A New High-Temperature Transition of Crystalline Cholesterol in Mixtures with Phosphatidylserine

Richard M. Epand,* Diana Bach,† Raquel F. Epand,* Nina Borochov,‡ and Ellen Wachtel§
*Department of Biochemistry, McMaster University, Hamilton, ON L8N 3Z5, Canada; †Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 58102, Israel; ‡Center for Technological Education, Holon 58102, Israel; and §Chemical Services Unit, Weizmann Institute of Science, Rehovot 76100, Israel

ABSTRACT Phosphatidylserine and cholesterol are two major components of the cytoplasmic leaflet of the plasma membrane. The arrangement of cholesterol is markedly affected by the presence of phosphatidylserine in model membranes. At relatively low mol fractions of cholesterol in phosphatidylserine, compared with other phospholipids, cholesterol crystallites are formed that exhibit both thermotropic phase transitions as well as diffraction of x-rays. In the present study we have observed and characterized a novel thermotropic transition occurring in mixtures of phosphatidylserine and cholesterol. This new transition is observed at 96°C by differential scanning calorimetry (DSC), using a heating scan rate of 2°C/min. Observation of the transition requires that the hydrated lipid mixture be incubated for several days, depending on the temperature of incubation. The rate of formation of the material exhibiting a transition at 96°C is more rapid at higher incubation temperatures. At 37°C the half-time of conversion is ~7 days. Concomitant with the appearance of the 96°C peak the previously known transitions of cholesterol, occurring at ~38°C and 75°C on heating scans of freshly prepared suspensions, disappear. These two transitions correspond to the polymorphic transition of anhydrous cholesterol and to the dehydration of cholesterol monohydrate, respectively. The loss of the 75°C peak takes a longer time than that of the 38°C peak, indicating that anhydrous cholesterol first gets hydrated to the monohydrate form exhibiting a transition at 75°C and subsequently is converted by additional time of incubation to an altered form of the monohydrate, showing a phase transition at 96°C. After several weeks of incubation at 37°C, only the form with a phase transition at 96°C remains. If such a sample undergoes several successive heating and cooling cycles, the 96°C peak disappears and the 38°C transition reappears on heating. For samples of 1-palmitoyl-2-oleoyl phosphatidylserine or of 1-stearoyl-2-oleoyl phosphatidylserine having mol fractions of cholesterol between 0.4 and 0.7, the 38°C transition that reappears after the melting of the 96°C component generally has the same enthalpy as do freshly prepared samples. This demonstrates that, at least for these samples, the amount of anhydrous cholesterol crystallites formed is indeed a property of the lipid mixture. We have also examined variations in the method of preparation of the sample and find similar behavior in all cases, although there are quantitative differences. The 96°C transition is partially reversible on cooling and reheating. This transition is also scan rate dependent, indicating that it is, at least in part, kinetically determined. The enthalpy of the 96°C transition, after incubation of the sample for 3 weeks at 37°C is dependent on the ratio of cholesterol to 1-palmitoyl-2-oleoyl phosphatidylserine or to 1-stearoyl-2-oleoyl phosphatidylserine, with the enthalpy per mole cholesterol increasing between cholesterol mol fractions of 0.2 and 0.5. Dimyristoyl phosphatidylserine at a 1:1 molar ratio with cholesterol, after incubation at 37°C, exhibits a transition at 95°C that reverses on cooling at 44°C, instead of 60°C, as observed with either 1-palmitoyl-2-oleoyl phosphatidylserine or 1-stearoyl-2-oleoyl phosphatidylserine. These findings along with the essential absence of the 96°C transition in pure cholesterol or in cholesterol/phosphatidylcholine mixtures, indicates that the phospholipid affects the characteristics of the transition, and therefore the cholesterol crystallites must be in direct contact with the phospholipid and are not simply in the form of pure crystals of cholesterol. These observations are particularly important in view of recent observations of the presence of cholesterol crystals in biological systems.

INTRODUCTION

There is increasing evidence for the existence of cholesterol-rich domains in membranes (Pralle et al., 2000; Schutz et al., 2000). The existence and properties of these domains, termed rafts, is currently an area of considerable interest and activity (Brown and London, 1998; Harder et al., 1998; Simons and Ikonen, 2000). In addition to cholesterol, these domains are also enriched in sphingomyelin (Rietveld and Simons, 1998). However, sphingomyelin is largely found on the extracellular side of the plasma membrane (Dekkers et al., 2000). It remains unknown what the lipid composition of the cytoplasmic face of these domains is. There is indirect evidence that the cytoplasmic face of rafts also contains considerable amounts of cholesterol because the cholesterol-to-phospholipid ratio of the detergent-insoluble fraction, thought to be largely composed of rafts, is very high. Cholesterol is therefore unlikely to be only on one side of the membrane. The cytoplasmic face of the membrane is also enriched in the amino-containing phospholipids. Cholesterol has lower solubility in both of the major aminophospholipids, phosphatidylethanolamine (Cheetham et al., 1989) and particularly in phosphatidylserines (Bach et al., 1998; Epand et al., 2000). It is therefore of interest to

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Address reprint requests to Dr. Richard M. Epand, Department of Biochemistry, McMaster University Health Sciences Centre, Hamilton, ON L8N 3Z5, Canada. Tel.: 905-525-9140; Fax: 905-521-1397; E-mail: epand@mcmaster.ca.

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understand the behavior of cholesterol in membranes rich in phosphatidylserine.

Cholesterol can form crystallites at moderate mol fractions of cholesterol in bilayers of phosphatidylserine. These crystallites are sufficiently large to give rise to x-ray diffraction (Wachtel and Bach, 1987). There is also evidence that cholesterol crystallites may form in biological specimens (Jacob et al., 1999; Kellner-Weibel et al., 1999) and that their formation may be associated with pathological consequences (Jacob et al., 2001; Guo et al., 2000; Klinkner et al. 1995; Tulenko et al., 1998).

An important question is what is the equilibrium state of cholesterol-phospholipid mixtures. There are several aspects to be considered. The first is whether the observed phase behavior is dependent on the manner in which the lipids are mixed and hydrated. Artifacts can potentially develop as a consequence of preferential precipitation of one of the lipid components during the evaporation of organic solvents in which they are dissolved, as for example, when chloroform alone is used. A special solvent exchange procedure has been developed to avoid this problem (Buboltz and Feigenson, 1999), but this procedure is not devoid of artifacts caused by the incomplete removal of solvent. There is really no ideal way to assure that there will not be lipid demixing during sample preparation. With regard to the use of benzene for the preparation of the lipid mixtures, we have previously shown that benzene is particularly slow to evaporate from films of phosphatidylserine and cholesterol. As a result of the interaction between cholesterol and benzene, cholesterol crystals cannot form (Bach et al., 1992). When cholesterol crystals are present, the formation of defects, as well as the size of the domains, will affect the ability to detect them. The amount of phase-separated cholesterol that is measured will depend on the method of detection. We have shown for example that differential scanning calorimetry (DSC) is a more sensitive method to detect cholesterol crystallites than is x-ray diffraction (Epand et al., 2000). These problems result in some quantitative uncertainty, but replicate samples, even those prepared at different times with different batches of lipid and in different laboratories, have generally resulted in quite similar phase behavior.

Another possible source of non-equilibrium behavior in this system results from the slow interconversion among different forms of cholesterol crystals. There are two well-documented thermotropic phase transitions of cholesterol crystals (Loomis et al., 1979). One is a polymorphic phase transition between two crystalline forms of anhydrous cholesterol that occurs at ~38°C. This transition exhibits a marked hysteresis, with the interconversion between the two forms being particularly slow in the temperature region between ~25°C and 35°C (Epand et al., 2000). The other transition is a result of the dehydration of crystals of cholesterol monohydrate, which usually occurs as a broad transition between 70°C and 80°C. Even in the presence of excess water, the hydration of anhydrous cholesterol is very slow, requiring ~24 h to complete at room temperature and much more than 2 days at 40°C (Loomis et al., 1979). In this manuscript we further explore equilibrium forms of cholesterol and identify a new transition involving cholesterol crystals.

MATERIALS AND METHODS

Materials

Cholesterol (Sigma grade, 99+%) was purchased from the Sigma Chemical Co. (St. Louis, MO), Northern Lipids (Vancouver, Canada) and Nu Chek Prep (Elysian, MN). The phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL).

Preparation of hydrated mixtures of phosphatidylserine and cholesterol

Phosphatidylserine (PS) and cholesterol were co-dissolved in chloroform/methanol (2/1, v/v). The solvent was evaporated under a stream of nitrogen with constant rotation of a test tube so as to deposit a uniform film of lipid over the bottom third of the tube. Last traces of solvent were removed by placing the tube under high vacuum for at least 2 h. The lipid film was then hydrated with one of the following buffers (as indicated in the text): 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.40 (for DSC studies); 0.01 M Tris/HCl, 150 mM NaCl, pH 7.4; or 0.01 M Tris/HCl, 500 mM NaCl, pH 7.4 (for x-ray studies). Using DSC, we have never observed a difference in the phase behavior of lipid mixtures of PS and cholesterol as a consequence of the use of buffers of different salt concentrations. The lipid film was suspended and hydrated by intermittent vortexing and heating to 60°C over a period of 30–60 min. Lipid suspensions were always hydrated or stored under argon.

Alternative methods of preparation of hydrated mixtures of phosphatidylserine and cholesterol

Unless otherwise indicated, the studies described in this manuscript were performed with samples prepared as described above. However, there have been reports in the literature suggesting that cholesterol crystals may be formed only as a consequence of solvent evaporation (McMullen et al., 2000). To ascertain whether the behavior we observed with mixtures of cholesterol and PS was dependent on the method of sample preparation, we employed two modifications of the method we had been using. One variation was equivalent to the method of McMullen et al. (2000) in which the solution of PS and cholesterol, co-dissolved in chloroform/methanol (2/1, v/v), was deposited by solvent evaporation at ~50°C. The lipid film was then treated as described in the previous section. This method will be referred to as the McMullen method. Another minor variation was to prepare the lipid film as described in the previous section but then to hydrate it with buffer by vortexing at room temperature instead of at 60°C. This will be referred to as the room-temperature method.

Differential scanning calorimetry

Measurements were made using a Nano differential scanning calorimeter (Calorimetry Sciences Corp., Provo, UT). Unless otherwise stated, the scan rate was 2°C/min and there was a delay of 5 min between sequential scans in a series to allow for thermal equilibration. The features of the design of this instrument have been described (Privalov et al., 1995). DSC curves

were analyzed by using the fitting program DA-2 provided by Microcal (Northampton, MA) and plotted with Origin, version 5.0.

X-ray diffraction experiments

Phosphatidylserine and cholesterol suspensions in either 0.15 or 0.5 M NaCl in Tris/HCl buffer at a concentration of ~6 mg lipid/ml were prepared as described above. Just before the experiment, the dispersion was centrifuged in an Eppendorf centrifuge for 15 min and the precipitate was loaded into a 1.5-mm quartz capillary. In addition to these phospholipid-cholesterol mixtures, pure crystalline cholesterol, either anhydrous or after hydration with water, was inserted into 1.5-mm quartz capillaries. Low-angle x-ray diffraction measurements on fresh or incubated samples were performed at room temperature as described in Cheetham et al. (1989). Two-dimensional wide-angle x-ray diffraction measurements were performed at room temperature as described in Wachtel et al. (1998). Profiles of intensity versus scattering angle were generated by averaging the two-dimensional data about the pattern center.

^{31}P NMR

The ^{31}P NMR spectra, from suspensions of ~50 mg of lipid in PIPES buffer, were obtained using a Bruker AM-500 spectrometer operating at 202.45 MHz in a 10-mm broad band probe over a 30-kHz sweep width in 16 × 1024 data points. A 90° pulse width of 16.6 μs was used. Composite pulse decoupling was used to remove any proton coupling. Generally, 800 free induction decays were processed using an exponential line broadening of 100 Hz before Fourier transformation. Probe temperature was maintained at 37 ± 0.2°C by a Bruker B-VT 1000 variable temperature unit. Temperatures were monitored with a calibrated thermocouple probe placed in the cavity of the NMR magnet.

RESULTS

Freshly prepared suspensions of 1-palmitoyl-2-oleoyl PS (POPS) or 1-stearoyl-2-oleoyl PS (SOPS) containing mol fractions of cholesterol of 0.4 or 0.5, i.e., above the solubility limit of cholesterol in the presence of these phospholipids (Bach et al., 1992), which have been cooled below 15°C, exhibit two thermotropic transitions, one at 38°C and another broad transition in the range 70°C to 80°C on the first DSC heating scan. The former is the polymorphic phase transition of anhydrous cholesterol, and the latter is due to the dehydration of the monohydrate. In this work, we find that when such samples are incubated for a period of ~3 weeks at 0°C, 25°C, or 37°C, the endotherms at these temperatures markedly weaken or disappear and a new transition appears at 96°C. Most samples of pure anhydrous cholesterol suspended in water will convert after incubation either at 0°C, 25°C, or 37°C to the form of cholesterol monohydrate having a temperature of dehydration around 75°C. However, some samples are observed to have a few percent of the form that undergoes a transition at 95°C. Hydration of cholesterol at low temperatures proceeds more slowly in the presence of phospholipid than in its absence. In the presence of PS, the conversion to the form with a transition temperature at 96°C is more complete when the incubation is carried out at higher temperature (Table 1). This is in opposition to the temperature dependence of the hydration of anhydrous pure cholesterol to the form that dehydrates at 75°C. The latter is more rapid at lower temperatures (Loomis et al., 1979). A series of successive heating and cooling scans of a sample of SOPS with 0.5 mol fraction of cholesterol that had been incubated for 2 weeks at 0°C is shown (Fig. 1). The first heating scan shows endotherms at 14°C, 38°C, 85°C, and 96°C. In the second

TABLE 1 Dependence of the formation of the 96°C transition on the incubation temperature measured in the first heating scan

Lipid	Mol fraction cholesterol	Incubation temperature (°C)	ΔH at 96°C (cal/mol)
POPS	0.4	0	453
POPS	0.4	22	1225
POPS	0.4	37	1675
POPS	0.5	0	600
POPS	0.5	22	1792
POPS	0.5	37	1788
SOPS	0.4	0	810
SOPS	0.4	22	1350
SOPS	0.4	37	1816
SOPS	0.5	0	613
SOPS	0.5	22	1400
SOPS	0.5	37	1620

Lipid suspensions were incubated for 3 weeks at the indicated temperature.

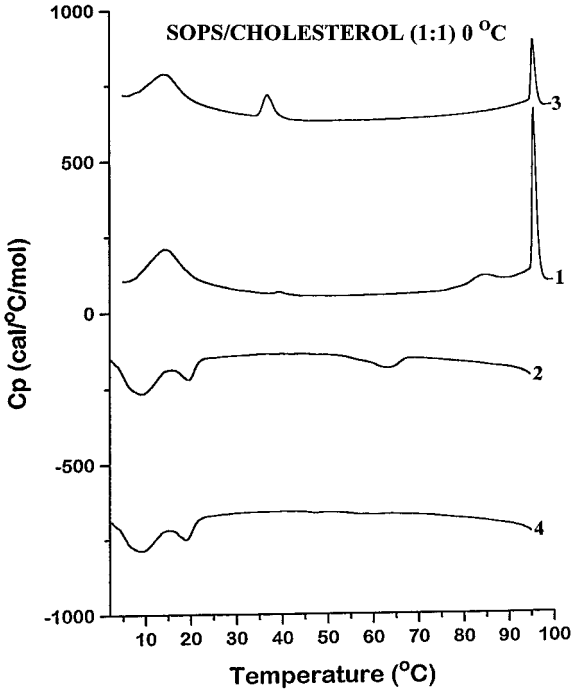

FIGURE 1 DSC scans of SOPS with 0.5 mol fraction cholesterol that had been incubated for 2 weeks at 0°C. SOPS concentration was 10 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.40. Scan rate was 2°C/min. The top two curves are heating scans and bottom two curves are cooling scans. The numbers indicate the order in which the scans were run. Curves have been displaced along the y axis for presentation. Excess heat capacity is expressed per mole of cholesterol.

heating scan (scan 3), the 96°C endotherm decreases in size, and the peak at 38°C grows stronger; the 85°C endotherm has disappeared. This indicates the conversion of the 96°C dehydrating form to the anhydrous form after sequential heating and cooling. The presence of the anhydrous form of cholesterol is shown by the appearance, at this scan rate, of the polymorphic transition at 38°C in heating scans and at 18°C in cooling scans (Epand et al., 2000). The phospholipid chain melting transition is observed in all scans in the temperature range 10–15°C. The DSC scans were started at 0°C, with the phospholipid in the gel state. It has been shown previously (Epand et al., 2000) that the polymorphic transition of anhydrous cholesterol exhibits considerable hysteresis and that the sample has to be undercooled to convert the cholesterol crystallites to the low-temperature form. We also showed in that work that cooling the sample below the phase transition temperature of the lipid had no effect on the appearance of cholesterol crystallites.

Unlike the dehydration of cholesterol monohydrate at ~70–80°C, the transition at 96°C is partially reversible. This is shown by the observation that in the first cooling scan (Fig. 1, scan 2) one can see an exotherm at ~60°C and by the presence, albeit at lower enthalpy, of the 96°C transition on the second heating scan (Fig. 1, scan 3). In scan 4 (the second cooling scan) the exotherm at 60°C is not seen.

The dependence of the phase behavior of samples of POPS with 0.5 mol fraction cholesterol on the time of incubation at 37°C is shown in Fig. 2. The enthalpy of the peak at 96°C increases with time of incubation (Table 2). A broad transition of low enthalpy at ~11°C, corresponding to the residual chain melting transition of the phospholipid is also detected. In addition, in the scans after 0 or after 91 h of incubation, there are clearly observed a relatively sharp transition at 38°C and another broad transition centered at 77°C or at 83°C after 0 or 91 h of incubation, respectively. With increasing time of incubation at 37°C, both the 38°C and the ~80°C transitions disappear. In addition to the first heating scans shown in Fig. 2, we have also sequentially measured a series of five additional scans for each sample, three cooling scans and two additional heating scans for a total of six scans (not shown). After the final, third heating scan, the only observable transition is that corresponding to the polymorphic transition of anhydrous cholesterol at 38°C. The enthalpy of this transition, after the complete disappearance of the forms with a higher transition temperature, is 420 ± 25 cal/mol cholesterol, independent of the time of incubation at 37°C. A similar value of 536 ± 26 was found previously by us for samples of 1:1 cholesterol:POPS that were not aged (Epand et al., 2000).

We also tested the scan rate dependence of the DSC thermogram of a sample of POPS with 0.5 mol fraction cholesterol that had been incubated at 37°C for 3 weeks. There is a marked effect of scan rate, with the transition at 96°C broadening and shifting to lower temperatures with slower scan rates (Fig. 3). Even at the more rapid scan rate of 2°C/min there clearly is a shoulder toward lower temperature that becomes more prominent and broader at slower scan rates. The equilibrium transition appears to be lower than 96°C, but the rate of the transition increases rapidly with increasing temperature. The change in the characteristics of the transition at slow scan rate is not caused by chemical degradation of the lipid. A sample of POPS with 0.5 mol fraction cholesterol that had been incubated at 37°C for 3 weeks was kept at 70°C for 5 h and then scanned from 0°C to 100°C by DSC at a rate of 2°C/min. The shape of the 96°C transition was unaltered by the 70°C incubation, indicating that the change in transition shape with scan rate is not a consequence of lipid degradation.

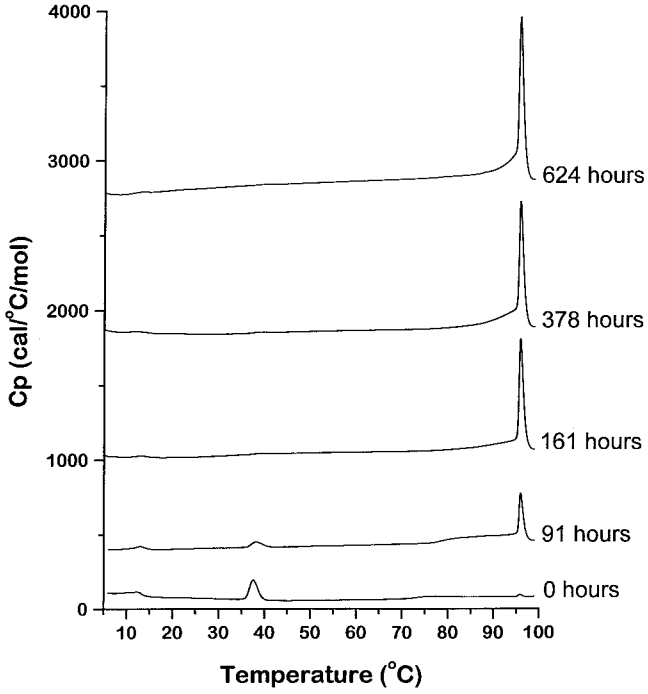

FIGURE 2 First DSC heating scan of a sample of POPS with 0.5 mol fraction of cholesterol after incubation at 37°C for the times indicated. POPS concentration was 6.7 mg/ml in 20 mM Pipes, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.4. Scan rate was 2°C/min. C_p is given per mole of cholesterol, and curves have been displaced along the *y* axis for presentation.

TABLE 2 Time dependence of the formation of the 96°C transition by incubation of POPS and 0.5 mol fraction cholesterol at 37°C

Time (h)	ΔH (cal/mol cholesterol)
0	25
91	435
161	1135
378	1395
624	1790

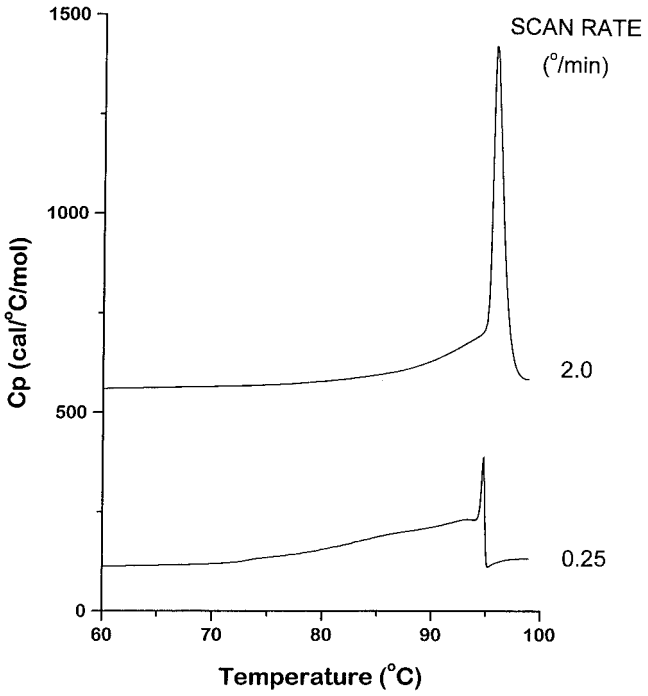

FIGURE 3 Dependence on scan rate of the thermotropic properties of POPS with 0.5 mol fraction of cholesterol that had been incubated at 37°C for 3 weeks. POPS concentration was 6.7 mg/ml in 20 mM Pipes, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.4. First DSC heating scan of a sample was at the indicated scan rate. C_p is given per mole of cholesterol, and curves have been displaced along the y axis for presentation.

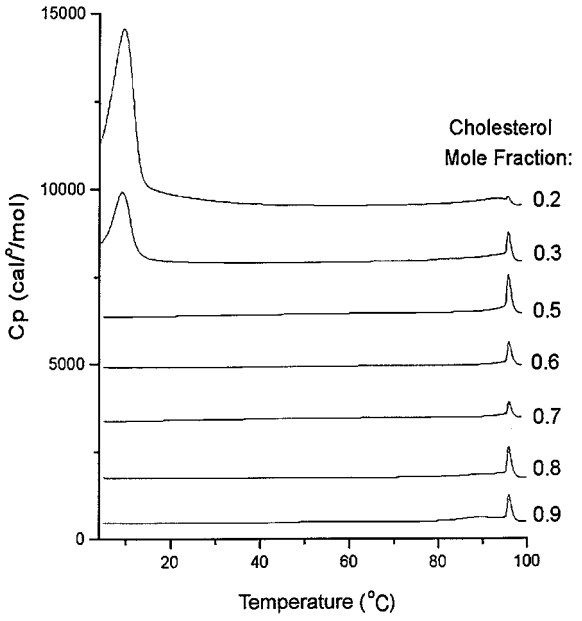

FIGURE 4 Dependence of the enthalpy of the 96°C transition on the mol fraction of cholesterol in mixtures with POPS. All samples have been incubated at 37°C for 3 weeks. First DSC heating scan was at a scan rate of 2°C/min of a sample at the indicated cholesterol mol fraction. C_p is given per mole of cholesterol, and curves have been displaced along the y axis for presentation.

The corresponding phosphatidylcholines (PCs) behave very differently with regard to their ability to induce formation of cholesterol crystallites after incubation. Neither 1-palmitoyl-2-oleoyl PC (POPC) nor 1-stearoyl-2-oleoyl PC (SOPC) with 0.5 mol fraction cholesterol exhibited any transition corresponding to cholesterol crystals either before or after incubation at 37°C for 3 weeks (not shown).

There is a strong dependence of the formation of the 96°C transition on the mol fraction of cholesterol present in mixtures with POPS. We incubated samples of POPS containing between 0.2 and 0.9 mol fraction of cholesterol for 3 weeks at 37°C. The first heating DSC curve showed a gradual increase in the enthalpy of the 96°C transition between a mol fraction of 0.2 and 0.5 (Fig. 4). One should note that unlike in the case of SOPS incubated at 0°C for 2 weeks (Fig. 1), samples of POPS incubated at 37°C for 3 weeks exhibit no residual endotherm at 38°C (Figs. 2 and 4). At higher mol fractions of cholesterol, the low-temperature shoulder on the 96°C peak moves to lower temperature and finally becomes a separate peak at 0.9 mol fraction cholesterol. This portion of the transition also becomes gradually more prominent with increasing mol fractions of cholesterol. We have analyzed this portion of the DSC curve as the sum of two transitions. The temperature and enthalpy of each of these components is given in Table 3. The enthalpy of the polymorphic transition at 38°C of anhydrous cholesterol both for fresh samples and for incubated and heated samples is given in Table 4 for a number of mixtures of POPS or SOPS with different mol fractions of cholesterol.

To investigate whether any other phases were present in the lipid samples with cholesterol either before or after incubation, we measured the ^{31}P NMR powder patterns of POPS containing 0.5 mol fraction of cholesterol as either a fresh sample or one that had been incubated at 37°C for 3 weeks. Both samples gave similar spectra, typical of a bilayer with no indication of any isotropic component that would indicate a change in lipid morphology to smaller particles or to structures with a high degree of curvature.

TABLE 3 Components of the high-temperature transition in mixtures of POPS and cholesterol after incubation for 3 weeks at 37°C

Mol fraction*	$T1$ (°C)	ΔH_1 (cal/mol cholesterol)	$T2$ (°C)	ΔH_2 (cal/mol cholesterol)
0.6	93.5	200	96.0	740
0.7	93.0	270	96.0	540
0.8	90.1	1027	95.9	1074
0.9	88.8	1590	95.9	870

*Mol fraction of cholesterol in POPS.

TABLE 4 Enthalpy of the polymorphic phase transition of anhydrous cholesterol in PS-cholesterol mixtures measured after two heating and cooling cycles between 0°C and 100°C

Mol fraction cholesterol	Fresh sample (cal/mol cholesterol)	Incubated and heated sample (cal/mol cholesterol)
SOPS		
0.7	280 ± 25	280 ± 50
0.6	335 ± 25	200 ± 50
0.5	728 ± 24	400 ± 50
0.4	425 ± 24	380 ± 40
POPS		
0.7	215 ± 20	215 ± 50
0.6	235 ± 20	190 ± 50
0.5	536 ± 26	420 ± 25
0.4	300 ± 17	325 ± 25

Different forms of cholesterol can be distinguished from one another by differences in their wide-angle x-ray diffraction patterns (Loomis et al., 1979). To identify the form of cholesterol present in incubated mixtures, two-dimensional x-ray diffraction patterns were measured and averaged about the pattern center. The wide-angle x-ray diffraction pattern of POPS with 0.5 mol fraction cholesterol in 0.01 M Tris/HCl, 150 mM NaCl, pH 7, following a 3-week incubation at 37°C is shown in Fig. 5. Diffraction patterns of cholesterol monohydrate and anhydrous cholesterol are also shown for comparison. Only a few reflections can be clearly distinguished in the incubated mixed system. These must be due primarily to the cholesterol, because the low-salt buffer prevents multilamellar stacks of phospholipids from forming (Hauser, 1984). Due to the small number of reflections, it is not possible to unambiguously identify the cholesterol phase. However, the positions of the reflections that are observed are more consistent with reflections of cholesterol monohydrate than those of anhydrous cholesterol.

We determined whether the observed behavior is sensitive to the method of preparation of the sample. We compared samples of POPS with 0.45 mol fraction cholesterol that had been prepared by the standard method used in this manuscript involving evaporation of the organic solvent at room temperature with the McMullen method of solvent evaporation at ~50°C and finally by the room-temperature method in which the sample is hydrated at room temperature instead of at 60°C. The temperatures of the observed transitions of fresh samples prepared by these three methods were identical, as were replicate samples that had been incubated for 3 weeks at 37°C. However, there are quantitative differences in the average enthalpy of these transitions (Table 5). Similar samples were also studied by low-angle x-ray diffraction. The presence of the 34-Å diffraction peak of cholesterol crystallites was detected in both fresh and incubated samples, prepared using either the standard method or the McMullen method. These two methods of preparation were also compared by x-ray diffraction using

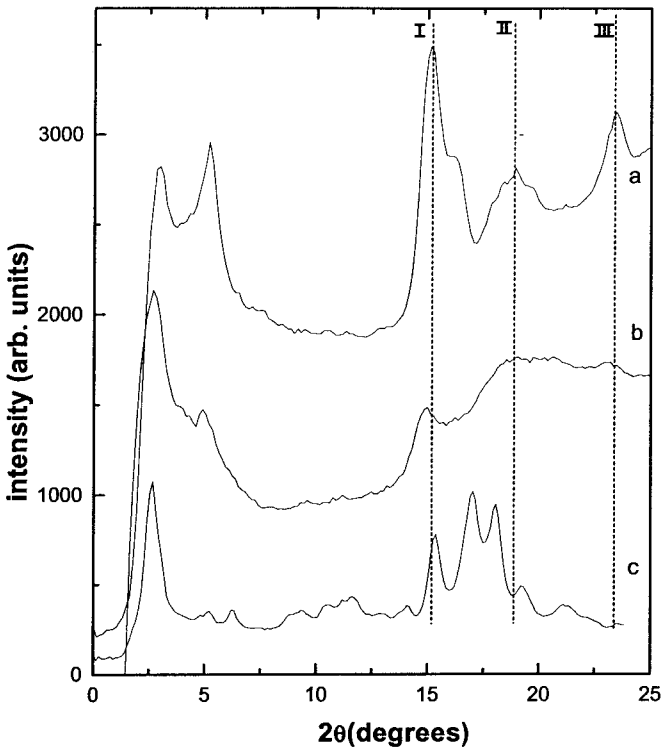

FIGURE 5 X-ray diffraction intensity versus the scattering angle 2θ for cholesterol monohydrate (*a*); POPS with 0.5 mol fraction cholesterol in 0.01 M Tris-HCl, 150 mM NaCl, pH 7, following 3 weeks incubation at 37°C (*b*); and anhydrous cholesterol (*c*). Wide-angle intensity maxima for cholesterol monohydrate are at 0.58 nm (*I*); 0.47 nm (*II*); and 0.38 nm (*III*). Room-temperature experiments and data analysis were performed as described in Materials and Methods.

both fresh and incubated samples of SOPS with 0.36 mol fraction cholesterol. Diffraction from cholesterol crystallites was demonstrated in samples prepared by both methods.

We also studied the acyl chain dependence of the behavior of PS-cholesterol mixtures by measuring the thermotropic properties of a mixture of dimyristoyl PS (DMPS) with 0.5 mol fraction of cholesterol that had been incubated at 37°C for 3 weeks (Fig. 6). The transition at 37°C on heating may arise from both the chain melting transition of DMPS as well as the polymorphic phase transition of anhydrous cholesterol. There is also a prominent transition at 95°C, similar to the 96°C transition observed with POPS and SOPS. This transition is partly reversible, as we have seen with the other forms of PS, but the results of subsequent scans are different with DMPS compared with the other two forms of PS studied here. On the first cooling scan there is an exotherm centered at 44°C (Fig. 6), compared with 60°C observed with SOPS (Fig. 1). The expected exotherm for the polymorphic transition of anhydrous cholesterol is also observed in the cooling scans but with DMPS it is broadened and of lower enthalpy and is not readily discerned in Fig. 6. It may, however, be seen on an expanded scale. The high-temperature transition shifts from

TABLE 5 Comparison of enthalpy of cholesterol crystallite transitions of samples of POPS with 0.45 mol fraction cholesterol prepared by different methods

Method of sample preparation	Fresh sample (cal/mol cholesterol), polymorphic transition of anhydrous cholesterol*	Sample incubated for 3 weeks at 37°C (cal/mol cholesterol)	
		Polymorphic transition of anhydrous cholesterol*	96°C transition†
Standard method	450 ± 25	300 ± 35	1770 ± 150
McMullen method	250 ± 30	230 ± 25	1025 ± 125
Room-temperature method	490 ± 30	355 ± 35	1300 ± 150

Reported enthalpy is the average of two independently prepared samples.
*Average of 38°C transition on heating and 18°C transition on cooling, after the melting of the 96°C transition in the incubated sample.
†Enthalpy of the transition on the first scan, which appears only in incubated samples.

95°C on the first scan to 91°C on the second heating scan, in contrast to this transition with SOPS that does not shift temperature on the second scan.

DISCUSSION

Pure cholesterol can form three-dimensional crystals that exhibit crystalline polymorphism as well as converting from an anhydrous to a monohydrate form in the presence of water. Such crystals may be present in certain pathological conditions such as lipid deposits in arterial plaques. However, in the presence of phospholipids there may also be periodically organized domains of cholesterol that are intimately associated with a bilayer. The close association between cholesterol aggregates and the bilayer is suggested by the observation that in some cases cholesterol crystals are not readily separable from the phospholipid by centrifugation.

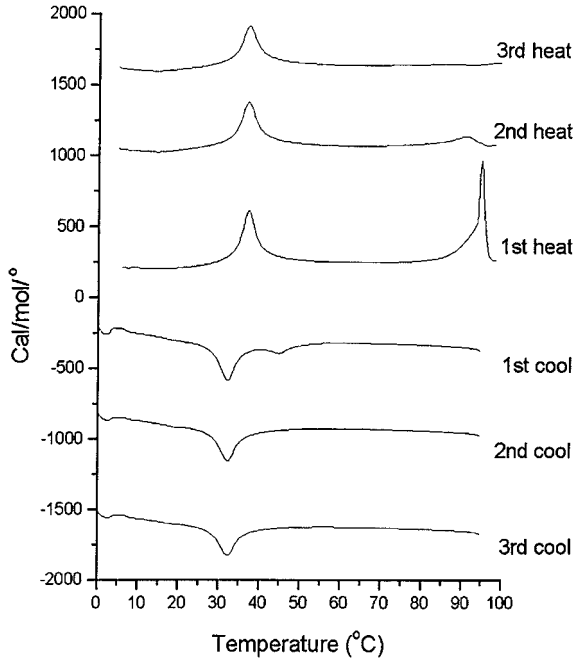

FIGURE 6 DSC scans of DMPS with 0.5 mol fraction cholesterol. DMPS concentration was 9 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.40. Scan rate was 2°C/min. The top three curves are heating scans and bottom three curves are cooling scans. The numbers indicate the order in which the scans were run. Curves have been displaced along the *y* axis for presentation. Excess heat capacity is expressed per mole of cholesterol.

The type of transition we observe in the present study in the temperature region of 95–96°C has never been previously observed with pure cholesterol (Loomis et al., 1979) or with mixtures of cholesterol with any phospholipid. Freshly prepared samples of pure cholesterol exhibit a transition at 38°C, corresponding to a polymorphic transition of anhydrous cholesterol. In addition, in fresh samples occasionally a broad transition at ~75°C is also observed, corresponding to the dehydration of cholesterol monohydrate. With increasing time of incubation the anhydrous form is gradually converted into the monohydrate. In the absence of phospholipid, no further change occurs. However, in the presence of PS there is a further change, with the enthalpy of the 75°C transition decreasing, whereas that of the new 96°C transition increases (Fig. 2).

Some of the characteristics of the 96°C transition are different from other cholesterol transitions. Unlike the other transition involving the dehydration of cholesterol in fresh samples, this transition is partially reversible within a relatively short period of minutes. An exotherm is observed on the first cooling scan and a significant fraction of the 96°C transition is retained in the second heating scan (Fig. 1). However, after several heating and cooling scans the 96°C transition disappears and again requires several days to re-form. Despite the relatively rapid partial reversibility on cooling of the transition at 96°C, it exhibits kinetics that is slow relative to the scan rate. At a rapid scan rate of 2°C/min, the transition appears sharp, but at slower scan rates the transition is broadened toward lower temperature (Fig. 3). It is not known how slowly one has to scan to obtain equilibrium-phase behavior. This transition is complex, having at least two components. It is possible that if one could scan sufficiently slowly, the transition properties after incubation would be indistinguishable from the dehydration of cholesterol monohydrate. This is also suggested

by the fact that the x-ray diffraction pattern of the cholesterol crystallites measured after incubation of the mixtures is quite similar to cholesterol monohydrate. In addition the cross-polarization magic-angle spinning nuclear magnetic resonance (CP-MAS) NMR has been used to detect cholesterol crystals (Guo and Hamilton, 1996). We have observed that the cholesterol crystals formed after long incubation has the same spectra as cholesterol monohydrate (unpublished observation). It has not been determined if the 96°C transition is solely a consequence of a kinetic effect or only partially so.

The cholesterol transitions observed at 38°C and at ~75°C exhibit the same transition temperature and kinetics whether the transition is measured with pure cholesterol or with fresh mixtures of cholesterol with phospholipids. This is not the case with the 96°C transition, which is observed, as a dominant feature, only in the presence of phospholipid. The very minor peak at 96°C, which occasionally appears in pure cholesterol, both in our own hands as well as in the published report of Loomis et al. (1979) has very small enthalpy, does not grow with time, and is not very reproducible. Our observation that the 96°C transition is partially reversible is also a property that is different from pure cholesterol crystals. These observations strongly suggest that the cholesterol crystallites are not physically separated from the phospholipid.

There are also some effects that can be discerned as a consequence of different acyl chain compositions of the phosphatidylserine. Although the characteristics of the 96°C transition are almost the same in mixtures of cholesterol with POPS or with SOPS, there are differences observed when DMPS is used (Fig. 6). There is a slight decrease in the transition temperature from 96°C to 95°C at the scan rate of 2°C/min and a large decrease in the transition temperature on cooling, i.e., from 60°C to 44°C. These dependencies on the nature of the phospholipid suggest that the transition observed at 96°C involves cholesterol monohydrate that is interacting with the surrounding phospholipid.

The transition observed at 96°C involves, at least in part, the dehydration of cholesterol. The low-temperature form is almost certainly cholesterol monohydrate because anhydrous cholesterol converts to the monohydrate form after incubation in water for several days. In addition, the diffraction pattern from this form is very similar, if not identical, to that of known cholesterol monohydrate crystals. The high-temperature form is likely to be the high-temperature polymorph of anhydrous cholesterol because on recooling and heating, the transitions characteristic of anhydrous cholesterol are observed. The form of cholesterol that has an endotherm at 96°C evolves from the known cholesterol monohydrate that dehydrates at 75°C. The enthalpy for the dehydration of cholesterol monohydrate at ~75°C is 2.35 kcal/mol (Loomis et al., 1979). After incubation for 3 weeks at 37°C, the enthalpy of the 96°C transition of SOPS with 0.4 mol fraction cholesterol, or POPS with 0.5 mol fraction cholesterol, is ~1.8 kcal/mol (Table 1), only slightly less than the value for the dehydration of cholesterol monohydrate. If the enthalpy of dehydration of cholesterol monohydrate were the same in these samples with PS as it is for pure cholesterol monohydrate crystals, it would suggest that cholesterol monohydrate is very sparingly soluble in PS membranes at certain mol fractions of cholesterol, once nucleation of the crystal is initiated. However, the phospholipid chain melting transition after incubation is still shifted to lower temperatures and has a lower enthalpy, indicating that the cholesterol monohydrate and PS are not completely phase separated. A possible explanation for the slow kinetics, the higher temperature, and the high enthalpy of dehydration of cholesterol monohydrate after incubation may be that the nature of the interaction between the phospholipid and the phase-separated cholesterol associated with the bilayer changes with time. It is an important question because it is the monohydrate form of cholesterol crystals that is found in biological specimens.

With SOPS and with POPS containing between 0.4 and 0.7 mol fraction cholesterol, a similar final amount of anhydrous cholesterol is observed from either freshly prepared samples after dehydration of the form of cholesterol giving the 75°C transition or from aged samples after dehydration of the form of cholesterol giving the 96°C transition (Table 4). This indicates that the amount of cholesterol crystals formed is largely a property of the system and not an artifact of sample preparation because two different paths generally achieve almost the same end result. However, the case of SOPS containing 0.5 mol fraction cholesterol appears to be an exception. In addition, cholesterol crystallites are observed in the incubated samples at mol fractions of cholesterol of 0.3 and even 0.2 with POPS, unlike the case with fresh samples in which no crystallites were observed at these mol fractions (Bach et al., 1992). Despite these differences, however, it is clear that the formation of these crystallites is not simply a result of sample preparation and the evaporation of organic solvent as had been suggested by others (McMullen et al., 2000; Buboltz and Feigenson, 1999).

DMPS was used in the study of McMullen et al. (2000). This lipid has a chain melting transition that overlaps the polymorphic transition of anhydrous cholesterol. We have also prepared samples of DMPS and cholesterol by the McMullen procedure (McMullen et al., 2000) as well as with the procedure used for most of the samples studied in this work. In all cases we clearly observe the presence of cholesterol crystallites both by DSC and by x-ray diffraction.

We have compared the amount of cholesterol crystallites present in samples of POPS with 0.45 mol fraction cholesterol, prepared by various methods (Table 5). We find similar qualitative behavior in the observed transition behavior. However, there are some quantitative differences. In particular, the samples made by the McMullen method have

a smaller amount of cholesterol crystals. There are several possible explanations for this. One is that cholesterol crystals are formed during solvent evaporation. This cannot be a significant contribution because such cholesterol crystals would be separated from the phospholipid and we have shown that the characteristics of the 96°C peak are influenced by the presence of the phospholipid. There are other sources for this quantitative difference. It may be that as a consequence of heating the solvent and the resultant rapid evaporation, the cholesterol crystals that eventually form are either smaller or less perfect than those observed using the traditional method of preparation. These less perfect crystals, having a smaller coherent size, would diffract more weakly and also have a broader transition in the DSC. In addition, there may be trace contaminants formed during the prolonged incubation or during heating. Hence, there are several factors that make it difficult to accurately determine the amount of cholesterol crystallites present in these samples at equilibrium. However, the major features of the phase behavior of these lipid mixtures are independent of these factors. In addition, we have demonstrated (Bach et al., 1992) that cholesterol has a different solubility in POPS and SOPS. It would be difficult to explain this observation in terms of preferential precipitation of one of the components because POPS and SOPS would have virtually identical solubility in bulk organic solvents.

We also consider what form the cholesterol is in subsequent to the formation and disappearance of the 96°C peak. After the final heating scan of the samples that had been incubated at 37°C for 3 weeks, the only observable transition was that at 38°C, corresponding to the polymorphic phase transition of anhydrous cholesterol. Taking the enthalpy of this transition to be 910 cal/mol (Loomis et al., 1979), we can calculate what fraction of the cholesterol is in the form of anhydrous cholesterol crystals. The final amount of anhydrous cholesterol crystallites varies in a complex manner with the mol fraction of cholesterol, first rising up to a total mol fraction of cholesterol of 0.5, then falling, and then rising again with increasing cholesterol concentration (Table 6). The difference between the measured amount of cholesterol crystallites and the total cholesterol in the sample is the amount of non-crystalline cholesterol. The mol fraction of cholesterol not detected as crystallites reaches a maximum at a total cholesterol mol fraction of 0.6–0.7 with POPS (Table 6). A similar amount of non-crystalline cholesterol is also observed at this composition with SOPS. If we compare incubated samples of POPS and SOPS with mol fraction cholesterol 0.4 (Table 6) with comparable fresh samples (Epand et al., 2000), we find a similar amount of non-crystalline cholesterol. We suggest that this non-crystalline cholesterol must include more than just the cholesterol dissolved in the membrane. It is possible that some of the cholesterol is present as very small clusters that do not constitute a large enough cooperative unit to be detected by calorimetry. In fresh samples of POPS containing 0.2 or 0.3 mol fraction cholesterol, the cholesterol is completely miscible (Bach et al., 1992). However, we find that in such samples, upon incubation, some crystalline cholesterol monohydrate forms, with a dehydration temperature of 96°C, and after dehydration a small peak due to anhydrous cholesterol is still seen. This may occur due to the coalescence with time of very small clusters that initially are not detected by DSC or x-ray but after 3 weeks at 37°C constitute a sufficiently large cooperative unit to be detected as a small peak with DSC.

TABLE 6 State of cholesterol after incubation at 37°C for 3 weeks and heating to 100°C to dehydrate cholesterol as derived from the enthalpy of the 38°C peak

Mol fraction cholesterol	% Cholesterol as anhydrous crystals*	Mol fraction of cholesterol not crystalline
SOPS		
0.7	31	0.48
0.6	22	0.47
0.5	44	0.28
0.4	42	0.23
POPS		
0.9	68	0.29
0.8	53	0.38
0.7	24	0.53
0.6	21	0.47
0.5	46	0.27
0.4	36	0.26
0.3	22	0.23
0.2	7	0.19

*Calculated from the right hand column of Table 4 and the known enthalpy per mol of the polymorphic transition of anhydrous cholesterol.

We have shown that cholesterol monohydrate in the presence of phosphatidylserine can exhibit two kinds of crystalline forms, one of which is formed more rapidly and undergoes dehydration at ~75°C and the other which forms after several days of incubation of the cholesterol-phosphatidylserine suspension. This latter form, at a scan rate of 2°C/min, does not undergo dehydration until ~95°C, indicating that it is in a different state than crystals of cholesterol monohydrate that form in the absence of phospholipid.

Cholesterol is much less polar than phospholipids. It also has a relatively rigid fused ring structure. It is therefore not surprising that cholesterol can self-associate to form crystalline domains in membranes. Crystallites of cholesterol monohydrate have been recently found in biological materials including human atherosclerotic plaque tissue (Guo et al., 2000), arterial smooth muscle membranes (Tulenko et al., 1998), macrophage foam cells (Klinkner et al., 1995; Kellner-Weibel et al., 1999), and human ocular lens fiber cell plasma membranes (Jacob et al., 1999, 2001). It remains to be determined how common this arrangement is in cholesterol-rich regions in biological membranes. An understanding of the types of crystals that can form with cholesterol and their properties will facilitate an understanding of their putative role in biology.

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REFERENCES

Bach, D., N. Borochov, and E. Wachtel. 1998. Phase separation of cholesterol in dimyristoyl phosphatidylserine cholesterol mixtures. *Chem. Phys. Lipids.* 92:71–77.

Bach, D., E. Wachtel, N. Borochov, G. Senisterra, and R. M. Epand. 1992. Phase behaviour of heteroacid phosphatidylserines and cholesterol. *Chem. Phys. Lipids.* 63:105–113.

Brown, D. A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* 14:111–136.

Buboltz, J. T., and G. W. Feigenson. 1999. A novel strategy for the preparation of liposomes: rapid solvent exchange. *Biochim. Biophys. Acta.* 1417:232–245.

Cheetham, J. J., E. Wachtel, D. Bach, and R. M. Epand. 1989. Role of the stereochemistry of the hydroxyl group of cholesterol and the formation of nonbilayer structures in phosphatidylethanolamines. *Biochemistry.* 28:8928–8934.

Dekkers, D. W., P. Comfurius, R. G. Van Gool, E. M. Bevers, and R. F. Zwaal. 2000. Multidrug resistance protein 1 regulates lipid asymmetry in erythrocyte membranes. *Biochem. J.* 350:531–535.

Epand, R. M., D. Bach, N. Borochov, and E. Wachtel. 2000. Cholesterol crystalline polymorphism and the solubility of cholesterol in phosphatidylserine. *Biophys. J.* 78:866–873.

Guo, W., and J. A. Hamilton. 1996. ^{13}C MAS NMR studies of crystalline cholesterol and lipid mixtures modeling atherosclerotic plaques. *Biophys. J.* 71:2857–2868.

Guo, W., J. D. Morrisett, M. E. DeBakey, G. M. Lawrie, and J. A. Hamilton. 2000. Quantification in situ of crystalline cholesterol and calcium phosphate hydroxyapatite in human atherosclerotic plaques by solid-state magic angle spinning NMR. *Arterioscler. Thromb. Vasc. Biol.* 20:1630–1636.

Harder, T., P. Scheiffele, P. Verkade, and K. Simons. 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J. Cell Biol.* 141:929–942.

Hauser, H. 1984. Some aspects of the phase behaviour of charged lipids. *Biochim. Biophys. Acta.* 772:37–50.

Jacob, R. F., R. J. Cenedella, and R. P. Mason. 1999. Direct evidence for immiscible cholesterol domains in human ocular lens fiber cell plasma membranes. *J. Biol. Chem.* 274:31613–31618.

Jacob, R. F., R. J. Cenedella, and R. P. Mason. 2001. Evidence for distinct cholesterol domains in fiber cell membranes from cataractous human lenses. *J. Biol. Chem.* 276:13573–13578.

Kellner-Weibel, G., P. G. Yancey, W. G. Jerome, T. Walser, R. P. Mason, M. C. Phillips, and G. H. Rothblat. 1999. Crystallization of free cholesterol in model macrophage foam cells. *Arterioscler. Thromb. Vasc. Biol.* 19:1891–1898.

Klinkner, A. M., C. R. Waites, W. D. Kerns, and P. J. Bugelski. 1995. Evidence of foam cell and cholesterol crystal formation in macrophages incubated with oxidized LDL by fluorescence and electron microscopy. *J. Histochem. Cytochem.* 43:1071–1078.

Loomis, C. R., G. G. Shipley, and D. M. Small. 1979. The phase behavior of hydrated cholesterol. *J. Lipid Res.* 20:525–535.

McMullen, T. P., R. N. Lewis, and R. N. McElhaney. 2000. Differential scanning calorimetric and Fourier transform infrared spectroscopic studies of the effects of cholesterol on the thermotropic phase behavior and organization of a homologous series of linear saturated phosphatidylserine bilayer membranes. *Biophys. J.* 79:2056–2065.

Pralle, A., P. Keller, E. L. Florin, K. Simons, and J. K. Horber. 2000. Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. *J. Cell Biol.* 148:997–1008.

Privalov, G., V. Kavina, E. Freire, and P. L. Privalov. 1995. Precise scanning calorimeter for studying thermal properties of biological macromolecules in dilute solution. *Anal. Biochem.* 232:79–85.

Rietveld, A., and K. Simons. 1998. The differential miscibility of lipids as the basis for the formation of functional membrane rafts. *Biochim. Biophys. Acta.* 1376:467–479.

Schutz, G. J., G. Kada, V. P. Pastushenko, and H. Schindler. 2000. Properties of lipid microdomains in a muscle cell membrane visualized by single molecule microscopy. *EMBO J.* 19:892–901.

Simons, K., and E. Ikonen. 2000. How cells handle cholesterol. *Science.* 290:1721–1726.

Tulenko, T. N., M. Chen, P. E. Mason, and R. P. Mason. 1998. Physical effects of cholesterol on arterial smooth muscle membranes: evidence of immiscible cholesterol domains and alterations in bilayer width during atherogenesis. *J. Lipid Res.* 39:947–956.

Wachtel, E. J., and D. Bach. 1987. X-ray diffraction study of cholesterol-phosphatidylserine mixtures. *Biochim. Biophys. Acta.* 922:234–238.

Wachtel, E., N. Borochov, D. Bach, and I. R. Miller. 1998. The effect of ethanol on the structure of phosphatidylserine bilayers. *Chem. Phys. Lipids.* 92:127–137.