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Novel properties of cholesterol–dioleoylphosphatidylcholine mixtures

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Abstract

We have studied the properties of mixtures of cholesterol with dioleoylphosphatidylcholine (DOPC), and with several other phospholipids, including 1-stearoyl-2-oleoylphosphatidylcholine (SOPC) and dioleoleoylphosphatidylserine (DOPS), as a function of cholesterol molar fraction and of temperature. Mixtures of DOPC with a cholesterol molar fraction of 0.4 or greater display polymorphic behavior. This polymorphism includes the formation of structures that give rise to isotropic peaks in ^{31}P NMR at cholesterol molar fractions between 0.4 and 0.6, dependent on the thermal history of the sample. Cryo-electron microscopy studies demonstrate the formation of small globular aggregates that would contribute to a narrowing of the ^{31}P NMR powder pattern.

At molar fraction cholesterol 0.6 and higher and at temperatures above 70 °C, the mixtures with DOPC convert to the hexagonal phase. Lipid polymorphism is accompanied by the phase separation of cholesterol crystals in the anhydrous form and/or the monohydrate form. The crystals that are formed have substantially altered kinetics of hydration and dehydration, compared with both pure cholesterol monohydrate crystals and with crystals formed in the presence of the other phospholipids that do not form the hexagonal phase in the presence of cholesterol. This fact demonstrates that these cholesterol crystals are in intimate contact with the DOPC phospholipid and are not present as morphologically separate structures.

Keywords: ^{13}C CP/MAS NMR; Phosphatidylserine; Phosphatidylcholine; Cholesterol crystallite; Differential scanning calorimetry; Lipid morphology; X-ray diffraction; Cryo-TEM

1. Introduction

There is currently considerable interest in determining the arrangement of cholesterol in biological membranes. Studies of mixtures of cholesterol with individual phospholipids have contributed to our understanding of the dependence of the solubility of cholesterol in membranes containing phospholipids of different structure [1]. Cholesterol crystals have been observed in membranes of living cells [2]. The formation of crystals of cholesterol has also been demonstrated in the lens of the eye where it may function to protect against the formation of protein deposits and cataract formation [3]. In addition, cholesterol crystals have been implicated in pathological conditions such as the formation of atherosclerotic plaques [4–7]. Release of the cholesterol crystals from these plaques can result in their dissemination to many organs, forming embolisms that affect the functioning of these organs [8,9], including renal involvement [10]. In addition to cholesterol crystals in atherosclerotic plaques, these crystals can also form in the intestine and result in the obstruction of the small bowel [11,12]. Hence, cholesterol crystals occur more commonly than is generally appreciated. In the case of the lens of the eye, they may have a physiological function, but generally, cholesterol crystals are associated with a variety of pathological situations. It is important to understand the conditions required for the formation of these crystals.

Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; DO, dioleoyl; PO, 1-palmitoyl-2-oleoyl; SO, 1-stearoyl-2-oleoyl; CP, cross-polarization; MAS, magic angle spinning; DSC, differential scanning calorimetry; cryo-TEM, cryo-transmission electron microscopy

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Since cholesterol has a rigid structure, because of its fused ring system, it has been suggested that cholesterol should be less miscible with phospholipids containing more highly unsaturated acyl chains [13–16]. There is also evidence from fluorescence studies on pyrene-labeled cholesterol that cholesterol oligomers form more readily in bilayers containing unsaturated lipids [17]. We have recently shown that an equimolar mixture of cholesterol and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) do not result in the formation of any cholesterol crystals [18]. In the present study, we demonstrate that phosphatidylcholine (PC) having two acyl chains with only a single double bond, that is, dioleoylphosphatidylcholine (DOPC), when mixed with cholesterol has several properties that are markedly different from mixtures of cholesterol with other phospholipids that we have used for comparison, that is, 1-stearoyl-2-oleoylphosphatidylcholine (SOPC), dioleoleoyl-phosphatidylserine (DOPS) and 1-stearoyl-2-oleoylphosphatidylserine (SOPS).

2. Materials and methods

2.1. Materials

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Avanti or from NuChek Prep (Elysian, MN).

2.2. Preparation of hydrated mixtures of phospholipid and cholesterol for differential scanning calorimetry (DSC) and NMR experiments

Phospholipid and cholesterol were codissolved 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 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.40 and suspended by intermittent vortexing and heating to 50–60 °C over a period of 30 min under argon. Samples used for NMR analysis were incubated at least 24 h at 4 °C to allow conversion of anhydrous cholesterol crystals to the monohydrate form.

2.3. Sample preparation for X-ray diffraction experiments

Phospholipids and cholesterol were dissolved in chloroform/methanol 2:1(v/v) and mixed to give the desired ratios of the lipids. Solvents were driven off by a stream of nitrogen and the resulting lipid films were kept under high vacuum for 3 h. The samples were prepared in one of two ways. Method 1: Phospholipids or phospholipid–cholesterol mixtures were dispersed in salt solution: 500 or 150 mM NaCl in 10 mM Tris buffer, pH 7.4 in the case of samples of DOPC or 500 mM NaCl in the case of DOPS, at a concentration of about 10 mg/ml. All samples were incubated at 50 °C for 1 h. The dispersions were centrifuged in an Eppendorf centrifuge for 15 min and the resulting precipitate loaded into 1.5 mm diameter quartz X-ray capillaries. Method 2: The dry phospholipids or phospholipid–cholesterol mixtures were introduced into X-ray capillaries and an excess of salt solution (as above) was added, followed by incubation at 50 °C. Diffraction patterns obtained were found not to depend on the method of preparation nor on the particular buffer used. In one experiment, following incubation, the sample was kept in boiling water for 5 min and then cooled immediately under tap water. Buffers with either 500 or 150 mM NaCl were used. The former allows direct comparison with our earlier results with charged lipids, where the higher salt concentration is necessary to obtain ordered multilamellar structures giving sharp diffraction peaks in X-ray. The 150 mM NaCl is more physiological and allows comparison with other studies in the present paper using other methods.

2.4. Sample preparation for cryo-transmission electron microscopy (cryo-TEM)

Dry mixtures of DOPC and of DOPC–cholesterol at 1:1 molar ratio prepared as described above were dispersed in 50 mM NaCl in 10 mM Tris–HCl buffer at a concentration of 10 mg/ml and incubated at 50 °C for 0.5 h. The lower salt concentration was dictated by the necessity of maximizing the contrast between the liposomes and the surrounding solvent. Samples were prepared for cryo-TEM observation in a controlled environment vitrification system (CEVS) [19]. A 4-μl drop of sample was deposited on a lacy carbon film supported on a 200-mesh electron microscope grid (SPI Supplies, USA). The drop was then thinned by blotting with filter paper down to a layer thickness of 1000–3000 Å. Specimens were vitrified by plunging into liquid ethane at its melting point.

2.5. Differential scanning calorimetry

Measurements were made using a Nano Differential Scanning Calorimeter (Calorimetry Sciences Corporation, American Fork, UT). 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 [20]. DSC curves were analyzed by using the fitting program, DA-2, provided by Microcal Inc. (Northampton, MA) and plotted with Origin, version 5.0.

2.6. ^{31}P NMR

The ^{31}P NMR spectra, from suspensions of about 25 mg of lipid in PIPES buffer, were obtained using a Bruker DRX-500 spectrometer operating at 202.45 MHz in a 5 mm broad band

probe over a 50 kHz sweep width in 32k data points. A 90° pulse width of 9.4 μs was used. A 3.0 s relaxation delay was used between acquisitions. The sample was contained in a 5-mm-diameter Shigemi NMR tube (Shigemi Co., Tokyo, Japan). 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 to ±0.2 °C by a Bruker BVT 3000 digital variable temperature unit. Temperatures were monitored with a calibrated thermocouple probe placed in the cavity of the NMR magnet.

2.7. ^{13}C cross-polarization (CP)/magic angle spinning (MAS) NMR

Lipid suspensions in buffer were spun in an Eppendorf centrifuge at room temperature. The resulting hydrated pellet was transferred to a 18 × 4 mm ZrO_2 rotor, attempting to pack the maximal amount of lipid into the rotor while maintaining it wet. The rotor was placed in a Bruker Avance 300 spectrometer operating at 75.48 MHz for ^{13}C and equipped with CP/MAS capabilities. The spectra were referenced to an external standard of glycine crystals, assigning a chemical shift of 176.14 ppm for the carbonyl carbon. Samples were spun at 5 kHz. The temperature inside the rotor was controlled by the variable temperature unit of the instrument and was calibrated by measuring the chemical shift of ethylene glycol as a function of spinning speed between 0 and 10 kHz. At 5 kHz, the temperature of the sample was about 1 °C warmer than the set temperature. The power levels used for cross-polarization corresponded to a 4-μs $\pi/2$ pulse. The Hartmann–Hahn match was established on the sample of glycine. Continuous-wave decoupling at an increased power level was used during acquisition. Some experiments were repeated to verify the stability and reproducibility of the cross-polarization. Generally, each spectrum was obtained with 12,000 scans and processed with a 1-Hz line broadening. Resonances were assigned based on reports of PC [21], unsaturated acyl chains [22], phosphatidylserine (PS) [23,24] and cholesterol [25].

2.8. X-ray diffraction

Low angle X-ray diffraction measurements were performed as a function of temperature as described in Bach et al. [26].

2.9. Cryo-TEM

Images were recorded using low dose cryo-conditions on a TVIPS slow scan 1k CCD camera utilizing an FEI Tecnai F20 microscope operating at 200 kV. Fast Fourier transforms of selected regions of the TEM images were performed using the public domain program NIH-Image.

3. Results

3.1. DSC

Mixtures of phospholipid and cholesterol exhibit phase separation of cholesterol crystallites beyond a particular molar fraction of cholesterol that is strongly dependent on the nature of the phospholipid. There are two kinds of cholesterol crystals that can form, anhydrous cholesterol and cholesterol monohydrate [27]. Anhydrous cholesterol can be detected by the presence of the thermotropic transition between two polymorphic forms that occurs at about 38 °C on heating and generally at about 20 °C on cooling [28]. Cholesterol monohydrate generally undergoes a dehydration at about 80 °C that is very slowly reversible, requiring many hours for rehydration [27].

Fresh samples of mixtures of DOPS and cholesterol do not exhibit DSC transitions indicating the formation of cholesterol monohydrate crystals, but at molar fractions of 0.3 and higher, the polymorphic transition of anhydrous cholesterol can readily be observed (Fig. 1). This transition is also observed at 18 °C on cooling and again at 37 °C on subsequent reheating with similar enthalpy (not shown). After incubation for 3 weeks at 37 °C, it was found that the anhydrous cholesterol crystals had converted to cholesterol monohydrate in mixtures of DOPS with 0.4 molar fraction

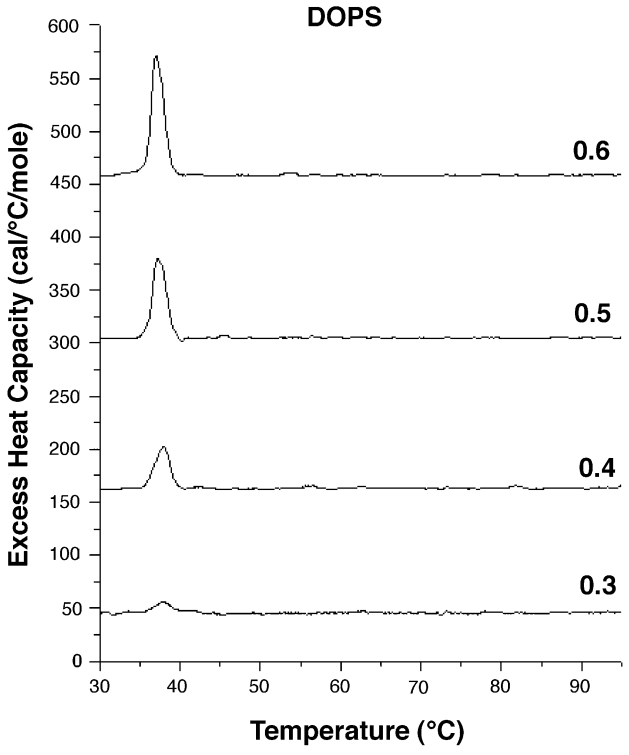

Fig. 1. First DSC heating scan of a sample of DOPS with molar fractions of cholesterol of 0.3, 0.4, 0.5 and 0.6 as indicated on the graph. Lipid concentration 1.5 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.4. Scan rate = 2 °C/min. C_p is given per mole cholesterol and curves have been displaced along the *y*-axis for presentation.

cholesterol and higher, but not at 0.3 molar fraction (data not shown). The dehydration transition after incubation occurs at 96 °C at a scan rate of 2 °C/min (not shown) as previously observed in mixtures of cholesterol with POPS and SOPS [29]. The enthalpy observed at 96 °C containing 0.4, 0.5 and 0.6 molar fraction cholesterol with DOPS is 75, 240 and 890 cal/mol cholesterol, respectively. Taking into account that the polymorphic transition of anhydrous cholesterol has an enthalpy of 910 cal/mol and assuming that the dehydration of cholesterol monohydrate at 96 °C has the same enthalpy, 2350 cal/mol, as for pure cholesterol monohydrate crystals, we calculate that in fresh samples of DOPS with 0.4 and 0.5 molar fractions of cholesterol (see Table 1), 13% and 24%, respectively, of the cholesterol is in the form of anhydrous crystals. This reduces to 3% and 10%, respectively, in the form of cholesterol monohydrate crystals after 3 weeks of incubation. In contrast, at 0.6 molar fraction of cholesterol, the 25% of cholesterol in the form of anhydrous crystals in fresh samples is increased to 38% as cholesterol monohydrate crystals after incubation.

The thermotropic behavior of mixtures of DOPC and cholesterol is novel. At 0.6 molar fraction cholesterol, there is a transition at 38 °C whose enthalpy is only 26 cal/mol cholesterol and which disappears on subsequent thermal cycling (Fig. 2). At this molar fraction of cholesterol, there is also a transition at 84 °C on heating that shifts to 75 °C on cooling. The enthalpy of this transition is about 10% larger in the first scan but on subsequent heating and cooling scans, it remains at 360 ± 10 cal/mol lipid. The fact that the enthalpy of these two cooling and heating transitions are the same suggests that the cooling transition is the reverse of the heating one, only shifted to lower temperature because of a kinetic hysteresis effect. At cholesterol molar fractions of 0.7 (Fig. 3) and 0.8 (Fig. 4), there is also a high-temperature transition with similar hysteresis but occurring at 5–10 °C lower temperature. The enthalpy of the transition on heating at 78 °C in the first scan is about 50% larger than in subsequent scans, when it becomes constant and equal to 560 cal/mol for DOPC samples containing 0.7 and 0.8 molar fraction cholesterol (Table 2). Similarly, upon cooling, the exothermic peak appears at approximately 68 °C for both samples. In addition, for the sample with 0.7 molar fraction cholesterol, the sharp transition, typical of the anhydrous polymorph, is observed only in the first heating scan (there may be a broad transition at this temperature on subsequent heating scans with this molar fraction of cholesterol), whereas when the amount of cholesterol is raised to 0.8, this transition is observed as a cooperative transition in all heating and cooling scans. There also appears to be an additional broad transition in the heating scans of the DOPC sample with 0.7 molar fraction cholesterol at about 60 °C that we suggest corresponds to the dehydration of cholesterol monohydrate crystals (see Discussion). The DOPC sample with 0.8 molar fraction cholesterol exhibits transitions upon heating corresponding to the polymorphic tran-

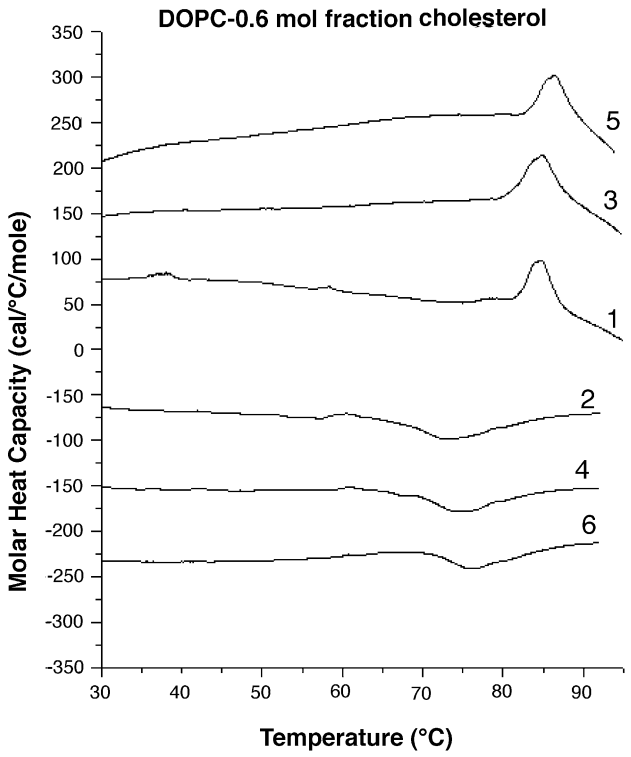

Fig. 2. DSC scans of DOPC with 0.6 molar fraction cholesterol. Lipid concentration 1.5 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.40. Scan rate = 2 °C/min. Top three curves are heating scans and bottom three curves are cooling scans. The numbers indicate the order in which the scans were run. Curves have been displaced along the *y*-axis for presentation. Excess heat capacity is expressed per mole of cholesterol.

Table 1
Enthalpy of anhydrous cholesterol crystals in mixtures with various lipids in the liquid crystalline state

Mole fraction cholesterol	Lipid			
	SOPS	DOPS	SOPC	DOPC
Enthalpy (cal/mol cholesterol)				
0.3	>0[a]	15 ± 10 (2%)	0	0
0.4	425 ± 24[b] (47%)[c]	120 ± 30 (13%)	0	0
0.5	728 ± 24[b] (80%)	220 ± 30 (24%)	0	0
0.6	335 ± 25[b] (37%)	230 ± 30 (25%)	Trace on first scan	Trace on first scan
0.7	280 ± 25[b] (31%)		180–50[d] (20–5%)	125[e] (14%)
0.8	–	–	300–130[d] (33–14%)	Heat 220 ± 15 (24%), Cool 145 ± 5 (16%)

[a] From Bach et al. [43]. Crystalline cholesterol is first detected by X-ray diffraction at 0.2 molar fraction cholesterol.
[b] From Epand et al. [29].
[c] Values in parentheses are the percentages of cholesterol in crystalline form.
[d] Gradually decreasing with each subsequent scan.
[e] Enthalpy on first scans. Transition in subsequent scans becomes broad and difficult to quantify.

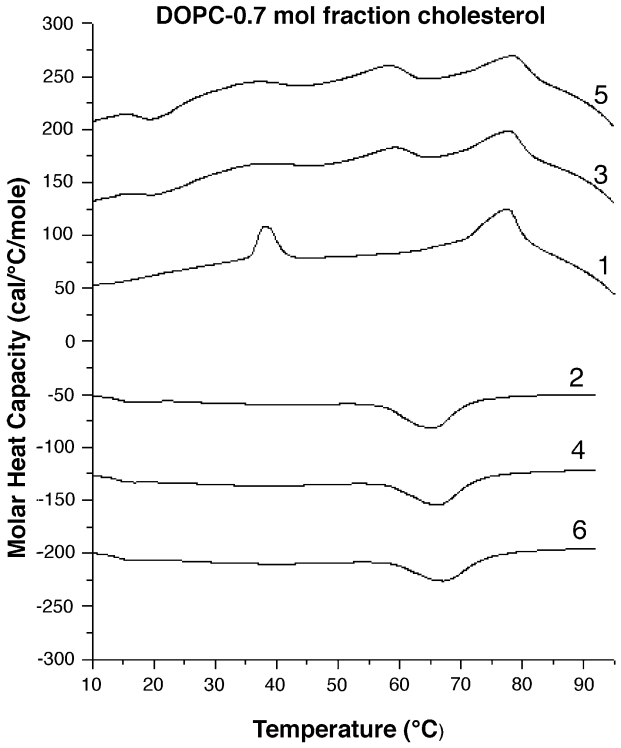

Fig. 3. Same as Fig. 2 using DOPC and 0.7 molar fraction cholesterol.

sition of anhydrous cholesterol at 38 °C, to the lamellar to hexagonal transition at 78 °C as well as the broad transition centered at about 60 °C.

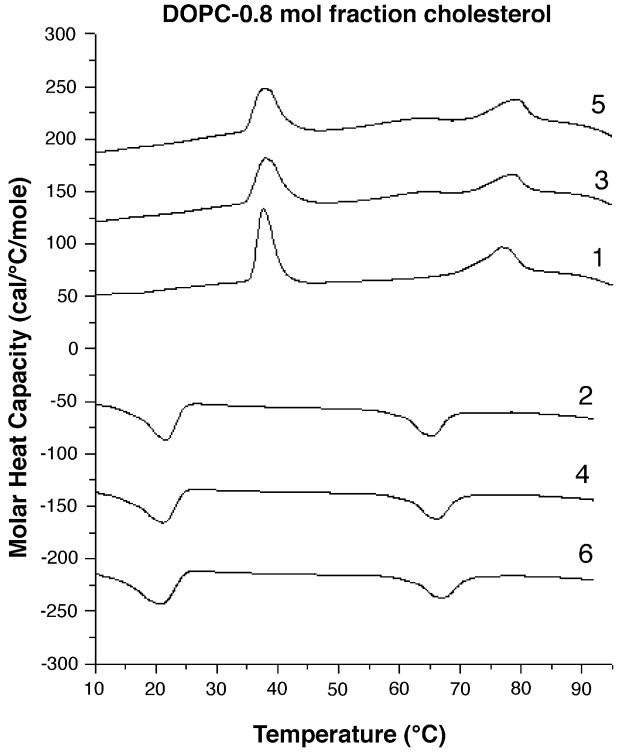

Fig. 4. Same as Fig. 2 using DOPC and 0.8 molar fraction cholesterol.

Table 2
Bilayer to hexagonal phase transition of DOPC–cholesterol mixtures

Molar fraction cholesterol	T_H on heating (°C)	T_H on cooling (°C)	ΔH (cal/mol lipid)
0.6	84	75	360 ± 10
0.7	78	65	560 ± 20
0.8	78	65	560 ± 50

Values from DSC studies. T_H is the bilayer to hexagonal transition temperature.

The small change in chemical structure associated with replacing 18:1 with 18:0 on the C1 of the glycerol moiety to make SOPC results in large changes in the thermotropic behavior of mixtures with cholesterol. At 0.6 molar fraction cholesterol, there is only a weak endotherm corresponding to the polymorphic transition of anhydrous cholesterol with associated enthalpy of 45 cal/mol cholesterol only in the first heating scan and not on subsequent scans (not shown). At 0.7 molar fraction cholesterol, the most prominent transition on the first scan is the polymorphic transition of anhydrous cholesterol. However, this transition behaves differently from other systems we have studied and differently from pure anhydrous cholesterol [27,28]. The transition in the cooling direction occurs at the relatively high temperature of 31 °C and the enthalpy of this transition decreases with each successive scan, until finally in the third cooling scan almost no transition is observed (Fig. 5). This does not happen with SOPC at 0.8 molar fraction cholesterol in which the first heating scan shows a peak at about 75 °C that may correspond to the dehydration of cholesterol monohydrate crystals that is only partially converted to anhydrous cholesterol crystals after dehydration (Fig. 6). With mixtures of cholesterol with either SOPC or DOPC, at molar fractions of cholesterol where crystals form, there is a decrease in the quantity of cholesterol crystals detected by DSC with each sequential scan. This is particularly dramatic for mixtures of SOPC with 0.7 molar fraction cholesterol (Fig. 5). In the case of SOPC, it may be a consequence of the mixture slowly reaching an equilibrium state in which less cholesterol is present as crystals.

We summarize the enthalpy values determined for the polymorphic transition of anhydrous cholesterol in mixtures with the various phospholipids used and from this value calculate what percentage of the cholesterol is in crystalline form (Table 1). The most dramatic difference is between PC and PS with the former showing the presence of cholesterol crystals only at much higher molar fractions of the sterol. In general, with PC, the enthalpy of the polymorphic transition of anhydrous cholesterol is less constant in sequential scans than is the case for PS. This may be caused by a slow dissolution of cholesterol into the membrane and/or to a more rapid conversion from anhydrous cholesterol crystals to crystals of cholesterol monohydrate. It is not clear how substitution of 1-oleoyl for 1-stearoyl affects the amount of anhydrous cholesterol crystals in PC but it does reduce the

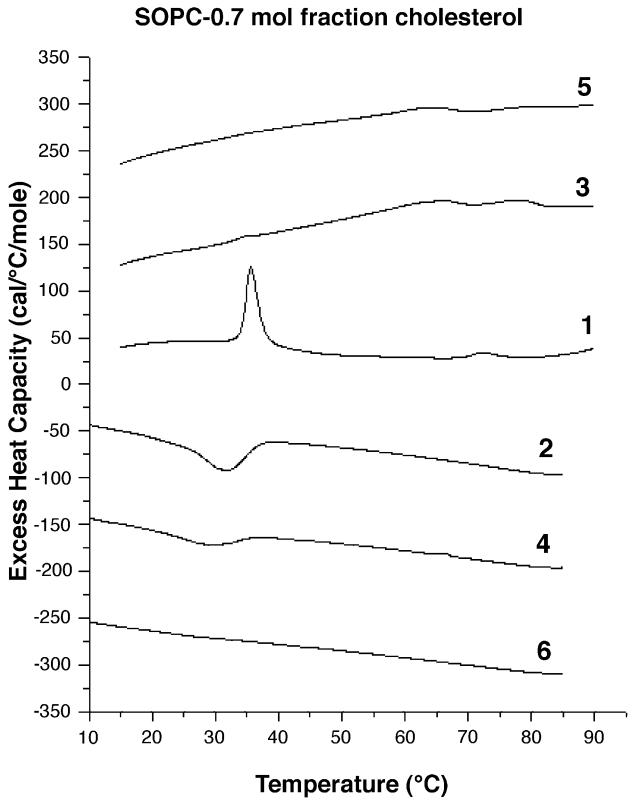

Fig. 5. DSC scans of SOPC with 0.7 molar fraction cholesterol. Lipid concentration 1.5 mg/ml in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.40. Scan rate = 2 °C/min. Top three curves are heating scans and bottom three curves are cooling scans. The numbers indicate the order in which the scans were run. Curves have been displaced along the *y*-axis for presentation. Excess heat capacity is expressed per mole of cholesterol.

amount present with PS. This issue is discussed further below.

3.2. ^{31}P NMR

It has been shown that cholesterol can promote the formation of the hexagonal phase with PC when the acyl chains of the PC are polyunsaturated [30]. Studies by Chen and Rand [31] showed that DOPC/cholesterol mixtures remain lamellar up to the highest temperature investigated, 50 °C, and the highest molar fraction of cholesterol used, 0.5. We have investigated if the lipid mixtures used in the present study exhibited polymorphic behavior using ^{31}P NMR as well as, for some samples, X-ray diffraction (see below). In the case of the pure PC or PS, we did not observe the formation of non-lamellar structures up to a temperature of 100 °C. Addition of cholesterol to SOPC at molar fractions between 0.5 and 0.8 did not result in a change in the ^{31}P powder pattern between 40 and 90 °C. However, with DOPC, there were dramatic changes with temperature that depended on the molar fraction of cholesterol in the mixture (Fig. 7). For pure DOPC, the ^{31}P NMR powder pattern corresponded to that of a bilayer structure up to the highest temperature. At 0.3 molar fraction cholesterol, there is a very small fraction of isotropic signal, particularly in the recooled sample. At 0.4 molar fraction cholesterol, a large fraction of the sample shows an isotropic pattern in addition to the bilayer pattern, while at 0.5 molar fraction cholesterol, the sample starts out as a bilayer, but upon heating to 90 °C, the signal characteristic of an isotropic phase appears and remains after recooling. The isotropic pattern remains for the DOPC sample with 0.5 molar fraction cholesterol down to 0 °C (not shown). At 0.6 and 0.7 molar fractions of cholesterol, hexagonal phase powder patterns appear at higher temperatures and these revert back to a bilayer powder pattern on recooling. Interestingly, at 0.7 molar fraction, there is little isotropic structure, only a reversible interconversion between lamellar and hexagonal phases as a function of temperature.

3.3. Low angle X-ray diffraction

The room temperature low angle X-ray diffraction pattern of DOPS corresponds to that of a multilamellar structure with spacing between lamellae of 55 Å (data not shown). The addition of cholesterol results in an increase in this spacing. This increase reaches approximately 4 Å for a 1:1 molar ratio, as has been observed for mixtures with other PS molecules in the L_α phase [1]. Increasing the molar fraction of cholesterol to approximately 0.4 results in the appearance of the characteristic 34 Å diffraction peak of cholesterol

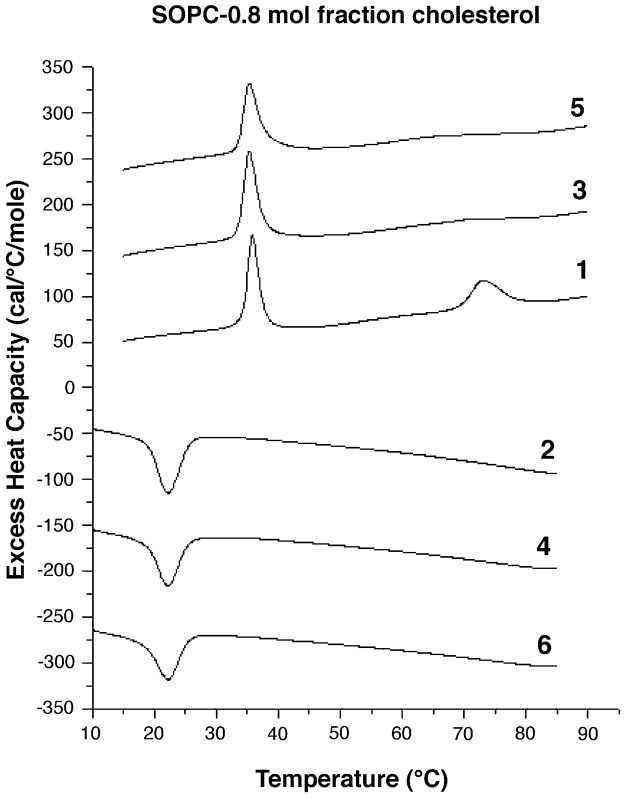

Fig. 6. Same as Fig. 5 using SOPC and 0.8 molar fraction cholesterol.

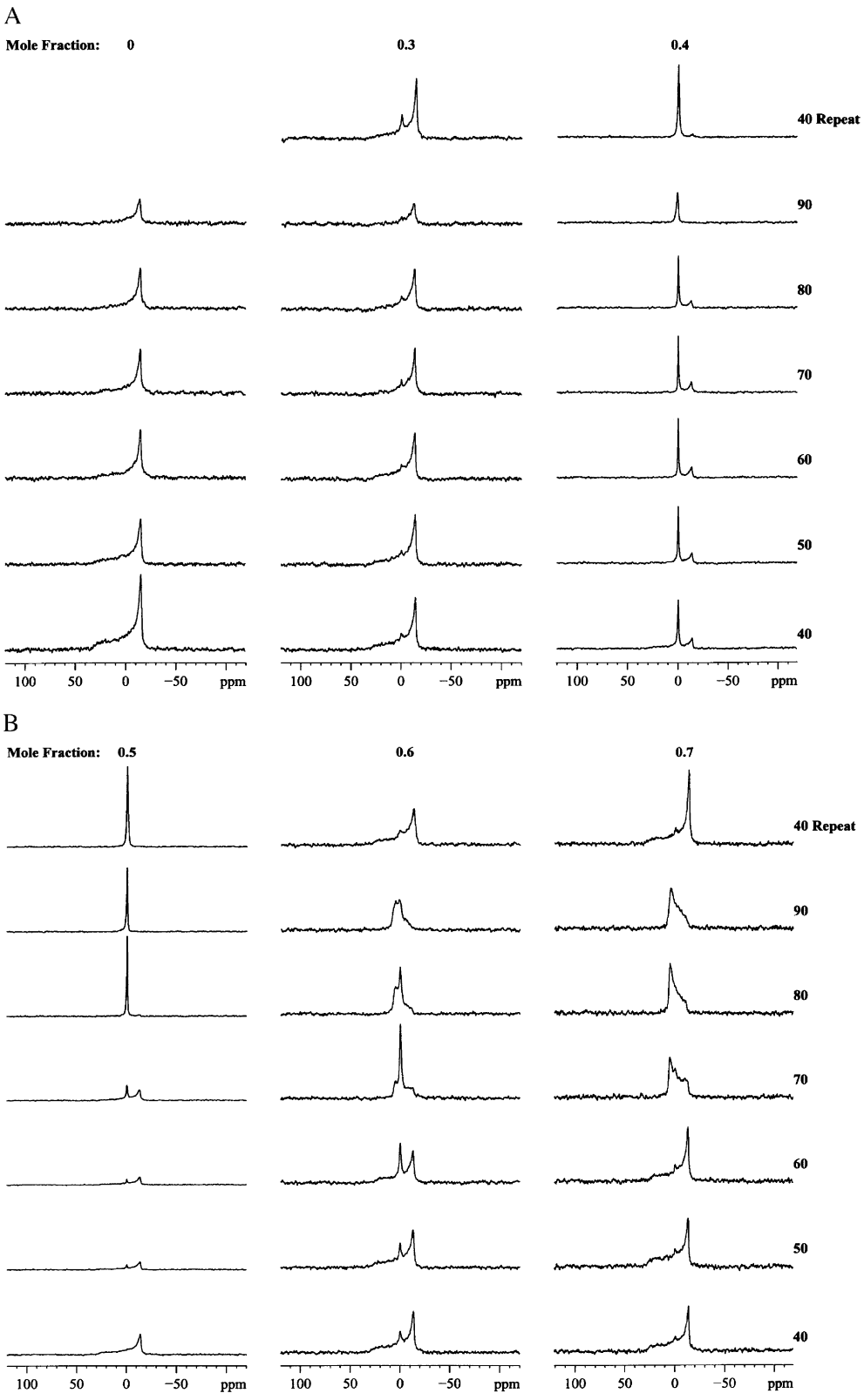

Fig. 7. ^{31}P NMR spectra of DOPC with different molar fractions of cholesterol as a function of temperature. (A) DOPC alone and with cholesterol molar fractions of 0.3 and 0.4. (B) DOPC with cholesterol molar fractions of 0.5, 0.6 and 0.7. For each sample, the spectrum was first accumulated at 40 °C (lower spectrum) over a period of about 30 min and then the sample heated in approximately 10 °C increments. This process was repeated over a range of temperatures. Temperatures are indicated on the right hand side of the spectra. The order of spectral acquisition is from bottom to top. The top spectrum at 40 °C was measured after heating to 100 °C. In all samples, the lipid concentration was 50 mg in 400 μl.

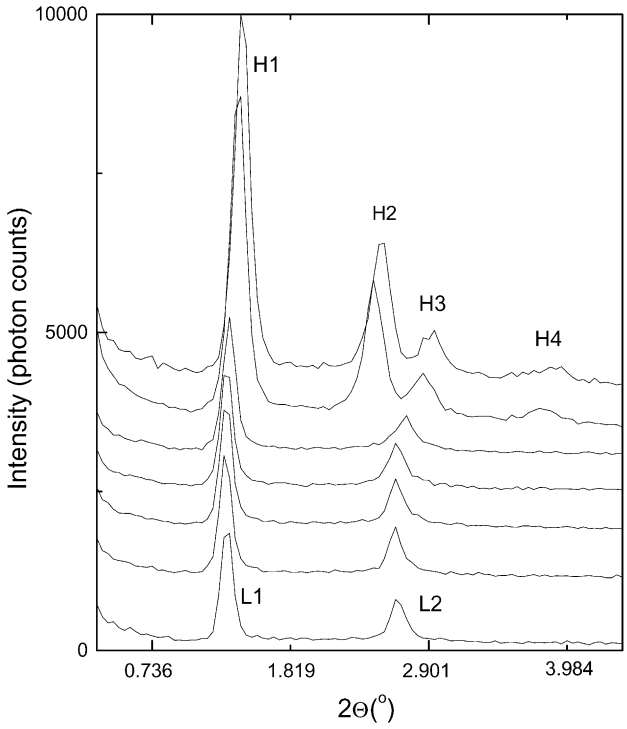

Fig. 8. X-ray diffraction profiles of a DOPC–cholesterol mixture, molar fraction cholesterol 0.7, as a function of temperature. Scan temperatures are (from top to bottom) 90, 81, 67, 62, 53, 43 and 23 °C. L1 and L2 refer to the first two diffraction orders of the lamellar structure; H1–H4, to the first four diffraction orders of the two-dimensional hexagonal lattice. 2Θ is the scattering angle.

crystallites (not shown). Heating to 40 °C can cause these samples to become inhomogeneous: two sets of lamellar diffraction peaks are observed, the first corresponding to a lamellar spacing ~ 72 Å and the second, ~ 59 Å. DOPS with molar fraction cholesterol 0.52 remains lamellar until about 60 °C. Above this temperature, the sample disorders and diffraction orders higher than the first can no longer be observed.

X-ray diffraction confirms that DOPC alone forms only lamellar structures. The lamellar spacing of 61 Å measured at room temperature decreases to approximately 55 Å at 54 °C. Addition of cholesterol causes an increase in the spacing between lamellae that is significantly larger than in the case of DOPS. At room temperature, DOPC with 0.5 molar fraction cholesterol has spacing of 68 Å. It should be noted that with 0.4 or 0.45 molar fraction cholesterol, DOPC sometimes displays an inhomogeneous lamellar phase at room temperature: one lamellar spacing is characteristic of DOPC only and one spacing is characteristic of DOPC with higher molar fractions of cholesterol. A DOPC–cholesterol mixture at molar ratio 1:1, which has been kept in boiling water for 5 min and then cooled under tap water, shows no diffraction signal indicative of an ordered phase when measured at room temperature.

The polymorphism of DOPC with molar fraction cholesterol 0.6 and above can be detected in X-ray profiles. Room temperature lamellar periodicity decreases weakly from 70 Å for molar fraction cholesterol 0.7 to 67 Å for molar fraction

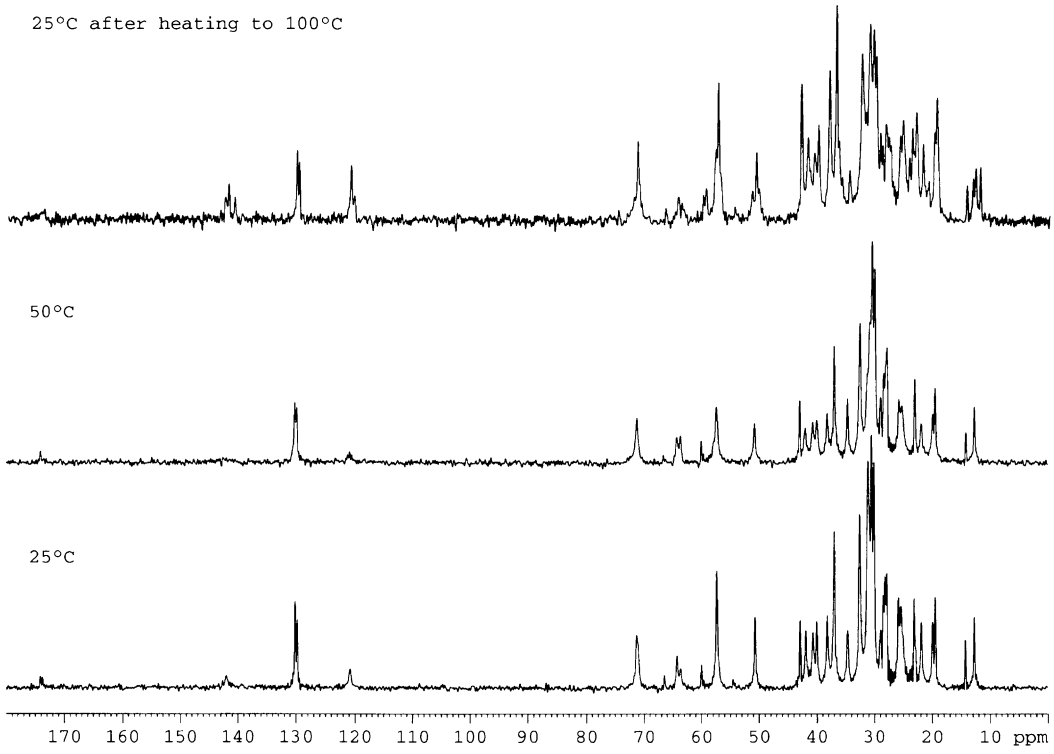

Fig. 9. ^{13}C CP/MAS NMR spectra of DOPC with 0.7 molar fraction cholesterol. The bottom spectrum corresponds to a hydrated but unheated sample at 25 °C, the middle spectrum is the same sample measured at 50 °C and the top spectrum was measured after the sample was heated in boiling water for 2 min and then recooled to 25 °C. Measurement made at 75.48 MHz with sample spinning at 5 kHz using a contact time of 1 ms.

Table 3
Assignment of observed resonances in ^{13}C CP-MAS NMR spectra

Assignment	Chemical shift (ppm)
C=O	174.1
Cholesterol C5	141.9 and 140.8
Acyl C=C	130.1
Acyl C=C	129.6
Cholesterol C6	120.8
Terminal CH_3	14.26
Crystalline cholesterol C18	13.23
Cholesterol C18	12.77
Crystalline cholesterol C18	11.95

0.8. For these samples, the diffraction pattern is independent of temperature until the lamellar–hexagonal transition, in the vicinity of 70 °C, is reached (Fig. 8). Then three or four diffraction peaks appear which can be indexed on a two-dimensional hexagonal lattice, with lattice constants 71–72 Å. As can be seen in Fig. 8, the hexagonal phase persists until at least 90 °C. Cooling to 30 °C restores the lamellar phase.

3.4. ^{13}C CP/MAS NMR

The ^{13}C CP/MAS spectra of DOPC with 0.7 molar fraction of cholesterol shows little change between 25 and 50 °C (Fig. 9). The identification of some of the resonances is indicated in Table 3. Resonances were assigned based on reports of PC [21], unsaturated acyl chains [22], PS [23,24] and cholesterol [25]. After heating to 100 °C and cooling, there are several distinct changes in the spectrum, indicative of the formation of crystallites of cholesterol monohydrate. The peaks corresponding to carbons 5 and 6 of the cholesterol double bond (at about 141 and 121 ppm, respectively) increase in intensity and the C5 resonance is clearly split, characteristic of cholesterol monohydrate crystals [25]. Even more indicative of the formation of cholesterol monohydrate crystals is the behavior of the C18 peaks of cholesterol [18]. The resonance position for this carbon is different in cholesterol dissolved in bilayers and cholesterol monohydrate crystals. It is clear that after heating to 100 °C and recooling to 25 °C, there are peaks at 13.23 and 11.94 ppm, corresponding to crystals of cholesterol monohydrate that were not present either at 25 °C before heating or at 50 °C (the highest temperature with which we could obtain reliable measurements with our instrument and sample rotor) (Fig. 10).

3.5. Cryo-TEM

Cryo-TEM images were obtained from a DOPC dispersion and also from a DOPC/cholesterol mixture, molar fraction cholesterol 0.5, each frozen rapidly from room temperature. In these images, the lipids appeared predominantly in the form of unilamellar or multilamellar vesicles with mean diameter around 3000 Å. A mixture of DOPC/cholesterol that had been heated to 100 °C, rather than being a turbid liquid as in the case of the nonboiled samples, displayed in part a gel-like consistency. In this case, only the

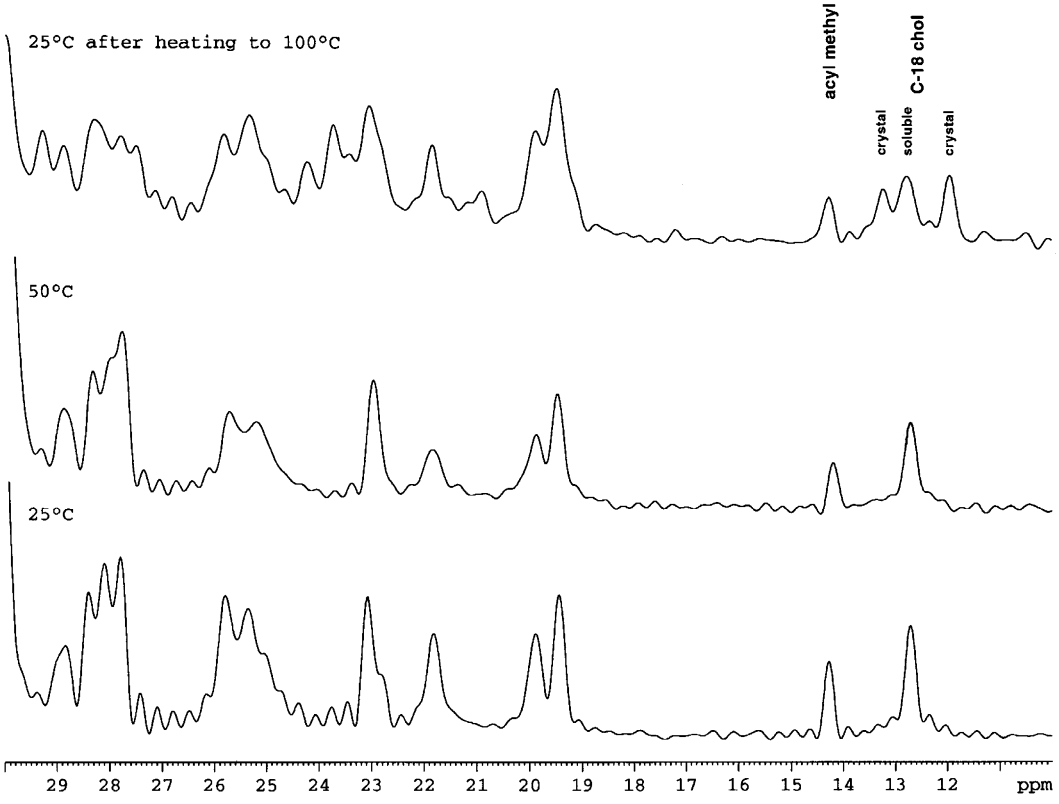

Fig. 10. Low-frequency (upfield) region of the ^{13}C CP/MAS NMR spectra shown in Fig. 9.

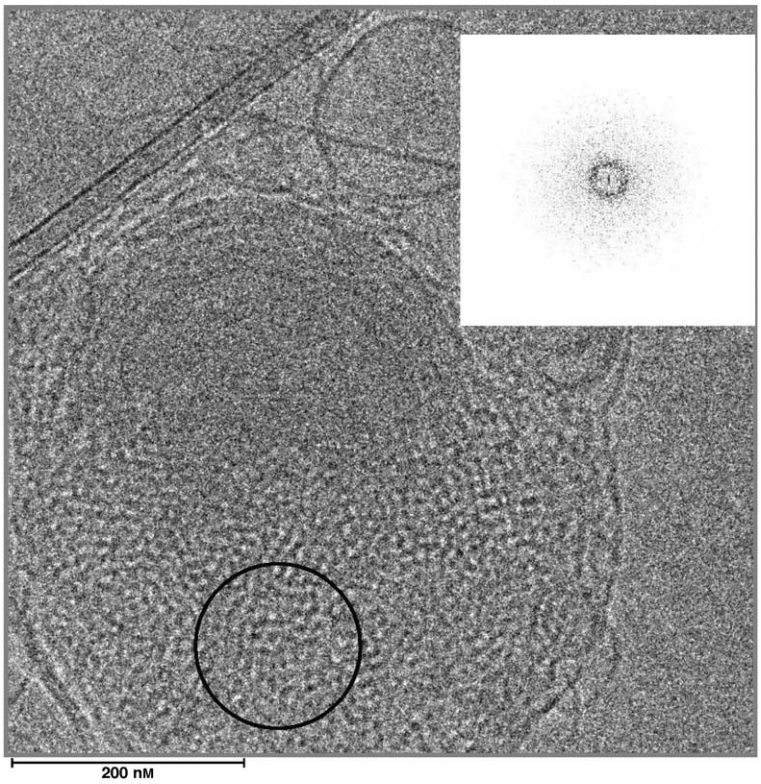

Fig. 11. Cryo-TEM image of a DOPC–cholesterol mixture, molar fraction cholesterol 0.5 after heating to 100 °C. The area selected for FFT is enclosed in the circle and the resulting transform appears in the inset in the upper right corner.

supernatant surrounding the gel could be sampled on the microscope grid. Here too vesicles were present, but in addition, arrays of small globular aggregates were observed (Fig. 11). Fast Fourier transform of selected areas within these arrays showed that the small aggregates are arranged without any long-range order. From the diameter of the diffuse ring, the mean center to center distance between aggregates was calculated to be 110–120 Å.

4. Discussion

The present study shows that the miscibility of cholesterol with DOPC (18:1/18:1-PC) is comparable to that of other forms of PC having one (Table 1, this paper) or two [32,33] saturated acyl chains. Introducing a C9–C10 double bond in the *sn*-1 chain of the phospholipid might be expected to result in better accommodation of the cholesterol molecule [34]. However, although *N*-palmitoyl-D-sphingomyelin forms membrane domains with cholesterol, *N*-oleoyl-D-sphingomyelin does not [35], indicating that saturated acyl chains interact more favorably than oleoyl chains with cholesterol. However for PCs, the difference between a saturated acyl chain and an oleoyl chain is not very great. This is not the case with PS where we show that a higher cholesterol molar fraction is required for the formation of crystallites in DOPS, as compared with SOPS, and that the amount of crystalline cholesterol formed is greater with SOPS (Table 1). Thus, one cannot make a simple generalization about the effect of acyl chain unsaturation on cholesterol solubility, since the effect of acyl chain unsaturation is also dependent on the nature of the lipid headgroup.

There is a clear difference in cholesterol solubility between PS and PC, with the latter dissolving more cholesterol. This would suggest that in biological membranes, there should be a higher cholesterol concentration in the outer leaflet of the plasma membrane than in the cytoplasmic leaflet. Furthermore, the outer leaflet contains sphingomyelin that also has a high affinity for cholesterol [36]. However, there is evidence to suggest the reverse, that is, the cytoplasmic leaflet, at least in erythrocytes, has more cholesterol [37]. Of course a biological membrane is more complex than the simple lipid mixtures we are using. In the work of Schroeder et al. [37], a fluorescent analog of cholesterol was used that may not in fact behave in the same way as cholesterol itself. Brasaemle et al. [38] used ^{3}H-cholesterol and digestion of the cholesterol in the outer leaflet with cholesterol oxidase to probe the membrane asymmetry. If the conclusion is correct, however, it would suggest that at least some cholesterol is present in segregated clusters in the cytoplasmic leaflet.

Another feature of DOPC/cholesterol is the marked polymorphic behavior of these lipid mixtures beginning at cholesterol molar fractions as low as 0.4 (Fig. 7). Such behavior is not observed with either pure DOPC or with

mixtures of SOPC and cholesterol up to a cholesterol molar fraction of 0.8. It is known that cholesterol markedly increases the negative curvature of DOPC [31]. However, sufficient unsaturation in the lipid acyl chains is required for mixtures of cholesterol and PC to spontaneously convert to the hexagonal phase in the absence of tetradecane. It has also been previously observed that 22:6/22:6-PC [30] will undergo a conversion from lamellar to hexagonal phase at about 60 °C in the presence of an equimolar concentration of cholesterol in the absence of tetradecane. The presence of a two-dimensional hexagonal lattice in mixtures of DOPC with 0.7 molar fraction cholesterol is directly demonstrated by X-ray diffraction (Fig. 8). The value of d_{hex} that we find, 71–72 Å, is in the range also found by Chen and Rand [31] at high cholesterol concentration, although the two values cannot be compared quantitatively since the earlier studies only extended up to equimolar cholesterol and DOPC. In addition, there are differences in temperature and in the presence of tetradecane in the earlier study. The current study was done at higher temperatures that would result in a decrease in d_{hex}, to approximately a 20% lower value [39]. The presence of tetradecane has little effect on the hexagonal phase spacings [39]. Our findings therefore agree with those of Chen and Rand that at sufficiently high cholesterol concentrations, the curvature properties of DOPC become similar to those of DOPE [31].

In addition to forming a hexagonal phase, mixtures of DOPC with lower molar fractions of cholesterol give rise to an isotropic phase. We have investigated the morphological changes responsible for the appearance of the isotropic ^{31}P NMR signal. Neither X-ray diffraction studies nor cryo-TEM gave any indication of the formation of a cubic phase. However, the cryo-TEM did demonstrate a large change in morphology in a sample of DOPC/cholesterol (1:1) that had been heated to 100 °C (Fig. 11). We see that vesicles still remain but some structures have smaller aggregates inside them. This is not a universal feature but it is not uncommon. With specimens of DOPC/cholesterol (1:1) that have not been heated, this morphology can also be found, but it is much less common than with the heated sample. This is consistent with the fact that the isotropic signal is more prominent in the DOPC–cholesterol sample after heating rather than before. It does not explain why the entire ^{31}P NMR signal appears to be isotropic. The spontaneous formation of small vesicles from large unilamellar vesicles of DOPC has been previously observed by cryo-TEM [40]. This phenomenon does not commonly occur with multilamellar vesicles, but it has been previously observed with polyunsaturated PC and cholesterol [30] as well as with sphingomyelin [41] and mixtures of POPC and POPS [42]. Many of the smaller particles that we observe with cryo-EM are too small to be vesicles. Because of the lack of long-range order, we have been unable to characterize the nature of the molecular arrangements in these particles.

An additional interesting feature of the formation of a hexagonal phase in DOPC is that it appears to promote the phase separation of cholesterol crystals. In freshly prepared samples of DOPC with 0.7 molar fraction cholesterol, there is a small amount of anhydrous crystalline cholesterol present (Fig. 3). However, these anhydrous cholesterol crystals are metastable and disappear in subsequent scans. Furthermore, after equilibration of the sample for several hours to allow conversion of any existing crystals to the monohydrate form, there are no crystals present at 25 °C that can be detected by CP/MAS NMR (Fig. 9). However, after the sample is heated to 100 °C and recooled, the presence of cholesterol monohydrate crystals is evident (Fig. 9). Thus, cholesterol monohydrate crystals appear subsequent to or concomitant with the formation of the hexagonal phase. It should be noted that a temperature as high as 100 °C is not required for cholesterol crystals to form on heating and in other specimens, cholesterol crystals were observed in samples heated to only 50–60 °C.

Furthermore, for DOPC with 0.7 molar fraction cholesterol, the crystals that form are largely in the monohydrate form and the interconversion between the monohydrate and anhydrous forms of cholesterol is rapid. This is indicated by the observation that the freshly heated and cooled sample of DOPC with 0.7 molar fraction cholesterol gives a ^{13}C CP/MAS NMR spectrum corresponding to the monohydrate. Some hydration would have occurred during the measuring time of about 6 h, but there was no contribution to the spectrum from anhydrous cholesterol crystals, indicating that they were not present at the start of spectral accumulation. This is also consistent with the DSC results. In addition to the DSC transition, identified from ^{31}P NMR as arising from conversion between lamellar and hexagonal phases, there is also a transition at 58 °C in the second heating scan that we suggest arises from the dehydration of cholesterol monohydrate. This peak does not appear in the sample with 0.6 molar fraction cholesterol. This is consistent with the fact that no cholesterol crystals are observed by ^{13}C CP/MAS NMR at this molar fraction of cholesterol. Furthermore, this peak is not seen in the first heating scan of the sample of DOPC with 0.7 molar fraction cholesterol where no crystals of cholesterol monohydrate appear in the NMR. The loss of the small sharp peak, corresponding to the polymorphic transition of anhydrous cholesterol, requires that the sample be heated to a temperature in which it converts to the H_{II} phase. If we heat and cool the sample between 0 and 70 °C, the same small transition at 38 °C is observed for each heating scan, as well as a transition of similar magnitude at 21.8 °C for each cooling scan (not shown). In contrast, if the DOPC sample with 0.7 molar fraction cholesterol is heated to 100 °C on the first heating scan and then cooled to 0 °C, followed by repeated scanning between 0 and 70 °C, the only observed polymorphic transition of anhydrous cholesterol, observed at 38 °C, is seen in the first heating scan and not in subsequent scans. Thus, the large domains of anhydrous cholesterol crystals are destroyed by interconversion between the lamellar and H_{II} phase. There is no

distinct transition that we can identify in DSC as that of the rehydration of the anhydrous cholesterol on cooling despite the fact that the peak at 58 °C appears in both the second and the third heating scan of DOPC with either 0.7 or 0.8 molar fraction cholesterol. The 58 °C peak is not prominent in the sample with 0.8 molar fraction cholesterol as shown in Fig. 4, but this transition can be observed in an expanded plot. Hence, the dehydration process is reversed at some stage between the completion of the dehydration, heating to 100 °C, cooling to 0 °C and reheating to 58 °C. The relatively rapid reversibility of the dehydration transition and the lower temperature at which it occurs compared with the 80–95 °C in other lipids or as pure cholesterol monohydrate, is indicative of the fact that the crystallites in the case of 0.7 molar fraction cholesterol are in intimate contact with the phospholipid. At 0.8 molar fraction cholesterol, on the other hand, the polymorphic transition of anhydrous cholesterol appears at the temperatures observed for pure cholesterol crystals, both upon heating and cooling, due to the fact that at this ratio, there is insufficient lipid present to influence either the kinetics or the thermotropic properties of the crystallites.

We thus demonstrate that mixtures of DOPC and cholesterol exhibit several novel features that are not observed with SOPC, SOPS or DOPS alone or in mixtures with cholesterol. At cholesterol molar fractions higher than 0.5, mixtures with DOPC convert to the H_{II} phase. It has been shown that cholesterol enhances the negative curvature tendency of phospholipids and will facilitate the formation of the H_{II} phase [31]. This phenomenon occurs with mixtures of cholesterol and DOPC but not with cholesterol and SOPC because of the decreased negative curvature tendency of SOPC due to its saturated *sn*-1 acyl chain. Formation of an H_{II} phase is also not observed with mixtures of DOPS and cholesterol. This may be attributed to the phase separation of cholesterol crystals that already commences at a molar fraction of cholesterol of 0.3–0.4.

Another particular property of cholesterol crystals in the presence of DOPC is that much larger amounts of crystals form on heating, either as cholesterol monohydrate or as anhydrous cholesterol, depending on the molar fraction of cholesterol. The sample of DOPC with 0.7 molar fraction cholesterol exhibits a much more rapid rate of hydration of the cholesterol crystals (compared with other PC–cholesterol mixtures) and these crystals become dehydrated at a lower temperature. One would have expected DOPC to behave similarly to other PCs since it has the same headgroup, which is the region of the molecule where the cholesterol hydroxyl group is located. In addition, the carbonyl group of DOPC has a chemical shift for the ^{13}C at 174.1 compared with 174.0 for POPC, indicating little difference in the hydrogen bonding of this group between these two lipids. The different behavior of cholesterol crystals in DOPC is likely related to the fact that these mixtures form the hexagonal phase, resulting in a different environment for cholesterol.

Our results demonstrate that in model liposome studies, DOPC cannot necessarily be interchanged with other PCs just because it is a bilayer-forming lipid at room temperature. Many of its other properties, particularly with regard to mixtures with cholesterol, will be different as compared with those of SOPC, for example.

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