Properties of Mixtures of Cholesterol with Phosphatidylcholine or with Phosphatidylserine Studied by ^{13}C Magic Angle Spinning Nuclear Magnetic Resonance

Richard M. Epand,* Alex D. Bain,† Brian G. Sayer,† Diana Bach,‡ and Ellen Wachtel§
*Department of Biochemistry and †Department of Chemistry, McMaster University, Hamilton, Ontario L8S 4M1, Canada; and
‡Department of Biological Chemistry and §Chemical Services Unit, Weizmann Institute of Science, Rehovot, Israel

ABSTRACT The behavior of cholesterol is different in mixtures with phosphatidylcholine as compared with phosphatidylserine. In ^{13}C cross polarization/magic angle spinning nuclear magnetic resonance spectra, resonance peaks of the vinylic carbons of cholesterol are a doublet in samples containing 0.3 or 0.5 mol fraction cholesterol with 1-palmitoyl-2-oleoyl phosphatidylserine (POPS) or in cholesterol monohydrate crystals, but a singlet with mixtures of cholesterol and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC). At these molar fractions of cholesterol with POPS, resonances of the C-18 of cholesterol appear at the same chemical shifts as in pure cholesterol monohydrate crystals. These resonances do not appear in samples of POPS with 0.2 mol fraction cholesterol or with POPC up to 0.5 mol fraction cholesterol. In addition, there is another resonance from the cholesterol C18 that appears in all of the mixtures of phospholipid and cholesterol but not in pure cholesterol monohydrate crystals. Using direct polarization, the fraction of cholesterol present as crystallites in POPS with 0.5 mol fraction cholesterol is found to be 80%, whereas with the same mol fraction of cholesterol and POPC none of the cholesterol is crystalline. After many hours of incubation, cholesterol monohydrate crystals in POPS undergo a change that results in an increase in the intensity of certain resonances of cholesterol monohydrate in ^{13}C cross polarization/magic angle spinning nuclear magnetic resonance, indicating a rigidification of the C and D rings of cholesterol but not other regions of the molecule.

INTRODUCTION

High molar fractions of cholesterol in bilayers composed of phosphatidylcholine result in the formation of liquid-ordered phases in which the acyl chains of the phospholipid have a higher degree of order but a rapid rate of lateral diffusion (Huang et al., 1993; Ipsen et al., 1987; Vist and Davis, 1990). This phase has been referred to as the liquid-ordered phase and is thought to exist in biological membranes in sequestered "raft" domains (Brown and London, 1998; Harder et al., 1998; Rietveld and Simons, 1998; Fielding and Fielding, 2000). At higher concentrations in the membrane, cholesterol readily forms crystallites, presumably as a consequence of its relatively rigid fused ring structure. Interestingly, the molar fraction of cholesterol required to initiate the formation of crystallites is dependent on the nature of the phospholipid. Cholesterol is less soluble in bilayers of phosphatidylserine (PS) and phosphatidylethanolamine than phosphatidylcholine (PC) (Bach et al., 1998). This is of particular relevance to the cytoplasmic monolayer of "raft" domains of mammalian plasma membranes that contain lipids that are less miscible with cholesterol. It is possible that the formation of more stable forms of stacked cholesterol aggregates is responsible for the very slow rate of transbilayer diffusion of a fraction of cholesterol on the cytoplasmic leaflet (Schroeder et al., 1991).

Cholesterol crystals may play a role in certain pathological conditions. 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).

With regard to measuring the miscibility of cholesterol with phospholipids, there are several important considerations. The first is how the phospholipid and cholesterol are mixed. Both lipids are soluble in organic solvents, and they can be deposited as a film by solvent evaporation. It is possible that during solvent evaporation there is separation of the two lipid components, and modified procedures have been proposed to avoid this (Buboltz and Feigenson, 1999; McMullen et al., 2000). However, these methods introduce other potential complications, and there is no ideal way of making these mixtures. We have shown that even the small structural difference between 1-palmitoyl-2-oleoylphosphatidylserine (POPS) and 1-stearoyl-2-oleoylphosphatidylserine results in a detectably different miscibility of cholesterol (Bach et al., 1992). The extent of formation of cholesterol crystals is strongly dependent on the molar fraction of cholesterol (Epand et al., 2001a). These observations demonstrate that cholesterol crystals do not simply form as a consequence of sample preparation.

More critical than the method of preparation is the thermal history of the sample. This is largely a consequence of

Submitted January 15, 2002, and accepted for publication June 13, 2002.
Address reprint requests to Richard M. Epand, Department of Biochemistry, McMaster University Health Sciences Centre, Hamilton, ON L8N 325, Canada. Tel.: 905 - 525 - 9140; Fax: 905 - 521 - 1397; E-mail: epand@mcmaster.ca.
© 2002 by the Biophysical Society
0006-3495/02/10/2053/11 $2.00

the extremely slow interconversion of the two polymorphs of anhydrous cholesterol around room temperature (Epand et al., 2000). In addition, anhydrous cholesterol crystals slowly convert over a period of hours to form crystals of cholesterol monohydrate (Loomis et al., 1979).

^{13}C cross polarization/magic angle spinning nuclear magnetic resonance (CP/MAS NMR) has been used for the study of lipids, including mixtures with cholesterol (Forbes et al., 1988). This technique has been shown by Hamilton and coworkers to be an excellent method for detecting cholesterol crystallites (Guo and Hamilton, 1993, 1996). This is because the efficiency of cross polarization increases as the molecule becomes more solid-like (Alemany et al., 1983a, b). Hence the intensity of ^{13}C resonances in a cross-polarized spectrum is greater from cholesterol crystallites than from cholesterol dissolved in a membrane. The technique has also been used to develop a method to study the order and dynamics in membranes using inter-proton pair order parameters (Urbina et al., 1998). We wanted to compare mixtures of cholesterol with POPS and with 1-palmitoyl-2-oleoyl phosphatidyl choline (POPC) both above and below the concentration required for the detection of cholesterol crystals by differential scanning calorimetry (DSC) or by x-ray diffraction. In addition, we have recently observed that after becoming hydrated, the crystals of cholesterol monohydrate undergo a further change that modifies their dehydrating properties (Epand et al., 2001a). We investigated the nature of the structural changes in the crystals of cholesterol monohydrate as a consequence of the time of incubation that are responsible for the changes in their dehydrating properties.

MATERIALS AND METHODS

Materials

Cholesterol and phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL).

Preparation of hydrated mixtures of POPS and cholesterol

POPS 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 piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 1 mM EDTA, and 150 mM NaCl with 0.002% NaN_3, pH 7.40. The lipid film was suspended and hydrated by intermittent vortexing and heating to 60°C over a period of 30 min under argon. Except for the experiment determining the dependence of the spectra on time of incubation, all samples were incubated at least 24 h at 4°C to allow conversion of the cholesterol to cholesterol monohydrate.

Preparation of cholesterol monohydrate crystals

Cholesterol was dissolved in ethanol and distilled, deionized water was added dropwise until a white precipitate appeared. The resulting cloudy solution was warmed until it clarified, and then the solution allowed to cool gradually to room temperature and then to 4°C. The resulting white solid crystalline material was separated by centrifugation.

^{13}C CP/MAS NMR

Lipid suspensions or cholesterol monohydrate crystals 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. Similar spectra were obtained for some of the samples on a Bruker Avance 500 spectrometer. 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 generally spun at 5 kHz and at a temperature of 25°C, but spinning at rates between 2 and 10 kHz had little effect on the spectrum, except for some changes in the resolution. The power levels used for cross-polarization were not ramped during the contact and 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. In the contact time dependence studies, the spectra were collected in random order to avoid biases due to drift in spectrometer performance. If spectra were obtained on different days, then duplicates of previous runs were included to normalize the intensities.

The temperature inside the rotor, controlled by the variable temperature unit of the instrument, 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 ~1° warmer than the set temperature. Contact times between 0.02 and 4 ms were used with a recycle time of 5 s. Generally each spectrum was obtained with 12,000 scans and processed with a 1-Hz line broadening. Resonances were assigned based on reports of phosphatidylcholine (Forbes et al., 1988), unsaturated acyl chains (Batchelor et al., 1974), phosphatidylserine (Holwerda et al., 1981; McLaughlin, 1982), and cholesterol (Guo and Hamilton, 1996).

Direct polarization

Single pulse excitation with high power proton decoupling was used with a 4-μs pulse for ^{13}C and the proton frequency optimized for decoupling. Recycle times of 100 s were used for the spectra in Fig. 1. Preliminary experiments with delays of 5 and 10 sec were also performed.

RESULTS AND DISCUSSION

The CP in ^{13}C CP/MAS NMR is useful for studying carbon atoms in a more rigid, solid-like environment, because the polarization transfer allows the experiment to be repeated at the proton relaxation time. This allows ^{13}C NMR spectra to be acquired at natural abundance within a reasonable time (Fig. 1). However, there is always some uncertainty about the quantitative nature of ^{13}C CP/MAS NMR (Montez et al., 1993; Warschawski and Devaux, 2000). Even for a spin system in a pure rigid solid, small motions can change the effective dipolar coupling, which is responsible for the polarization transfer. The situation in a lipid bilayer with mixed components is much more complex. At high magnetic fields and fast spinning rates, the spectra can be quite sensitive to the experimental parameters. However, the

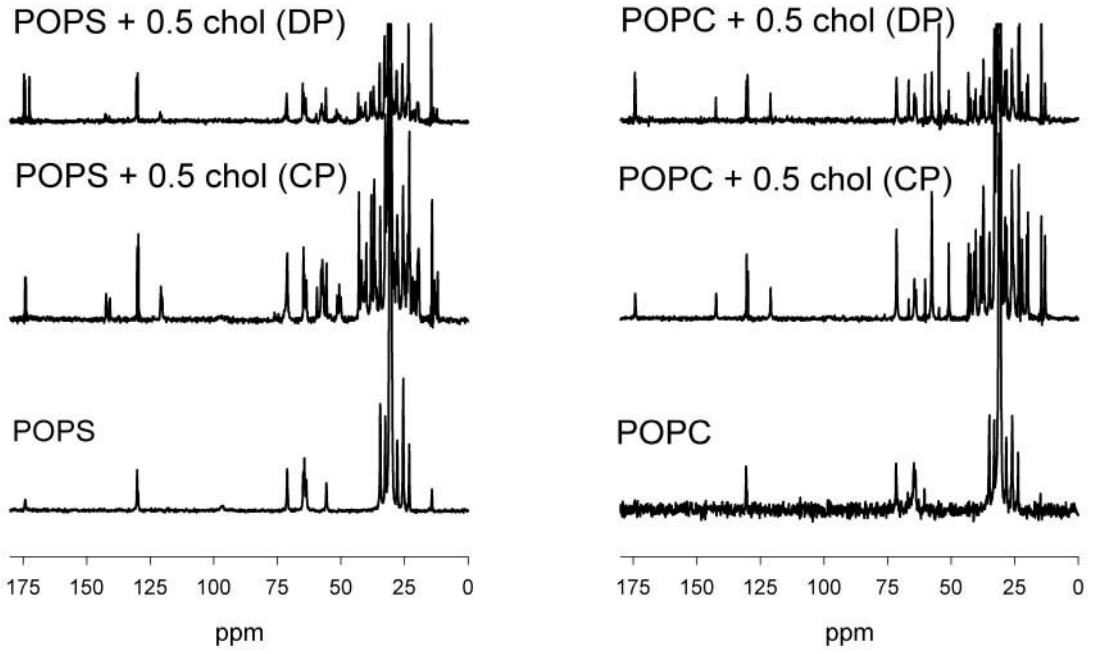

FIGURE 1 ^{13}C CP/MAS NMR (CP) NMR spectra of POPS and POPC with and without 0.5 mol fraction cholesterol and direct polarization (DP) spectra of the samples with cholesterol. Measurement made at 75.48 MHz with sample spinning at 5 kHz at 25°C. For the CP/MAS spectra, the contact time was 1 ms, except for the POPC sample in which the contact time is 0.5 ms. For the direct polarization measurements the delay time was 100 s.

spectra reported here were obtained at 7.0 T and a spinning rate of 5 kHz, so the variation will be less pronounced. Sample spinning can also distort intensities. If the carbons have substantial chemical shielding anisotropy (CSA), intensity can be lost to spinning sidebands. However, for a 120-ppm CSA, spinning at 5 kHz at 7.0 Tesla should leave more than 90% of the intensity in the center band. In this work, all the samples were run under very similar conditions, and the spectral intensities were reproducible within approximately ±10%. The conclusions are drawn from relative changes in the intensities as well as from the chemical shifts of particular resonances.

Chemical shifts of the C═O of POPC and POPS

There are two C═O groups from the acyl chains on each of the phospholipids, which are esterified to the C-1 and the C-2 positions of the glycerol moiety. The resonance of the C═O group has a chemical shift of ~174 ppm. The peaks from each of the two carbonyls are not resolvable in the case of either of the pure phospholipids, nor with samples of POPC with molar fractions of cholesterol up to 0.5 (Fig. 2). However, the resonance of the C═O is split into a doublet, at 174.26 and 173.96 ppm, for all of the samples of POPS containing cholesterol (Fig. 2). These results indicate that cholesterol has a different interaction with POPS bilayers than with POPC bilayers, even at molar fractions at which no cholesterol crystals are detected by other means.

Chemical shifts of the C═C of POPC and POPS

The carbons 9 and 10 at the double bond of the oleoyl group of POPC or POPS have chemical shifts that are almost identical, i.e., 130.11 and 129.68 ppm. For the pure phospholipid, either POPC or POPS (Fig. 2), the intensities of the two resonances (at a contact time of 1 ms) appear to have unequal intensity with the resonance at higher frequency (lower field) being more intense. This is not the case in the presence of cholesterol, where the two resonances appear to be almost equal in intensity for both lipids (Fig. 2). However, at shorter contact times, below 0.5 ms, the intensity of the lower field resonance is again larger in mixtures of cholesterol with either of the two lipids (see Fig. 4 below for the case of POPS with 0.5 molar fraction cholesterol).

Chemical shifts of the C═C of cholesterol

The ring numbering system of cholesterol is shown in Fig. 3. The carbons at the double bond position of cholesterol are well resolved in the ^{13}C CP/MAS NMR spectrum and do not overlap with any signals from the phospholipid. When cholesterol is mixed with POPC, these resonances are not detectable at cholesterol molar fraction of 0.2 but are observed as two single peaks at cholesterol molar fractions of 0.3 and 0.5 (Fig. 2). However, in the case of mixtures of POPS with cholesterol, these resonances are clearly observed at 0.2 mol fraction cholesterol (Fig. 2). At cholesterol

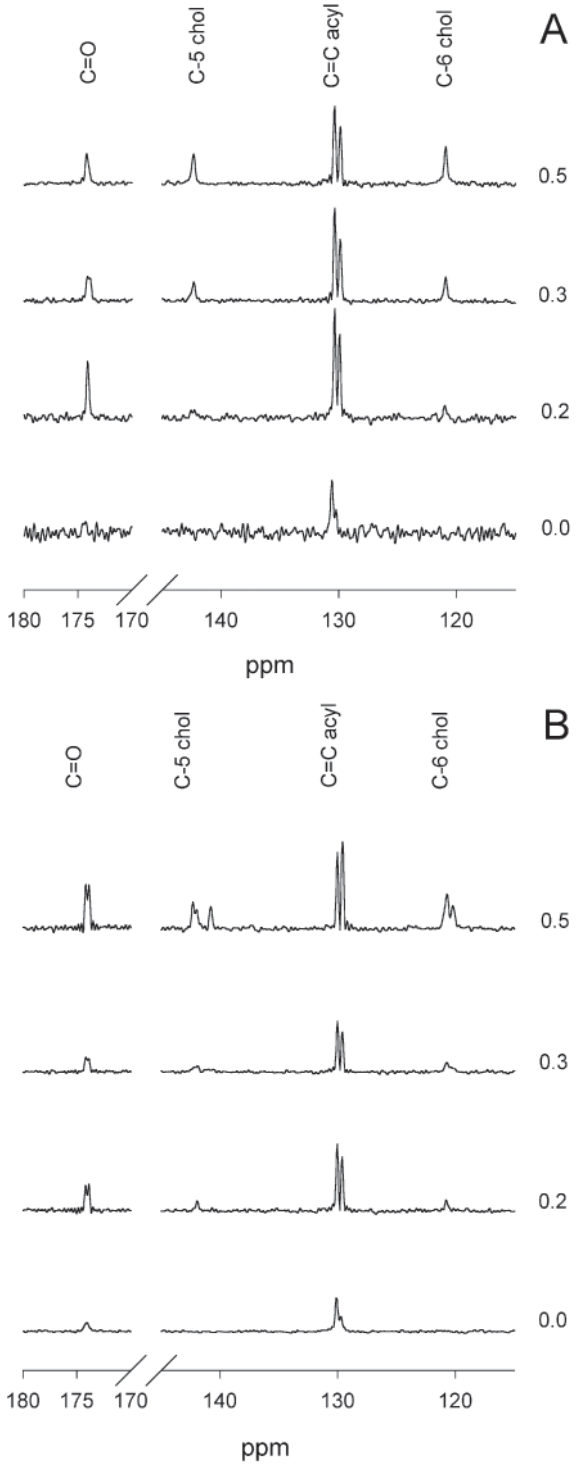

FIGURE 2 High frequency (downfield) region of the ^{13}C CP/MAS NMR spectra of POPC (section A) and POPS (section B) with varying molar fractions of cholesterol indicated by the numbers on the right hand side of the spectra. Contact time 1 ms.

molar fractions of 0.3 and especially at 0.5, it is clear that these resonances are split into doublets (Fig. 2), as they are in authentic crystals of cholesterol monohydrate (Guo and Hamilton, 1996). The doublet for C5 appears at 142.39 and 140.88 ppm and for C6 at 120.81 and 120.32 ppm. These and other peaks arising from crystals of cholesterol monohydrate have a similar dependence on contact time in mixtures with POPS as they do in pure cholesterol monohydrate crystals (Fig. 4). This indicates that the motional properties of the cholesterol crystals in POPS membranes are similar to those of cholesterol monohydrate crystals.

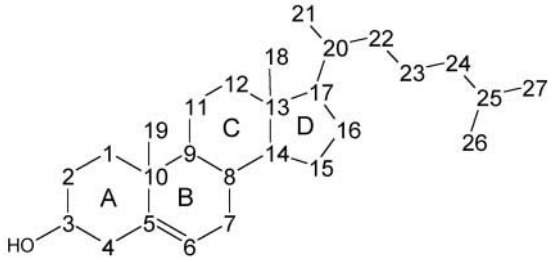

FIGURE 3 Chemical structure of cholesterol showing the ring numbering system and the letter designation of each of the four rings.

Methyl groups

The terminal methyl groups of the acyl chains, and C18 of cholesterol give clear ^{13}C resonances on the low frequency side (upfield) of 15 ppm. The terminal methyls appear at 14.15 ppm for both POPC and POPS (Fig. 5) and are not resolved. The lowest frequency signal(s) is (are) that (those) from the C18 methyl group of cholesterol (Guo and Hamilton, 1996). In the monohydrate crystal, there are two C18 resonances at 13.17 and 11.9 ppm. Resonances at these chemical shifts also appear in samples of POPS containing 0.3 or 0.5 mol fraction cholesterol, indicating the presence of cholesterol crystals in the samples. This is in accordance with DSC studies (Epand et al., 2001a). There is also a

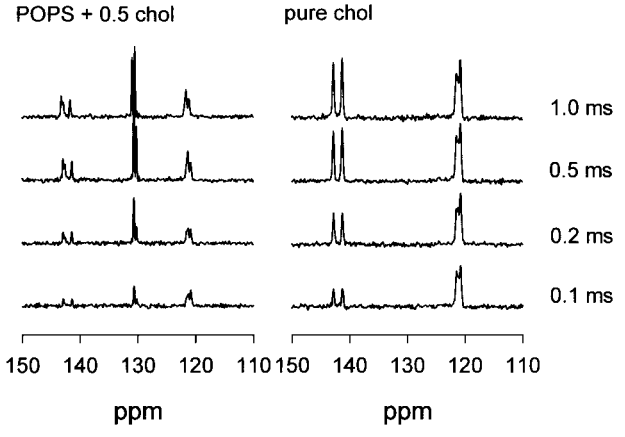

FIGURE 4 Contact time dependence of the high frequency (downfield) region of the ^{13}C CP/MAS NMR spectrum of pure cholesterol monohydrate crystals (*right*) and of POPS with 0.5 mol fraction cholesterol (*left*). Contact times are indicated on the right and are the same for both sets of spectra.

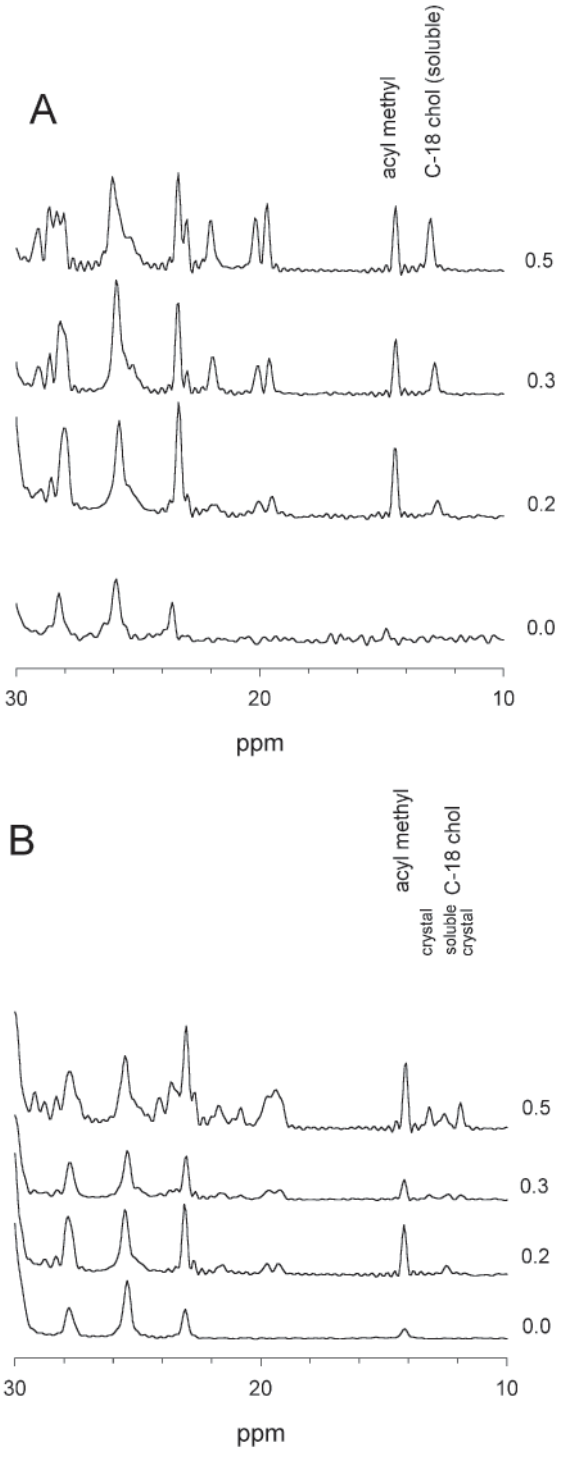

FIGURE 5 Low frequency (upfield) region of the ^{13}C CP/MAS NMR spectrum of POPC (section A) and POPS (section B) with varying molar fractions of cholesterol indicated by numbers on the right hand side of the spectra. Contact time 1 ms.

resonance at 12.6 ppm in all of the samples of either POPC or POPS with cholesterol. This resonance arises from non-crystalline cholesterol in the membrane.

Contact time

Peak intensity, and even the ability to detect a signal in CP/MAS will depend on the contact time used. This is because the carbon signals arise from being in "contact" with protons through a dipolar coupling. The dipolar coupling decreases as the inverse cube of the distance to the protons and may be further reduced by molecular motion. In a free liquid, the coupling averages to zero, and there is no cross-polarization. The rate of cross-polarization is indicative of the rigidity of the environment with samples that are more solid-like having more rapid rates of cross polariza-

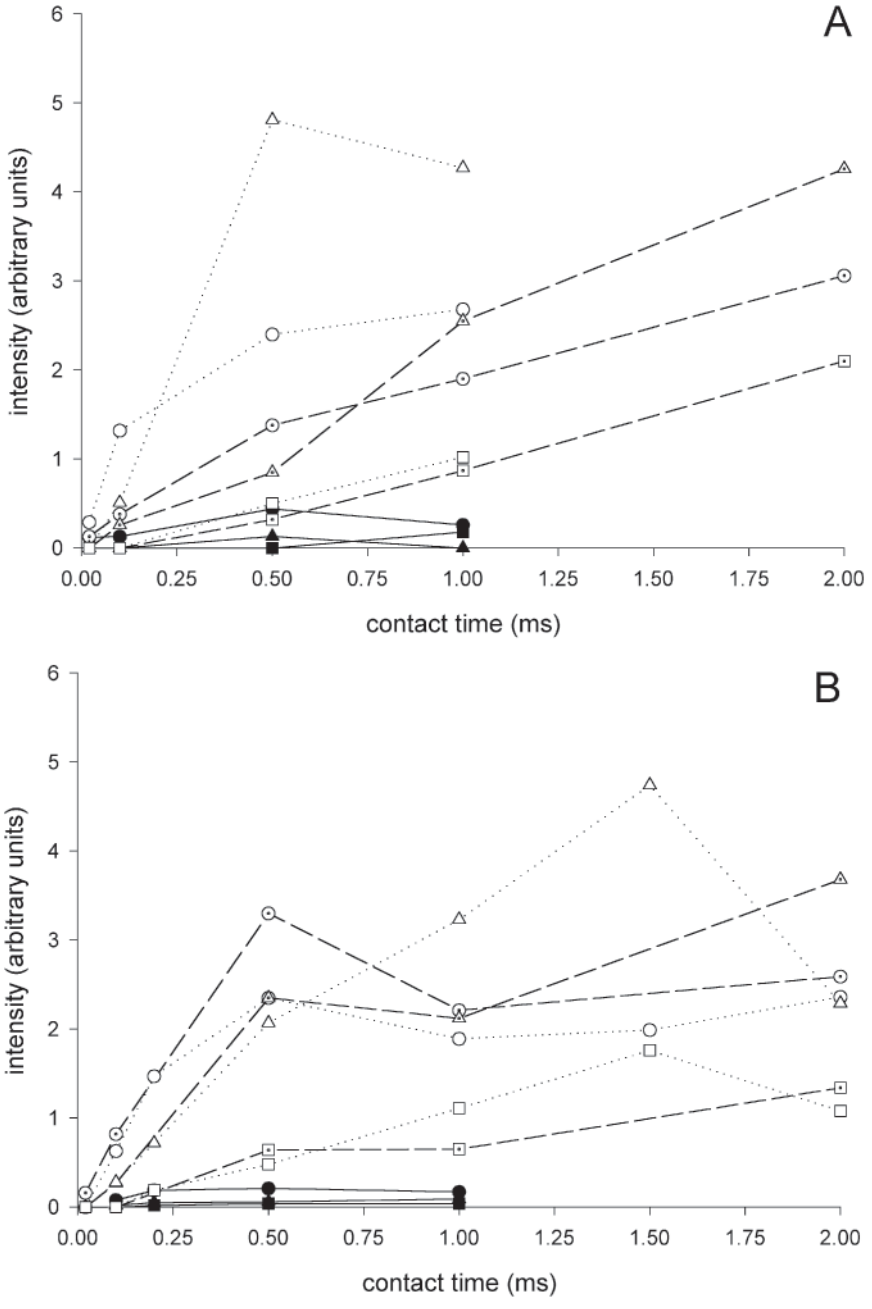

FIGURE 6 Intensity of several resonances as a function of contact time for samples with POPC (section A) and with POPS (section B). Phospholipid alone (*filled symbols, solid lines*), phospholipid with 0.2 mol fraction cholesterol (*dotted symbols, dashed lines*) and phospholipid with 0.5 mol fraction cholesterol (*open symbols, dotted lines*). (*Squares*) C═O at 174 ppm; (*circles*) C═C at 130.1 ppm; (*Triangles*) methyl at 14.2 ppm.

tion. Higher signal intensity is indicative of a greater degree of cross polarization.

To characterize the cross polarization for various samples, we systematically varied the contact time in the spectrum of each sample from 0.02 to 4 ms. The cross-polarized intensity will rise to a maximum with increasing contact times and then start to fall because of spin relaxation.

The rate of cross polarization of the carbon atoms from POPC generally increases approximately fourfold with the addition of 0.2 mol fraction cholesterol (Fig. 6). Increasing cholesterol to 0.3 (not shown) or 0.5 mol fraction further raises the rate of cross polarization in the case of POPC (Fig. 6). This is in accord with the well known ordering effect of cholesterol on phospholipid bilayers in the liquid crystalline state.

In the absence of cholesterol, the rate of cross polarization is similar for both POPC and POPS. However, upon adding 0.2 mol fraction cholesterol to either lipid, there is a

TABLE 1 Peak intensity by direct polarization for mixtures of 0.5 mol fraction cholesterol with POPC or POPS

		POPC		POPS	
Assignment	Chemical shift (ppm)	Number of carbon atoms	Relative observed intensity*	Number of carbon atoms	Relative observed intensity†
C═O	174.2	-	-	1	1.03
C═O	174.0	2	0.94	1	0.91
Serine COOH	172.2	-	-	1	1†
Cholesterol C5	142.3 to 140.8	1	0.45	1	0.26
Acyl C═C	130.1	1	0.82	1	0.94
Acyl C═C	129.6	1	0.95	1	1.05
Cholesterol C6	120.7	1	0.54	1	0.19
Quaternary CH_3	54.5	3	3‡	-	-
Terminal CH_3	14.22	2	2.08	2	2.12
Crystalline cholesterol C18	13.26	0	0	a§	0.305
Cholesterol C18	12.72	1	0.71	b§	0.147
Crystalline cholesterol C18	11.99	0	0	c§	0.284

*Relative to the quaternary ammonium methyl groups.
†Relative to the serine carboxyl group.
‡By definition.
§Sum of all three C18 resonances, a + b + c, should equal 1 (see text).
Delay time 100 sec

more rapid rate of cross polarization with POPS (Fig. 6) than with POPC. This indicates that with 0.2 mol fraction cholesterol there is less molecular motion in the POPS sample compared with the POPC sample.

Direct polarization

In principle it should be possible to quantify the number of carbon atoms from Bloch decay measurements (direct polarization, as in liquids, rather than cross-polarization). However, with more rigid groups this may become difficult to accomplish because of the long carbon T_1 s, requiring a long delay time to measure full peak intensity (Brainard and Cordes, 1981). To approach full peak intensity within a practical amount of accumulation time, we used a delay time of 100 s and an accumulation of approximately 3000 scans (3.5 days of acquisition time) (Fig. 1).

The spectra of POPC with 0.5 mol fraction cholesterol show a resonance at 54.46 ppm, corresponding to the three quaternary ammonium methyl groups of POPC. This resonance is not observed in the CP/MAS spectra, indicating that it has high mobility. With the long 100-s delay time, the carbon atoms of the POPC have close to the expected intensity relative to the quaternary ammonium methyl groups, except for the ester carbonyl (Table 1). However, most of the carbon atoms of cholesterol have about half of the expected intensity, suggesting that 100 s is still not long enough to measure the full resonance intensity. It would be expected that cholesterol would have more restricted rotation, and hence a longer T_1 in the slow motional regime, because of its fused ring system and slower axial rotation compared with the phospholipid. In the case of mixtures of POPS with 0.5 mol fraction cholesterol, all carbon resonances of POPS have intensities equal to those expected on the basis of the signal from the COOH in the headgroup, even the ester carbonyl. However, the signals from the cholesterol are even weaker than they are in mixtures with POPC.

The exception is the C18 methyl group where the sum of the three resonances assigned to the C18 group is 0.74 of full intensity. This is about the same relative intensity as found for the 12.7 ppm signal from the C18 of cholesterol in POPC. This comparison suggests that the motion of most of the cholesterol molecule, except near the C18 position, is more restricted in POPS than it is in POPC. This conclusion is supported by the fact that intensities of the cholesterol resonances are more markedly reduced using a 5- or a 10-s delay time, compared with small decreases that occur in the phospholipid signal intensities at the shorter delay time (not shown). This is particularly marked for the vinylic carbons of cholesterol, where the 140.9 ppm resonance is not observed at the shorter delay times. It is also not observed in direct polarization measurements of cholesterol monohydrate crystals with 5- or 10-s delay times (not shown).

The C18 signals can be used to estimate the fraction of cholesterol that is in crystalline form. One signal from this group, at 12.7 ppm, is observed in all samples of cholesterol mixed with either of the two phospholipids, including at low molar fractions of cholesterol. This peak corresponds to cholesterol "dissolved" in the membrane. The C18 resonances at 13.26 and 11.99 ppm are observed only with mixtures of POPS with higher molar fractions of cholesterol or with crystals of cholesterol monohydrate. These resonances therefore correspond to cholesterol crystals or to aggregates of cholesterol in the membrane that closely resemble cholesterol crystals. The ratio of the sum of the 13.26 and 11.99 peaks to the sum of the three C18 peaks is 0.800 (Table 1). Using delay times of 5 and 10 s, this ratio

is calculated to be 0.805 and 0.794, respectively. The independence of this ratio over a 20-fold range of delay times indicates that even though the intensity is only 0.74 that of the phospholipid, this is likely to be a result of the different motional properties of the two molecules, rather than because of the presence of a fraction of cholesterol with a very long T_1. There is, thus, a dramatic difference between POPC and POPS. POPC bilayers have no crystalline cholesterol at a cholesterol molar fraction of 0.5 yet POPS bilayers have 80% of the cholesterol in the form of monohydrate crystals.

It is interesting to compare the fraction of cholesterol estimated to be present as crystallites from the MAS NMR study with the estimate obtained from calorimetry. It is well known that cholesterol crystals can exist in two anhydrous forms, as well as the monohydrate (Loomis et al., 1979). With calorimetry, the most accurate estimate of the amount of cholesterol crystals in mixtures with phospholipids comes from measuring the enthalpy of the polymorphic transition of anhydrous cholesterol crystals that occurs at approximately 38°C upon heating. This is because this transition is relatively sharp and the anhydrous form takes at least several hours to undergo hydration to the monohydrate form, even in the presence of excess water. There is an additional complication in trying to estimate the amount of crystalline cholesterol monohydrate in mixtures with phospholipids from calorimetry. This is a result of the fact that over a period of many hours the transition temperature corresponding to the dehydration of cholesterol crystals shifts from ~75°C to 96°C (Epand et al., 2001a). The enthalpy of the transition at 96°C with POPS and 0.5 mol fraction cholesterol is 1.8 kcal/mol. Taking the value of 2.35 kcal/mol for the dehydration of pure cholesterol monohydate crystals (Loomis et al., 1979) it would be estimated that 77% of the cholesterol is in crystalline form. However, this estimate is uncertain because the dehydration transition of cholesterol in the presence of POPS occurs at a different temperature from that observed with pure cholesterol monohydrate crystals, and therefore it may also have a different enthalpy. After dehydration of cholesterol monohydrate by heating, only 46% of the cholesterol is detected as anhydrous cholesterol suggesting that either there is less crystalline cholesterol after dehydration or that DSC overestimates the amount of cholesterol monohydrate crystals in the samples having a dehydration transition at 96°C. The estimate from NMR for the amount of crystalline cholesterol monohydrate agrees well with that from calorimetry in aged samples. However, the samples used for MAS were not incubated for extended periods. We suggest that there are smaller domains of cholesterol monohydrate crystals that are difficult to detect by DSC at early times because of the broadness of the transition, but can easily be detected by NMR. It is possible that upon aging, these small domains coalesce to produce the 96°C melting form that has a higher degree of cooperativity and a higher observed transition enthalpy. This indicates that cholesterol monohydrate crystals are less miscible with POPS than are crystals of anhydrous cholesterol.

Time-dependent changes of cholesterol crystals

It has been shown that the ^{13}C CP/MAS NMR spectrum of crystals of the low temperature polymorph of anhydrous cholesterol is different from that of cholesterol monohydrate crystals (Guo and Hamilton, 1996). We have studied the time-dependent changes that occur upon incubation of samples of POPS with 0.5 mol fraction cholesterol. Immediately after suspension of the lipid film in buffer these samples contain cholesterol crystals at 25°C, largely in the form of the low temperature polymorph of anhydrous cholesterol. The hydration of cholesterol crystals occurs over a period of several hours (Loomis et al., 1979) and then, upon further incubation, another change takes place (Epand et al., 2001a). The nature of this transformation is not well characterized. No changes occur with pure cholesterol monohydrate crystals in the absence of a phospholipid. The rate of the transformation is slow, requiring days to complete, except for one reported case in the presence of added protein (Epand et al., 2001b). We further characterized the nature of the changes that result in altered dehydration properties of cholesterol monohydrate using ^{13}C CP/MAS NMR.

To obtain a good signal to noise ratio in CP/MAS NMR spectroscopy requires the accumulation of spectra over a period of ~6 h or more. The first spectrum was measured for 6 h after the sample had been hydrated, incubated for 30 min at 60°C, cooled, centrifuged, the pellet packed into an MAS rotor and brought to the NMR instrument. During the time of sample preparation plus the first 6 h of spectral accumulation, the major fraction of anhydrous cholesterol crystals was converted to cholesterol monohydrate. The changes due to hydration are most clearly seen in the resonances for the C5 (~140 ppm) and C6 (~120 ppm) of cholesterol (Fig. 7). The first spectrum, taken over the first 6 h, actually represents a mixture of anhydrous cholesterol and cholesterol monohydrate, as the sample is continually changing during the measurement. We are not concerned here with the kinetics of this initial hydration process, but rather the subsequent series of spectra taken after 6 h when most of the cholesterol crystals are in the form of the monohydrate. These spectra were measured during sequential 12-h periods and then compared with the spectrum of a sample that had been incubated for 3 weeks at 37°C. The position and shape of the peaks did not change with increasing time of incubation. However, there were measurable changes in the intensities of the peaks with time of incubation for some, but not for all, of the peaks (Fig. 8). The results demonstrate that several of the carbons of the C-ring of cholesterol, e.g., C9 and 14 and several of the carbons of the D-ring, e.g., C14 and 17 as well as the C18 methyl group of crystalline cholesterol monohydrate increase in intensity

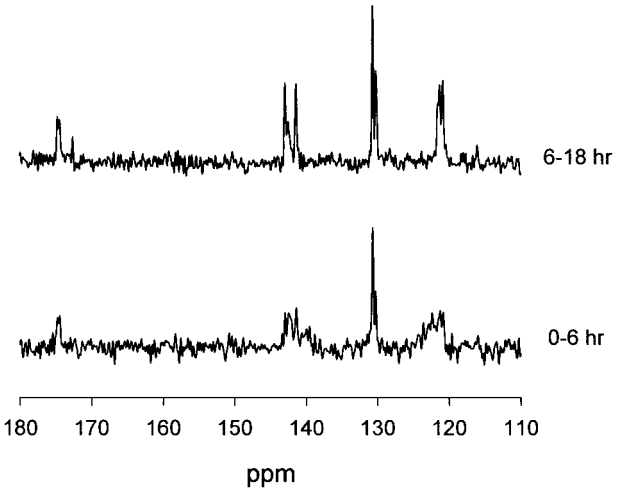

FIGURE 7 Conversion of anhydrous cholesterol to cholesterol monohydrate in a sample of POPS with 0.5 mol fraction cholesterol, maintained at a temperature of 25°C during spectral accumulation. The bottom spectrum was taken during the first 6 h after sample preparation and the top spectrum is of the same sample measured immediately following the first spectrum (*bottom*) and accumulating over the time period 6 to 18 h after sample preparation. Contact time was 1 ms.

with time of incubation, whereas resonances at either end of cholesterol, C3 of the A ring and C24 of the alkyl chain do not change with time, nor does the C18 methyl group of noncrystalline cholesterol. All of the spectra were recorded with a contact time of 1 ms, which is at or close to the contact time required for maximal intensity in most of the carbon atoms in most of the samples. The results indicate that the marked changes in DSC behavior of cholesterol monohydrate crystals incubated in the presence of POPS with excess buffer (Epand et al., 2001a) or in the presence of the protein NAP-22 (Epand et al., 2001b) does not involve a large change in structure. The cholesterol remains as the monohydrate, in agreement with x-ray diffraction results (Epand et al., 2001a). The observation that the intensity of certain resonances does not change with time suggests that there is no increase in the amount of cholesterol monohydrate crystals present in the samples but rather a change in the motional properties of certain groups. The change that occurs on incubation appears to be a tighter packing of the C and D rings in cholesterol stacks, resulting in a decrease in the molecular motion but only in this region of the cholesterol.

CONCLUSIONS

We have thus demonstrated that POPS and POPC interact differently with cholesterol, even at molar fractions in which cholesterol crystals are not formed. This is shown by the ability of cholesterol to induce splitting of the ester C═O of POPS but not of POPC. In addition, there is evidence for the presence of cholesterol crystals and/or immobilized cholesterol aggregates at 0.3 and 0.5 mol fraction cholesterol with POPS but no indication of this even at 0.5 mol fraction cholesterol with POPC. The more crystalline-like arrangement of cholesterol in POPS is indicated by the splitting of the vinylic carbon resonances of cholesterol (C5 and C6) as well as the appearance of cholesterol C18 resonances at chemical shifts coincident with those of authentic crystals. In addition, the lower intensity of the peaks from the vinylic carbon resonances of cholesterol in Bloch decays also indicates the greater rigidity of the sterol with POPS.

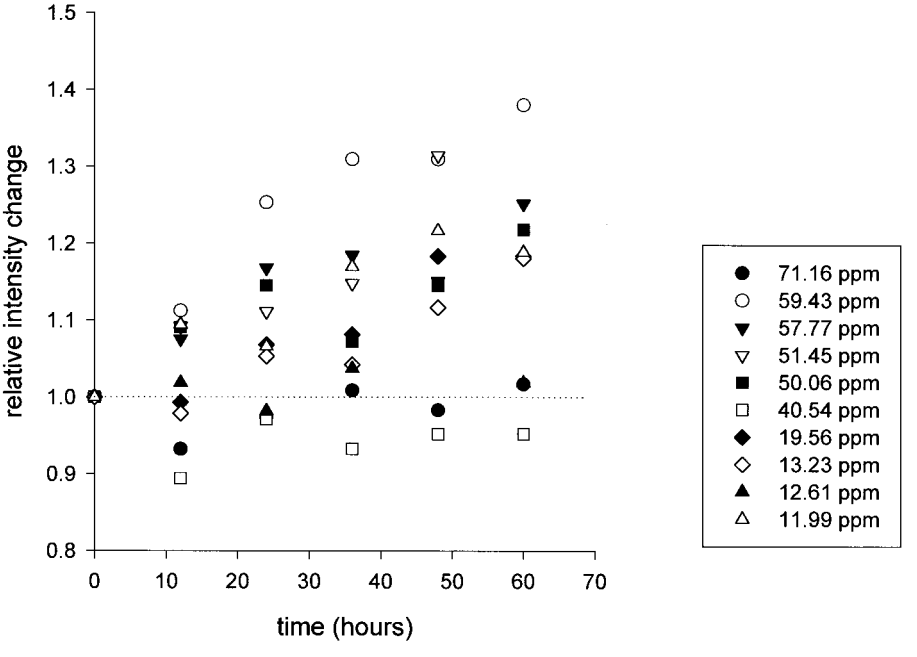

FIGURE 8 Time dependence of cholesterol ^{13}C CP/MAS NMR peak intensities of a sample of POPS with 0.5 mol fraction of cholesterol incubated at 25°C. The intensities are calculated as relative changes, normalized to 1 at zero time. Contact time 1 ms. The chemical shift corresponding to each symbol is indicated in the insert in the figure. These peaks are assigned to the following carbons of cholesterol: 71.16 ppm, C3; 59.43 ppm, C14; 57.77 ppm, C17; 51.45 ppm and 50.06 ppm, C9; 40.54 ppm, C24; 19.56 ppm, C16; 13.25 ppm, C18 crystalline; 12.61 ppm, C18 noncrystalline cholesterol; 11.99 ppm, C18 crystalline.

The molecular basis of the difference in behavior between POPC and POPS in its interactions with cholesterol is not simple to explain. The difference between these two lipids must be a consequence, either directly or indirectly, of the difference in the headgroup. There is evidence that cholesterol transfers less readily from PS-containing vesicles than from those containing PC (Leventis and Silvius, 2001; Niu and Litman, 2002). This has been interpreted to suggest that cholesterol has a stronger affinity for PS than for PC. However, the results of the present study indicate that the cholesterol has lower miscibility with PS than with PC. The reason that the sterol dissociates more slowly from PS-containing vesicles is because it is forming ordered, more stable, crystalline-like domains of sterol. It has been shown that PS has a relatively rigid headgroup structure compared with PC (Browning and Seelig, 1980). This may explain why the two C═O groups of POPS are more easily resolved in the presence of cholesterol, i.e., because exchange between the two environments is slow. However, there is evidence that the effect of lower solubility of cholesterol is not specific for PS but is also seen with other anionic lipids (Bach and Wachtel, 1989). In addition, the solubility of cholesterol is also sensitive to the acyl chain composition (Huster et al., 1998) and is not solely determined by the headgroup. Even a minor change, in going from POPS to 1-stearoyl-2-oleoyl phosphatidylserine, results in a significant change of cholesterol solubility (Bach et al., 1992). Thus, a much larger variety of structurally different lipids would have to be studied with regard to their interactions with cholesterol to further define the molecular details of these phenomena.

We are grateful to Bruker Canada and to Martine Monette for running several samples on the Avance 500 instrument. We are also grateful to Dr. Raquel F. Epand for helpful discussion. This work was supported by a grant from the Canadian Institutes of Health Research (MT-7654). Richard M. Epand is a Senior Investigator of the Canadian Institutes of Health Research.

REFERENCES

Alemany, L. B., D. M. Grant, R. J. Pugmire, T. D. Alger, and K. W. Zilm. 1983a. Cross polarization and magic angle spinning NMR spectra of model organic compounds: I. Highly protonated molecules. *J. Am. Chem. Soc.* 105:2133–2141.

Alemany, L. B., D. M. Grant, R. J. Pugmire, T. D. Alger, and K. W. Zilm. 1983b. Cross polarization and magic angle spinning NMR spectra of model organic compounds: II. Molecules of low or remote protonation. *J. Am. Chem. Soc.* 105:2142–2147.

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., and E. Wachtel. 1989. Thermotropic properties of mixtures of negatively charged phospholipids with cholesterol in the presence and absence of Li^+ or Ca^{2+} ions. *Biochim. Biophys. Acta.* 979:11–19.

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.

Batchelor, J. G., R. J. Cushley, and J. H. Prestegard. 1974. Carbon-13 fourier transform nuclear magnetic resonance: VIII. Role of steric and electric field effects in fatty acid spectra. *J. Org. Chem.* 39:1698–1705.

Brainard, J. R., and E. H. Cordes. 1981. Carbon-13 nuclear magnetic resonance studies of cholesterol-egg yolk phosphatidylcholine vesicles. *Biochemistry.* 20:4607–4617.

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

Browning, J. L., and J. Seelig. 1980. Bilayers of phosphatidylserine: a deuterium and phosphorus nuclear magnetic resonance study. *Biochemistry.* 19:1262–1270.

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.

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.

Epand, R. M., D. Bach, R. F. Epand, N. Borochov, and E. Wachtel. 2001a. A new high-temperature transition of crystalline cholesterol in mixtures with phosphatidylserine. *Biophys. J.* 81:1511–1520.

Epand, R. M., S. Maekawa, C. M. Yip, and R. F. Epand. 2001b. Protein-induced formation of cholesterol-rich domains. *Biochemistry.* 40: 10514–10521.

Fielding, C. J., and P. E. Fielding. 2000. Cholesterol and caveolae: structural and functional relationships. *Biochim. Biophys. Acta.* 1529: 210–222.

Forbes, J., J. Bowers, X. Shan, L. Moran, E. Oldfield, and M. A. Moscarello. 1988. Some new developments in solid-state nuclear magnetic resonance spectroscopy studies of lipids and biological membranes, including the effects of cholesterol in model and natural systems. *J. Chem. Soc. Faraday Transactions.* 84:3821–3849.

Guo, W., and J. A. Hamilton. 1993. Molecular organization and motions of cholesteryl esters in crystalline and liquid crystalline phases: a 13C and 1H magic angle spinning NMR study. *Biochemistry.* 32:9038–9052.

Guo, W., and J. A. Hamilton. 1996. 13C 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.

Holwerda, D. L., P. D. Ellis, and R. E. Wuthier. 1981. Carbon-13 and phosphorus-31 nuclear magnetic resonance studies on interaction of calcium with phosphatidylserine. *Biochemistry.* 20:418–423.

Huang, T. H., C. W. Lee, S. K. Das Gupta, A. Blume, and R. G. Griffin. 1993. A 13C and 2H nuclear magnetic resonance study of phosphatidylcholine/cholesterol interactions: characterization of liquid-gel phases. *Biochemistry.* 32:13277–13287.

Huster, D., K. Arnold, and K. Gawrisch. 1998. Influence of docosahexaenoic acid and cholesterol on lateral lipid organization in phospholipid mixtures. *Biochemistry.* 37:17299–17308.

Ipsen, J. H., G. Karlstrom, O. G. Mouritsen, H. Wennerstrom, and M. J. Zuckermann. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta.* 905:162–172.

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 choles-

terol 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.

Leventis, R., and J. R. Silvius. 2001. Use of cyclodextrins to monitor transbilayer movement and differential lipid affinities of cholesterol. *Biophys. J.* 81:2257–2267.

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

McLaughlin, A. C. 1982. Phosphorus-31 and carbon-13 nuclear magnetic resonance studies of divalent cation binding to phosphatidylserine membranes: use of cobalt as a paramagnetic probe. *Biochemistry.* 21: 4879–4885.

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 [In Process Citation]. *Biophys. J.* 79:2056–2065.

Montez, B., E. Oldfield, J. A. Urbina, S. Pekerar, C. Husted, and J. Patterson. 1993. Editing 13C-NMR spectra of membranes. *Biochim. Biophys. Acta.* 1152:314–318.

Niu, S.-L., and B. J. Litman. 2002. Lipid dependence of membrane cholesterol partitioning; a direct probe to differential cholesterol-lipid interactions. *Biophys. J.* 82:153a.

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.

Schroeder, F., G. Nemecz, W. G. Wood, C. Joiner, G. Morrot, M. Ayraut-Jarrier, and P. F. Devaux. 1991. Transmembrane distribution of sterol in the human erythrocyte. *Biochim. Biophys. Acta.* 1066:183–192.

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.

Urbina, J. A., B. Moreno, W. Arnold, C. H. Taron, P. Orlean, and E. Oldfield. 1998. A carbon-13 nuclear magnetic resonance spectroscopic study of inter-proton pair order parameters: a new approach to study order and dynamics in phospholipid membrane systems. *Biophys. J.* 75:1372–1383.

Vist, M. R., and J. H. Davis. 1990. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: 2H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry.* 29:451–464.

Warschawski, D. E., and P. F. Devaux. 2000. Polarization transfer in lipid membranes. *J. Magn. Reson.* 145:367–372.