From Lanosterol to Cholesterol: Structural Evolution and Differential Effects on Lipid Bilayers

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ABSTRACT Cholesterol is an important molecular component of the plasma membranes of mammalian cells. Its precursor in the sterol biosynthetic pathway, lanosterol, has been argued by Konrad Bloch (Bloch, K. 1965. *Science.* 150:19–28; 1983. *CRC Crit. Rev. Biochem.* 14:47–92; 1994. Blonds in Venetian Paintings, the Nine-Banded Armadillo, and Other Essays in Biochemistry. Yale University Press, New Haven, CT.) to also be a precursor in the molecular evolution of cholesterol. We present a comparative study of the effects of cholesterol and lanosterol on molecular conformational order and phase equilibria of lipid-bilayer membranes. By using deuterium NMR spectroscopy on multilamellar lipid-sterol systems in combination with Monte Carlo simulations of microscopic models of lipid-sterol interactions, we demonstrate that the evolution in the molecular chemistry from lanosterol to cholesterol is manifested in the model lipid-sterol membranes by an increase in the ability of the sterols to promote and stabilize a particular membrane phase, the liquid-ordered phase, and to induce collective order in the acyl-chain conformations of lipid molecules. We also discuss the biological relevance of our results, in particular in the context of membrane domains and rafts.

INTRODUCTION

Cholesterol is the sterol molecule universally present in mammalian cells. It is predominantly distributed in the cell plasma membrane, amounting to 30–50 mol % of the total lipid fraction of the membrane. Given the fact that a plasma membrane consists of >200 lipid species and that the lipid composition varies greatly from one cell type to another, the presence of cholesterol certainly appears strikingly singular. At the level of molecular structure, cholesterol, while being amphiphilic, also differs significantly from the other lipid species. Its hydrophobic part consists chemically of a planar steroid ring and a short hydrocarbon tail (Fig. 1 *a*). Consequently, this part of the molecule is physically rigid and smooth at atomic scale (Fig. 1 *b*). This molecular characteristic has important implications for the interactions of cholesterol with other lipid species.

A considerable amount of research has been carried out during the last few decades to elucidate the biological functions of cholesterol in cells, to understand the physical basis for the biological functions by investigating the role of cholesterol in modulating physical properties of artificial and biological membranes, and to unravel the relationship between the functions and the molecular structure of cholesterol (Finegold, 1993; Vance and Van den Bosch, 2000). However, an equally important and intriguing question, the question of the origin of cholesterol in the context of cell evolution, has received relatively less attention, perhaps due to the apparent overwhelming complexity that must be involved in answering the question.

Nevertheless, a hypothesis, based on the pioneering work of Konrad Bloch, has been put forward (Bloch, 1965, 1983, 1994; Bloom and Mouritsen, 1995): cholesterol has been selected over the almost unimaginably long time scale of natural evolution for its ability to optimize certain physical properties of cell membranes with regard to biological functions. As a working hypothesis, it underlines the need to do comparative studies of the physical effects of cholesterol and its evolutionary precursors on cell membranes.

Although no fossils exist of evolutionary precursors to cholesterol, living "fossils" have been argued by Bloch to be present in the contemporary biosynthetic pathways of sterols (Bloch, 1965, 1983, 1994). In other words, the temporal sequence of the biosynthetic pathway can be taken to represent the evolutionary sequence. The evidence comes from a series of studies of sterol biochemistry and organism evolution (Bloch, 1994) and appear highly convincing. This concept of living molecular fossils offers the possibility of an experimental laboratory program to identify the physical properties that are relevant to evolutionary "optimization."

Inspired by Konrad Bloch's idea of sterol evolution, we have chosen to study cholesterol in comparison with its precursor, lanosterol. Lanosterol occupies an important position in the sterol pathways, as it is a common precursor in both the mammalian and fungal sterol pathways. A comparative analysis of the molecular structures of the two sterols (see Fig. 1) clearly shows that lanosterol, with three additional methyl groups, can be characterized as being

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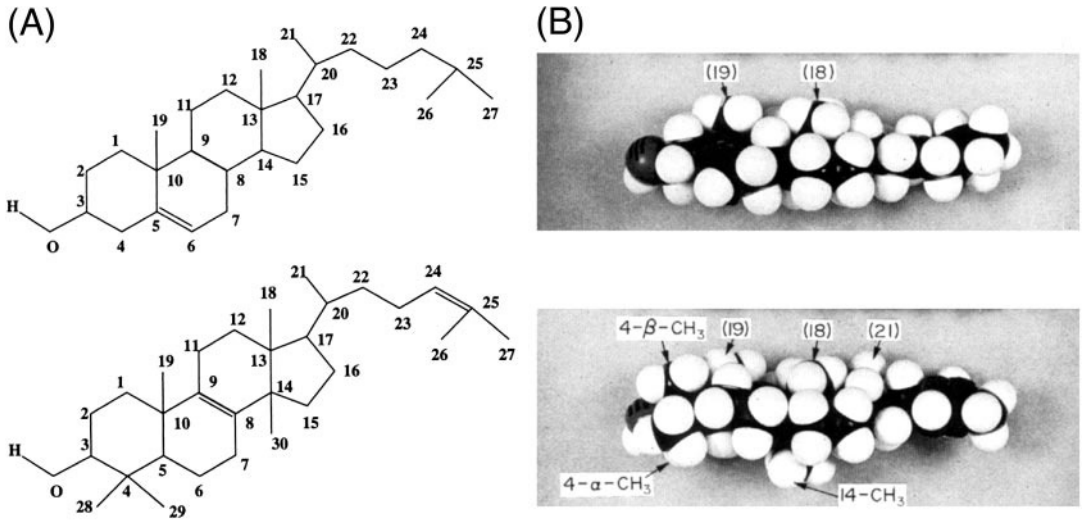

FIGURE 1 Molecular structures of cholesterol (*top*) and lanosterol (*bottom*). (*A*) Chemical structures; (*B*) space-filling models. The three additional methyl groups on lanosterol are indicated in (*B*) as 14-CH_3, 4-α-CH_3, and 4-β-CH_3.

structurally less smooth in its hydrophobic part than cholesterol: the methyl group at position 14, in particular, protrudes from the steroid surface. In fact, the biochemical process of converting lanosterol to cholesterol is essentially a process of streamlining the steroid surface by successive removal of the three methyl groups, where the C-14 methyl group is the first to be removed (Bloch, 1983, 1994). This structural differentiation will turn out to have consequences in terms of the physical roles each of the two sterols plays as a molecular component in lipid membranes.

Being fully aware of the fact that the issue of sterol evolution in its full biological context is not an easy one to approach by purely physical techniques, we have taken on a somewhat smaller task of posing a well-defined question that can be answered quantitatively by use of physical concepts and techniques. Because artificial bilayer membranes are important model systems for biological membranes, we have studied model membranes composed of a single species of phosphatidylcholine and either one of the two sterols. Due to their amphiphilic nature, both cholesterol and lanosterol easily intercalate in a lipid-bilayer membrane, with their hydroxyl group positioned on the average at the hydrophobic-hydrophilic interface and with the hydrophobic steroid skeleton embedded in the bilayer core. The molecular ordering and the phase equilibria of these lipid-sterol systems provide a specific context within which differential effects of lanosterol and cholesterol can be unambiguously defined and quantitatively assessed through experimental and theoretical investigations.

Several other comparative studies of lanosterol-lipid and cholesterol-lipid membranes have been reported, each focusing on a particular aspect of the effects of the sterols on the physical properties of the lipid membranes. In particular, the "microviscosities" (or more precisely, the conformational order of the lipid acyl chains) of cholesterol-lipid and lanosterol-lipid membranes were measured and compared (Dahl et al., 1980), and it was shown that cholesterol increased the "microviscosity" of the membranes much more effectively than lanosterol. The differential effects of lanosterol and cholesterol on the permeability of lipid membranes to small sugar molecules (Yeagle et al., 1977; Bloch, 1983) were also investigated, and it was again demonstrated that cholesterol reduced the membrane permeability more effectively than lanosterol. Moreover, molecular ordering and dynamics of phosphatidylcholine bilayers in the presence of either cholesterol (or ergosterol) or lanosterol were investigated experimentally by use of NMR techniques at a couple of specific temperatures and sterol concentrations (Yeagle, 1985; Urbina et al., 1995). Recently, the effects on membrane domain formation of the molecular structures of several sterols, including both lanosterol and cholesterol, were studied in both fluorescence-quenching and detergent-solubilization experiments and compared (Xu and London, 2000). These studies showed that cholesterol has a stronger ability than lanosterol to induce conformational order in lipid chains and promote domain formation in membranes. Even more recently, a theoretical study of lipid-lanosterol and lipid-cholesterol bilayers by use of molecular dynamics simulation methods was carried out (Smondyrev and Berkowitz, 2001). With simulations performed over microscopic time scales (nanoseconds), the study focused on investigating differences in physical properties of the two types of lipid-sterol bilayers, which include lipid-chain ordering, the dynamics, and the location in the bilayer transverse direction of the sterol molecules. At a relatively modest sterol concentration ($\sim$10%), subtle differences in sterol location and mobility in the bilayers were identified, which indicate that lanosterol is more mobile than cholesterol as a

bilayer constituent. At a sterol concentration of 50%, cholesterol was observed to have an overall slightly stronger condensing effect on the bilayer than lanosterol. The objective of our study is to establish the systematics of thermodynamic behavior of lanosterol-lipid and cholesterol-lipid membranes in terms of phase equilibria, and furthermore, to understand the molecular basis underlying the phase equilibria by combining theoretical modeling with experimental investigations.

The phase equilibria of many different types of model membranes have been understood. A bilayer composed of a single lipid species displays several phase transitions when driven thermally. We will be concerned with one of them, the main transition. The transition is first-order, involving two simultaneous macroscopic processes corresponding to the ordering of the translational and the internal chain conformational variables that pertain to lipid molecules. The transition takes the pure lipid bilayer from a solid-ordered (*so*) phase, where the bilayer is a two-dimensional crystal of the lipids with their chains in conformationally ordered states, to a liquid-disordered (*ld*) phase, where the bilayer is a two-dimensional liquid of the lipids with their chains in conformationally disordered states (Mouritsen, 1991). In other words, the two ordering processes are strongly coupled in the main transition, even though no fundamental principles dictate that they should be so.

The main transition is of key importance for understanding the influence of sterols in membranes. Sterol molecules such as lanosterol and cholesterol are, in contrast to phospholipids, essentially rigid and relatively smooth in their hydrophobic parts. Their mode of interaction with lipid molecules is of dual nature. On the one hand, they prefer to have next to them acyl chains that are ordered as in the *so* phase, thereby inducing chain ordering. On the other hand, because the sterols have different molecular shapes from a conformationally ordered lipid chain, they tend to break the lateral packing order of the *so* phase. Consequently, these sterols can uncouple the two types of macroscopic order in the translational and conformational variables of the lipid molecules. This effect can lead to the emergence of a physical state of lipid-sterol membranes, which has characteristics intermediate between the *so* and the *ld* states: the liquid-ordered (*lo*) state. This phenomenology underlies the first simple theory of the phase equilibria in cholesterol-lipid systems (Ipsen et al., 1987), which yielded predictions consistent with subsequent experimental observations (Vist and Davis, 1990; Thewalt and Bloom, 1992; Silvius et al., 1996). There is mounting evidence that the lipid-bilayer component of many biological membranes needs to be in this liquid-ordered state to perform its biological functions properly (Bloom et al., 1991).

The nature of the phase equilibria of lipid-cholesterol membranes has been a particularly elusive problem, and to some researchers the problem still remains controversial. Specifically, a few other different pictures of the phase equilibria of lipid-cholesterol membranes have been put forward: one picture involving the concept of molecular complex formation is based on experimental studies of monolayers formed at air-water interfaces from binary mixtures of cholesterol and a charged phospholipid (dimyristoylphosphatidylserine) (Radhakrishnan and McConnell, 1999; Radhakrishnan et al., 2000). Another picture is derived from differential scanning calorimetry (DSC) measurements of bilayer membranes of binary mixtures of cholesterol and dipalmitoylphosphatidylcholine and relies on a particular interpretation of observed features in the specific heat (McMullen and McElhaney, 1995). A theoretical study has also been carried out, which suggests a microscopic interaction model that involves many-body interactions between constituent molecules in membranes of cholesterol-lipid binary mixtures at very high cholesterol concentrations (Huang and Feigenson, 1999).

Our studies of simple bilayers of binary mixtures of phospholipids and the sterols consist of two parts: experiments using solid-state deuterium-NMR spectroscopy and calorimetry, which investigate and characterize the collective conformational ordering of the lipid molecules and the thermal behavior of the bilayer membranes, respectively, and parallel statistical mechanical modeling and computer simulations, which focus on understanding and relating the observed macroscopic phenomena to molecular interactions, and in turn, to the different molecular chemistries of the two sterol molecules. Our most important conclusion is that cholesterol, with its streamlined molecular structure, interacts more effectively with lipid chains with conformational order and stabilizes the liquid-ordered state of the lipid bilayers more effectively than lanosterol. This main finding has been briefly reported in a recent letter publication (Nielsen et al., 2000). In the present full article we will present a complete account of our studies, which includes additional data and results not yet reported. We will also discuss the results in connection to the biological functions of plasma membranes.

MATERIALS AND METHODS

Experimental

Chemicals and sample preparations

1-palmitoyl-2-petroselinoyl-*sn*-glycero-3-phosphatidylcholine (PPetPC) is *cis*-unsaturated at position C6–7 of the *sn*-2 chain, and was obtained from Avanti Polar Lipids Inc. (Birmingham, AL). This lipid differs from POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine)—a lipid species common in biological membranes—in one respect: its single unsaturated C–C bond is closer to the hydrocarbon-water interface. The advantage of using PPetPC instead of POPC is that its main-transition temperature is above the freezing point of water, and this offers us an advantage in carrying out experiments. For NMR experiments, PPetPC-d_{31}, of which the palmitoyl chain is perdeuterated, was obtained by custom synthesis from Avanti. It contained ~15% of the lipid with chains interchanged (PetPPC-d_{31}) as a byproduct of the synthesis. Cholesterol, lanosterol, and deuterium-depleted water were obtained from Sigma Chemical Co. (St. Louis, MO)

and used without further purification. The main impurity in lanosterol is 24-dihydrolanosterol.

To prepare multilamellar lipid dispersions, aliquots of $CHCl_3$ stock solutions of phospholipid and sterol were mixed in the appropriate quantities. The $CHCl_3$ was evaporated under a thin stream of N_2 and the samples were then placed under high vacuum overnight. The resulting films were dissolved in benzene/methanol (95:5 vol/vol) and then lyophilized overnight. Finally, the samples were hydrated at room temperature with a buffer (20 mM Hepes and 300 mM NaCl in deuterium-depleted water at pH 7.4) and mixed thoroughly; 50 mg PPetPC-d_{31} and 600 μl buffer, along with an appropriate mass of sterol, were used to make the samples for NMR measurements. In DSC measurements the total sample volume was 0.7 ml, containing ~20 mg phospholipid, along with an appropriate amount of sterol.

NMR and DSC measurements

Deuterium NMR spectra were obtained by using the quadrupolar echo technique with a locally built spectrometer operating at 46 MHz. A typical spectrum resulted from 10,000 repetitions of the two-pulse sequence with a 90° pulse length of 4 μs, inter-pulse spacing of $\tau = 40$ μs, and dwell time of 2 μs. The delay between acquisitions was 300 ms, and data were collected in quadrature with Cyclops 8-cycle phase cycling. Occasionally the spectra were symmetrized before subtraction by zeroing the out-of-phase channel to improve the signal-to-noise ratio. If the quadrupolar echo spectra of dispersions having different sterol concentrations were not uniformly affected by relaxation, a correction was performed to ensure correct weighting of the spectral components (Thewalt et al., 1992).

From the spectral data, the average half first moment, M_1, was calculated as

$$M_1 = \frac{1}{2A} \sum_{\omega=-x}^{x} |\omega| f(|\omega|), \tag{1}$$

where A is the area under the spectrum and $f(\omega)$ is the signal at a given frequency separation ω from the central (Larmor) frequency. The non-zero values of $f(\omega)$ are found between the points $-x$ and x where the spectrum ends. In practice, x and $-x$ were chosen to be $\pm$125 kHz. The first moment, M_1, is related to the chain-averaged carbon-deuteron order parameter $\langle S_{CD} \rangle$ as

$$M_1 = \frac{\pi}{\sqrt{3}} \frac{e^2 qQ}{h} \langle S_{CD} \rangle , \tag{2}$$

where e^2qQ/h is the static quadrupolar coupling. Hence, M_1 is a measure of the conformational order of the chain with respect to the membrane normal.

The spectra were also used in the determination of phase diagrams of the membrane systems by means of spectral subtractions. The spectral subtraction procedure is discussed in detail below.

No hysteresis was observed in the obtained NMR data. There was no dependence of spectra on equilibration time in the range from 15 min to 2 h. The spectra also appeared to be independent of the direction of temperature change (cooling/heating).

DSC measurements were made on a MicroCal 2 calorimeter with a MicroCal 1 control unit that was computer-interfaced via Optomux digitizer modules taking data at the rate of 1 point/10 s. The scans performed were only heating scans. To minimize the kinetic effects, the heating rate was chosen to be 9°C/h, the slowest feasible heating rate. As a check, a second scan of a sample was performed, in general, following overnight equilibration of the first scan. No significant differences in the two scans were observed.

Theoretical modeling

As a parallel to the experiments, the theoretical modeling of the phase equilibria of the lipid-sterol systems is used to provide insight into the microscopic physics underlying the experimental observations. In this part, basic microscopic models for lipid-sterol interactions are first proposed and the thermodynamic phase equilibria are then investigated by the statistical mechanical studies of the models by use of Monte Carlo computer simulations.

Model

Recently, a microscopic model was proposed for bilayer membranes of phospholipid-cholesterol binary mixtures to describe the phenomenology of the molecular interactions between cholesterol and lipids, and the equilibrium phase behavior of the membrane systems predicted by the model is entirely consistent with the experimental observations of the same systems (Nielsen et al., 1999). In the present work, we have extended that theoretical modeling to investigate systematically the phase equilibria of lipid-sterol bilayers as a function of the evolutionary chemistry of the sterols.

Our model description of a lipid-sterol membrane consists of three basic ingredients: a representation of the translational degrees of freedom, a description of molecular conformational degrees of freedom, and a minimal model of the interactions between the molecules. A complete presentation of the model is given in the Appendix.

The physical essence of our modeling of molecular interactions may deserve some emphasis here. Both the lipid and sterol molecules are modeled as hard-core particles that interact with each other. The molecular interactions are modeled by combinations of square-well potentials that are shown schematically in Fig. 2. The model is proposed based on the established phenomenology of lipid-cholesterol interactions (Ipsen et al., 1987; Nielsen et al., 1999) and the comparative analysis of the molecular structures of cholesterol and lanosterol. The figure captures the dual nature of the microscopic interactions between a sterol molecule and a lipid molecule, which is at the center of the microscopic interpretations of the lipid-cholesterol phenomenology; it also emphasizes the difference in the lanosterol-lipid and cholesterol-lipid interactions (see Fig. 2 *B*). Within the model, the depth of the longer-range potential well in Fig. 2 *B*, J_{chol} or J_{lan}, is a measure of the strength of cohesive interactions between a sterol and a lipid molecule with conformationally ordered chains. Thus, our modeling proposes that the *predominant* (though not the only) physical effect of varying molecular structure of a sterol is to modulate the cohesive interactions, i.e., the packing, between the sterol and a lipid molecule (Bloch, 1983). In other words, J may be taken as a principal physical representation of the degree of molecular smoothness of sterols: from lanosterol to cholesterol there is an increase in the value of J, as illustrated both qualitatively in Fig. 2 *B* and quantitatively in Table 1. Hence, the microscopic physical consequences of the evolution in the sterol chemistry are described by a single-parameter-tuned potential of sterol-lipid interactions. In this sense our approach is a minimal-model approach. Details on how the values of J_{chol} or J_{lan} and the values of other parameters are chosen are provided in the Appendix.

Simulation methods and data analysis

The models presented above are studied by using the statistical mechanical method of Monte Carlo simulations (Frenkel and Smit, 1996). From the simulation data, the phase equilibria of the model systems are established in terms of both phase diagrams and thermodynamic characterizations of the phases by molecular ordering and lateral distributions (structures).

The basic simulation method is that of Monte Carlo importance sampling (Frenkel and Smit, 1996). The straightforward application of the sampling method directly uses the microscopic Hamiltonian describing a system, and each sampling run generates a statistical ensemble of microscopic states according to the Boltzmann distribution. In our simulation studies of the lipid-sterol systems this direct sampling method suffices when we explore regions in the parameter space where no phase transitions

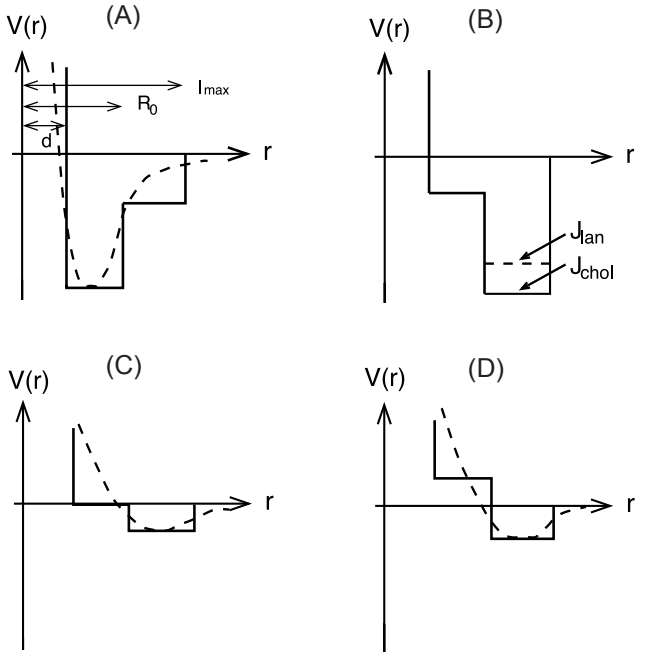

FIGURE 2 Schematic illustration of the model interaction potentials. (*A*) $V_{o\text{-}o}(R)$, where subscript "o" refers to a lipid chain in the conformationally ordered state; (*B*) $V_{o\text{-}s}(R)$, where the depth of the potential corresponding to s = chol is represented by a solid line and a parameter J_{chol}, and that corresponding to s = lan is represented by a dotted line and a parameter J_{lan}; (*C*) $V_{d\text{-}s}(R)$, where subscript "d" refers to a lipid chain in the disordered state; (*D*) $V_{s\text{-}s}(R)$. d is the hard-disk radius, R_0 is the range of the short-range potentials, and l_{max} is the effective range of the long-range potentials. The ratio of R_0/d is chosen so that the average surface area of a sterol molecule is ~30% larger than that of a lipid chain in the ordered state. The dashed lines illustrate the more realistic interaction potentials that the model potentials approximate.

are encountered. To deal with the difficulty of barrier crossing associated with strongly first-order phase transitions, however, a modified sampling method is used, where the "Hamiltonian" directly controlling the sampling process consists of the physical Hamiltonian and an artificial part designed to reduce the energy barrier separating two coexisting phases. Consequently, the statistical ensemble generated by a sampling run does not follow the Boltzmann distribution corresponding to the physical Hamiltonian. Efficient sampling, however, is achieved, and the physical Boltzmann distribution can be constructed from the data collected. This type of non-Boltzmann sampling methods are discussed in detail elsewhere (Besold et al., 1999; Nielsen et al., 1999).

In each simulation run, periodic boundary conditions and planar geometry are imposed on the system, and the following parameters are fixed: the total number of molecules (lipid plus sterol molecules) N, the temperature T, and a lateral "pressure" Π, which represents the self-stabilizing force of a bilayer membrane. In particular, Π is kept fixed in *all* simulations. The chemical composition of the lipid-sterol mixtures is either fixed absolutely by fixing the respective numbers of the lipid and sterol molecules or fixed only on average via an effective "chemical potential." The former case is referred to as the canonical ensemble and the latter case as the semi-grand canonical ensemble (Frenkel and Smit, 1996). To derive phase diagrams, many different simulation runs are performed systematically by varying T and the chemical composition. At each fixed setting of T and chemical composition, many simulation runs are carried out for different values of N, such that phase transitions can be identified through finite-size scaling analysis.

TABLE 1 Interaction parameters for the model potentials for the two types of lipid-sterol membranes

	$V_{o\text{-}o}$	$V_{o\text{-}d}$	$V_{d\text{-}d}$	$V_{s\text{-}s}$	$V_{s\text{-}d}$
Long range*	0.40	−0.15	0.20	0.20	0.00
Short range	0.45	0.40	−0.20	−0.15	−0.065
		Cholesterol			Lanosterol
$V^1_{o\text{-}s}(J)^\dagger$		0.85			0.75
$V^s_{o\text{-}s}$		−0.625			−0.525

*The parameter values for the interaction potential, $V_{o\text{-}o}$, between two lipid chains in the ordered state, the interaction potential, $V_{o\text{-}d}$, between a lipid chain in the ordered state and a lipid chain in the disordered state, the interaction potential, $V_{d\text{-}d}$, between two lipid chains in the disordered state, the interaction potential, $V_{s\text{-}s}$, between two sterol molecules, and the interaction potential, $V_{s\text{-}d}$, between a lipid in the disordered state and a sterol molecule. Note that these values are identical for both the lipid cholesterol and the lipid-lanosterol systems.

†The parameter values for the interaction potential, $V_{o\text{-}s}$, between a lipid chain in the ordered state and a sterol molecule, where the subscript "s" corresponds to either chol or lan, respectively. Note that the parameter $V^1_{o\text{-}s}$ is the same as the parameter J discussed in Theoretical Modeling. All the parameters are given in units of J_0.

A simulation run consists of preparing the system in a high-temperature liquid state, then quenching it down to a lower temperature desired, equilibrating the system to that temperature, and finally taking a sufficiently large number of "measurements" of relevant quantities for statistical analysis. In the data analysis that leads to the determination of phase diagrams several different techniques are used, including histogram and thermodynamic reweighting techniques and finite-size scaling analysis (Nielsen et al., 1999).

Various thermodynamic quantities are calculated to explicitly characterize different physical phases. Among them is ϕ, the equilibrium conformational order parameter of the lipid chains. This parameter, which is defined in the Appendix, is within our model the theoretical counterpart of $\langle S_{CD}\rangle$, the experimental chain order parameter defined in Eq. 2. Another class of the calculated quantities are the so-called structure factors, $S_T(\vec{q})$ and $S_{sterol}(\vec{q})$, which are also defined in the Appendix as functions of Fourier wave vector, $\vec{q}$. The two structure factors are useful for identifying and quantifying the lateral structure and ordering in the membranes, i.e., the actual average patterns of lateral distributions of the different molecular constituents of the bilayer.

RESULTS

Experimental results

DSC data, NMR spectra, and data analysis

We performed DSC measurements on multilamellar dispersions of PPetPC/cholesterol and PPetPC/lanosterol mixtures as functions of sterol concentration. Representatives of the obtained data are shown in Fig. 3.

In the absence of sterol, the DSC scan of PPetPC displays a single endothermic peak of width (at half-maximum height) 1.6°C and peak temperature (T_m)16.8°C. Upon the addition of a small amount (3 mol %) of sterol, the peak temperature drops significantly (to 15.3°C for cholesterol and 14.7°C for lanosterol). In addition, the peak broadens and apparently consists of more than one component.

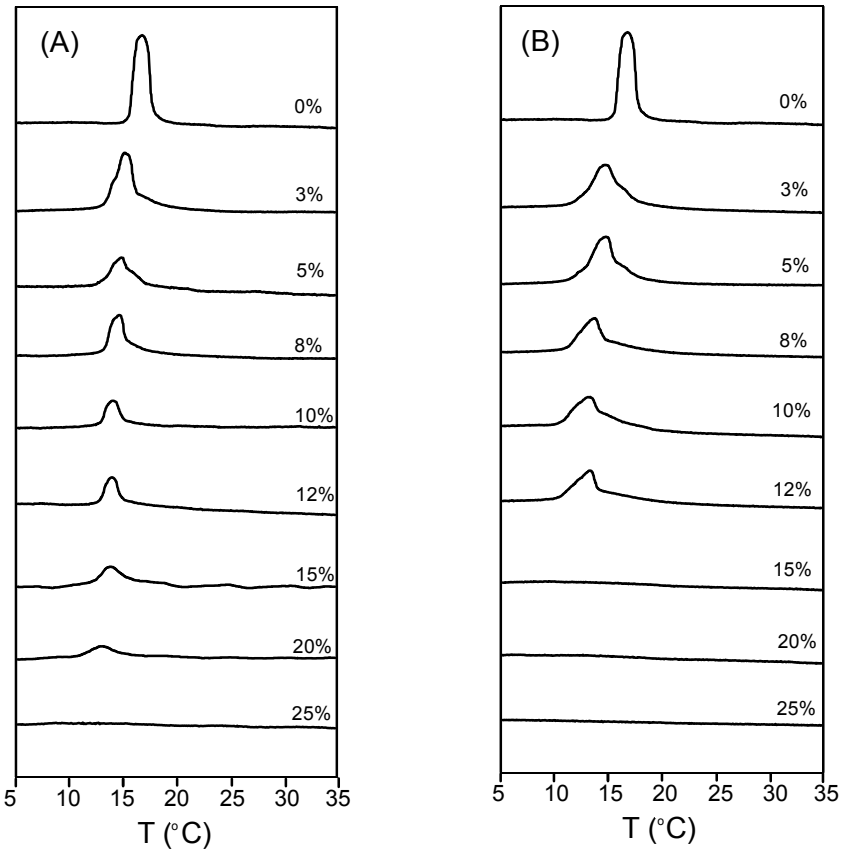

FIGURE 3 DSC traces for aqueous multilamellar dispersions of (*A*) PPetPC/cholesterol and (*B*) PPetPC/lanosterol. The membrane sterol concentration (mol %) is indicated. Trace intensities have been normalized according to the amount of PPetPC present in the samples.

Adding more cholesterol to a concentration of 10 mol % causes the peak temperature to drop a further 1.1°C, to 14.2°C. At cholesterol concentrations between 10 and 20 mol %, the peak temperature lies between 12.0°C and 14.2°C, with an average value of 13.2°C. Increasing the lanosterol concentration in the membranes has more dramatic effects on the observed endothermic behavior. At 10 mol % lanosterol the peak temperature already drops to 13.2°C, and at lanosterol concentrations of 15 mol % or greater, no peak is visible.

A comparison of solid-state NMR spectra of aqueous dispersions of PPetPC without sterol and with either of the two sterols at 30 mol % is shown in Fig. 4. The pure PPetPC membrane displays an *so* to *ld* transition, the signature of which is evident in the spectra obtained at temperatures below and above 15°C. At a lower temperature the spectrum is bell-shaped, reflecting the very limited rotational motion of deuterons in the perdeuterated lipid chains, which are closely packed and have very limited translational freedom. In other words, such spectra report the properties of a membrane in the *so* phase. At higher temperatures the spectrum is a superposition of Pake doublets, characteristic of the rapid reorientations of the lipid chains, which are axially symmetric about the membrane surface normal. As a rule, the splittings of Pake doublets are proportional to the degree of conformational ordering at particular positions along the lipid chain. The small magnitudes of the splittings observed for PPetPC indicate, therefore, that the membrane is in the *ld* phase. At 15°C (T_m) the spectrum consists of a superposition of *so* and *ld* spectra, and the fraction of the total intensity provided by each spectrum reflects the amount of each phase present.

For the sterol concentration of 30 mol % (Fig. 4, *B* and *C*) the spectral shape is that of a superposition of Pake doublets for temperatures ranging from 0 to 40°C. In the low-temperature spectra, the component Pake doublets have broadened intrinsic linewidths, characteristic of a liquid membrane that is very viscous. Upon heating, the spectra gradually narrow and their component Pake doublets sharpen. There is no dramatic change in spectral shape, indicating that no phase boundaries are crossed. In other words, both types of the PPetPC/sterol membranes appear liquid-like in this temperature range. Furthermore, the quadrupolar splittings observed for the sterol-containing membranes are much larger than those in the pure PPetPC membranes (compare the 40°C spectra in Fig. 4, *A–C*), indicating higher degrees of conformational ordering in the lipid chains induced by the sterols. Thus, it is clear that the presence of the sterols at this concentration eliminates the *so–ld* transition and puts the membranes in another physical state: the liquid-ordered state. The major difference between the two types of sterol-rich membrane dispersions is that the

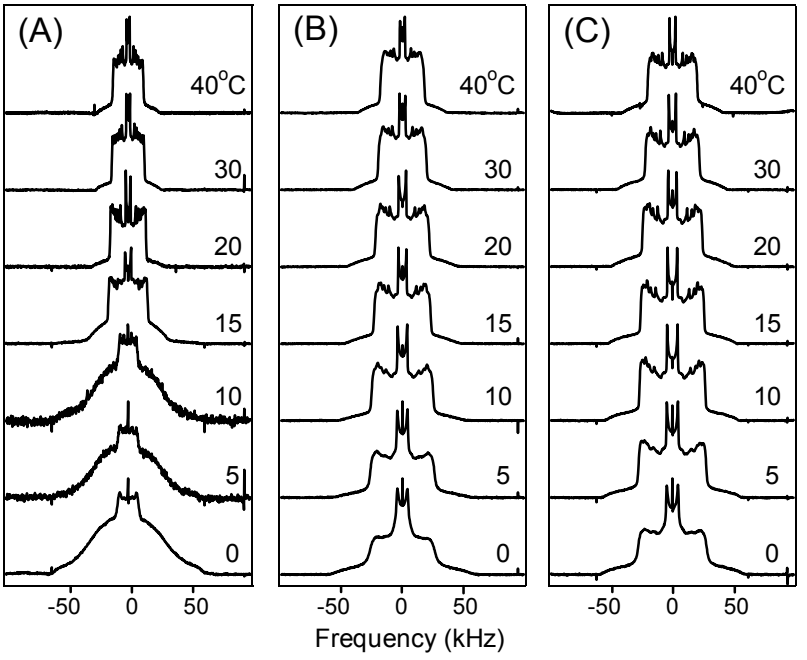

FIGURE 4 (*A*) Deuterium-NMR spectra of PPetPC as a function of temperature; (*B*) deuterium-NMR spectra of PPetPC/lanosterol (70:30) as a function of temperature; (*C*) deuterium-NMR spectra of PPetPC/cholesterol (70:30) as a function of temperature.

lipids in the lanosterol-containing membrane are significantly less ordered than those in the cholesterol-containing membrane.

For the sterol concentrations lower than 30 mol % and at low temperatures, the obtained NMR spectra are all superpositions of two components: one characteristic of the *so* phase and the other of the *lo* phase. These spectra are the manifestation of the coexistence of the *so* and the *lo* phase. Clearly, each spectrum of a membrane dispersion in the coexistence region reflects the amount of the labeled lipid in each phase. A spectral-subtraction procedure exploiting this fact enables an analysis of the spectra, which leads to the determination of the solidus (*so/so* + *lo*) and liquidus (*so* + *lo/lo*) phase boundaries. These two phase boundaries are essential parts of the whole phase diagrams of the PPetPC/sterol membranes (see below).

Phase diagrams

In deriving experimental phase diagrams, both the DSC data and the NMR spectra were used.

The apparent difference between the main-transition temperature reported by the DSC data (16.8°C) and that yielded by the NMR data (15°C) on the pure PPetPC membrane can easily be explained. The DSC data presented were obtained for non-deuterated PPetPC, while the NMR measurements required the deuterated PPetPC. The pure membranes made of the deuterated PPetPC were shown by DSC scans to have the main-transition temperature at 15°C (data not shown here). When phase diagrams were derived by combining the DSC data together with the NMR data, this difference was taken into account and properly offset by subtracting 1.8°C from the DSC data obtained for non-deuterated PPetPC.

The spectral-subtraction method mentioned above has been described thoroughly elsewhere (Vist and Davis, 1990; Thewalt et al., 1992). Briefly, two spectra corresponding to membrane systems of different sterol concentrations at a given temperature are used and analyzed together. For obtaining the solidus endpoint at the given temperature, a judiciously determined fraction, *K*, of the higher-concentration spectrum can be subtracted from the lower-concentration spectrum to yield just a spectrum of the purely *so* membrane. The sterol concentration corresponding to this *so* endpoint spectrum can then be calculated in a straightforward manner by using the two known sterol concentrations and the fraction, *K*. The sterol concentration marking the liquidus endpoint of the coexistence is obtained in a similar way, by subtracting a fraction of the lower-concentration spectrum from the high-concentration spectrum.

In the case of PPetPC/cholesterol membranes, subtractions were performed by using the spectra from membrane dispersions containing 10, 15, and 20 mol % cholesterol at each temperature in the range from 0 to 12°C, and resulted in nearly vertical solidus and liquidus boundaries at sterol concentrations of 8–9 mol % and 28–30 mol %, respectively. For PPetPC/lanosterol, subtractions were possible only up to 9°C (due to the emergence of the *ld* phase at higher temperatures) and resulted in solidus and liquidus lines that tended toward higher lanosterol mole fractions as the temperature was lowered (see Fig. 5).

The two phase diagrams thus derived, for PPetPC/cholesterol and PPetPC/lanosterol membranes, respectively, are

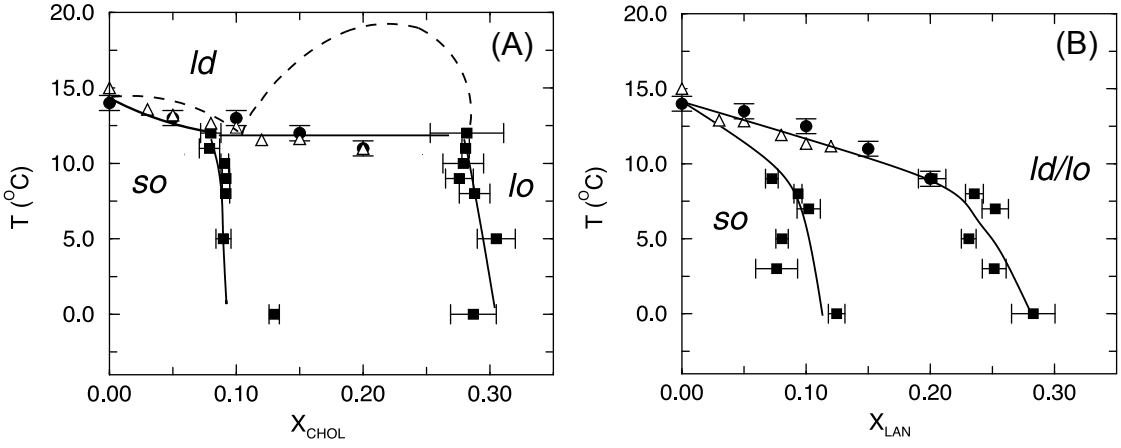

FIGURE 5 Experimental phase diagrams as determined by DSC (*open triangles*) and by NMR spectroscopy (*solid squares* and *solid circles*). (*A*) PPetPC-cholesterol systems; (*B*) PPetPC-lanosterol systems. The lines connecting the points are guides to the eyes. The phase boundaries indicated by the dashed lines in (*A*) are not derived from any experimental data and are shown only to illustrate the qualitative structure of the phase diagram, which is consistent with the thermodynamic phase rules.

shown in Fig. 5. They clearly indicate that the thermal phase equilibria of the two classes of lipid-sterol membranes are qualitatively different. The PPetPC/cholesterol membranes have the two usual thermodynamic phases: the *so* phase and the *ld* phase. In addition, when the concentration of cholesterol exceeds ~30%, a third distinct and stable thermodynamic phase appears, where the membranes are liquid-like, but at the same time have high degree of lipid conformational order. This *lo* phase is stable over a rather wide range of temperatures, extending both below and significantly above the main transition temperature of the pure lipid membrane. The evidence for the thermodynamic stability of the *lo* phase lies in the two-phase boundaries defining the *so–lo* coexistence and in the three-phase coexistence line, which are well resolved within experimental accuracy. The details of the *so–ld* coexistence could not, however, be determined as unambiguously, and the phase boundary of the *ld–lo* coexistence could not be resolved satisfactorily, either.

In the phase diagram for PPetPC/lanosterol membranes the *lo* states exist at low temperatures for high lanosterol concentrations. There are, however, no thermodynamic phase distinctions between them and the high-temperature *ld* states, as indicated by the absence of the three-phase coexistence line.

The phase diagrams are also corroborated by the first-moment M_1 data calculated from the NMR spectra according to Eq. 1. M_1 provides a gauge for the averaged conformational order of the lipid chains. Low M_1 values reflect low degrees of chain conformational order. Fig. 6, *A* and *B* show the derived M_1 as a function of temperature at various sterol concentrations for the lipid-cholesterol

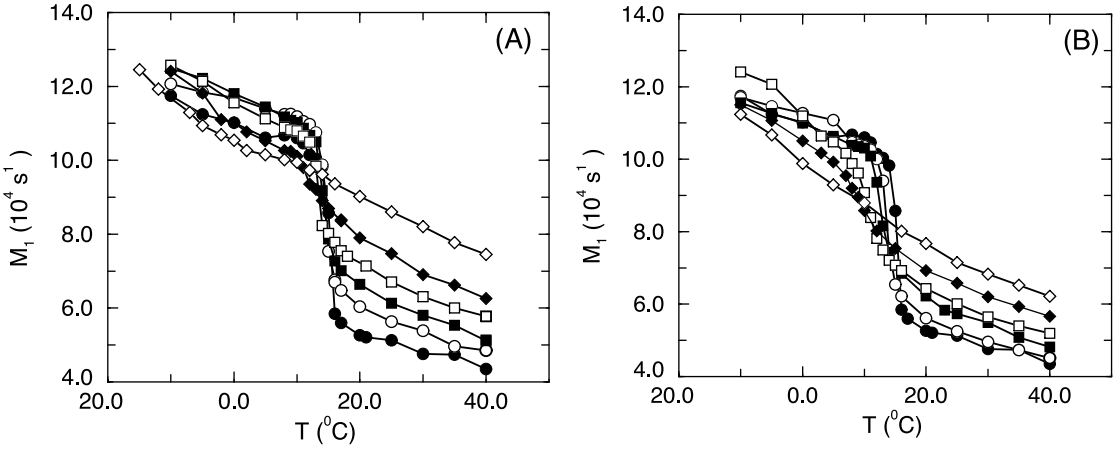

FIGURE 6 First moment of the quadrupolar spectrum, M_1, as determined from the NMR spectroscopy experiments. (*A*) PPetPC-cholesterol systems; (*B*) PPetPC-lanosterol systems. The sterol concentrations in (*A*) and (*B*) are counted up from the bottom of the curves, 0%, 5%, 10%, 15%, 20%, and 30%, respectively. Note that M_1 is essentially a measure of the acyl chain order parameter (see Eq. 2).

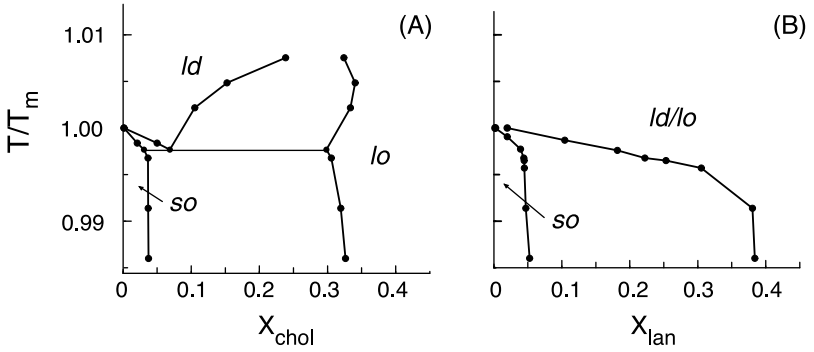

FIGURE 7 Theoretical phase diagrams determined from the Monte Carlo simulations of the microscopic model of interactions for the lipid-sterol membranes. (*A*) Lipid-cholesterol membranes; (*B*) lipid-lanosterol membranes.

and the lipid-lanosterol bilayers, respectively. Clearly, the thermally driven variations of M_1 in the two cases have qualitative differences. M_1 obtained for the lipid-cholesterol bilayer of a fixed concentration displays an abrupt change at a particular temperature, and this temperature shows very little sensitivity to variations in the cholesterol concentration. In contrast, M_1 for the lipid-lanosterol systems changes gradually with temperature for all lanosterol concentrations, and the temperature corresponding to the inflection point of each M_1 curve is increasingly suppressed with increasing concentration of lanosterol.

Theoretical results

In this section we present the results of the Monte Carlo simulations in terms of the equilibrium phase diagrams, collective conformational ordering of the lipid chains, and structural characterizations of the lipid-sterol membranes.

Phase diagrams

Shown in Fig. 7 are the equilibrium phase diagrams for the lipid-cholesterol and the lipid-lanosterol membranes, which are derived from a large amount of simulation data. The phase diagrams are given in the thermodynamic parameter space spanned by the sterol concentration x_{sterol} (where $x_{\text{sterol}} = x_{\text{chol}}$ or x_{lan}) and a reduced temperature, T/T_{m}, where T_{m} is the main transition temperature for the pure lipid system. In terms of microscopic interaction parameters, the lipid-cholesterol systems are distinguished from the lipid-lanosterol systems by a rather modest ($\sim$10%) increase in the strength of the cohesive interactions between a sterol molecule and a lipid chain in its conformationally ordered state (see Table 1).

It is clear from the figure that the small modification in the lipid-sterol interaction strength leads to considerable differences in the overall topologies of the two phase diagrams. In the case of lipid-cholesterol systems (Fig. 7 *A*), the most significant characteristics of the phase diagram is a stable region of coexistence between the *ld* and *lo* phases. Associated with this coexistence are necessarily a stable critical point and the existence of a three-phase line. The critical point is found to be located close to $T \approx 1.0075T_{\text{m}}$, $x_{\text{chol}} \approx 0.298$. The temperature of the three-phase coexistence is estimated to be $T = 0.9977T_{\text{m}}$, and the concentrations of cholesterol in the three coexisting phases is $x_{\text{chol,so}} = 0.030$, $x_{\text{chol,ld}} = 0.068$, and $x_{\text{chol,lo}} = 0.298$, respectively. In the phase diagrams of the lipid-lanosterol systems, no *ld–lo* coexistence can be identified; correspondingly, only a metastable critical point exists and there is no three-phase line.

The systematic development of the stable *ld–lo* coexistence as lanosterol "evolves" to cholesterol is a macroscopic signature of an increase in the capacity of the sterols to stabilize the *lo* phase. Another consistent signature is that with the sterol "evolution" the *lo* phase boundary of the low-temperature *so–lo* coexistence moves toward lower sterol concentrations, indicating a broadening of the region of stability of the *lo* phase in the cholesterol-lipid membranes.

Conformational ordering of the lipid molecules

To characterize quantitatively the differential effects of the two sterols on the physical properties of the mixture membranes, the lipid-chain order parameter, ϕ, as defined in the Appendix, is calculated and presented in Fig. 8. This thermal average represents the collective conformational ordering of the lipid molecules.

In Fig. 8 *A* ϕ is shown, for a fixed temperature $T = 1.0129T_{\text{m}}$, as a function of sterol concentration for both types of the lipid-sterol systems. This temperature is above that corresponding to the critical point of the *ld–lo* coexistence region of the lipid-cholesterol system. At this temperature, neither of the two types of the lipid-sterol systems undergoes any phase transitions as the sterol concentration is changed. In Fig. 8 *B*, ϕ is given for a fixed sterol concentration $x_{\text{sterol}} = 0.367$. Clearly, both cholesterol and lanosterol have order-inducing effects on the lipid chains, but cholesterol is much more potent. As shown in Fig. 8 *A*, for example, cholesterol at $x_{\text{chol}} = 0.40$ is able to rigidify close to 55% of the lipid chains, whereas lanosterol at the same concentration can only rigidify 30%.

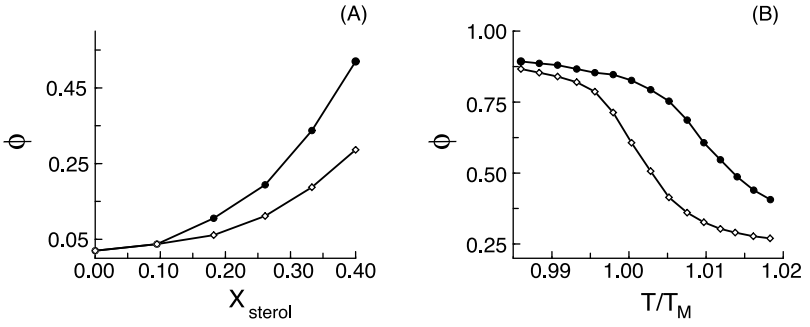

FIGURE 8 Theoretically calculated average of lipid-chain order parameter, ϕ, for the lipid-sterol membranes containing $N = 1600$ particles. The solid circles correspond to lipid-cholesterol membranes, and the open diamonds correspond to lipid-lanosterol membranes. (*A*) ϕ as a function of sterol concentration at a fixed temperature, $T = 1.0129T_m$; (*B*) ϕ as a function of temperature for a fixed sterol concentration, $x_{sterol} = 0.367$.

Molecular organization

The statistical mechanical modeling of the microscopic models also allows us to analyze quantitatively the lateral distribution of the molecules in the lipid-sterol membranes. The quantities characterizing lateral structures are the structure factors, the definitions of which are given in the Appendix.

Fig. 9 contains examples of the calculated structure factors. The data shown are calculated at $T = 0.9806T_m$ and $x_{sterol} = 0.367$, where the membranes are in an *lo* state. The figure shows the circular averages (over the directions of the Fourier wave vectors, $\vec{q}$) of the sterol structure factor $S_{sterol}(\vec{q})$, characterizing the distributions of the sterol molecules. Shown in the inset are the circular averages of the total structure factor $S_T(\vec{q})$. The total structure factor has the typical features common to systems of liquids. The partial, sterol structure factor, however, reveals more interesting structural information. Specifically, there appears a distinct low-$\vec{q}$ at $\simeq 0.35 \cdot 2\pi/d$, where d is the radius of the hard-disk cross-section of each particle, as defined in the Appendix. This particular q-value corresponds to a real-space length scale that covers several molecules, if a quantitative value of 5 Å for d may be used. The signal almost disappears in $S_{lan}(\vec{q})$ for the lipid-lanosterol systems.

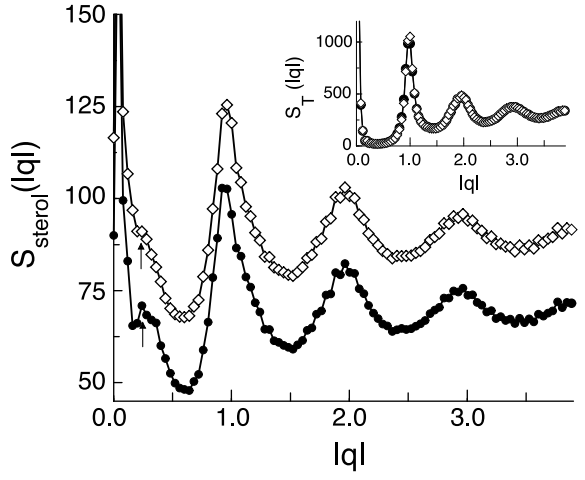

FIGURE 9 The partial structure factor describing the distribution of the sterol molecules in the membranes, $S_{sterol}(q)$, calculated at $T = 0.9806T_m$ and $x_{sterol} = 0.367$. $S_{chol}(q)$ is shown by the filled circles and $S_{lan}(q)$ by the open diamonds. For clarity, the curve for $S_{lan}(q)$ has been shifted along the y-axis. The values of q are given in units of $2\pi/d$, where d is the hard-core diameter assigned to the particles. The arrows indicate an unusual structural signal in addition to the usual peaks characteristic of liquid structure. The inset shows the total structure factor, $S_T(q)$, for the two lipid-sterol systems.

Analysis of microscopic configurations such as those shown in Fig. 10 suggests that the pattern is a microstructure consisting of aligned "threads" of the cholesterol molecules, intervened by "threads" of lipid molecules with conformationally ordered chains (see Fig. 11). The stronger the cohesive interaction between a cholesterol molecule and an ordered lipid chain is, the more likely such microdomains are to appear. This rationalization explains our observation through simulations that such microstructures disappear almost entirely in the lipid-lanosterol systems, where the lipid-sterol cohesive interaction becomes weaker. In the *lo* phase regions where the cholesterol concentration is high, the microdomains coexist with cholesterol domains (data not shown).

DISCUSSION

The "thread-like" microdomains

It is useful to compare the last result on the microscopic domains with the results of a Monte Carlo simulation study by Huang and Feigenson (1999). Their study focused on understanding the *high-concentration* solubility limit of cholesterol in lipid membranes, and it was based on a model which, in addition to the pairwise interactions, introduced cholesterol multibody interactions. Furthermore, the model used an underlying lattice representation for molecular positions. In contrast, our modeling is based on an off-lattice representation and does not in any way deal with the issue of solubility. The two-body interactions in Huang and Feigenson's model were nevertheless qualitatively very similar to the microscopic interactions in our model, and produced a molecular distribution pattern on a microscopic scale that resembles the "thread-like" structure observed in our study. The cholesterol multibody interactions were only introduced as a possible mechanism for the removal of choles-

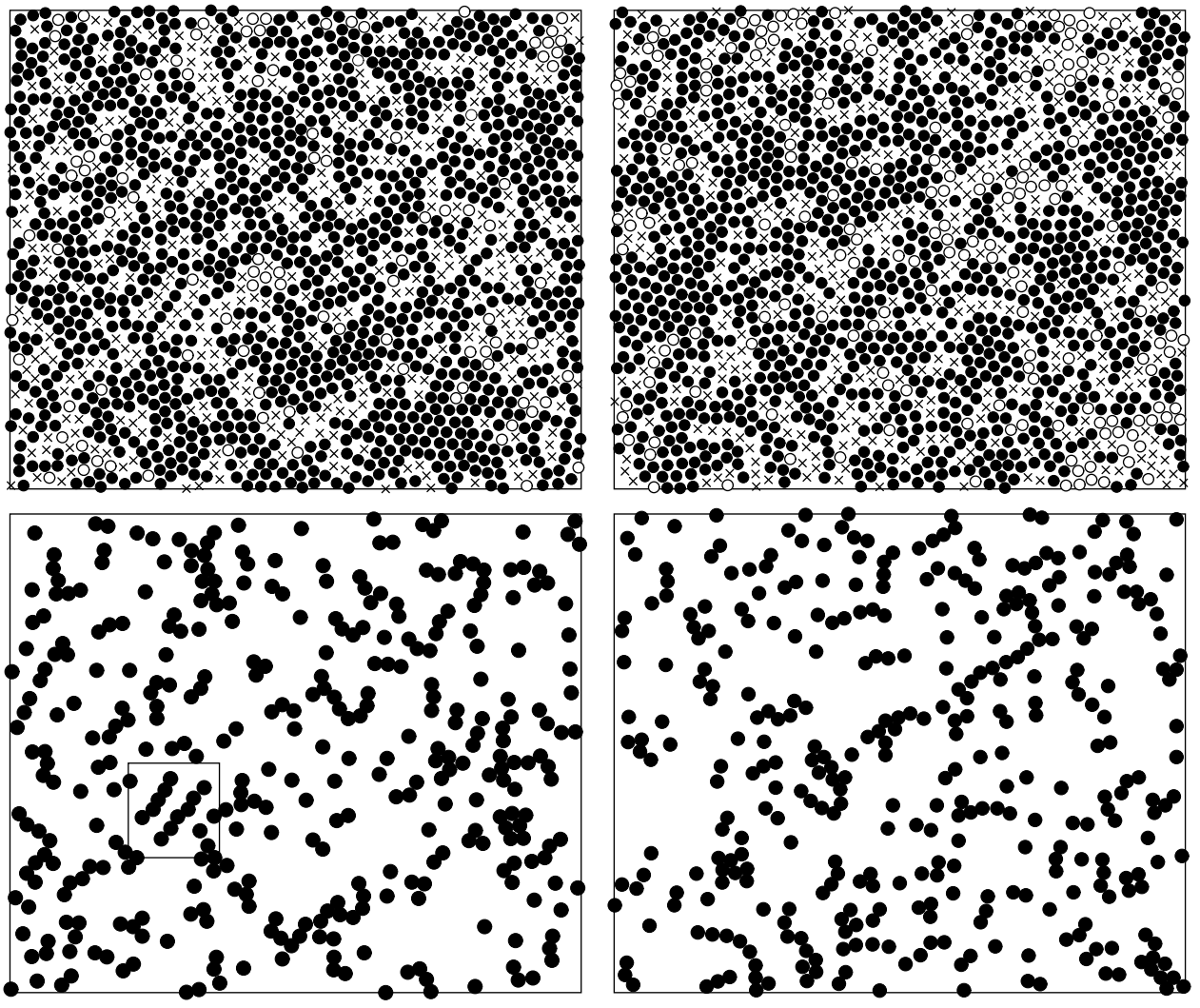

FIGURE 10 Snapshots of microconfigurations for lipid membrane systems containing cholesterol (*left*) and lanosterol (*right*), calculated at $T = 0.9806T_m$ and $x_{sterol} = 0.367$. The upper panel shows all particles, with lipid chains in the ordered state shown by filled circles, lipid chains in the disordered state shown by open circles, and sterol molecules shown by crosses. The lower panel shows only the corresponding sterol molecules. The part highlighted by the box in the lower left snapshot is shown in detail in Fig. 11.

terol domains from the membranes, thus accounting for the solubility limit of cholesterol. They also induced some super-lattice structures close to and at the solubility limit, into which the microscopic structures appeared to organize. It is, however, difficult to distinguish unequivocally the super-lattice structures from artifacts, given the underlying lattice representation specifically and given the lack of clear experimental evidences for super-lattice structures in general.

Naturally, the "thread-like" microstructures found in the present study are yet to be verified experimentally. They may, however, provide a *microscopic* basis for the notion of "cholesterol-lipid" complexes, which underlies a model for the thermodynamics of multicomponent lipid-cholesterol systems (Radhakrishnan and McConnell, 1999; Radhakrishnan et al., 2000).

Lipid-sterol interactions, the *lo* domains, and the rafts

We have in the previous sections presented both the results of our experimental studies and the results of our theoretical modeling of the phase equilibria of the lipid-cholesterol and the lipid-lanosterol membrane systems. The focus of the studies is to identify and characterize the differential effects of cholesterol and its (evolutionary) precursor, lanosterol, on equilibrium physical properties of the membranes and to relate those effects to the difference in the molecular chemistries of the two sterols.

Clearly, the theoretically calculated phase diagrams (shown in Fig. 7) agree with those determined experimentally (shown in Fig. 5) for both types of the lipid-sterol membranes; there is also an agreement between the theo-

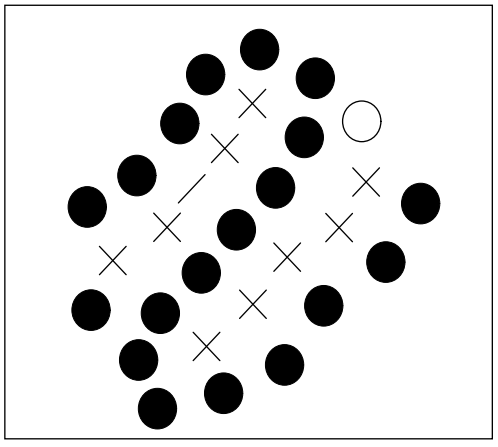

FIGURE 11 Enlarged version of the box highlighted in Fig. 10, showing a local "thread-like" distribution of lipid and cholesterol molecules. Lipid chains in the ordered state are shown by filled circles, lipid chains in the disordered state are shown by open circles, and cholesterol molecules are shown by crosses.

retical and experimental results for the conformational ordering of the lipid molecules (see Figs. 6 and 8). Because the theoretical results are based on our microscopic model of sterol-lipid interactions with no extensive or specific parameter-fitting, the agreement provides concrete support for the model which proposes a physical interpretation of the differential molecular chemistry of cholesterol and lanosterol. The streamlined molecular structure of cholesterol (when compared to that of lanosterol) may indeed enable it to interact more strongly with conformationally ordered lipid chains (Bloch, 1965; Yeagle et al., 1977; Yeagle, 1985; Slotte, 1995; Urbina et al., 1995), thereby rendering it more effective than its precursors in the dual act of inducing high conformational order in the lipid chains and breaking the lateral packing order of the lipid molecules. This microscopic effect is, in the membrane systems that we have studied, macroscopically manifested both as the enhanced stability of the *lo* phase with respect to both temperature and sterol concentrations, as indicated by the phase diagrams, and as an increased conformational order of the lipid chains, as quantified also in Figs. 6 and 8.

Our physical interpretation of the different molecular chemistry of cholesterol and lanosterol in terms of a simple model of sterol-lipid interactions appears to receive additional support from other experimental studies, where it has been demonstrated that cholesterol shows a stronger ability than lanosterol to promote and stabilize the *lo* structure in membranes formed of lipids other than the specific lipid (PPetPC) used in our studies. In an earlier comparative study of the effects of cholesterol, ergosterol, and lanosterol on molecular order and dynamics of phosphatidylcholine (PC) bilayer membranes (Urbina et al., 1995), it was shown that at 30 mol % sterol content and at several temperatures, cholesterol was significantly more effective than lanosterol at increasing lipid order in both membranes formed of saturated DMPC and membranes formed of mono-unsaturated POPC. The difference in the effectiveness of the sterols was suggested to be caused by the additional steric constraints imposed by the three bulky methyl groups of lanosterol on its interactions with the PC lipids. In a more recent study of the effect of sterol structure on domain formation in membranes containing both a saturated PC (DPPC) and an unsaturated PC (either a fluorescent analog or DOPC) (Xu and London, 2000), domains with characteristics of the *lo* structure and enriched in the saturated lipid were shown to exist in cholesterol-lipid membranes, while no discernible domain formation was observed in lanosterol-lipid membranes. These observed effects were again rationalized in terms of the ability of cholesterol, and the lack of ability of lanosterol, to participate in strong close-packing with saturated lipids that are conformationally ordered. However, the work of Urbina et al. (1995) concerning ergosterol also suggests that the structure of the steroid rings of a sterol may not be the only structural factor influencing the packing of the sterol with lipids. The bulky and stiff C-24 methylated side chain of ergosterol seems to render the sterol unable to interact effectively with phospholipids with one mono-unsaturated acyl chain.

Regarding our use of PPetPC as the phospholipid in our membrane systems, there arises a question concerning the difference between lipids with saturated chains and lipids with mono-unsaturated chains, and the effect of the position of the double bond along an monounsaturated chain. In this context there is considerable experimental evidence that the phase behavior observed in DPPC-cholesterol systems is generic for a large number of PC-cholesterol systems (Thewalt et al., 1992; Silvius et al., 1996). In particular, different binary mixtures of cholesterol with saturated lipids of different chain length display qualitatively similar phase diagrams. Furthermore, changing the lipid from a saturated species to one of a number of mono-unsaturated lipids either having a *cis* double bond at either 6–7 position or 9–10 position, or having a *trans* double bond at 9–10 position preserves the qualitative topology of the DPPC-cholesterol phase diagram. This suggests that certain specific details in the molecular chemistry of lipids are not of primary importance to the qualitative nature of the macroscopic phase equilibria. Our theoretical model of lipid-sterol interactions, being minimal, naturally neglects such details. Within the context of the model it is not the absolute strength of the lipid-lipid and lipid-sterol interactions, but rather their relative strength that primarily determines the qualitative features of phase equilibria of lipid-sterol systems.

In relation to the hypothesis on the molecular evolution of cholesterol, it is interesting to discuss our results in terms of the biological relevance of the *lo* structure in membranes. As discussed in the previous paragraph, the *lo* structure is generic for lipid-cholesterol mixtures in that it appears as a stable equilibrium structure in a large number of different

mixture membranes over a wide range of compositions and temperatures (Thewalt et al., 1992; Almeida et al., 1992; Mateo et al., 1995; Silvius et al., 1996). Because cholesterol is abundant in plasma membranes of mammalian cells, and saturated and mono-unsaturated lipids make up a major fraction among the rest of lipid constituents in the membranes (White, 1973), it may be expected that the physical properties of the *lo* structure play a crucial role in supporting some of the essential biological functions of the membranes.

Among their many different functions, cell plasma membranes must act as physical protection to the intracellular organelles and biochemical processes, and must therefore have the kind of mechanical properties that ensure their structural coherence, flexibility, and integrity. The membranes must also act as an effective physical barrier to passive cross-membrane transport of molecules to secure the active transport processes that are regulated by molecular pumps, channels, and carriers. At the same time, the membranes must maintain fluidity, a physical property essential for the necessary lateral mobility of lipids and membrane-associated proteins required for many membrane-related cellular processes (Bloch, 1965; Bloom et al., 1991; Bloom and Mouritsen, 1995). Although a physical understanding that would unify these different, unusual, some even seemingly mutually conflicting, aspects of the cell membranes is still missing, a unifying picture may be emerging for the simpler systems of model lipid-cholesterol membranes. In particular, phospholipid bilayers containing ~20–30% cholesterol are known to be fluid (Evans and Needham, 1986) and to have elasto-mechanical strengths comparable to the membrane of red blood cells (Evans and Needham, 1986; Needham and Nunn, 1990; Düwe et al., 1990; Meleard et al., 1997). It has also been reported that 50% cholesterol in lipid bilayers almost completely abolishes the cross-membrane permeation of small molecules and ions (Bloch, 1983; Bhattacharya and Haldar, 2000), while lanosterol has hardly any effect on the permeation (Bloch, 1983). Our results suggest that the notion and the existence of the *lo* structure in the lipid-sterol bilayer membranes may be the unifying basis underlying the various types of observations.

In recent years, cholesterol-rich *lo* structures have received much attention within another context of cell biology. Detergent-resistant membrane fragments (DRMs) have been isolated from detergent treatment of a variety of eukaryotic cells. These membrane domains are rich in cholesterol and saturated sphingolipids, and are suggested to have the physical characteristics of the *lo* phase (Ostermeyer et al., 1999). Based on the studies of DRMs, a hypothesis has been put forward that *rafts*, which are domain structures enriched in cholesterol and sphingolipids, exist in cell membranes (Simons and Ikonen, 1997; Brown and London, 1997). This hypothesis is entirely consistent with the known facts that cholesterol strongly favors interacting with lipids with saturated acyl chains and that sphingolipids have fully saturated acyl chains and constitute a significant fraction of the membrane lipids. The potential functional importance of rafts in processes such as intracellular membrane sorting and signal transduction at the cell surface has been proposed (Brown and London, 2000). The existence of rafts in cell membranes is yet to be unambiguously verified. However, rafts have been observed in multicomponent model membranes formed of unsaturated phosphatidylcholines, cholesterol, and sphingomyelin (Dietrich et al., 2001; Rinia and de Kruijff, 2001). Moreover, in ternary mixtures of cholesterol with both a saturated lipid and an unsaturated lipid, domains, which are in the *lo* phase and are enriched in the saturated lipid, and which show resistance to detergent solubilization, have been demonstrated to coexist with domains in the *ld* phase that are enriched in the unsaturated lipid (Xu and London, 2000). It is also suggested that cholesterol concentration is higher in the *lo* domains than in the *ld* domains. This observed phenomenon is rationalized by a better structural fitting, or in our terms, a stronger cohesive interaction, between cholesterol and the saturated lipid than between the sterol and the unsaturated lipid. The comparative study of the different *lo* domains corresponding to different sterols (including both cholesterol and lanosterol) used in the mixtures further supports the notion that the structural smoothness of a sterol is one of the key parameters in domain formation: the better a sterol can form tight packing with lipids with saturated acyl chains, the more strongly it promotes the formation of the detergent-insoluble domains (Xu and London, 2000). This structure-based rationalization is indeed at the center of our theoretical model of sterol-lipid interactions. Furthermore, the experimental work on rafts underlies the crucial role of cholesterol in promoting and stabilizing the *lo*-type domains in membranes, which is also the main conclusion of our study.

APPENDIX

Theoretical microscopic model

The model proposed in this paper for the systems of lipid-sterol mixtures is in essence similar to a model earlier proposed by us for the lipid-cholesterol mixture system (Nielsen et al., 1999). The model consists of three basic ingredients: a representation of the translational degrees of freedom, a description of molecular conformational degrees of freedom, and a minimal model of the interactions between the molecules.

In representing the translational degrees of freedom, the model uses an algorithm that formulates a spatial distribution of molecules in the two-dimensional space as a triangulation of a plane. By randomly accessing all forms of triangulations, the algorithm explores all possible configurations of molecular distribution. It therefore allows for local density fluctuations and molecular diffusion in the space, which are the essential elements in a realistic description of molecular translational degrees of freedom. The details of this triangulation algorithm are given elsewhere (Nielsen et al., 1996).

The conformational degrees of freedom associated with the lipid chains are represented by two states (Doniach, 1978). One state, the "ordered" state, has zero internal energy and is non-degenerate, characteristic of the conformational state of a lipid chain in the "gel" phase. The other state, the "disordered" state, has a high internal energy, reflecting that energy is

required for conformational excitations, and a large degeneracy, effectively representing the large number of conformational excitations that a lipid chain can assume. Both cholesterol and lanosterol molecules are modeled as rigid particles with no conformational degrees of freedom. All particles are considered as hard-core particles.

The energy function that models molecular interactions in the lipid-sterol mixture systems is given by

$$H = H_0 + H_{\text{o-s}} + H_{\text{d-s}} + H_{\text{s-s}}. \tag{3}$$

Here $H_{\text{o-s}}$, $H_{\text{d-s}}$, and $H_{\text{s-s}}$, as indicated by the various subscripts, represent the pairwise interaction potentials between an ordered chain and a sterol molecule, a disordered chain and a sterol molecule, and two sterol molecules, respectively. They are defined as follows:

$$\begin{aligned} H_{\text{o-s}} &= \sum_{\langle \text{i<j} \rangle} V_{\text{o-s}}(R_{\text{ij}})\{\mathcal{L}_{\text{io}}\mathcal{L}_{\text{js}} + \mathcal{L}_{\text{jo}}\mathcal{L}_{\text{is}}\} \\ H_{\text{d-s}} &= \sum_{\langle \text{i<j} \rangle} V_{\text{d-s}}(R_{\text{ij}})\{\mathcal{L}_{\text{id}}\mathcal{L}_{\text{js}} + \mathcal{L}_{\text{jd}}\mathcal{L}_{\text{is}}\} \\ H_{\text{s-s}} &= \sum_{\langle \text{i<j} \rangle} V_{\text{s-s}}(R_{\text{ij}})\{\mathcal{L}_{\text{is}}\mathcal{L}_{\text{js}}\}. \end{aligned} \tag{4}$$

Here $\langle i < j \rangle$ denotes a summation over nearest neighbors, and i is an index labeling the particles in the system. $\mathcal{L}_{\text{io}}$, $\mathcal{L}_{\text{id}}$, and $\mathcal{L}_{\text{is}}$ are occupation variables which are unity when the ith particle is a lipid chain in the ordered state, a lipid chain in the disordered state, and a sterol molecule, respectively, and which are zero otherwise. The energy of interaction between two chains that are both in the disordered state is approximated to be zero, which sets the reference point for the interaction energies.

H_0 is the energy function describing the interactions in the pure lipid system. It is formulated as

$$\begin{aligned} H_0 = \sum_{\text{i}} E_{\text{d}}\mathcal{L}_{\text{id}} + \sum_{\langle \text{i<j} \rangle} V_{\text{o-o}}(R_{\text{ij}})\mathcal{L}_{\text{io}}\mathcal{L}_{\text{jo}} \\ + \sum_{\langle \text{i<j} \rangle} V_{\text{o-d}}(R_{\text{ij}})\{\mathcal{L}_{\text{io}}\mathcal{L}_{\text{jd}} + \mathcal{L}_{\text{jo}}\mathcal{L}_{\text{id}}\} + \Pi \cdot A. \end{aligned} \tag{5}$$

Here E_{d} is the excitation energy of the conformationally disordered state, and $V_{\text{o-o}}(R)$ and $V_{\text{o-d}}(R)$ are the distance-dependent, chain-conformation-dependent interaction potentials between two neighboring lipid chains. Π is, in effect, a lateral surface pressure stabilizing the system against lateral expansion, and A is the total area of the system.

All of the microscopic interaction potentials are approximated by a sum of a hard-core repulsive potential of range d, a short-range square-well potential of range R_0, $V^{\text{s}}(R)$, and a longer-range attractive square-well potential of range $l_{\max}$, $V^{\text{l}}(R)$. $V^{\text{s}}(R)$ and $V^{\text{l}}(R)$ are given by

$$V^{\text{s}}(R) = \begin{cases} -V^{\text{s}}, & d < R \le R_0 \\ 0, & \text{otherwise}, \end{cases} \tag{6}$$

$$V^{\text{l}}(R) = \begin{cases} -V^{\text{l}}, & d < R \le l_{\max} \\ 0, & \text{otherwise}, \end{cases} \tag{7}$$

where the specific values of V^{l} and V^{s} depend on both the molecular type and conformational state of the interacting particles.

Some of the microscopic interactions are sketched in Fig. 2 to illustrate our minimal modeling of the dual molecular mechanism of sterol molecules in lipid bilayers. A comparison between Fig. 2, *A* and *B* illustrates the "crystal-breaker" mechanism, as the interactions involved imply that a sterol molecule dissolved in an ordered-chain environment tends to have a larger surface area than that of a lipid chain, thus disrupting the lateral packing of the ordered chains. Similarly, a comparison between Fig. 2, *B* and *C* makes the "chain-rigidifier" mechanism clear, as the given interactions are such that a sterol molecule prefers its neighboring chains to be in the conformationally ordered state.

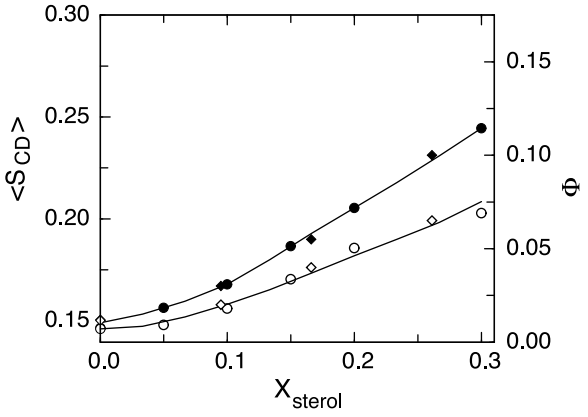

FIGURE 12 Fitting of the theoretical (ϕ) order parameter to the equilibrium averages of the experimental order parameter (S_{CD}, as given in Eq. 2), which leads to the specific value of J used for lipid-lanosterol interactions in the model. The experimental data are obtained at $T = 40°\text{C}$. The theoretical data are calculated for a system containing $N = 1600$ particles at a temperature of $T = 1.0359T_{\text{m}}$. Corresponding to cholesterol and lanosterol, the experimental data are shown by filled and open circles, respectively, and the theoretical data are given by filled and open diamonds, respectively. The lines connecting the points are guides to the eye.

The unit of length scales in the model is for convenience set at the hard-core diameter, d, and the unit of energy is defined to be J_0. To convert the energy and length scales into units relevant for lipid bilayer systems, J_0 should be of the order 10^{-20} J and d of the order 5 Å. The surface pressure, Π, is fixed at $\Pi d^2/J_0 = 3.0$. The radius of the short-range potential, Eq. 6, is set at $R_0/d = 1.3$. The values of Π and R_0 are chosen such that the change in surface area across the main transition is comparable to that of a pure PC (DPPC) bilayer system. Other parameters in the definition of the interaction potentials are summarized in Table 1. The values for the lipid-cholesterol and the lipid-lipid interaction parameters are chosen such that the theoretical phase diagram is similar to that of the DPPC-cholesterol system.

The excitation energy of the disordered state of lipid chains is chosen to be $E_{\text{d}} = 2.78J_0$, and the degeneracy, D_{d}, of the disordered state, is taken to be $\ln D_{\text{d}} = 12.78$. These values are the same as for the 10th state of the Pink Model (Pink et al., 1980) and give a latent heat of the main transition comparable to that measured experimentally for the pure bilayer system.

In our minimal modeling the molecular interactions for the lipid-cholesterol and lipid-lanosterol systems differ only in the part that describes the interaction between a sterol molecule and an ordered lipid chain, $V_{\text{o-s}}$. The values of the relevant parameters are listed in Table 1. The particular values defining the strength of the lipid-lanosterol interaction potential relative to that of lipid-cholesterol were determined by fitting the theoretically calculated value of the lipid chain order parameter to the acyl-chain order parameter derived from the NMR data as a function of sterol concentration for both types of the lipid-sterol systems at a single temperature. The temperature was relatively high so that no phase boundary lines were crossed as the sterol concentration was varied. The fit is shown in Fig. 12. From the figure it is clear that with the set of the parameter values used we are able to obtain, over a large range of sterol concentrations, a fit of the theoretical data to the experimental values for the order parameter.

From the simulation data the equilibrium conformational order parameter, ϕ, is calculated from its definition given by

$$\phi = \frac{1}{2}\left(\left\langle \frac{\sum_{\text{i}=1}^{\text{N}}(\mathcal{L}_{\text{io}} - \mathcal{L}_{\text{id}})}{\sum_{\text{i}=1}^{\text{N}}(\mathcal{L}_{\text{io}} + \mathcal{L}_{\text{id}})} \right\rangle + 1\right), \tag{8}$$

where $\langle \ldots \rangle$ denotes the equilibrium (ensemble) average. To characterize the lateral ordering among the different molecular species, the structure factors, $S_T(\vec{q})$ and $S_{sterol}(\vec{q})$, are calculated as functions of Fourier wave vector, $\vec{q}$, as follows:

$$S_T(\vec{q}) = \frac{1}{N}\{\langle \rho_T(\vec{q})\rho_T(-\vec{q})\rangle - \langle \rho_T(\vec{q})\rangle^2 \delta_{\vec{q},\vec{0}}\}$$

$$S_{sterol}(\vec{q}) = \frac{1}{N}\{\langle (\rho_{sterol}(\vec{q})\rho_{sterol}(-\vec{q})\rangle - \langle \rho_{sterol}(\vec{q})\rangle^2 \delta_{\vec{q},\vec{0}}\}. \quad (9)$$

$\rho_T(\vec{q})$ is the Fourier transform of the total particle-number density operator at a spatial point $\vec{r}$, $\rho_T(\vec{r}) \equiv \sum_{i=1}^{N} \delta(\vec{r} - \vec{r}_i)$, and $\rho_{sterol}(\vec{q})$ is the Fourier transform of the partial density operators, $\rho_{sterol}(\vec{r}) \equiv \sum_{i=1}^{N} \delta(\vec{r} - \vec{r}_i)\mathcal{L}_{is}$, for the sterol molecules, respectively.

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