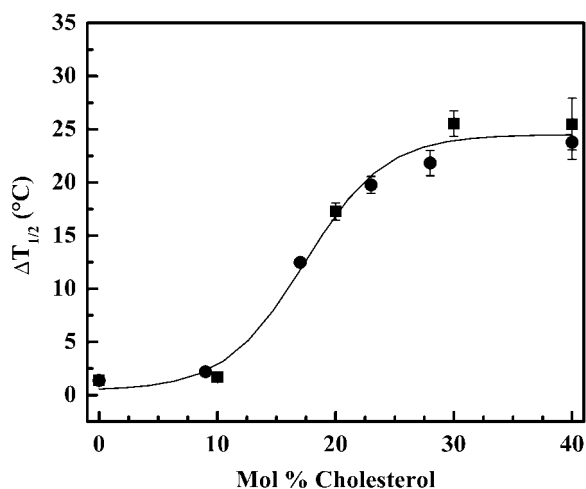


FIGURE 5 Plots of the apparent cooperativity of the DSC heating endotherms of large MLVs generated from binary mixtures of natural (●) or enantiomeric (■) cholesterol and egg SpM as a function of sterol concentration. The cooperativity of the endothermic components are expressed as $\Delta T_{1/2}$, the width of heating endotherm measured at half-height.

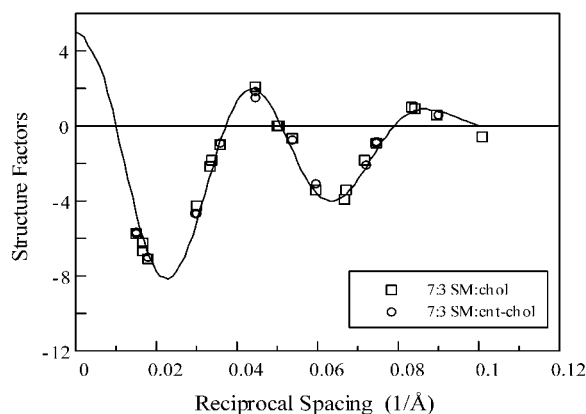


FIGURE 6 X-ray structure factors plotted versus reciprocal spacing for bilayers composed of natural cholesterol and egg SpM (□) and enantiomeric cholesterol and egg SpM (○) at a molar ratio of 3:7. The solid line represents the continuous Fourier transform for the natural cholesterol/Egg SpM bilayers calculated by use of the sampling theorem.

The structure factors (Fig. 6) were used to calculate electron-density profiles across the bilayers. Fig. 7 compares the electron-density profiles calculated at the same resolution ($d/2h_{\max} \approx 7 \text{ \AA}$) for natural or enantiomeric cholesterol/SpM bilayers (molar ratio, 3:7), with Fig. 7 *A* showing profiles for unoriented MLVs in excess water and Fig. 7 *B* showing profiles from oriented multilayers at 66% relative humidity. For each profile, the center of the bilayer is located at the origin, the low-electron-density trough in the center of the profile corresponds to the terminal methyl groups at the ends of the hydrocarbon chains, the medium-density regions on either side of this trough correspond to the methylene-chain regions of the bilayer, and the high-electron-density peaks near the edge of the profile correspond to the lipid headgroups. It can be seen that in both excess water and at 66% relative humidity, the electron-density profiles are identical within experimental error for SpM bilayers containing either natural or enantiomeric cholesterol. In particular, the head-group peak separation across the bilayer (dpp) is similar for the two systems (Table 1), so that $dpp = 45.4 \text{ \AA} \pm 1.1 \text{ \AA}$ (mean \pm standard deviation, $n = 4$ experiments) and $dpp = 45.9 \text{ \AA} \pm 1.0 \text{ \AA}$ ($n = 3$) for SpM bilayers containing 30 mol % natural or enantiomeric cholesterol, respectively. These

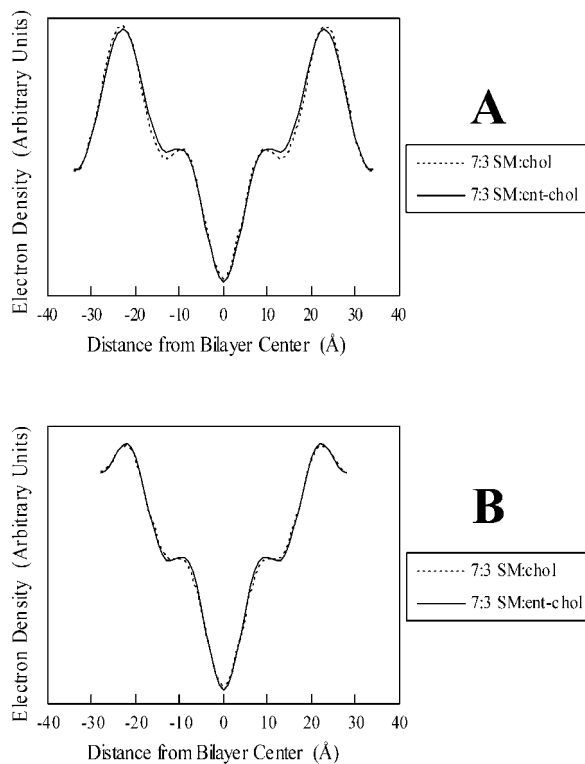


FIGURE 7 Electron-density profiles (resolution of $d/2h_{\max} = 7 \text{ \AA}$) for natural cholesterol/egg SpM and enantiomeric cholesterol/egg SpM (molar ratio, 3:7) bilayers in (A) excess water and (B) a 66% relative humidity atmosphere. For each profile, the center of the bilayer is at the origin, and the high-density peaks near the outer edges of the profile correspond to the sphingomyelin headgroups.

results can be compared to the value of $dpp = 47.8 \text{ \AA} \pm 1.0 \text{ \AA}$ ($n = 8$) that was recently obtained for bilayers of natural cholesterol and bovine brain SpM (molar ratio, 1:2) (Gandhavadi et al., 2002). The larger value of dpp for bilayers containing bovine brain SpM compared to those containing egg SpM is undoubtedly due to the higher percentage of long fatty acid chains in bovine brain SpM. According to Avanti Polar Lipids, the fatty acid composition of bovine brain SpM is 1.7% of 16:0; 45.5% of 18:0; 5.1% of 20:0; 7.2% of 22:0, and 23.3 wt % of 24:0 and 24:1.

Neutral buoyant-density measurements

MLVs of either natural or enantiomeric cholesterol and egg SpM (molar ratio, 3:7) both pelleted at an $\text{H}_2\text{O}/\text{D}_2\text{O}$ ratio of 1:0.5476 (density, 1.0361 g/ml), whereas both floated when centrifuged at an $\text{H}_2\text{O}/\text{D}_2\text{O}$ ratio of 1:0.575 (density, 1.0373 g/ml). Therefore, the density of both MLVs was between 1.0361 and 1.0373 g/ml. These values can be compared to densities of 0.9852 and 1.0616 measured by Wiener et al. (1988) for DPPC in the liquid-crystalline and gel phases, respectively. Thus, our measured density for cholesterol/egg SpM MLVs is between that of a liquid-crystalline and gel-phase PC without cholesterol. This would be expected based on the known effects of cholesterol on area per molecule and hydrocarbon chain orientational order described in the Introduction.

DISCUSSION

The results of our DSC studies of egg SpM alone generally agree quite well with those of previous studies of the thermotropic phase behavior of this sphingolipid. In particular, our measured phase transition temperature of 39°C is close to the values of $37\text{--}38^\circ\text{C}$ reported previously (McKeone et al., 1986; Chien et al., 1991; Chi et al., 1992). Similarly, our enthalpy value of 6.0 kcal/mol is similar to the values of 5.3 and 6.8 kcal reported by McKeone et al. (1986) and by Chien et al. (1991), respectively. The variations in the phase-transition temperatures and enthalpies reported to date could be at least partially due to variations in the fatty acid compositions of the various egg SpM preparations studied.

The results of our DSC studies of binary mixtures of natural or enantiomeric cholesterol and egg SpM are also in excellent agreement with previous DSC studies of the thermotropic phase behavior of binary mixtures of natural cholesterol with various naturally occurring SpMs (Oldfield and Chapman, 1971; Calhoun and Shipley, 1979; Estep et al., 1979; McKeone et al., 1986; Chien et al., 1991; Chi et al., 1992; McIntosh et al., 1992b). In all of these studies, the incorporation of increasing quantities of cholesterol into SpM bilayers results in small shifts in the temperature and large decreases in the enthalpy and cooperativity of the gel/liquid-crystalline phase transition, with this phase transition being completely abolished at 50 mol % cholesterol. How-

ever, neither our present results nor the previous results of other investigators agree with those recently reported by Shaikh et al. (2001). These workers report that the incorporation of up to 10 mol % of natural cholesterol into egg SpM bilayers results in a small downward shift in the temperature, only a slight decrease in the cooperativity, but a major decrease in the enthalpy of the gel/liquid-crystalline phase transition. These discrepant findings are almost certainly due to the fact that these workers failed to detect and accurately monitor the broad component of the egg SpM phase transition, which was lost in the baseline, and in fact actually monitored only the sharp component of their DSC thermograms. As we have discussed previously (McMullen et al., 1993), the same error has also produced artifactual DSC results in previous studies of MLVs composed of natural cholesterol and PC (Singer and Finegold, 1990a,b) or PE (Singer and Finegold, 1990b).

It is interesting to compare the effects of the incorporation of natural (or enantiomeric) cholesterol on the thermotropic phase behavior of egg SpM bilayers, which consists primarily of N-16:0-SpM, reported in the present work, with the effects of the incorporation of natural cholesterol on the thermotropic phase behavior of bilayers composed of dipalmitoyl molecular species of PC (Vist and Davis, 1990; McMullen et al., 1993), PE (McMullen et al., 1999), or PS (McMullen et al., 2000) reported previously. In all four binary systems, the incorporation of increasing quantities of cholesterol decreases the temperature of the sharp component of the gel/liquid-crystalline phase transition slightly and decreases its enthalpy markedly, such that this component is abolished at sterol levels >20 mol %. Similarly, the incorporation of progressively increasing quantities of cholesterol initially increases and then decreases the enthalpy of the broad component of the chain-melting phase transition and progressively decreases its cooperativity, such that the broad component is abolished at 50 mol % sterol. These results indicate that the stoichiometry of the interactions of cholesterol in egg SpM and phospholipid bilayers are similar, and that cholesterol is freely miscible in all of these lipids up to a level of at least 50 mol %. However, the incorporation of increasing quantities of cholesterol does have a different effect on the temperature shift of the broad component, increasing it moderately in DPPC bilayers (Simons and Ikonen, 2000; McMullen et al., 1993), decreasing it slightly in dipalmitoylphosphatidylserine bilayers (McMullen et al., 2000) and decreasing it markedly in dipalmitoylphosphatidylethanolamine bilayers (McMullen et al., 1999). Interestingly, the incorporation of increasing quantities of natural or enantiomeric cholesterol into egg SpM bilayers also shifts the temperature of the broad component of the gel/liquid-crystalline phase transition moderately upward, as observed previously for DPPC bilayers (McMullen and McElhaney, 1995; McMullen et al., 1993). In fact, the magnitude of the upward temperature shift observed in the cholesterol/egg SpM system is slightly

greater than that observed in the cholesterol/DPPC system ($+5$ – 6° versus $+3^\circ\text{C}$ at 40 mol % sterol, respectively). This occurs despite the fact that the average length of the two hydrocarbon chains is slightly greater in egg SpM than in DPPC, which should reduce the magnitude of this upward temperature shift due to a reduction in hydrophobic mismatch between the host SpM bilayer and the cholesterol molecule (see McMullen et al., 1993). This finding is thus compatible with previous suggestions that cholesterol may interact more strongly with SpM than with PC molecules of comparable hydrocarbon chain length and structure and therefore preferentially stabilize the gel relative to the liquid-crystalline state (Kinnunen, 1991; Brown, 1998).

The x-ray and density measurements indicate that the structures of bilayers composed of either natural or enantiomeric cholesterol and egg SpM at a molar ratio of 3:7 are very similar. Electron density profiles at 7 Å resolution (Fig. 7) are nearly identical, showing that the thicknesses of these bilayers are, within experimental error, the same both in excess-water (Fig. 7 *A*) and partially hydrated (Fig. 7 *B*) systems. The mass densities of the fully hydrated SpM bilayers that contain 30 mol % of either natural or enantiomeric cholesterol are also the same within 0.1%, which indicates that the partial molar volumes of the two bilayers are essentially identical. Because the thickness and volume of these bilayers are similar, the area per lipid molecule must also be very similar. These results strongly suggest that natural and enantiomeric cholesterol interact with egg SpM in an essentially identical fashion in lipid bilayer systems.

The results of the present DSC and x-ray diffraction study generally agree well with previous studies of the interaction of natural cholesterol with enantiomeric PCs and their structural analogs in bilayer systems (Ghosh et al., 1971; Arnett and Gold, 1982; Hermetter and Paltauf, 1982; Guyer and Block, 1983; Agarwal et al., 1986a,b) in that significant enantioselective sterol-lipid interactions were not detected. However, there is an apparent discrepancy between our bilayer results and the previous monolayer study, which appeared to show enantioselective cholesterol/egg SpM interactions (Lalitha et al., 2001). We consider two possible explanations for this discrepancy: 1) differences in the properties of monolayers and bilayers; and 2) potential problems with the monolayer studies. In terms of the first possibility, a lipid monolayer spread at an air-water interface may not have the same properties as one-half of a lipid bilayer because of the different boundary conditions of hydrocarbon-air and hydrocarbon-hydrocarbon interfaces. In terms of the second possibility, we note that the mean molecular areas reported for monolayers composed of mixtures of enantiomeric cholesterol and egg SpM at biologically relevant lateral pressures are actually significantly lower than the minimum molecular areas measured for each component separately in the liquid-condensed state. Specifically, at 24°C and a lateral surface pressure of 30 mN/

m, the cross-sectional areas of enantiomeric cholesterol and egg SpM alone were reported as 37.3 \AA^2 and $\sim 45 \text{ \AA}^2$, respectively, whereas the mean molecular area of a binary mixture of 30 mol % enantiomeric cholesterol and egg SpM was reported as $36\text{--}37 \text{ \AA}^2$. This result is not physically plausible, because cholesterol and other sterols are known to induce a liquid-ordered state in lipid monolayers and bilayers with a degree of organization and tightness of packing that is intermediate between that of the liquid-condensed (gel) and liquid-expanded (liquid-crystalline) states (Vist and Davis, 1990; Kinnunen, 1991; McMullen et al., 1993; Brown, 1998). To address these and other concerns, a reinvestigation of the force/area curves of monolayers composed of natural and enantiomeric cholesterol and egg SpM would seem to be in order.

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