

# Cholesterol Crystalline Polymorphism and the Solubility of Cholesterol in Phosphatidylserine

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**ABSTRACT** There is a marked hysteresis between the heating and cooling polymorphic phase transition of anhydrous cholesterol. At a scan rate of 0.05°C/min the difference in transition temperatures between heating and cooling scans is ~10°C. This phenomenon also occurs with mixtures of cholesterol with phosphatidylserine and can result in an underestimation of the amount of crystalline cholesterol in a sample that has not been cooled sufficiently. With 1-palmitoyl-2-oleoyl phosphatidylserine and 1-stearoyl-2-oleoyl phosphatidylserine the cholesterol crystallites form while the lipid remains in the L<sub>α</sub> phase. Sonication of dimyristoyl phosphatidylserine with a 0.4 mol fraction cholesterol results in the loss of cholesterol crystallite diffraction, but only a partial loss of the polymorphic transition detected by calorimetry. We therefore conclude that the thermal history of the sample can have profound effects on the appearance of the polymorphic phase transition of cholesterol by differential scanning calorimetry. Depending on the morphology of the vesicles, diffraction methods may underevaluate the amount of cholesterol crystallites present.

## INTRODUCTION

It has been demonstrated that cholesterol is much less soluble in phosphatidylserine (PS) (Bach, 1984; Wachtel et al., 1991) than it is in phosphatidylcholine. Recently, the validity of results using mixtures of cholesterol and phospholipids made by depositing lipid films from organic solvent has been questioned on the basis of the reproducibility of the specimens (Huang et al., 1999). The problem that is always encountered in making mixtures of lipids is that both components are insoluble in water, hence there is a problem of how to ensure complete mixing of the two components to make it more likely that the observed phase behavior reflects an equilibrium state. There is no ideal way to solve this problem. If the two lipids are codissolved in organic solvent, as is the case in the present manuscript, there is a possibility that they will separate during solvent removal. Even if the solvent is removed in the frozen state, such as from benzene, separation of lipid components may occur during freezing of the solvent, even if it is less likely to occur during solvent evaporation. Another danger with prior dissolution in another solvent is that the solvent may not be completely removed before hydration. In the case of removal of benzene from cholesterol-containing lipid samples, it was found that 72 h under high vacuum were required to remove the benzene (Bach et al., 1992). A novel

protocol has recently been suggested in which the organic solvent is rapidly exchanged for water using a specially constructed apparatus (Buboltz and Feigenson, 1999). Although it was demonstrated that this method gives very reproducible results, it would be difficult with any method to ascertain that this represents the lowest-energy equilibrium state. Rearrangements leading to cholesterol crystallite formation may be very slow, and procedures giving greater lipid mixing do not necessarily produce equilibrium states.

In the present manuscript we demonstrate that a complicating factor in the appearance of thermotropic cholesterol transitions in phospholipid/cholesterol mixtures is the marked hysteresis in the polymorphic transition of anhydrous cholesterol crystallites. In addition, we demonstrate that cholesterol crystallites that can easily be detected by differential scanning calorimetry (DSC) do not necessarily give rise to observable diffraction in x-ray diffraction experiments.

## EXPERIMENTAL PROCEDURES

### Materials

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

### Preparation of multilamellar vesicles (MLV) for DSC

PS and cholesterol were codissolved in chloroform/methanol (2:1, v/v) and the solution placed in a 10 × 1.5-cm test tube. The solvent was evaporated under a stream of nitrogen with constant rotation of the 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 in a vacuum chamber for at least 2 h. The lipid film was then hydrated with one of two buffers, either with 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN<sub>3</sub>, pH

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Abbreviations used: DMPS, dimyristoyl phosphatidylserine; POPS, 1-palmitoyl-2-oleoyl phosphatidylserine; SOPS, 1-stearoyl-2-oleoyl phosphatidylserine.

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7.40; or with 0.01 M Tris-HCl, 0.5 M NaCl, pH 7.4. Two different buffers were used for a combination of reasons. The PIPES buffer is the same as that used in our earlier DSC work with some of these lipids (Bach et al., 1992) and the 0.5 M salt buffer has also been used by us in previous diffraction studies. The high salt concentration is required to obtain multiple lamellar reflections from MLVs of charged lipids (Hauser, 1984). Using DSC, we have never observed a difference in lipid phase behavior between lipid mixtures suspended in these two buffers. The lipid film was suspended and hydrated by intermittent vortexing and heating to 50°C over a period of 30 min.

### Preparation of sonicated vesicles (SUV) for DSC

Lipid films were prepared and dried as described above for MLVs. The lipid films were hydrated in distilled water with vortexing and heating to 50°C, as above. The resulting suspension was then placed in a bath-type sonicator and sonicated for a period of 30 min. The slightly hazy suspension was then lyophilized. The lyophilized material was then suspended in salt buffer and vortexed with heating to 50°C over a period of 30 min.

### DSC

Measurements were made using a Nano differential scanning calorimeter (Calorimetry Sciences Corporation, Provo, UT). 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 Inc. (Northampton, MA).

### Preparation of samples for x-ray diffraction measurements

DMPS and cholesterol were dissolved in chloroform/methanol (2:1 v/v) and mixed together with mol fraction cholesterol of 0.4 and 0.5. Both samples were divided into two portions, the solvents were driven off by a stream of nitrogen gas, and the samples were kept under high vacuum (0.1–0.07 mm Hg) for 3 h. To one portion of each sample, water was added to give a concentration of ~6 mg/ml; to the other portion 0.5 M NaCl in 0.01 M Tris-HCl buffer was added to give a similar lipid concentration. All the samples were incubated at 58°C with frequent vortexing. The water dispersions were sonicated for 30 min in a bath sonicator at room temperature. They were then frozen in liquid nitrogen and the water was driven off by lyophilization at high vacuum. To the dried lipid mixtures was added 0.5 M NaCl in Tris-HCl buffer and the samples were incubated as above. Subsequently, all the dispersions were centrifuged in an Eppendorf centrifuge for 15 min and the precipitate was loaded into 1.5-mm quartz capillaries. An excess of mother liquor was added to each capillary and the capillaries were sealed under argon gas.

### X-ray diffraction experiments

X-ray diffraction measurements were performed as described in Cheetham et al. (1989). Each sample was measured at intervals over a period of approximately one month. Exposure times were on the order of 16 h. During the entire procedure, the samples were kept at room temperature.

### Analysis of x-ray diffraction data

Integrated intensity of the 34 Å diffraction peak of cholesterol was determined using the computer program ORIGIN (Microcal, Inc., Northampton, MA) for baseline subtraction and area integration. The integrated intensity of the second-order bilayer diffraction peak was determined similarly and

the ratio of the two intensity values calculated to normalize in each case to the amount of phospholipid irradiated by the x-ray beam.

## RESULTS

### DSC

The thermal transitions of anhydrous cholesterol and cholesterol monohydrate are well known (Loomis et al., 1979). Below 100°C, two transitions occur. One of them, occurring in the region of 38°C, involves the conversion of one crystalline form of anhydrous cholesterol to another. The other transition occurs above 70°C and has been identified as the conversion of cholesterol monohydrate to anhydrous cholesterol. At a cooling scan rate of 5°C/min, it has been shown that the polymorphic transition of cholesterol undergoes an “under-cooling to ~20°C” (Loomis et al., 1979). We have further investigated this phenomenon.

We have prepared suspensions of cholesterol in a manner similar to that described above for MLVs, but in the absence of PS. These samples were scanned several times between 0°C and 90°C with a series of sequential heating and cooling scans. The first heating scan showed the dehydration of cholesterol monohydrate and the polymorphic phase transition of anhydrous cholesterol. At scan rates between 0.5 and 2°C/min the dehydration of cholesterol was only observed in the first heating scan. For slower scan rates this transition was observed in both the second and third heating scans, but in none of the cooling scans. We conclude, as has been observed before, that the conversion of anhydrous cholesterol into cholesterol monohydrate is a very slow process. Samples heated to 90°C to dehydrate cholesterol could be incubated at 0, 22, or 37°C for seven days, resulting in the essentially complete conversion of cholesterol back to the monohydrate form, giving a transition at 72°C (data not shown).

The temperature of the polymorphic phase transition occurred at 23°C lower on cooling than on heating, at a scan rate of 2°C/min. A difference between the temperature of the polymorphic transition on heating and on cooling was maintained even at very slow scan rates (Fig. 1). The transition temperatures taken from DSC heating and cooling scans are plotted as the reciprocal of the scan rate to more easily demonstrate the insensitivity of the phase transition temperature to scan rate at slow scan rates (high values of 1/(scan rate)). To be closest to equilibrium a slow scan rate is required. The results indicate that the kinetics of the interconversion of the two polymorphic forms of cholesterol is extremely slow between ~25 and 35°C. This is illustrated by the marked shift in transition temperature observed between heating and cooling even at the very slow scan rate of 0.05°C/min (Fig. 2).

For the transition from cholesterol monohydrate to the high-temperature polymorph of anhydrous cholesterol, the dependence of the transition temperature on heating scan

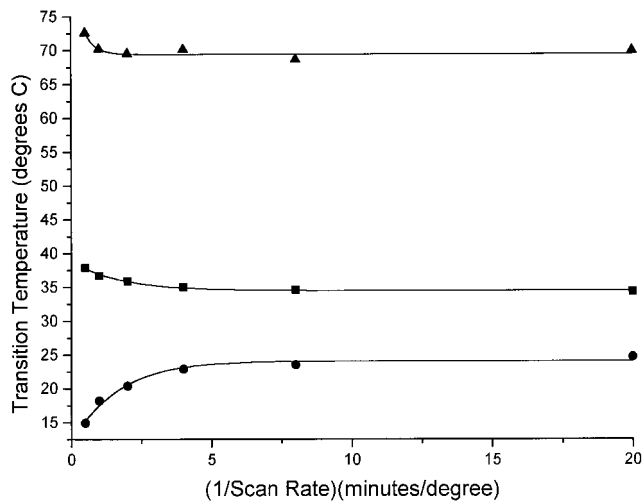


FIGURE 1 Effect of scan rate on the transition temperatures of pure cholesterol in excess buffer of 0.5 M NaCl, 10 mM Tris, pH 7.4. Cholesterol concentration, 26 mg/ml. *Triangles*: heating transition temperature for the dehydration of cholesterol; *squares*: heating transition temperature for the polymorphic phase transition of anhydrous cholesterol; *circles*: cooling transition temperature for the polymorphic phase transition of anhydrous cholesterol.

rate is small. However, no transition is observed on cooling at any scan rate, indicating that the dehydration of cholesterol is rapid above 70°C but that the rehydration is very slow over a range of temperatures.

The kinetics of the cholesterol transitions have marked effects on the magnitude of the polymorphic transition of cholesterol observed by DSC. One factor is the temperature

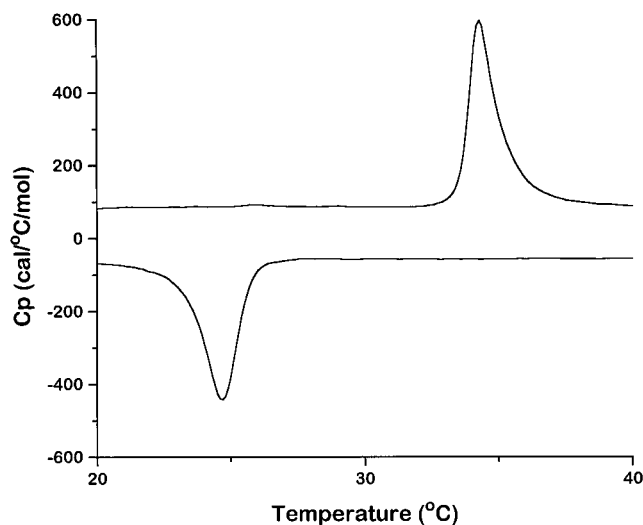


FIGURE 2 DSC of cholesterol dispersed in excess buffer of 0.5 M NaCl, 10 mM Tris, pH 7.4. Cholesterol concentration, 26 mg/ml. Scan rate 0.05°C/min. The top curve is a heating scan and the bottom curve is a cooling scan.

to which the lipid suspension has been cooled before DSC analysis. For example, if a sample of POPS with a mol fraction of 0.5 cholesterol is prepared by hydration at 50°C and is then scanned between 25°C and 90°C without having been cooled below room temperature, no polymorphic transition of anhydrous cholesterol is observed, only the transition of the monohydrate at 75°C (Fig. 3). With SOPS, which has been shown to have a lower solubility for cholesterol (Bach et al., 1992), a small transition is observed at 37°C upon heating a sample from 25°C which had never been cooled. The enthalpy of this transition decreases from 220 to 96 cal/mol cholesterol between the first and second heating scan (Fig. 4).

When either the same sample of POPS or SOPS with 0.5 mol fraction cholesterol is cooled in the DSC down to 0°C, an exotherm of 532 and 728 cal/mol cholesterol is observed for POPS and SOPS, respectively. The size of this peak does not change on subsequent heating and cooling between 0 and 90°C. The exotherm on cooling at 2°C/min occurs at 17°C, before the  $L_\alpha$  to  $L_\beta$  transition of the PS, which occurs at 10.7°C for SOPS and a lower temperature for POPS (scan 4 of Figs. 3 and 4). The polymorphic transition of cholesterol is not well resolved from the chain freezing transition of SOPS on cooling. Hence, more quantitative values of the transition enthalpy of the cholesterol polymorphic transition can be obtained from the heating scans for this lipid mixture. As will be shown below, in the case of DMPS it is the cooling scan that is better resolved and gives more reliable quantitation. Hence, because of the large hysteresis of the polymorphic transition of cholesterol, one can choose either

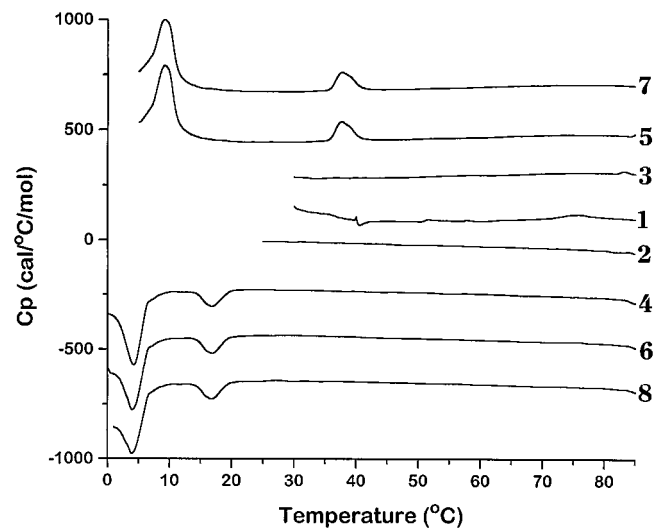


FIGURE 3 DSC scans of a single representative sample of POPS with 0.5 mol fraction cholesterol. POPS concentration 1.5 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002%  $\text{NaN}_3$ , pH 7.40. Scan rate 2°C/min. The top four curves are heating scans and the bottom four curves are cooling scans. 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 PS.

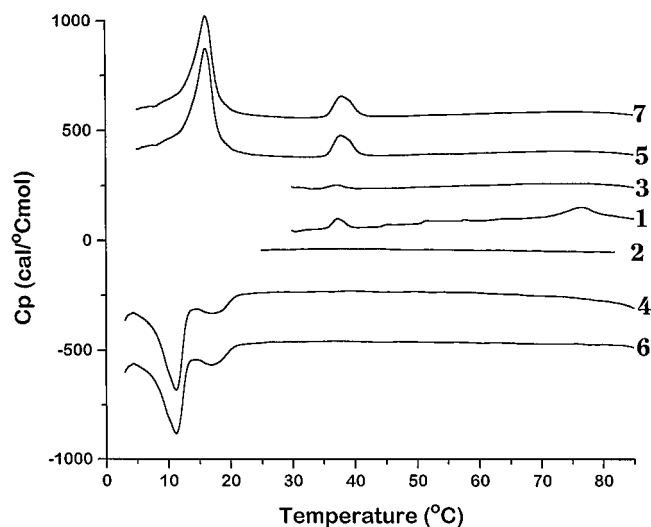


FIGURE 4 DSC scans of a single representative sample of SOPS with 0.5 mol fraction cholesterol. SOPS concentration 1.5 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002%  $\text{NaN}_3$ , pH 7.40. Scan rate 2°C/min. The top four curves are heating scans and the bottom three curves are cooling scans. 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 PS.

heating or cooling curves to have the cholesterol transition well separated from other transitions that may occur in the sample.

Another source of variability in the quantitation of cholesterol crystallites is the presence of cholesterol monohydrate in samples that have not been heated above 75°C. The enthalpy of the polymorphic transition of cholesterol is observed on the first scan if the sample is first cooled to 0°C. However, the magnitude of this enthalpy is only about half of that found with subsequent heating or cooling scans for both POPS (Fig. 5) or SOPS (Fig. 6) with 0.5 mol fraction cholesterol. After the first heating scan, repeated scanning between 0 and 90°C shows no further change.

At a cholesterol mol fraction of 0.4 at a heating scan rate of 2°C/min, an endothermic transition was observed at 38°C. The enthalpy of this transition was 425 and 275 cal/mol cholesterol for SOPS and POPS, respectively. It was noted with POPS that the enthalpy of the cooling polymorphic transition of cholesterol was ~20% lower than the heating transition. This discrepancy was not observed with pure cholesterol or with a cholesterol mol fraction of 0.5 with either POPS or with SOPS. We therefore investigated this phenomenon further. The concentration of lipid was increased fivefold to obtain a more accurate characterization of the transition. We observed a definite high temperature shoulder in the cooling scans for the cholesterol polymorphic transition of POPS with 0.4 mol fraction cholesterol (Fig. 7). The enthalpy of this shoulder amounted to ~5–10% of the enthalpy of the main component at 17.5°C. However, this shoulder would also result in an additional

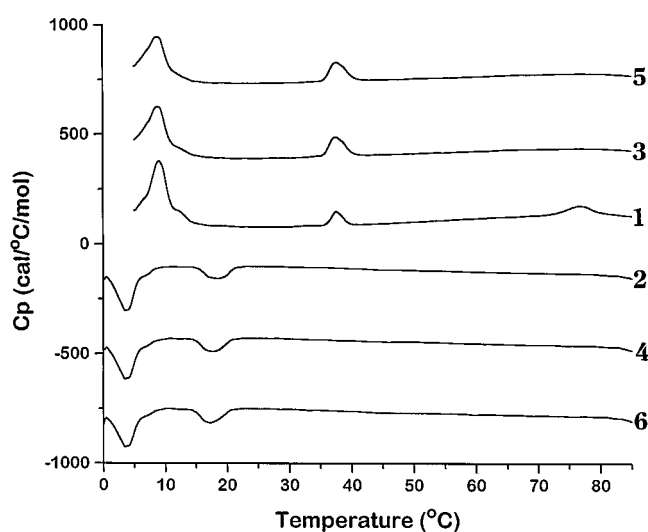


FIGURE 5 DSC scans of a single representative sample of POPS with 0.5 mol fraction cholesterol. POPS concentration 1.5 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002%  $\text{NaN}_3$ , pH 7.40. Scan rate 2°C/min. The top three curves are heating scans and the bottom three curves are cooling 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 PS.

underestimation of the transition enthalpy because it would lead to an inaccurate placement of the baseline. There was no comparable phenomenon in the heating scans (not shown) or at a cholesterol mol fraction of 0.5. To better

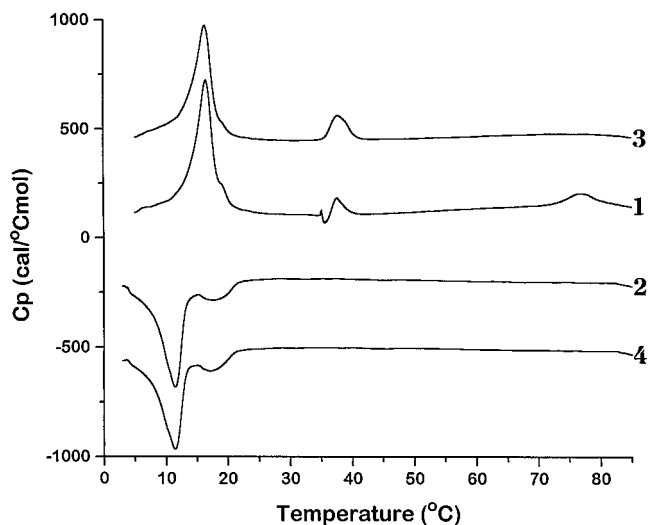


FIGURE 6 DSC scans of a single representative sample of SOPS with 0.5 mol fraction cholesterol. SOPS concentration 1.5 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002%  $\text{NaN}_3$ , pH 7.40. Scan rate 2°C/min. The top two curves are heating scans and the bottom two curves are cooling scans. 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 PS.

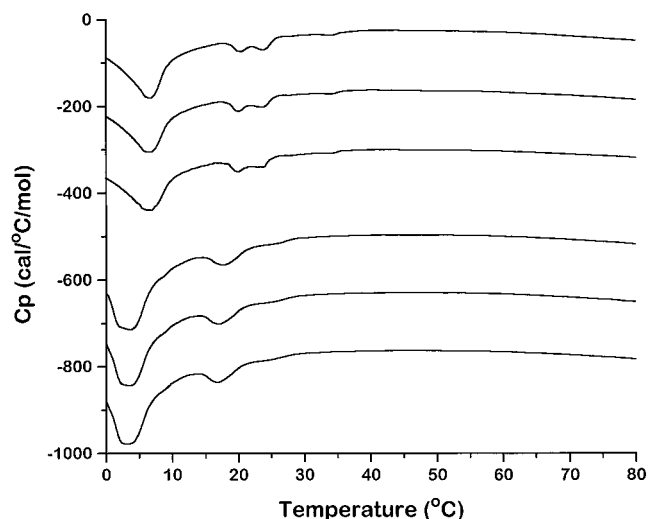


FIGURE 7 Cooling DSC scans of a single representative sample of POPS with 0.4 mol fraction cholesterol. POPS concentration 7.5 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN<sub>3</sub>, pH 7.40. Scan rate 2°C/min. The top three curves are at a cooling scan rate of 0.5°C/min and the bottom three curves are at a cooling scan rate of 2°C/min. Several replicate scans are shown to illustrate the excellent reproducibility of this complex phase behavior. Curves have been displaced along the y axis for presentation. Excess heat capacity is expressed per mole of PS.

resolve these components and to determine the effect of scan rate on their behavior, the DSC of POPS with 0.4 mol fraction cholesterol was repeated using a scan rate of 0.5°C/min. At this slower scan rate the exothermic cooling transition was clearly resolved into two major components at 20 and at 23°C, each with an enthalpy of close to 150 cal/mol cholesterol (Fig. 7). There is also a minor component centered at ~34°C with an enthalpy of 25 cal/mol cholesterol.

We also tested the effect of sonication on the thermotropic phase transitions of PS/cholesterol mixtures. Sonication was performed in distilled water, followed by lyophilization and resuspension in buffer with 0.5 M NaCl, as described in the Methods section. With SOPS and POPS containing 0.3 mol fraction cholesterol, we observed little difference between the sonicated and unsonicated samples (data not shown). However, in the case of DMPS with 0.4 mol fraction cholesterol, the samples became clearer upon sonication, compared with the POPS and SOPS mixtures, and the DSC showed marked broadening of the L<sub>β</sub>-L<sub>α</sub> phase transition (Fig. 8). The polymorphic transition of cholesterol can be readily observed in the cooling scans. However, the chain melting transition of DMPS is at the same temperature as the polymorphic transition of cholesterol observed on heating. The enthalpy of the cholesterol polymorphic transition was 225 cal/mol cholesterol in the unsonicated sample and 100 cal/mol in the sonicated sample. Storage of the sonicated samples overnight either at room temperature or at 4°C resulted in little change in the thermotropic behavior.

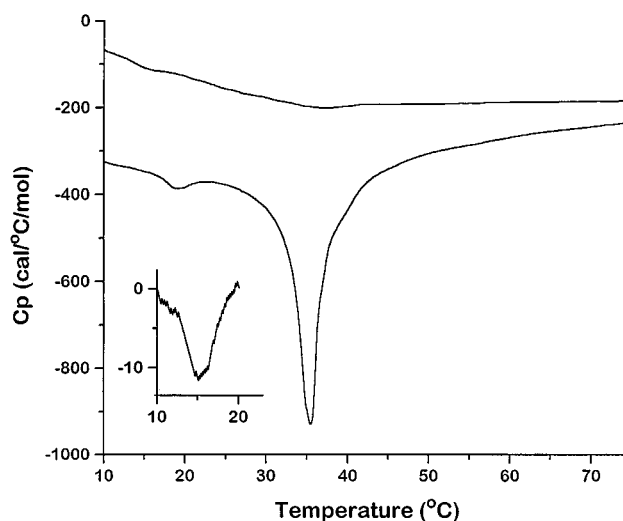


FIGURE 8 Cooling DSC scans of DMPS with 0.4 mol fraction cholesterol. DMPS concentration 1.5 mg/ml in 0.5 M NaCl, 10 mM Tris, pH 7.40. Scan rate 2°C/min. The top curve is a sample that had been sonicated, the bottom curve is an identical sample that had not been sonicated. The inset shows an expansion of the low-temperature region of the sonicated sample. Curves have been displaced along the y axis for presentation.

## X-ray diffraction

Fig. 9 presents the x-ray diffraction profiles of DMPS/cholesterol mixtures in both the sonicated and unsonicated forms with mol fraction cholesterol 0.4 and 0.5. In unsonicated samples (Fig. 9, curves *c* and *d*), the patterns are typical of diffraction from MLVs, i.e., sharp peaks with spacing in the ratios 1:2:3. A diffraction peak at 34 Å

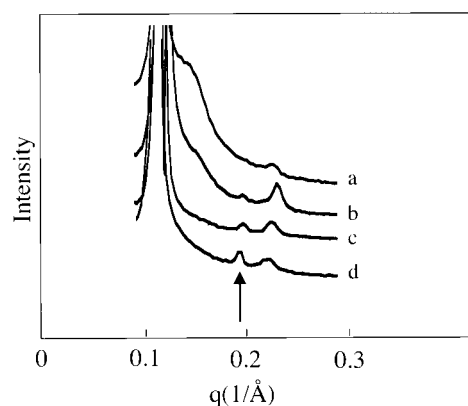


FIGURE 9 Low-angle x-ray diffraction profiles plotted as intensity versus the amplitude of the scattering vector  $q$ . Curve *a*: sonicated sample of DMPS with mol fraction cholesterol 0.4, prepared as described in the Experimental section. Curve *b*: sonicated sample of DMPS with mol fraction cholesterol 0.5. Curve *c*: unsonicated sample of DMPS with mol fraction cholesterol 0.4. Curve *d*: unsonicated sample of DMPS with mol fraction cholesterol 0.5. The arrow marks the 34 Å diffraction peak of cholesterol. Two orders of the lamellar diffraction are observed in each trace at ~60 Å and 30 Å, and an additional shoulder in the case of the sonicated samples.



(indicated by the arrow), characteristic of cholesterol crystals, is also observed. For sonicated samples (Fig. 9, curves *a* and *b*), the phospholipid lamellar diffraction is less well defined. In addition to the lamellar diffraction peaks, a broad shoulder is observed at a  $q$  of  $\sim 0.15 \text{ \AA}^{-1}$ , indicative of a poorly ordered sample. No cholesterol diffraction peak was observed for mol fraction cholesterol 0.4 (Fig. 9, curve *a*). In Fig. 10 is shown the time dependence of the ratio ( $R$ ) of the integrated intensity of the diffraction peak at  $34 \text{ \AA}$  normalized to the integrated intensity of the second-order lamellar diffraction calculated as described in the Experimental section. For the unsonicated sample with mol fraction cholesterol 0.4, the ratio  $R$  decreases by  $\sim 30\%$  during the first five days, and after that there is no significant change. For the unsonicated sample with mol fraction cholesterol 0.5,  $R$  decreases  $<15\%$  over the course of three weeks. The sonicated sample with 0.5 mol fraction cholesterol does not display any time dependence.

## DISCUSSION

A general problem with studying the properties of lipids is to ascertain whether the system is at equilibrium. There are many examples of slow equilibration with these systems, particularly for gel and crystalline phases. This can also be a potential source of error when studying lipid mixtures. If the rate of mixing of the two lipids or their rate of segregation is slow, then the observed properties of the mixture may be a consequence of the manner in which the sample was prepared. The usual way to prepare lipid mixtures is by first depositing a film containing the two lipids from organic solvent. Although this is likely to produce specimens that after hydration are not completely at equilibrium, the method has never been shown to result in specimens that are far from equilibrium. In contrast, we show in the present

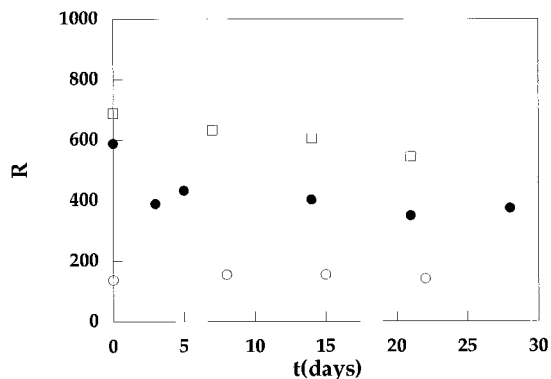


FIGURE 10 Time dependence of the ratio ( $R$ ) of the integrated intensity of the  $34 \text{ \AA}$  diffraction peak of cholesterol normalized to the integrated intensity of the second-order lamellar diffraction peak. *Open squares*: unsonicated sample of DMPS, mol fraction of cholesterol 0.5; *closed circles*: unsonicated sample of DMPS, mol fraction of cholesterol 0.4; *open circles*: sonicated sample of DMPS, mol fraction of cholesterol 0.5.

study that the thermal history of the sample can result in qualitatively different results, even for the same specimen.

The variability in the amount of cholesterol crystallites detected in a sample by DSC is primarily the result of the hysteresis of the polymorphic transition of cholesterol. Even at an extremely slow scan rate of  $0.05^\circ\text{C}/\text{min}$ , there is about a  $10^\circ\text{C}$  difference between the heating and the cooling scan for the polymorphic transition of cholesterol. To span this temperature range at this scan rate requires over 3 h, over which time there is no interconversion of one form of cholesterol into another. It is difficult to determine what the equilibrium phase of anhydrous cholesterol is within this temperature range because prolonged incubation leads to the formation of cholesterol monohydrate, which must be the more stable form of cholesterol at these temperatures.

The effect of the hysteresis of the polymorphic phase transition of cholesterol on the detection of cholesterol crystallites in PS/cholesterol mixtures is dramatically demonstrated by the fact that samples of POPS with 0.5 mol fraction of cholesterol show no polymorphic transition of anhydrous cholesterol if they have not been cooled below room temperature (Fig. 3). However, once the samples are cooled, even though they are still in the  $L_\alpha$  phase, this polymorphic transition of crystalline cholesterol appears and remains constant through a series of repetitive scans (Figs. 3 and 5).

The formation of cholesterol monohydrate can also reduce the amount of cholesterol crystallites detected by the  $38^\circ\text{C}$  polymorphic transition. In every sample of cholesterol/PS mixtures, the first DSC heating scan always showed a smaller polymorphic phase transition at  $\sim 38^\circ\text{C}$ , along with the transition to the high-temperature polymorph of anhydrous cholesterol at  $\sim 72^\circ\text{C}$ . Once the cholesterol monohydrate was dehydrated at  $72^\circ\text{C}$  it required several hours of incubation for it to rehydrate. With relatively rapid scan rates, after the first scan, one can avoid the complication of having two forms of cholesterol crystallites, i.e., the monohydrate and the anhydrous form, since there will be insufficient time for the cholesterol hydrate to reform. However, if cholesterol-PS mixtures are not first heated to over  $72^\circ\text{C}$ , one will underestimate the amount of cholesterol crystallites present in a sample from the enthalpy of the  $38^\circ\text{C}$  transition.

The factors discussed above can have major, qualitative effects on the appearance of cholesterol crystallites, particularly as a consequence of the hysteresis of the polymorphic transition of anhydrous cholesterol. In addition, however, there are additional complexities for certain systems. In the case of POPS with 0.4 mol fraction cholesterol, the polymorphic transition of cholesterol is split into several components in cooling scans, making it more difficult to obtain an accurate value for the transition enthalpy. However, the presence of a multicomponent transition is itself interesting, since in other samples there were only transitions that could be ascribed to only two domains, viz. PS mixed with cholesterol and pure cholesterol crystallites. This is still the case

with the heating scans of POPS with 0.4 mol fraction cholesterol, but the cooling curves exhibit definite contributions from two or more distinct cholesterol components. One possible cause for the existence of multiple forms of cholesterol is that there are cholesterol crystallites both within the bilayer and physically separated from the phospholipid.

There is also the question of the precision of the measurements using samples prepared in an identical manner. For each type of sample listed in Table 1 we prepared three separate lipid films and hydrated each separately. The precision between identical samples was  $\pm 5\%$ , which is only slightly greater than the reproducibility of sequential scans using the same sample. The reproducibility of independent samples can be seen by comparing Figs. 3 and 5 or Figs. 4 and 6. After the initial scan(s), the DSC curves are seen to be very similar for each of the two independently prepared duplicate pairs.

An indication that quantitative aspects of the phase miscibility of cholesterol and PS is complex is the finding that the amount of cholesterol not in the form of crystallites that can be detected by DSC is dependent on the total mol fraction of cholesterol in the sample (Table 1). The total mol fraction of cholesterol in the lipid mixture is known from the amount of phospholipid and cholesterol that was made into a lipid film. The amount of crystalline cholesterol was determined from the area under the DSC peak for the polymorphic transition of anhydrous cholesterol, as described in the footnote to Table 1. The enthalpy for this transition was taken from the report of Loomis et al. (1979) for the polymorphic transition of anhydrous cholesterol. This value of 910 cal/mol was also in agreement with our measurements of cholesterol suspended in water. It should be noted that although the measured transition is for anhydrous cholesterol, it can be measured in the presence of excess water because of the slow rate of hydration of anhydrous cholesterol. The difference between the total amount of cholesterol and that detected as crystallites from the DSC curve is taken as the fraction in the membrane not detected as crystallites. With SOPS and a mol fraction of cholesterol of 0.5, the amount of noncrystalline cholesterol corresponds to a mol fraction of cholesterol in the mem-

brane of 0.10, while at an overall mol fraction of cholesterol of 0.4, the fraction of noncrystalline cholesterol is 0.21. It is possible that at lower mol fractions of cholesterol a portion of the cholesterol is present neither as detectable crystallites nor is it dissolved in the membrane. It is possible that the average size of the crystallites becomes smaller as the total cholesterol concentration decreases, making it appear that more cholesterol is dissolved in the membrane. Consequently, DSC may not detect all forms of the crystallites.

The amount of cholesterol detected as crystallites can depend on the method used for detection. Quantitation of cholesterol crystallites by x-ray diffraction may lead to a lower estimate of crystalline cholesterol than DSC. Sonication of samples of DMPS and cholesterol, which produces predominantly SUVs, resulted in a somewhat greater solubility of cholesterol in DMPS compared with unsonicated samples. Nevertheless, there was still a significant amount of cholesterol detected by DSC even for a cholesterol mol fraction of 0.4. As shown above, no x-ray diffraction peak was detected at  $d = 34 \text{ \AA}$  for the sonicated sample with 0.4 mol fraction cholesterol. Thus, the limit of solubility of cholesterol in membrane bilayers may be overestimated if it is based on diffraction measurements only. We suggest that in the case of single-lamella vesicles, i.e., LUVs and SUVs, the dimension of cholesterol crystallites normal to the plane of the bilayer is much smaller than in the case of MLVs. As it is commonly considered that the cholesterol molecule lies with its long axis close to the normal to the plane of the bilayer (Marsan et al., 1999), this would also be the direction of the  $34 \text{ \AA}$  repeat of the cholesterol crystals (Shieh et al., 1977; Craven, 1976). Consequently, without phospholipid lamellar stacking, the  $34 \text{ \AA}$  diffraction peak and its higher orders will be very difficult to observe. In the case of the SUV sample with 0.5 mol fraction cholesterol, cholesterol diffraction is observed, perhaps because some cholesterol crystals are free in solution, as has been previously suggested (Huang and Feigenson, 1999).

Finally, the present results are in excellent agreement with previous findings (Bach et al., 1992, 1998) that acyl chain composition has an effect on the solubility of cholesterol in PS. Together they provide clear evidence that the properties of the mixtures of cholesterol with PS made by

**TABLE 1** Acyl chain dependence of cholesterol crystallization in PS membranes

LIPID	Mol fraction cholesterol	$\Delta H$ /mol cholesterol (calories)*	% cholesterol as crystals <sup>†</sup>	Mol fraction cholesterol in membrane not detected as crystallites
POPS	0.5	536 $\pm$ 26	59 $\pm$ 3	0.21 $\pm$ 0.02
SOPS	0.5	728 $\pm$ 24	80 $\pm$ 3	0.10 $\pm$ 0.03
POPS	0.4	300 $\pm$ 17	33 $\pm$ 2	0.27 $\pm$ 0.02
SOPS	0.4	425 $\pm$ 24	47 $\pm$ 2	0.21 $\pm$ 0.03
DMPS	0.4	225 $\pm$ 16	25 $\pm$ 2	0.30 $\pm$ 0.02

\*Enthalpies are average values for several heating and cooling scans with POPS, or heating scans with SOPS, or cooling scans with DMPS performed on independent samples in triplicate.

<sup>†</sup>Calculated using  $\Delta H$  for the polymorphic transition of cholesterol as 910 cal/mol (Loomis et al., 1979).

solvent evaporation are quite reproducible. It is likely that the solubility limits determined for cholesterol in membranes will depend on the manner that the crystallites are detected, as well as on the method of preparation of the samples. However, a major cause of variation in the amount of cholesterol crystals observed by DSC can easily be avoided by taking into account the marked hysteresis of the polymorphic transition of anhydrous cholesterol.

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