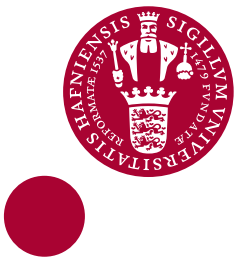

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Theory of thermal anomalies in the specific heat of lipid bilayers containing cholesterol

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ABSTRACT A theoretical explanation of the experimentally observed characteristic thermal anomalies in the specific heat of lipid bilayers containing cholesterol is provided in terms of the phase equilibria in the phosphatidylcholine-cholesterol system. The phase equilibria are calculated via a microscopic interaction model that takes proper account of both the conformational and the crystalline degrees of freedom of the lipid acyl chains. It is shown that the characteristic double-peaked specific heat, with a narrow and a broad component, is a natural consequence of the topology of the phase diagram. Some results for the enthalpy of the mixed system are also reported. It is suggested that there is no need for invoking special mechanisms such as lipid-cholesterol complexing or formation of special interfacial regions in the bilayer in order to explain the specific-heat anomalies.

INTRODUCTION

Cholesterol is one of the most important lipid components of eucaryotic plasma membranes. It not only plays a biochemical role in a variety of processes associated with membranes (Demel and de Kruyff, 1976; Bloch, 1983; Yeagle, 1985) but also acts as an indispensible regulator of the physical properties of lipid membranes (Presti, 1982; Bloom and Mouritsen, 1988). The physical interactions between cholesterol and lipids and the macroscopic manifestations of these interactions in membranes have been the subject of intensive experimental investigations using a great variety of techniques, notably calorimetry (Ladbrooke et al., 1968; Estep et al., 1978; Mabrey et al., 1978; Melchior et al., 1980; Blume, 1980; Imaizumi and Hatta, 1984; Genz et al., 1986), magnetic resonance spectroscopy (Recktenwald et al., 1981; Presti et al., 1982; Jacobs and Oldfield, 1979; Vist, 1984; Davis, 1988), micromechanics (Needham et al., 1988), neutron and x-ray scattering (Knoll et al., 1985; Mortensen et al., 1988; McIntosh, 1978; Hui and He, 1983), Raman spectroscopy (Pink et al., 1981; O'Leary and Levin, 1986), electron microscopy (Copeland and McConnell, 1980), and fluorescence depolarization (Smutzer and Yeagle, 1985; van Ginkel et al., 1986).

A substantial part of the experimental work has focussed on a quantitative evaluation of the thermodynamic effects of various amounts of cholesterol on the pure lipid bilayer main phase transition. The reason for this approach has been the observation that the main phase transition is very sensitive to the nature of the molecular interactions. The main phase transition takes the lipid bilayer from a solid gel phase to a fluid (liquid-crystalline) phase. Among the thermodynamic effects of cholesterol on this transition the following have been considered as important in the case of the dipalmitoyl phosphatidylcholine (DPPC)-cholesterol system: (*a*) the sharp component of the specific heat anomaly in the pure system, $x = 0$, is diminished and slightly broadened up to $\sim x = 10$ mol-%, and at the same time there is hardly any freezing-point depression, (*b*) around this concentration a second and broad specific-heat component can be discerned, (*c*) the sharp component steadily diminishes between ~ 10 mol-% and a particular limit $x^* \simeq 20 - 25$ mol-%. The sharp component is absent above x^* and then decreases as the cholesterol content is further increased; (*d*) the enthalpy of the sharp component decreases linearly with x, and the enthalpy of the broad component has a maximum around x^* and vanishes above $x \sim 50$ mol-%.

The double-peaked nature of the specific heat has been observed not only for cholesterol in DPPC membranes, but also in dimyristoyl phosphatidylcholine (DMPC) (Mabrey et al., 1978; Blume, 1980) dimyristoyl phosphatidylethanolamine (DMPE) (Blume, 1980), and dimyristoyl phosphatidic acid (DMPA) (Blume and Hillmann, 1986) membranes. Here we hypothesize that it is a general property of binary mixtures of lecithin and cholesterol with phase diagrams similar to that of the DPPC-cholesterol system (Fig. 1). This implies that it is not necessary for an explanation of the specific-heat anomalies to invoke special mechanisms, such as formation of

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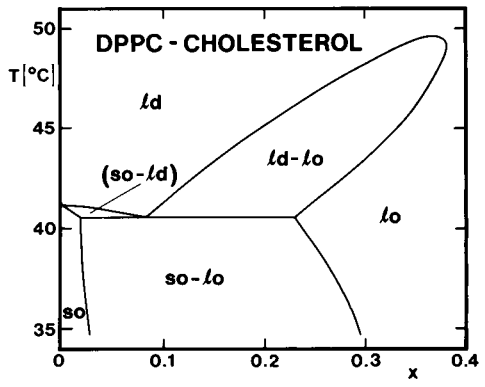

FIGURE 1 Theoretical phase diagram for DPPC-cholesterol bilayers. The various phases are denoted by so (solid-ordered), ld (liquid-disordered), and lo (liquid-ordered).

lipid-cholesterol complexes[1] (Melchior et al., 1980; Mabrey et al., 1978) or formation of special interfacial regions in the membrane (Estep et al., 1978; Imaizumi and Hatta, 1984; Genz et al., 1986). Instead, we point out that the thermal anomalies are simple consequences of the phase behavior and we present this explanation as a resolution of the controversy caused by the many different and often conflicting theoretical interpretations of the experimental data (for a review, see e.g., Presti, 1985). Our approach is made possible only by the recent advances in obtaining a reliable experimental phase diagram for the DPPC-cholesterol system using a combination of nuclear magnetic resonance spectroscopy and calorimetry (Vist, 1984; Davis, 1988) and the theoretical modeling of this phase diagram using a microscopic interaction model as well as a phenomenological thermodynamic model (Ipsen et al., 1987). This theoretical modeling has provided the first consistent description of the phase equilibria and has shown that the experimental phase behavior can be rationalized by means of a few basic assumptions. These assumptions, described below, take account of the different interactions between cholesterol and the internal (conformational) and lateral (crystalline) degrees of freedom of the lipid molecules. It was shown (Ipsen et al., 1987) that the unusual phase behavior of the DPPC-cholesterol system is due to the interplay between these two sets of degrees of freedom. Earlier theoretical work (Pink and Carroll, 1978; Pink and Chapman, 1979; Jähnig, 1981; O'Leary, 1983; Mouritsen et al., 1983) did not take into account the coupling between the conformational and crystalline degrees of freedom and hence could not provide a satisfactory explanation of the thermal anomalies in the specific heat.

[1]We interpret the term "complex" as a long-lived association between lipid and cholesterol molecules implying the presence of an effectively infinite strong interaction.

MODEL AND METHODS

The microscopic interaction model on which our results are based was first proposed in the context of pure lipid systems to describe the effects of acyl chain ordering and crystallization on the phase transitions in lipid monolayers (Mouritsen and Zuckermann, 1987) and bilayers (Zuckermann and Mouritsen, 1987). Subsequently, the model was extended to account for bilayers (Ipsen et al., 1987) and monolayers (Ipsen et al., 1989) containing cholesterol. The model used for the pure system is a combination of Pink's multi-state model (Pink et al., 1980; Caillé et al., 1980) which describes acyl-chain conformational degrees of freedom and their mutual interactions, and of a generalized multi-state Potts model (Ipsen et al., 1987) that is used to account for the translational degrees of freedom and the effects of crystallization in an approximate manner. The model therefore assumes that condensation and ordering in lipid bilayers involves two different sets of degrees of freedom. In pure lipid bilayers these two sets appear to be coupled and the acyl-chain conformational ordering and the two-dimensional crystallization take place at the same temperature (Caffrey, 1985; Zuckermann and Mouritsen, 1987). The main phase transition hence transforms the bilayer from a solid-ordered (so) gel phase to a liquid-disordered (ld) fluid phase, cf. Fig. 1, where the first label refers to the lateral nature of the two-dimensional membrane phase and the second label refers to the average internal conformational state of the acyl chains.

Pink's multi-state model is a two dimensional lattice model that accounts for the intra-chain conformational energy, the rotational isomerism of the chains, and the van der Waals interactions between chains. The ten chain conformations allowed for in the model include an all-*trans* ground state, eight gel-like excited states, and a highly excited state of low conformational order characteristic of the fluid phase. Each conformational state is characterized by a cross-sectional area per molecule, an internal conformational energy which is related to the number of gauche defects and an internal entropy that can be regarded as a density of states. Numerical values for these parameters are given for DPPC in Caillé et al (1980). The ten-state model on its own describes a phase transition from a conformationally ordered to a conformationally disordered phase which is driven by the difference between the high conformational entropy of the excited state and the considerably lower conformational entropies of the gel-like states. The generalized multi-state Potts model is a modification of the standard high-Q-state Potts model which has had great success in the description of grain growth in polycrystalline aggregates. The standard Potts model accounts for the energy of the grain boundaries of a metastable distribution of crystalline domains, each of which is characterized by a Potts state. In the modified Potts model, each lattice site represents an acyl chain of the lipid molecule and carries two independent sets of states i.e., Q Potts states and ten configurational states. The Potts variables again describe the orientation of crystalline domains with which the chain on the lattice site is associated. A domain boundary energy is modeled by allowing neighboring acyl chains to interact with a positive energy if they are in different Potts states and at the same time if each chain is in one of the first nine gel-like states. Otherwise the interaction is zero. This is reasonable since the tenth conformational state is representative of the fluid phase which cannot have a granular nature. The generalized Potts model therefore replaces in a very approximate manner the translational degrees of freedom of the solid phase.

The unusual behavior of cholesterol in relation to lipid bilayers can be

understood in the following terms: (*a*) cholesterol dissolves most easily in liquid phases, and (*b*) cholesterol prefers neighboring acyl chains to be orientationally ordered, i.e., with conformations characteristic of the solid phase. Cholesterol resolves this dilemma by preferring both lipid phases equally up to a certain cholesterol concentration. This leads to almost horizontal phase lines for low x and very little phase separation, cf. Fig. 1. Above ~10 mol-%, the cholesterol-induced acyl-chain ordering in the liquid phase and the concomitant cholesterol-induced crystal-breaking in the solid phase reaches a point where the conformational order is similar to that of the lipid gel phase. Cholesterol then prefers a liquid-ordered (lo) phase which is characterized by positional disorder and conformational order. This leads to massive phase separation, cf. Fig. 1. At this point the conformational and crystalline degrees of freedom decouple giving way to the unusual phase behavior of lipid-cholesterol mixtures.

The conformational part of the model originally used by Ipsen et al. (1987) included only two conformational chain states. This was sufficient to produce the correct topology of the phase diagram. However, to go beyond this pure free energy level and to obtain reliable derivatives of the free energy, e.g., the specific heat, a more detailed model is required. Hence, we use the ten-state model of Pink (Pink et al., 1980) which takes into account the statistical properties of several intermediate chain conformers. The hydrophobic interaction between the cholesterol molecules and the acyl chains is modeled by a molecular shape factor that is a simple function of the molecular cross-sectional area, i.e., a constant for the rigid cholesterol molecule. The crystal-breaking properties of cholesterol are accounted for by decoupling the Potts interaction between nearest-neighbor sites whenever a cholesterol molecule is involved.

The free energy, $G(T, x)$, of our microscopic interaction model is obtained in the mean-field approximation which is described elsewhere (Ipsen et al., 1987). The phase diagram can then be obtained from the free energy. The enthalpy and the specific heat are derived using the standard thermodynamic relations, i.e.,

$$H(T, x) = -T^2 \frac{\partial}{\partial T}\left(\frac{G(T, x)}{T}\right) \tag{1}$$

$$C_P(T, x) = \left(\frac{\partial H}{\partial T}\right). \tag{2}$$

In order to facilitate the numerical differentiation of the free energy, we have convoluted the free energy function obtained from the model calculations with an "instrumental window function" which is taken to be a Gaussian of width σ (Ipsen and Mouritsen, 1988). This procedure also facilitates a comparison with experimental data that are invariably subject to finite resolution due to instrumental limitations. Hence we obtain a smoothed free energy $\tilde{G}$

$$\tilde{G}(T, x) = \frac{T}{\sqrt{2\pi\sigma^2}} \int_0^\infty \cdot \frac{G(T', x)}{T'} \exp\left[-(T - T')^2/2\sigma^2\right] dT' \tag{3}$$

and a corresponding smoothed enthalpy, $\tilde{H}(T, x)$, and specific heat, $\tilde{C}_P(T, x)$, by using Eqs. 1 and 2. At a first-order phase transition, C_P has a delta-function singularity which is broadened into a Gaussian by our smoothening procedure.

RESULTS

In Fig. 1 the theoretical phase diagram for a set of model parameters relevant to DPPC is shown. This diagram is in close agreement with experimental data (Vist, 1980; Davis, 1988; Ipsen et al., 1987). The diagram shows a narrow coexistence region (so-ld) at low x, a three-phase line at intermediate x, and the occurrence of a new phase, lo, at high x. A distinct feature of the phase behavior is the occurrence of an eutectic point at x_{eu}. The (ld-lo) coexistence region at high temperatures terminates in an upper critical point.

The structure of the phase diagram is clearly reflected in the specific heat scans that are shown in Figs. 2 and 3. Fig. 2 shows the overall behavior for two different widths σ of the resolution function, cf. Eq. 3, whereas Fig. 3 gives a more detailed picture of the behavior at low concentrations and in the neighborhood of the eutectic point. The more narrow the resolution function is, the more details become available about $\tilde{C}_P$. From these figures we make the following observations about the specific-heat anomalies as the cholesterol content is increased:

(*a*) The sharp peak at $x = 0$ with the initial width of the resolution function decreases slightly in intensity as x is increased and the peak position moves towards somewhat lower temperatures as the three-phase line is approached. The peak position closely follows the ld-(so-ld) phase boundary. There is a small amount of asymmetry toward the so-(so-ld) boundary.

(*b*) As x is increased along the three-phase line towards the eutectic point, x_{eu}, the peak intensity first decreases and then increases again. For high resolution, the specific heat can be resolved into two distinct peaks following the two phase boundaries. These two peaks have a maximum

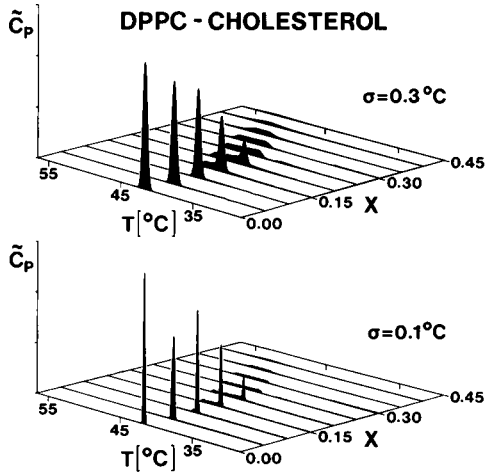

FIGURE 2 Theoretical specific heat, $\tilde{C}_P(T, x)$ in Eq. 3, v. s. temperature and composition for DPPC-cholesterol bilayers. Results are shown for two different widths, σ, of the resolution function: *a* σ = 0.3°C and *b* σ = 0.1°C. $\tilde{C}_P$ is in arbitrary units and the data in case (*a*) have been expanded vertically by a factor of 2.5. For the sake of clarity the area between the curve and the baseline has been shaded.

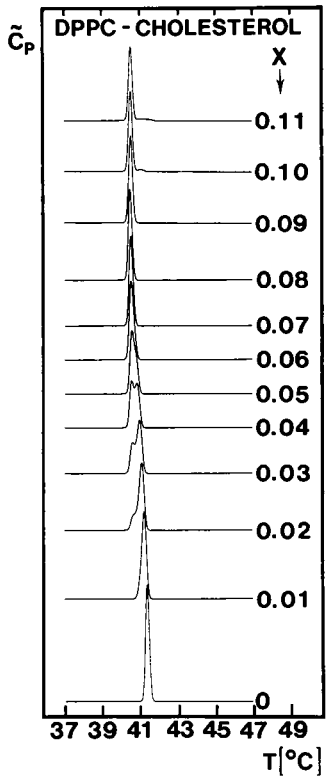

FIGURE 3 Theoretical specific heat, $\tilde{C}_P(T, x)$ in Eq. 3 v. s. temperature for different compositions of DMPC-cholesterol bilayers at low concentrations and around the eutectic point, cf. Fig. 1. The width of the resolution function used is $\sigma = 0.1$°C. $\tilde{C}_P$ is in arbitrary units.

splitting at the low-x terminus of the three-phase line. The splitting decreases towards the eutectic point. For a lower resolution, the specific heat peak will appear as a broadened peek in this concentration range, and the width of the peak first increases and then decreases again as the eutectic point is approached.

(*c*) At the eutectic point, the peak passes through a maximum and it attains the width of the Gaussian resolution function. The intensity of the peak is down to a factor much greater than $(1 - x)$ of the intensity at $x = 0$ due to the unusual properties of the lo-phase.

(*d*) Above the eutectic point, a second broad component of the specific heat can be discerned. For poor resolution (see e.g., Fig. 2 *a*) this new feature reduces to an upper shoulder of the main peak. When x is increased, the sharp specific-heat component steadily decreases in intensity without appreciably changing in width and with a position that follows the three-phase line. At $x = x^*$, which marks the high-x terminus of the three-phase line, the sharp component has vanished. At the same time, the broad component first increases in width and then decreases again while its intensity steadily decreases. The broad component matches the extent of the (ld-lo) coexistence region. Beyond the high-x terminus of this coexistence region the broad component is almost gone. However, there is still a measurably finite specific heat immediately above the terminus due to critical fluctuations. The center of this contribution moves towards higher temperatures as x is increased.

(*e*) The (so-lo)-lo and so-(so-lo) phase lines give only very weak signals in the specific heat since these lines do not involve acyl-chain disordering entropy.

The enthalpy $\tilde{H}(T, x)$ of the lipid-cholesterol mixture is shown in Fig. 4. The finite width of the resolution function used to construct $\tilde{H}$ implies a smearing of the jump-discontinuity at the first-order phase boundaries. The variation of $\tilde{H}$ with temperature clearly has two distinct regions, one with a rapid variation across the three-phase line and another with an approximate linear variation across the (ld-lo) coexistence region. These two regions reflect the same underlying phase behavior as do the two components of the specific heat.

We have extracted the heat content of each of the two specific-heat contributions separately. Obviously, there is no unique way of performing such a separation since the two components overlap. However, in view of the experimental interest in such a separation (Estep et al., 1978; Genz et al., 1986) we have carried out such a separation by the following simple rationale: The total heat content in the range from 32° to 52°C is obtained by integration. The heat content in the sharp component is approximated by the integral of a Gaussian fitted to the sharp component. The heat content in the broad component is then taken to be the difference between the total heat content in the specified range and that of the sharp component. The results obtained by this procedure are given in Fig. 5 which gives the enthalpy of the two components relative to the transition enthalpy of the pure system. It can be seen that the enthalpy of the sharp component decreases linearly and vanishes at a cholesterol concentration corre-

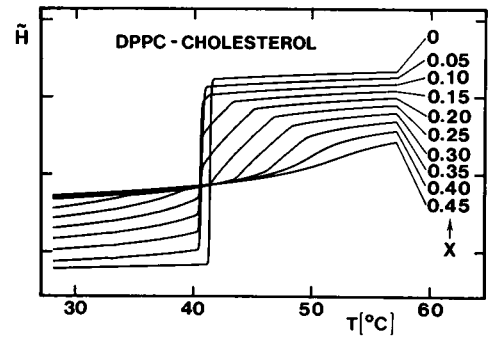

FIGURE 4 Theoretical enthalpy, $\tilde{H}(T, x)$, v. s. temperature for different compositions of DPPC-cholesterol bilayers. The width of the resolution function used is $\sigma = 0.1$°C. $\tilde{H}$ is in arbitrary units.

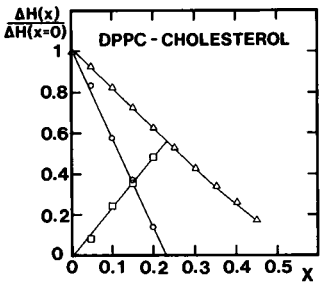

FIGURE 5 Relative enthalpy content $\Delta H(x)/\Delta H(x = 0)$, v.s. composition of DPPC-cholesterol-bilayers for the sharp (O) and the broad (□) components of the specific heat, cf. Fig. 3, shown together with the total enthalpy (△) in the range from 32° to 52°C.

sponding to the terminus, x^*, of the three-phase line, cf. Fig. 1. In contrast, the broad component has an increasing enthalpy content up to $\sim x^*$ and then decreases again and becomes vanishingly small above $x \simeq 0.5$. Fig. 5 also shows the total enthalpy of the mixture as obtained by integrating over the specified range from 32° to 52°C.

DISCUSSION

We have shown here that the unusual phase equilibria, cf. Fig. 1, of the DPPC-cholesterol bilayer system give rise to a set of specific-heat thermal profiles with distinct anomalies that reflect the topology of the underlying phase diagram. A number of specific-heat peaks can be discerned, depending on the thermal resolution and the composition of the membrane, cf. Figs. 2 and 3. Our results are based on a theoretical calculation on a microscopic interaction model for lipid bilayers containing cholesterol and hence the specific heat is basically derived from first principles and no special mechanisms are assumed. We therefore argue that an interpretation of the experimentally observed specific-heat anomalies does not require specific molecular mechanisms to be operative, such as complexing (Melchior et al., 1980; Mabrey et al., 1978; Snyder and Freire, 1980) or formation of special interfacial regions (Estep et al., 1978; Imaizumi and Hatta, 1984; Genz et al., 1986).

As earlier pointed out (Ipsen et al., 1987), the theoretical phase diagram as derived from the microscopic model is in close agreement with the most reliable experimental measurements of the phase equilibria in the DPPC-cholesterol system, including nuclear magnetic resonance spectroscopy and scanning calorimetry (Vist, 1984; Davis, 1988), electron spin resonance spectroscopy (Recktenwald and McConnell, 1980), and freeze-fracture microscopy (Copeland and McConnell, 1980). It should be mentioned, however, that there are still some experimental controversies regarding the low-temperature structure of the phase diagram and the interference with the ripple phase (Mortensen et al., 1988). We will not be concerned with these complications here. It is notoriously difficult to determine accurately the equilibrium phase diagram for mixtures using bulk techniques such as calorimetry or local-probe techniques such as magnetic resonance (for a recent discussion, see e.g., Ipsen and Mouritsen, 1988). In the case of calorimetry, this is due to the difficulty of relating the shape of the specific-heat scan to the phase boundaries, given a finite thermal resolution. Often, not all of the anomalies can be resolved. This is clearly demonstrated by the present work, cf. Figs. 2 and 3. In the case of the local-probe techniques, the probe molecules may report on local density fluctuations rather than macroscopic equilibrium phases and hence lead to a misinterpretation of the phase lines. Our results in Fig. 3 suggest that a careful calorimetric study with very slow scan rates (and consequently high resolution) at low cholesterol contents may reveal new features in the specific heat that should prove useful to accurately determine the extent of the (so-ld) coexistence region and the location of low-x terminus of the three-phase line.

The specific heat traces shown in Figs. 2 and 3 and their thermal anomalies are very similar, even quantitatively, to experimental calorimetry measurements of a variety of groups (Mabrey et al., 1980; Estep et al., 1980; Vist, 1984; Imaizumi and Hatta, 1984; Genz et al., 1986). In particular, the calculations reproduce the second broad component rather accurately. Moreover, the separation of the total heat content into contributions attributed to the two peaks, Fig. 5, is in quantitative accordance with experimental findings (Estep et al., 1980).

The results reported here were obtained for a set of model parameters pertinent to DPPC bilayers. However, our general findings for the specific-heat anomalies will also apply to other lipid membranes containing cholesterol if they have a phase diagram of the type in Fig. 1. Specifically, we expect the results to apply to DMPC membranes for which it is found experimentally (Mabrey et al., 1980; Imaizumi and Hatta, 1984) that two sharp specific-heat peaks can be discerned $\sim x \simeq 0.15$. This can within the present model be rationalized by a phase diagram similar to that in Fig. 1 where the ld-(ld-lo)-phase line is more horizontal (and the (ld-lo)-coexistence region more narrow) and therefore gives rise to a sharper and more intense second component.

Finally, we point out that the present theoretical calculations were performed within a mean-field approximation to the statistical mechanical problem posed by the microscopic interaction model. This approximation suppresses lateral density fluctuations that may have some effect on the phase equilibria, in particular close to $x = 0$ where the main phase transition in the pure system is

known to be strongly influenced by fluctuations (Mouritsen and Zuckermann, 1985). Consequently, we have neglected effects due to inhomogeneous lateral distribution of cholesterol and domain formation near the phase lines (Cruzeiro-Hansson et al., 1989). Such effects will lead to wings in the specific-heat peaks but are not expected to quantitatively change the overall behavior of the specific heat as a function of temperature and cholesterol content.

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