

Phospholipid-Cholesterol Bilayers under Osmotic Stress

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ABSTRACT Isothermal (27°C) phase behavior of dimyristoyl phosphatidyl choline-cholesterol mixtures at various osmotic pressures and cholesterol contents was investigated by means of isothermal sorption microcalorimetry and ^2H -nuclear magnetic resonance. The calorimetric method allows for simultaneous measurement of the partial molar enthalpy and the chemical potential (the osmotic pressure) of water, thus providing an almost complete thermodynamic description of the sorption process. From the experimental results, the $\Pi_{\text{osm}} - X_{\text{chol}}$ and the ternary composition phase diagrams are constructed. We note that there are strong similarities between the $\Pi_{\text{osm}} - X_{\text{chol}}$ phase diagram and the previously reported $T - X_{\text{chol}}$ phase diagram at excess water. At high cholesterol contents a single liquid ordered ($L_{\alpha}(o)$) phase is present over the whole range of water contents, implying that this phase has a remarkable stability not only at decreasing temperature but also at increasing osmotic pressure. At low cholesterol contents, the microcalorimetric experiments confirm the extraordinary property of cholesterol not to cause any substantial melting point depression. One important conclusion in the present study is that the P_{β} phase can dissolve cholesterol more readily than the L_{β} phase and that the addition of cholesterol induces the P_{β} phase. Finally, the putative $P_{\beta} - L_{\alpha}(o)$ periodic modulated structure is discussed.

INTRODUCTION

Cholesterol is a major constituent of many biological membranes. It comprises a lipid fraction of $\sim 30\%$ in, for example, the plasma membrane of eukaryotic cells (Yeagle, 1985) and the lipid matrix of stratum corneum, the outer part of human skin (Wertz et al., 1992). There is an intriguing interplay between the biological function of cholesterol and the physical-chemical properties of the membrane. This is reflected by the fact that cholesterol has a profound effect on the thermodynamic and mechanical properties of the lipid bilayers, thus influencing the membrane stability and the barrier properties (Yeagle, 1985; Bloom et al., 1991; Demel and de Kruffy, 1976). Cholesterol is also associated with some specific biological functions. Recently, the so-called lipid raft model has been proposed. It describes small size cholesterol-sphingolipid domains as membrane lipid rafts, which can serve as platforms for lipid and protein transport or as relay stations in intracellular signaling (Simons and Ikonen, 1997). The lipid rafts are closely related to the lipid domains around the protein caveolin, referred to as caveolae (Marx, 2001). It has further been demonstrated that the chemical potential of cholesterol in phospholipid bilayers depends on the lipid composition in a strongly nonideal way (Radhakrishnan et al., 2000). This is a consequence of the complex cholesterol-phospholipid intermolecular interactions, and it can be seen as a regulating mechanism, allowing for large variations in cholesterol con-

tent in adjacent compartments of the eukaryotic cells even though there is an equilibrium with respect to transfer of cholesterol. In other words, a small concentration difference in another lipid or protein component can result in a large difference in cholesterol content.

The effect of cholesterol on lipid bilayers has been extensively studied (Yeagle, 1985; Bloom et al., 1991). The majority of the studies undertaken to elucidate the effects of cholesterol on lipid morphology in bulk have been performed on systems containing phospholipids due to their frequent occurrence in biological membranes. Cholesterol has been referred to as a “crystal breaker” as it disturbs the translational order of the phospholipid molecules in the crystalline (gel) state (Vist and Davis, 1990; Ipsen et al., 1987). Cholesterol also causes a straightening of the disordered phospholipid acyl chains in liquid-like phases and reduces the mean headgroup area (Vist and Davis, 1990; Lafleur et al., 1990). This property is often referred to as the stabilizing effect of cholesterol.

The phase behavior in model systems of saturated phospholipids and cholesterol has been widely studied (Vist and Davis, 1990; Shimshick and McConnell, 1973; Ipsen et al., 1987; Anderson and McConnell, 2000; Nielsen et al., 1999). It has been demonstrated that phosphatidyl choline (PC)-cholesterol phase diagrams have a universal form with the main difference of translations along the temperature axis when varying the acyl-chain lengths (Thewalt and Bloom, 1992). Fig. 1 shows the $T - X_{\text{chol}}$ phase diagram of dimyristoyl phosphatidyl choline (DMPC) and cholesterol in excess water (Almeida et al., 1992). The phase diagram shows several remarkable features, indicating very specific PC-cholesterol interactions (Ipsen et al., 1987). In excess water pure DMPC goes through a phase transition from gel to liquid crystalline state at $T_m = 23.5^\circ\text{C}$. Cholesterol is to some extent soluble in phospholipids in the gel state. This is an unusual property for a solid, which is normally not a

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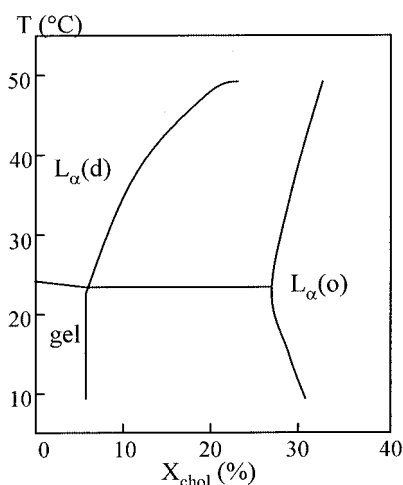


FIGURE 1 $T - X_{\text{choi}}$ phase diagram for DMPC-cholesterol in excess water. Phase diagram adapted from Almeida et al. (1992). The gel phase has been characterized as a P_{β} gel phase (Mortensen et al., 1988).

good solvent. In fact, the solubility of cholesterol is almost as high in the gel phase as in the liquid crystalline ($L_{\alpha}(d)$) phase, resulting in only very marginal melting-point depression. The notation, $L_{\alpha}(d)$, liquid disordered, refers to the common lamellar liquid crystalline phase observed above the chain melting temperature, which is generally called the L_{α} phase. The gel has been characterized as a rippled P_{β} gel phase (Mortensen et al., 1988).

At high cholesterol contents the system behaves like a liquid over the whole range of temperatures, and in contrast to the low concentration behavior, the cholesterol strongly favors the liquid phase over the solid phase at these compositions. The liquid phase at high cholesterol concentrations has been denoted a liquid ordered phase ($L_{\alpha}(o)$), motivated by the fact that several independent studies have demonstrated a high degree of the acyl-chain order in this lamellar liquid crystalline phase. Two two-phase regions are also present in the phase diagram in Fig. 1. Above T_m , two liquid phases, $L_{\alpha}(o)$ and $L_{\alpha}(d)$, coexist, and below T_m the $L_{\alpha}(o)$ and gel phases coexist. Furthermore, a narrow coexistence region ending in a eutectic point separating the gel and $L_{\alpha}(d)$ phases is expected on thermodynamic grounds.

In the majority of experimental work, phospholipid-cholesterol phase equilibria is studied in relation to variations in temperature. However, for many biological applications the chemical potential of water (osmotic pressure) is an equally relevant intensive variable as temperature for studying the relation between molecular interactions and phase behavior. The osmotic forces are important in regulating a number of biological membrane processes. Membrane fusion processes can be induced by creating a local osmotic stress (Hui et al., 1999). The effect of osmotic pressure on lipid phase behavior is also of uttermost importance in the case of human skin, due to the large difference in water chemical potential across the skin (Sparr and Wennerström, 2001).

Phase behavior of pure phospholipid-water binary systems have previously been studied both experimentally and theoretically (Guldbrand et al., 1982; Ulmuis et al., 1977; Gabriella-Madelmont and Perron, 1983). In a few cases, $T - \Pi_{\text{osm}}$ phase diagrams for phospholipid-water systems have been established (Smith et al., 1990; Markova et al., 2000). One of the outcomes of these studies is that a first order phase transformations from a gel to a liquid crystalline phase can be induced by a decrease in the osmotic pressure analogous to the transition induced by an increase of temperature in excess water. The response in phospholipid-cholesterol equilibria to changes in osmotic pressure has received much less attention, and no experimental phase diagrams of this kind can be found in the literature. Faure et al. (1997) presented a partial phase diagram for DMPC-cholesterol mixtures at varying water contents, but the authors made no connections to the intensive variable of the osmotic pressure.

In this paper, we investigate the phase behavior of DMPC-cholesterol mixtures at various osmotic pressures and cholesterol contents. An isothermal sorption microcalorimeter was used to monitor lipid hydration. This calorimeter allows for simultaneous measurement of the partial molar enthalpy and the chemical potential (the partial molar free energy or osmotic pressure) of the water (Wadsö and Markova, 2000). As a support to the calorimetric results the ^2H -nuclear magnetic resonance (NMR) quadrupolar splitting of heavy water is studied at various water contents. From the experimental results the $\Pi_{\text{osm}} - X_{\text{choi}}$ phase diagram is constructed. A ternary phase diagram based on molar compositions is also outlined.

MATERIALS AND METHODS

DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (98% pure, molecular weight = 678 g/mol) and cholesterol (molecular weight = 386.66 g/mol) were obtained from Larodan Fine Chemicals (Malmö, Sweden). DSC measurements showed no evidences of impurities. The Millipore water used was deionized, distilled, and filtrated through Millipore Q Purification System (Millipore Corporation, Bedford, MA). Samples of cholesterol and DMPC at different compositions were dissolved in 2:1 chloroform:methanol. The mixtures were heated for 10 min at 40°C, vortexed for 2 min, and then dried in vacuum at room temperature. The samples were thereafter dried in a vacuum pistol with a few drops of water to remove traces of the solvent. All samples were used directly after drying or annealed by prehydration and subsequent drying. According to Nilsson et al. (1991) and McIntosh et al. (1987) such a careful drying procedure is sufficient to remove all water from the lipid sample. The fact that the lipids were stored at -4°C before drying further insures the dry state of the lipids (Cevc and Marsh, 1987). However, other authors claim that one or maximal two water molecules per lipid are so strongly associated to the lipid headgroups that they are very difficult to remove in practice (Small, 1986). We report compositions based on the assumption that there is no water in the maximally dried sample (see below).

A novel double twin isothermal microcalorimeter was used to study the water vapor sorption of the phospholipids. A detailed description of the instrument is presented elsewhere (Wadsö and Markova, 2000). The calorimetric cell consists of two vessels connected by a steel tube. At the start of the measurements the bottom vessel contains 40 to 100 mg of a dry

sample. Approximately 100 μL of water is injected into the top vessel where it spreads over the hydrophobic porous membrane (Durapore (0.22 μm), Millipore). During the measurements water vaporizes in the top vessel and diffuses through the tube down to the bottom vessel where it is taken up by the sample. The experimental set-up could be looked upon as a continuous titration of an initially dry lipid with water vapor. During the experiments, the thermal powers of vaporization and sorption are measured separately in the double microcalorimeter. From the calorimetric measurements, the chemical potential of water in the sample cell, the water gain, and the differential enthalpy of sorption can be obtained. If equilibrium is maintained this enthalpy change represents the difference between the partial molar enthalpy of the water in the sample and in the liquid water. Each measurement was repeated three times with almost identical result.

Samples used for the NMR measurements were transferred into 5-mm diameter NMR ampoules. D_2O was added with a syringe and the ampoules were sealed. To avoid evaporation from the lipid- D_2O mixture, the sample filled as much as possible of the sealed ampoule. The water content in each sample can be converted to the corresponding relative humidity/osmotic pressure from the sorption isotherms. To prevent the samples from being hydrated during transfer, preparations were made in a glove bag under dry nitrogen atmosphere. The samples were homogenized by centrifugation back and forth, and left for at least 3 weeks to equilibrate. The NMR measurements were repeated after 2 months to ensure equilibrium. The ^2H -NMR spectra were recorded on a Bruker DM 100 NMR operating at 15.3 MHz at a temperature of $27 \pm 0.2^\circ\text{C}$. In addition, some control ^2H -NMR experiments were performed at Umeå University, Sweden, on a Chemagnetics CMX400 NMR operating at 61.48 MHz.

RESULTS AND DISCUSSION

Three independent properties of the DMPC-cholesterol system were measured for a range of cholesterol compositions between 0% to 37% ($X_{\text{chol}} = n_{\text{chol}}/(n_{\text{chol}} + n_{\text{DMPC}})$); the water chemical potential, the water partial molar enthalpy, and the water deuterium quadrupolar splitting as a function of the water content. For the pure phospholipid an increase in the osmotic pressure (corresponding to a decrease in water content) triggers a phase transition from $L_\alpha(d)$ lamellar liquid crystal to L_β gel phase. As discussed above, cholesterol has some rather unique effects on the phospholipid phase behavior at a range of temperatures, and we expect that the same intermolecular interactions will also affect the isothermal phase behavior at varying osmotic pressure. A first step in the interpretation of the measured physical parameters is to determine if the measuring point corresponds to a pure single phase or a two- (or three-) phase sample. There is a delicate interplay between the interpretation of the physical parameters and the phase behavior as such. Below, we first present separate discussions of the measured physical parameters. The results are then collected into a unified description of the phase behavior.

Chemical potential of water

The calorimetric sorption measurement provides a relation between the water content and the water chemical potential ($\Delta\mu_w$), expressed in terms of the osmotic pressure (Π_{osm}), or the relative humidity (RH); ($\Delta\mu_w = -V_w\Pi_{\text{osm}} = \text{RT}$

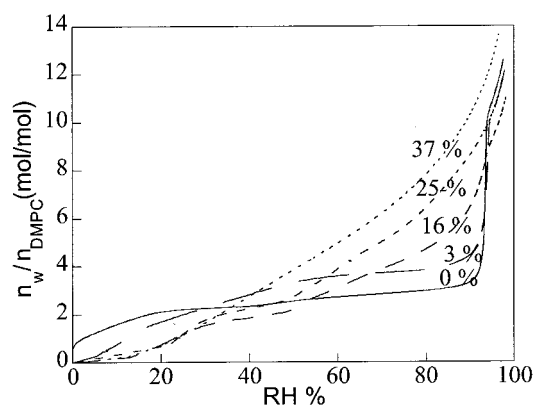


FIGURE 2 Microcalorimetric sorption data for DMPC-cholesterol mixtures at 27°C ; water content (n_w/n_{DMPC}) versus relative humidity (RH). $X_{\text{chol}} = 0\%$, 3% , 16% , 25% , and 37% .

$\ln(\text{RH}/100)$, RH is given in percents; R , gas constant; T , temperature). Fig. 2 shows the water uptake per phospholipid as a function of osmotic pressure for the five compositions studied.

For the samples containing 0% and 3% cholesterol, the sorption curves can be divided into four different regimes. At low relative humidities, there is a continuous uptake of water molecules. This is followed by a region of large changes in relative humidity with small changes in water content. At $\text{RH} = 93\%$ a step-wise sorption takes place at almost constant relative humidity. In the last regime there is again a continuous increase of the water content toward the swelling limit, as the relative humidity approaches saturation (i.e., as the osmotic pressure goes to zero).

Although the sorption curves for 0% and 3% cholesterol are qualitatively similar there are quantitative differences. The original dry samples are in a noncrystalline solid state. In the early stage of the sorption process the presence of cholesterol somewhat reduces the water uptake. A possible explanation for this is that the OH-groups of the cholesterol act as ligands for the phosphate on the DMPC, slightly reducing the water binding capabilities of these groups. For pure DMPC there is a composition of approximately 2.5 waters per lipid where a minute water uptake induces a large change in relative humidity from 20% to 90%. Such a relation between composition and chemical potential is typical for a stoichiometric compound. At 3% cholesterol a similar plateau is observed, although it occurs at approximately 3.5 water molecules per DMPC and it extends only between relative humidity of 50% and 85%.

The vertical part in the sorption isotherms occurring at $\text{RH} = 93\%$ signals a phase transition from a gel L_β phase to a liquid crystal $L_\alpha(d)$ phase for the pure DMPC (Markova et al., 2000). For the sample containing 3% cholesterol the corresponding transition occurs with a slightly lower slope and a significantly smaller uptake of water. The less steep slope can be due to slower kinetics of the transformation,

but a more plausible interpretation is that one crosses a narrow two-phase area. The sorption curve for pure DMPC shows a distinct step at approximately 10 waters per DMPC (RH = 93%), where the liquid crystal phase is formed. This indicates that the sorption process in this concentration regime is slow enough to ensure equilibrium during the experiment. For the swelling of the liquid crystalline phase above RH = 93%, there are no observable differences between the 0% and 3% cholesterol samples.

For the sample with the highest cholesterol content, $X_{\text{chol}} = 37\%$, the sorption curve shows a gradual uptake of water with decreasing osmotic pressure. There are no signs of phase changes over the whole range of osmotic pressures monitored. This is consistent with other measurements in the present investigation and with previous work (Faure et al., 1997; McIntosh, 1978), showing the existence of a so-called liquid ordered phase over the whole range of osmotic pressures studied at this cholesterol concentration. X-ray diffraction of PC-cholesterol mixtures containing 33% cholesterol have shown that the bilayers have nearly the same hydrocarbon chain structure over the whole range of relative humidity from 50% to 100% (McIntosh, 1978). The phospholipid-cholesterol phase behavior at high cholesterol contents in response to variation in osmotic pressure thus appears analogous to the phase behavior of the same system in response to variations in temperature (Fig. 1).

According to the phase diagram of Fig. 1, the $X_{\text{chol}} = 16\%$ and 25% samples are both in a two-phase area at zero osmotic pressure. The expected sorption curve for such two-phase areas is a linear combination of the sorption curves for the two pure phases weighted according to the lever rule. We do not have measurements for the systems at the exact phase boundaries, and we are not in a position to check for consistency. However, the sorption curves for the different liquid crystalline phases at 0% and 37% cholesterol, respectively, are very similar at high relative humidity. We should, therefore, not expect any strong effects from a two-phase character of the samples in this range. For the sample with $X_{\text{chol}} = 25\%$, the sorption curve is in fact very similar to that of the $X_{\text{chol}} = 37\%$ sample. There is, however, a small step in the curve at RH \approx 53%. This step also appears for the $X_{\text{chol}} = 16\%$ sample. For the latter sample we also observe a strong but continuous increase in the water content in the range RH = 85% to 93% and a distinct kink in the curve at the upper limit of this range. In an ideal experiment the passing of a three-phase line should be associated with a vertical step in water uptake, whereas the passing of the border between a one-phase and a two-phase area should be associated with a discontinuous change of slope of the sorption curve. In two-phase areas with sloping boundaries, the sorption curves can have a large but finite slope. From the data of Fig. 2, we can draw some preliminary conclusions about the underlying phase equilibria, but the interpretation can be made more reliable by also considering the enthalpy effects. Before turning to these we

should, however, compare our sorption isotherms with previous studies of the relation between water chemical potential and water content for phospholipid-cholesterol systems.

In a lamellar system, the water chemical potential is simply the interaction force per area ($\Pi_{\text{osm}} = -\Delta\mu/V_w = F/\text{area}$, V_w is the molar volume of pure water). There exists a large body of data on the relation between water content and water chemical potential in phospholipid systems, usually expressed in terms of force-distance curves (Rand and Parsegian, 1989). The influence of cholesterol on the repulsive interbilayer interaction between phospholipid bilayers has been investigated by means of the osmotic stress technique (Lis et al., 1982; McIntosh et al., 1989) and the climatic chamber technique (Jendrasiak and Mendible, 1976). The results from our DMPC-cholesterol sorption isotherms show, when comparisons can be made, quantitative agreement with the previous studies at higher values of RH, whereas there are some deviations for the lower water contents.

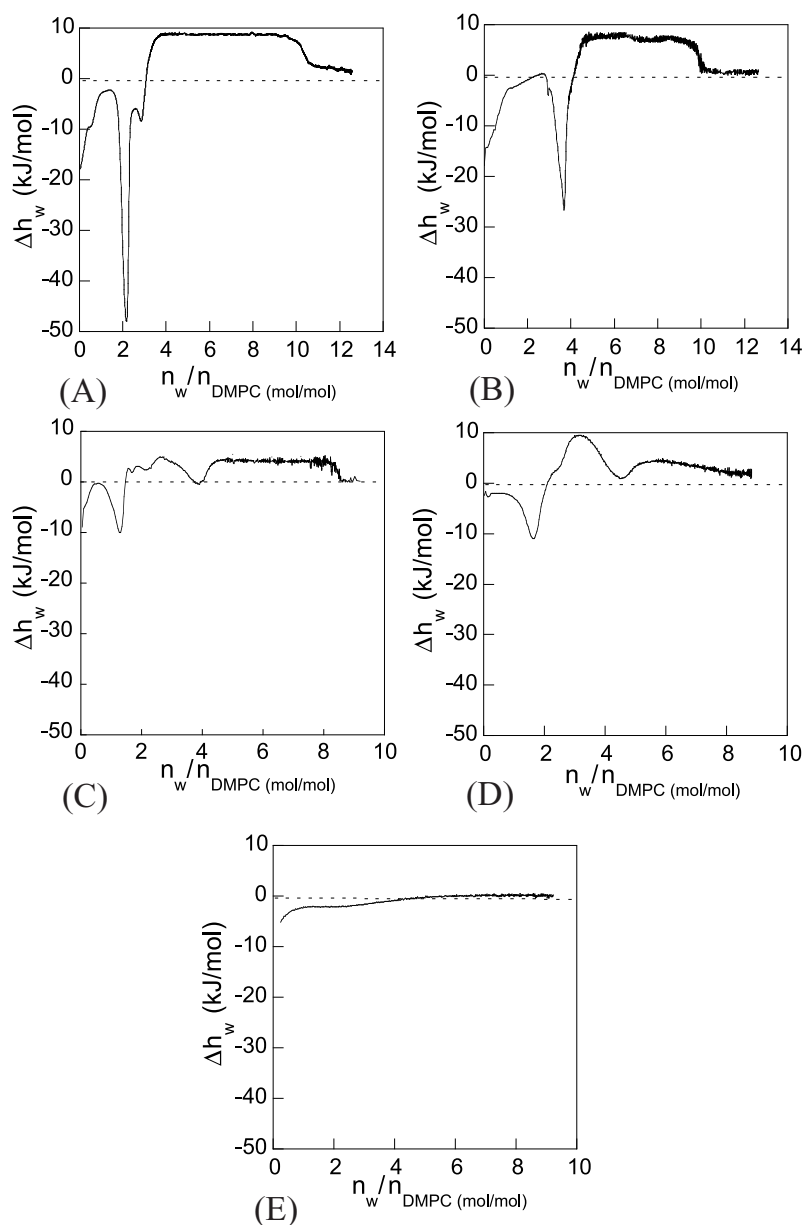
Partial molar enthalpy

A great advantage of the double calorimeter is that we can simultaneously monitor the water chemical potential (partial molar free energy) and the partial molar enthalpy of the water. In principle, this provides a complete thermodynamic description of the system at the given temperature and X_{chol} , on the basis of the Gibbs-Duhem relation (Markova et al., 2000). Whereas the chemical potential, by thermodynamic necessity, increases monotonically with increasing water content, there are many more features in the partial molar enthalpy curves shown in Fig. 3 for the five different samples. The calorimetric experiment demonstrates that even though two samples show very similar sorption isotherms, the enthalpy effects may be very different (e.g., the $X_{\text{chol}} = 25\%$ and $X_{\text{chol}} = 37\%$ samples) and vice versa (e.g., the $X_{\text{chol}} = 16\%$ and $X_{\text{chol}} = 25\%$ samples).

We have discussed the enthalpy effects during hydration of pure phospholipids in a previous publication (Markova et al., 2000). This study showed an endothermic transition from the L_β gel to the $L_\alpha(d)$ liquid crystal with an uptake of approximately seven water molecules per lipid (Fig. 3 A). At low water contents there is an exothermic water uptake, primarily associated with the hydration of the phosphate groups on the lipid (Pohle et al., 1998).

At $X_{\text{chol}} = 3\%$, the enthalpy curve (Fig. 3 B) shows the same qualitative features as the pure DMPC enthalpy curve, although there are some quantitative differences. As pointed out for the sorption isotherm, we do not observe any depression of the gel- $L_\alpha(d)$ transition nor any sign of a substantial two-phase coexistence region, as normally observed when a second component is added (Evans and Wennerström, 1999). This is analogous to the almost negligible melting point depression in the $T - X_{\text{chol}}$ phase diagram

FIGURE 3 Partial molar enthalpy of water (Δh_w) in the sorption process as a function of water content (n_w/n_{DMPC}), for DMPC-cholesterol mixtures at 27°C (A) $X_{\text{chol}} = 0\%$ (Markova et al., 2000), (B) $X_{\text{chol}} = 3\%$, (C) $X_{\text{chol}} = 16\%$, (D) $X_{\text{chol}} = 25\%$, and (E) $X_{\text{chol}} = 37\%$.



(Fig. 1), and it can be explained by the distribution coefficient of cholesterol between the gel and the liquid crystal being close to unity. However, in accordance with the observation from Fig. 2, the water uptake during the gel-liquid crystal transition has decreased to approximately five molecules. Furthermore, the enthalpy per water has decreased significantly from 8.8 to 7.8, implying a strong effect on Δh_w counted per cholesterol molecule. We interpret this as a combined effect of cholesterol disturbing the packing of the crystalline chains of the DMPC in the gel phase, increasing the enthalpy, and the straightening effect of cholesterol on the alkyl chains in the liquid crystal, decreasing the enthalpy. The presence of cholesterol also reduces the exothermic enthalpy effect at the lowest water contents, which can be tentatively interpreted as a manifesta-

tion of the competition between the OH of the cholesterol with water in the interaction between the DMPC phosphate groups.

The sorption isotherm of the $X_{\text{chol}} = 37\%$ sample demonstrates a continuous swelling of a single phase over the whole range of osmotic pressures studied. The simultaneously obtained enthalpy curve (Fig. 3 E) shows almost no features. At low water contents the swelling is slightly exothermic, whereas for water contents higher than four waters per DMPC molecule, the signal is very small and slightly positive. Thus, we confirm the conclusion that no large molecular changes occur on swelling and that a liquid ordered phase occurs at all water contents. The total enthalpy change, $H(n_{w1}) - H(n_{w0})$ per mole DMPC during sorption from n_{w0} to n_{w1} water at a given cholesterol/lipid

TABLE 1 Total enthalpy change of hydration determined from the integrals of the enthalpy curves (extrapolated to $RH = 100\%$); $\Delta H_{0 \rightarrow 100\%} = H(n_w/n_{DMPC}(100\%)) - H(n_w/n_{DMPC}(0\%))$

X_{chol}	$\Delta H_{0 \rightarrow 100\%}$ (kJ/mol)
0%	35
3%	17
16%	20
25%	22
37%	-7

ratio (X_{chol}), can be calculated from a numerical integration of the enthalpy curves;

$$H(n_{w1}) - H(n_{w0}) = \int_{n_{w0}}^{n_{w1}} \Delta h_w(n_w/n_{DMPC}) dn_w \quad (1)$$

The calculation gives an enthalpy change from the dry state to the fully hydrated (extrapolated value) state of -7 kJ/mol(DMPC) for the $X_{chol} = 37\%$ sample. This result is in strong contrast to the enthalpy change of 35 kJ/mol(DMPC) for the pure DMPC. Also, the DMPC-cholesterol samples with $X_{chol} \leq 25\%$ show a positive enthalpy change (see Table 1). The measurements demonstrate explicitly that the enthalpy of the swollen $L_\alpha(o)$ phase is lower than for the combined dry state and pure water from which it is formed. This further illustrates the rather special properties of the $L_\alpha(o)$ phase.

For samples with two or three phases, the measured partial molar enthalpy is a macroscopic effective value, and to be able to make a molecular interpretation, one needs to decompose Δh_w into the contributions from the different phases (Heerklotz and Binder, 1997). For a two-phase ternary sample, the measured Δh_w is determined from the enthalpies of hydration for each of the different phases and from the redistribution of mass between them. The magnitudes of these different contributions depend on the slope of the phase boundaries and the tie lines in the phase diagram and on the swelling behavior of the different phases. The addition of a small amount of water to such a sample gives rise to an enthalpy effect that is dependent on the exact composition of the sample. The measured Δh_w may therefore vary with the water content.

Addition of water to a three-phase ternary sample, on the other hand, only involves redistribution between phases and there are no swelling effects. When passing a three-phase triangle along the dilution-line, the compositions of the different phases remain constant and are given by the compositions at the corners of the three-phase triangle in the phase diagram. The change in enthalpy upon the addition of a small amount of water is independent of the position along the dilution-line in the three-phase triangle. This results in a “transition plateau” of constant molar enthalpy.

Even though their sorption curves are rather different, the samples with $X_{chol} = 16\%$ and $X_{chol} = 25\%$ show qualitatively similar enthalpy curves (Fig. 3, *c* and *d*). In both curves there is an endothermic peak around three water molecules per DMPC, which correlates with the small step in the sorption curve at $RH = 53\%$. The most likely interpretation of these observations is that a three-phase line is passed at $RH = 53\%$. For the $X_{chol} = 16\%$ sample, the Δh_w value appears constant at a water uptake between four and eight water molecules, indicating the passing of a three-phase line. However, the sorption curve shows a definite variation in the relative humidity in this range, indicating a two-phase area ending with a kink at $RH \approx 93\%$. Furthermore, at water contents corresponding to $RH 93\%$, the partial molar enthalpy goes practically to zero, which is consistent with a swelling of a single $L_\alpha(d)$ liquid crystalline phase. For the $X_{chol} = 25\%$ sample there is a positive Δh_w in the same range, but it varies considerably with composition, in this case demonstrating the two-phase character of the system. Furthermore, Δh_w does not go to zero even at the highest water contents studied, indicating that in a two-phase area persists up to the highest RH values monitored.

Deuterium NMR measurements

Magnetic resonance methods have proven to be very useful for probing the molecular properties of lipid systems. One particular aspect is the use of magnetic resonance for phase studies, because, if the molecules studied are present in different molecular environments with a slow exchange (relative to the inverse interaction strength), resolved signals from the separate phases should be observed. A particularly simple and versatile approach for amphiphile water systems is the use of deuterium NMR for samples containing heavy water (Ulmius et al., 1977; Khan et al., 1982). We have recorded deuterium NMR spectra for a range of samples at different DMPC-cholesterol ratios and with a set of different water contents at 27°C . Based on the sorption measurements we can estimate the osmotic pressures in the samples. For two-phase samples the water is distributed to give equal osmotic pressure in both phases, leaving an uncertainty about the water content in each phase.

Fig. 4, *A* and *B* show the NMR spectra for pure DMPC and for $X_{chol} = 3\%$ at $n_w/n_{DMPC} = 7.0$ mol/mol. At this water content, the L_β phase is present in the pure DMPC sample. The presence of a small amount of cholesterol has a dramatic effect on the quadrupolar splitting. In the $X_{chol} = 3\%$ spectrum, a narrow splitting typical for the P_β phase (Ulmius et al., 1977), is superimposed on the broader peak from the L_β phase. We can therefore conclude the P_β phase is induced by the addition of cholesterol.

For the single $L_\alpha(o)$ phase at high cholesterol contents, there is a clearly observed deuterium quadrupolar splitting of a magnitude typical for lipid systems (Fig. 4 *D*). It varies

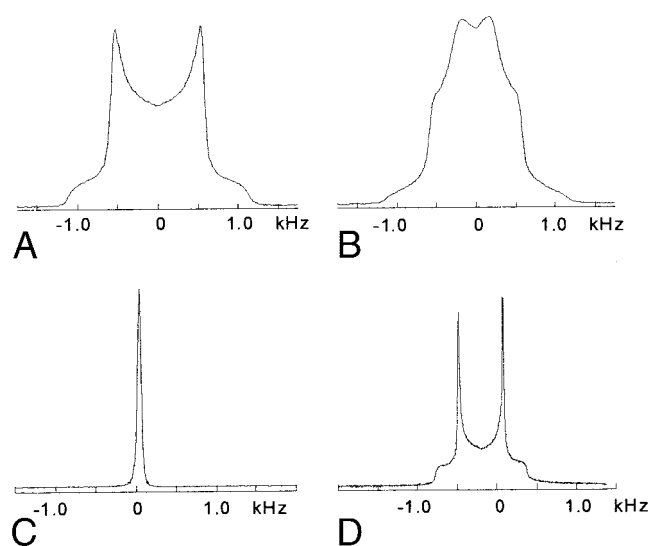


FIGURE 4 Selected ^2H -NMR spectra of DMPC-cholesterol mixtures at 27°C . (A) $X_{\text{chol}} = 0\%$: $n_{\text{w}}/n_{\text{DMPC}} = 7.0$ mol/mol; (B) $X_{\text{chol}} = 3\%$: $n_{\text{w}}/n_{\text{DMPC}} = 7.0$ mol/mol; (C) $X_{\text{chol}} = 25\%$: $n_{\text{w}}/n_{\text{DMPC}} = 7.0$ mol/mol; (D) $X_{\text{chol}} = 37\%$: $n_{\text{w}}/n_{\text{DMPC}} = 12.4$ mol/mol.

approximately inversely with water content, indicating that the main contribution to the splitting comes from the few most strongly interacting water molecules, presumably those close to the phosphate group. There is a remarkable difference between the NMR spectra of the samples containing 25% and 37% cholesterol, considering the very similar sorption curves of the two systems (Fig. 4, C and D). The narrow signals observed at $X_{\text{chol}} = 25\%$ can be due to either a weak average water-lipid interaction or to some averaging process over a sample that is heterogeneous on a colloidal length scale. With a quadrupolar splitting of approximately 1 kHz, motion between domains with different orientations on a time-scale of $(2\pi \cdot 10^3)^{-1}$ s will lead to motional narrowing of the NMR signal. From the calorimetric data, we have established that there are no major differences in the lipid-water interactions as the cholesterol content is varied. The most likely interpretation of the narrow deuterium NMR signals is thus that the samples have a structure on the mesoscopic scale in such a way that the diffusion of the water molecules through the sample results in a motional narrowing of the quadrupolar splitting. We also stress that our observations are reproducible and that the NMR signal remains constant for samples that have been stored for more than 2 months. There is thus no obvious influence of nonequilibrium defects introduced by the sample preparation.

Phase equilibria

In this work we are aiming at a thermodynamic characterization of the DMPC-cholesterol-water system at a constant temperature of 27°C . We have by measurements at several

DMPC-cholesterol ratios directly measured the partial molar free energy and enthalpy of water. A complete characterization would certainly also involve the determination of the phase diagram(s) for the system. It is in general a rather demanding task to establish the phase diagram for a three-component system and, in the present case, we encounter some extra difficulties, caused mainly by the physically very interesting and physiologically important molecular interplay between phospholipids and cholesterol in bilayer and monolayer arrangements (Ipsen et al., 1987; Radhakrishnan and McConnell, 1999).

One complication in the phase diagram arises from the relatively large number of phases that can be realized. For the binary lipid water system at 27°C two different phases, a gel L_β and a liquid crystalline $L_\alpha(d)$ phase are formed, excluding the very low water contents. However, by going down only two degrees in temperature a rippled gel P_β phase also appears (Smith et al., 1990; Janiak et al., 1979). Studies of the phospholipid-cholesterol system in excess water reveal the existence of two liquid crystalline phases $L_\alpha(d)$ with disordered chains at low cholesterol contents, and a more unusual $L_\alpha(o)$ phase with nearly straight phospholipid hydrocarbon chains at high cholesterol contents (Vist and Davis, 1990; Sankaram and Thompson, 1991). Thus, from the known limits we expect at least three phases to appear in the phase diagram. Additionally, there is both direct experimental evidence and structural arguments leading to the conclusion that cholesterol is more compatible with the rippled P_β gel phase than with the L_β gel phase (Matuoka et al., 1994; Rock et al., 1989; Mortensen et al., 1988). This was also confirmed for the DMPC-cholesterol system at low water contents by ^2H -NMR. Thus, it is concluded that the P_β phase is also present in the DMPC-cholesterol phase diagram at 27°C . For binary phospholipid-water systems the swelling properties of the P_β phase are intermediate between those of the $L_\alpha(d)$ and L_β phases, and this also applies to the enthalpies.

Unfortunately our study is not extensive enough to unequivocally establish a unique phase diagram, but it contains enough information to provide some main features. First, we note that there are, in the present case, two different choices of variables for the phase diagram. For the water component we can specify either the chemical potential/osmotic pressure of water or the water content. Both of these representations have their advantages, and we will discuss both because they contain different quantitative information. A main technical difference between the two representations is that the chemical potential is an intensive variable, which has the same value in two coexisting phases, and the $\Pi_{\text{osm}} - X_{\text{chol}}$ representation includes one-phase and two-phase areas and three-phase lines. In the ternary composition representation, the three-phase lines change into three-phase triangles, and furthermore, the direction of tie lines has to be specified.

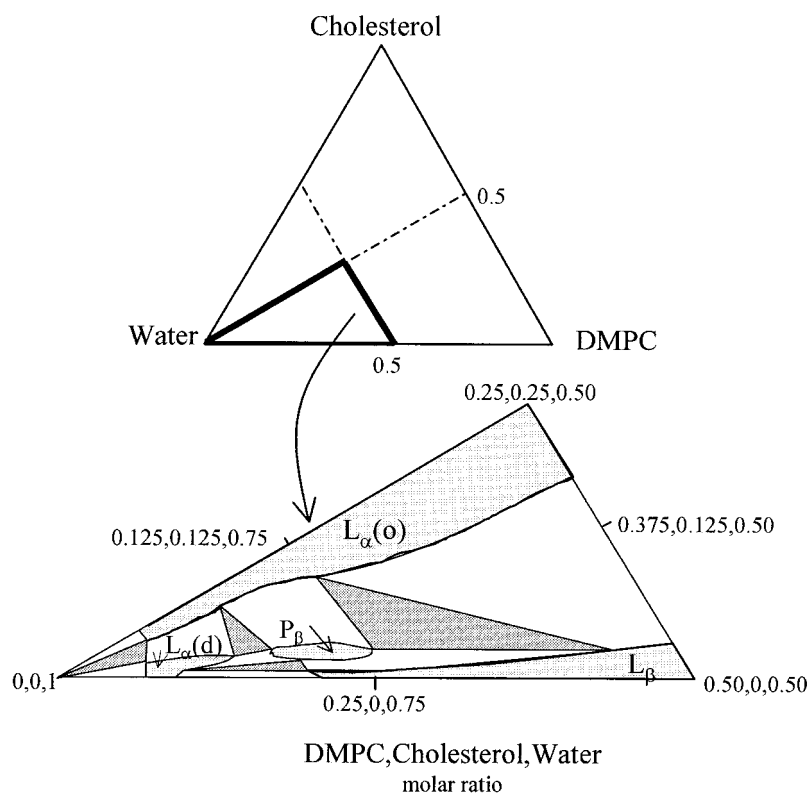
The main features of the phase diagram that emerge from the calorimetric measurements with support from the NMR observations are: 1) At high cholesterol contents (our sample $X_{\text{chol}} = 37\%$), there is a continuous swelling as water is added, or equivalently, as the osmotic pressure decreases. There is a smooth change in the partial molar enthalpy upon hydration and the NMR spectra show well-resolved quadrupolar splitting with a magnitude that varies in a regular way with water content. The clear conclusion is that a single liquid ordered ($L_{\alpha}(o)$) phase is present over the whole range of water contents investigated. 2) For $X_{\text{chol}} = 3\%$, the first significant observation is that there is virtually no “melting point depression,” which normally appears when a third component is added to a water-phospholipid system. This effect of cholesterol is well established for the excess water case when temperature is the intensive variable that is varied (Vist and Davis, 1990). The molecular explanation was elaborated by Ipsen et al. (1987), and the arguments are equally valid when we change the variable from T to Π_{osm} . However, there is an important difference relative to the excess water situation. For the pure phospholipid-water system at 27°C , the gel-liquid crystal transition is from L_{β} to $L_{\alpha}(d)$ rather than the P_{β} to $L_{\alpha}(d)$ transition induced by temperature (Fig. 1). One should expect substantial differences in the ability to solubilize cholesterol between the P_{β} and L_{β} phases, and it is improbable that the close to unity value of the distribution coefficient of cholesterol between the liquid crystal to gel phases applies to both the P_{β} and the L_{β} gel phases. The calorimetric and NMR data for $X_{\text{chol}} = 3\%$ show that the addition of cholesterol tends to stabilize P_{β} relative to L_{β} , and that the transition observed for $\text{RH} = 92\%$ to 93% is due to the passing of a three phase line, $L_{\beta} - L_{\alpha}(d) - P_{\beta}$, and a very narrow two-phase area of $L_{\alpha}(d)$ coexisting with P_{β} or L_{β} . We can thus conclude that cholesterol is more readily incorporated into the P_{β} phase than in the L_{β} phase. 3) For the $X_{\text{chol}} = 16\%$ and $X_{\text{chol}} = 25\%$ we observe a small step in the sorption curve at approximately $\text{RH} = 50\%$ and $n_{\text{w}}/n_{\text{DMPC}} = 3$. In this region the corresponding enthalpy curves have a distinct endothermic peak. This is most simply interpreted in terms of a three-phase line $L_{\beta} - P_{\beta} - L_{\alpha}(o)$ at this osmotic pressure. McIntosh (1978) has previously reported the presence of two coexisting lamellar phases in DPPC-cholesterol ($X_{\text{chol}} = 33\%$) mixtures at relative humidity below 50% . This indicates that the sample at this composition is in the two-phase $L_{\beta} - L_{\alpha}(o)$ region. 4) For the $X_{\text{chol}} = 16\%$ and $X_{\text{chol}} = 25\%$ we observe an endothermic enthalpy at compositions above four water molecules per DMPC. A comparison with the sorption curves reveals that there is a clear variation in osmotic pressure in this regime. For the $X_{\text{chol}} = 16\%$ sample, this indicates a two-phase $L_{\alpha}(d) - L_{\alpha}(o)$ coexistence area in the range of $\text{RH} = 85\%$ to 95% , and a one-phase $L_{\alpha}(o)$ area above $\text{RH} \approx 95\%$. This interpretation is not fully consistent with the phase diagram in Fig. 1, implying a two-phase coexistence also at $\text{RH} = 100\%$.

However, the phase boundary between the $L_{\alpha}(d) - L_{\alpha}(o)$ coexistence region and the $L_{\alpha}(d)$ region in Fig. 1 was given with a rather high uncertainty (Almeida et al., 1992). Relatively large variations in the location of this phase boundary are also revealed when comparing different studies (Vist and Davis, 1990; Sankaram and Thompson, 1990; Shimshick and McConnell, 1973). Our calorimetric results clearly indicate that the $L_{\alpha}(d)$ single phase is reached at excess water for the $X_{\text{chol}} = 16\%$ sample, which suggests a lower slope of the phase boundary between the $L_{\alpha}(d)$ and the $L_{\alpha}(d) - L_{\alpha}(o)$ regions in the phase diagram in Fig. 1. On thermodynamic grounds we also expect a three-phase line $P_{\beta} - L_{\alpha}(o) - L_{\alpha}(d)$ to occur, but the data do not give a clear indication of its presence.

If we combine this information with the established behavior at the boundaries $X_{\text{chol}} = 0\%$ (DMPC-water), and $\Pi_{\text{osm}} = 0$ (excess water) and make use of the limitations set by the phase rule, which implicitly assumes first order phase transitions, we arrive at the phase diagrams shown in Figs. 5 and 6. The ternary composition phase diagram contains four three-phase triangles (including the equilibrium with excess water), but we can only give the general locations of these triangles, rather than accurate coordinates for the corners. In the $\Pi_{\text{osm}} - X_{\text{chol}}$ phase diagram, the $L_{\beta} - L_{\alpha}(d) - P_{\beta}$ three-phase line is present at $\text{RH} = 92.5 \pm 1\%$ and the $L_{\beta} - P_{\beta} - L_{\alpha}(o)$ three-phase line is present at $\text{RH} = 53 \pm 3\%$. There is a larger uncertainty about the position of the $P_{\beta} - L_{\alpha}(d) - L_{\alpha}(o)$ three-phase line, which we set to $\text{RH} = 86 \pm 6\%$.

In general terms the phase diagrams presented here seem to be in agreement with previous observations on this and analogous systems. However, there is one important exception to this observation. Both Copeland et al. (1980) and Mortensen et al. (1988) have observed a more complex behavior in the $P_{\beta} - L_{\alpha}(o)$ coexistence region. By means of freeze-fracture electron microscopy and small-angle neutron scattering, they have demonstrated a continuous increase in the ripple amplitude when the cholesterol content is increased up to $20 \text{ mol}\%$, where the ripple separation in principle increases to infinity as approaching the phase boundary to the $L_{\alpha}(o)$ phase region. The interpretation of these results is that, instead of a true phase separation, there is a striation so that bands of the $L_{\alpha}(o)$ phase separates ripples in a superstructure that tends to disorder at higher cholesterol contents. The ripple DMPC-cholesterol bilayers can then be described as single phase bilayers where cholesterol-rich domains are regularly included as stripes in the “valleys” of the rippled structure, separated by stripes of DMPC-rich P_{β} gel domains (Fig. 7). The calorimetric measurements are primarily sensitive to the strong short-range interactions, and it is not possible to distinguish between true phase separation and separation of regions on a mesoscopic scale. It would lead too far to go into a detailed discussion of the true phase character of a situ-

FIGURE 5 Schematic partial ternary composition phase diagram of the water-rich corner for DMPC-cholesterol-water at 27°C. The phase diagram is outlined from the microcalorimetric data and the ^2H -NMR spectra. The phase diagram contains four three-phase triangles (dark gray), four one-phase regions (light gray), and several two-phase regions (white). The exact positions of triangle corners and the phase boundaries are not determined, and the figure only provides the main features of phase diagram.



ation where areas of one single phase are continuously inserted between areas of a different character. A reasonable description is to say that the phase boundary of the P_β phase toward the $L_\alpha(o)$ phase loses its strict thermodynamic significance and becomes more like a critical micelle concentration for surfactant solutions, indicating a boundary where there is no true thermodynamic phase transition but a change in the organization in the system (dotted line in Fig. 6). At the other end of the former two-phase area, where the ripple repeat tends to diverge, this constitutes a continuous transition with expected anomalies in the thermodynamic parameters caused by critical fluctuations (dashed line in Fig. 6). When this rather subtle effect is incorporated into the phase diagram in Fig. 6, the former three phase line $P_\beta - L_\alpha(d) - L_\alpha(o)$ is changed into a boundary between a one-phase and a two-phase area, and it is no longer strictly straight. This also goes for the three-phase line $L_\beta - P_\beta - L_\alpha(o)$.

CONCLUSIONS

In the present study we have investigated how a phospholipid-cholesterol mixed system responds to osmotic stress. This has an interest per se under physiologically stressed conditions like drying or freezing. A similar condition also occurs in the skin, which is usually exposed to a rather dry atmosphere. In skin, cholesterol is a major component,

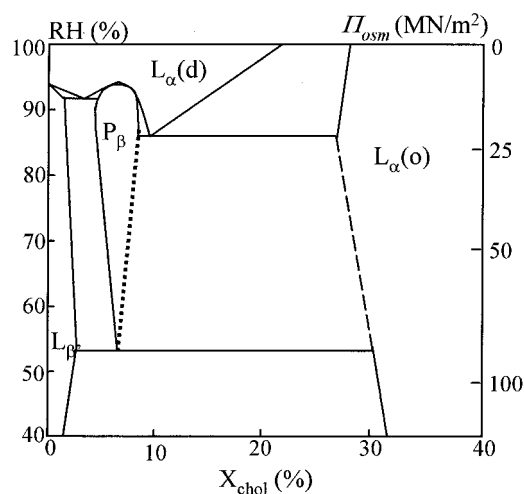


FIGURE 6 $\Pi_{\text{osm}} - X_{\text{chol}}$ phase diagram for DMPC-cholesterol at 27°C outlined from the calorimetric data and the ^2H -NMR spectra. The positions of the three-phase lines are determined to $\text{RH} = 92.5 \pm 1\%$ ($L_\beta - L_\alpha(d) - P_\beta$), $\text{RH} = 53 \pm 3\%$ ($L_\beta - P_\beta - L_\alpha(o)$) and $\text{RH} = 86 \pm 6\%$ ($P_\beta - L_\alpha(d) - L_\alpha(o)$). If the putative $P_\beta - L_\alpha(o)$ modulated structure is incorporated in this phase diagram, the character of the dashed and the dotted phase boundaries no longer represent first-order phase transitions. The phase boundary to the right of the $P_\beta - L_\alpha(o)$ coexistence region, represented with a dashed line, indicates a continuous transition between the $P_\beta - L_\alpha(o)$ modulated phase and the $L_\alpha(o)$ phase. The dotted line on the left-hand side of the $P_\beta - L_\alpha(o)$ coexistence region marks the position where the local character of the phase changes without a true thermodynamic phase transition. Left-hand axis, relative humidity (RH). Right-hand axis, the corresponding osmotic pressure of water (Π_{osm}).

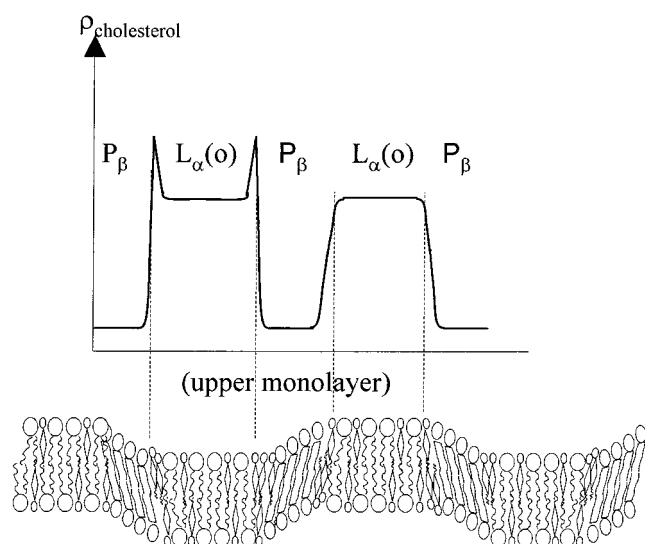


FIGURE 7 Schematic illustration of the $P_\beta - L_\alpha(o)$ modulated structure. $L_\alpha(o)$ domains are regularly incorporated in the “valleys” of the rippled structure and separated by stripes of P_β domains. The curve illustrates the cholesterol density in the upper monolayer of the bilayer structure. Due to its shape and lineactant properties, cholesterol is suggested to preferentially dissolve at the domain boundaries with a negative curvature.

whereas the lipids are ceramides and fatty acids rather than phospholipids (Wertz et al., 1992).

From a more general perspective the present study can be seen as a complement to the numerous studies of phospholipid-cholesterol system in excess water. In that case, phase transformations are induced by temperature changes. A variation of another intensive variable, the chemical potential of water/osmotic pressure, provides an additional perspective for understanding the molecular interactions in the system. An analogous approach has been taken by McConnell and coworkers, who have studied lipid-cholesterol interactions in monolayers by varying the surface pressure (Hagen and McConnell, 1997; Keller et al., 2000). We note that there are strong similarities between the phase diagram of Fig. 6 and those presented for phospholipid-cholesterol systems for excess water in Fig. 1. The microcalorimetric experiments demonstrate the extraordinary property of cholesterol not to cause any substantial melting point depression in the $\Pi_{\text{osm}} - X_{\text{chol}}$ phase diagram. Analogous behavior has previously been demonstrated with temperature as the intensive variable (Vist and Davis, 1990). One can also conclude that the $L_\alpha(o)$ liquid ordered phase, with $X_{\text{chol}} > 30\%$, has a remarkable stability, not only with decreasing temperature, but also with increasing osmotic pressure.

The P_β phase is absent in the binary water-phospholipid system at 27°C, but we find that this phase is induced by the addition of cholesterol. Theoretical models of the cholesterol-phospholipid phase behavior describe the gel state as a solid ordered phase, and they do not distinguish between the P_β and the L_β gel phases (Ipsen et al., 1987; Nielsen et al.,

1999). One conclusion from the present study is that the P_β phase can dissolve cholesterol readily, whereas the L_β phase might behave more like a conventional solid with a low solubilization capacity. This implies that the distinction between these different gel phases is an important factor in understanding the PC-cholesterol interactions. Based on previous studies of cholesterol’s line active (compare with surface active) ability in stabilizing two-dimensional domains (Sparr et al., 1999; Weis and McConnell, 1985) we can speculate about the molecular mechanisms leading to the relatively high solubility of cholesterol in the P_β phase. The basic driving force for the formation of the P_β phase in the binary water-phospholipid system is a mismatch between the cross-sectional area of the all trans alkyl-chains and the repulsive interaction between the phospholipid headgroups (Parsegian, 1983; Kirchner and Cevc, 1994). By forming a rippled bilayer the area per molecule, projected onto the bilayer repeat direction, can be kept small, whereas the nonprojected area per molecule of the headgroup region is larger. However, the curvature of the water bilayer interface is by necessity nonuniform in such a rippled structure, which introduces some degree of disorder. When cholesterol is presented to such a structure it is likely to preferentially dissolve in the more disordered regions, and it should have the highest solubility in the regions of negative curvature. Cholesterol can then act to relieve the curvature strain in the rippled structure. As the curved regions become saturated with cholesterol (at ~ 8 mol%) the chemical potential of the cholesterol reaches that of cholesterol in the cholesterol-rich liquid ordered $L_\alpha(o)$ phase, and the appearance of the $L_\alpha(o)$ phase is thus expected. If the formal line tension between the P_β and the $L_\alpha(o)$ phase is negative, i.e., if it is favorable free energy wise to keep the $P_\beta - L_\alpha(o)$ contact, this could lead to the formation of the modulated single phase of alternating domains of the two molecular arrangements. The main driving force for such a structure is then that the high energy curved regions of the P_β phase are eliminated as illustrated in Fig. 7.

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