brief communication

Phosphatidylcholine: cholesterol phase diagrams

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ABSTRACT Two mono-*cis*-unsaturated phosphatidylcholine (PC) lipid molecules, having very different gel-liquid crystalline phase transition temperatures as a consequence of the relative positions of the double bond, exhibit PC:cholesterol phase diagrams that are very similar to each other and to that obtained previously for a fully saturated PC:cholesterol mixture (Vist, M. R., and J. H. Davis. 1990. *Biochemistry* 29:451–464). This leads to the conjecture that PC:cholesterol membrane phase diagrams have a universal form which is relatively independent of the precise chemical structure of the PC molecule. One feature of this phase diagram is the observation over a wide temperature range of a fluid but highly conformationally ordered phase at bilayer concentrations of more than ~25 mol% cholesterol. This 'liquid ordered' phase is postulated to be the relevant physical state for many biological membranes, such as the plasma membrane of eukaryotic cells, that contain substantial amounts of cholesterol or equivalent sterols.

INTRODUCTION

The role of the bulk of cholesterol in a typical eucaryote is generally believed to be that of a passive modulator of membrane physical properties (Bloch, 1989) and biophysical studies of phospholipid:cholesterol model systems have been extensive in kind and number. The recent publication of a partial phase diagram for 1,2-di-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC): cholesterol (Vist and Davis, 1990) and the successful modeling of this phase diagram (Ipsen et al., 1987, 1989) indicate that the essential features of this particular membrane are now understood. The theoretical phase diagram of DPPC:cholesterol mixtures (Ipsen et al., 1989) is shown in Fig. 1 *a*. The major finding in the DPPC:cholesterol system is that at cholesterol concentrations above 22 mol% the membrane is in a 'liquid ordered' (*lo*) phase. In common with the gel or 'solid ordered' (*so*) phase of pure lipid bilayer systems, the acyl chains in the *lo* phase have restricted conformational freedom, i.e., the chains are relatively extended or "orientationally ordered". In contrast to the *so* phase, however, in which the molecular constituents of the membrane are locked into lattice positions for long times, the molecules in the *lo* phase have high diffusivity parallel to the plane of the membrane and undergo rapid rotational diffusion about the axis perpendicular to the plane of the membrane. In the absence of cholesterol, this enhanced diffusion is always accompanied by the onset of conformational freedom of the acyl chains, i.e., low orientational order, so that the normal fluid phase of pure lipid systems is appropriately described as the 'liquid disordered' (*ld*) phase. It is now well documented that the striking spectroscopic manifestations of two-dimensional fluidity (Vist and Davis, 1990) are correlated, in both the *lo* and *ld* phases, with the most distinctive and definitive feature of a fluid, namely the absence of shear restoring forces (Bloom et al., 1991). Thus, in the *lo* phase, which exists over a wide temperature range in the DPPC:cholesterol bilayer, the membrane is thick but not rigid. This is a state that biological membranes would likely favor since in such a membrane undesirable processes like passive ion leakage would be lessened with no loss in the lateral mobility needed for the proper functioning of membrane proteins.

It is natural to ask at this point whether the *lo* phase is really physiologically relevant or how one could systematically build up empirical evidence concerning this point. In addressing this question, it should be kept in mind that cholesterol or equivalent sterol molecules are mainly found in the plasma membranes of eucaryotic cells (Bloom and Mouritsen, 1988; Bloom et al., 1991). No procaryotic cell is known to synthesize sterols. Since mono-unsaturated lipids are commonly found in the plasma membranes of eukaryotic cells, we have extended the Vist and Davis (1990) study of the phase diagram of mixtures of a saturated lipid (DPPC) and cholesterol to mixtures of cholesterol with two different phosphatidylcholines (PCs) having one saturated and one *cis*-unsaturated chain: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or a related lipid, 1-palmitoyl-2-petroselinoyl-*sn*-glycero-3-phosphocholine (PPetPC). POPC is ubiquitous in plasma cell membranes of eucaryotes, but is difficult to study calorimetrically since its gel-to-liquid crystalline, or *so* to *ld* (liquid disordered), transition temperature (T_m) is below 0°C, more than fifty degrees less than its saturated analogue 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (Silvius, 1982). The second lipid studied, PPetPC, differs from POPC in the position of its unsaturation (at C6-7 instead of C9-10), which boosts its T_m to 16.8°C and makes its phase diagram amenable to determination in our laboratory both by deuterium nuclear magnetic resonance (^{2}H NMR) and differential scanning calorimetry (DSC). The variation of T_m with position of unsaturation has previously been observed for these lipids (Guyer and Bloch, 1983). We note, in passing, that of all possi-

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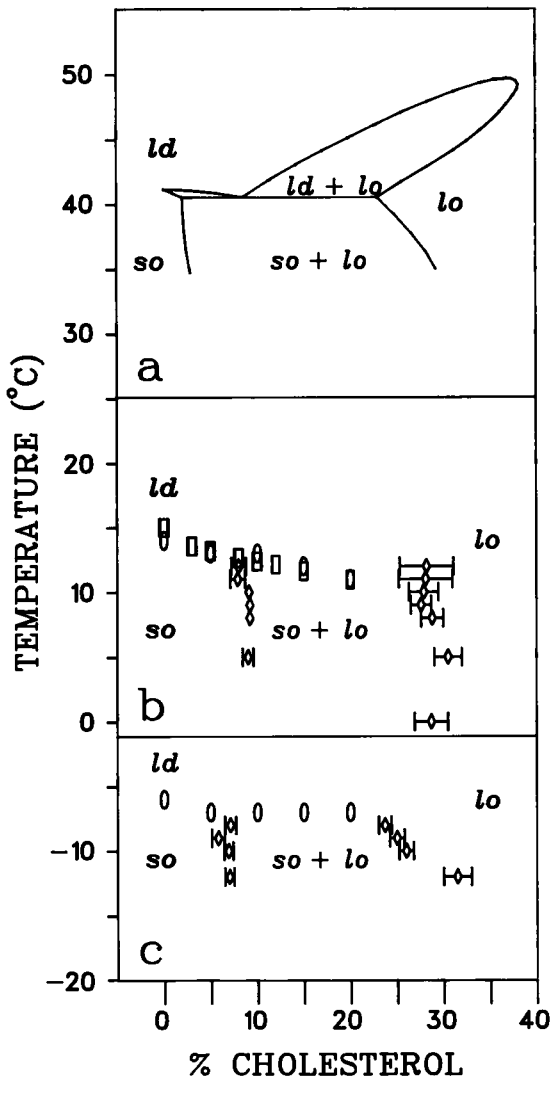

FIGURE 1 (*a*) Theoretical phase diagram for DPPC:cholesterol. (Fig. 1 of Ipsen et al., 1989, used with the authors' permission.) (*b*) Partial phase diagram of PPetPC-d_{31}:cholesterol obtained from ^{2}H NMR and DSC. (*c*) Partial phase diagram of POPC-d_{31}:cholesterol obtained from ^{2}H NMR. Note that the $T = -12$°C results may be inaccurate as the spectrum becomes distorted at these low temperatures. Phases are: *ld*, liquid disordered; *so*, solid ordered; *lo*, liquid ordered. (*Circles*) ^{2}H NMR T_m determined from examination of the spectra. (*Squares*) DSC T_m. (*Diamonds*) Phase boundaries determined from ^{2}H NMR spectral subtraction.

ble double bond positions in the homologous series of *cis*-dioctadecenoyl PCs, dioleoyl PC has the lowest T_m (Barton and Gunstone, 1975). It is likely that in analogous homologous lipids containing one saturated and one unsaturated acyl chain, the lipid having an oleic acid unsaturated chain has the lowest value of T_m. This may well be the reason why POPC is such a common lipid in the plasma membrane of eukaryotic cells.

As we shall show in the remainder of this paper, our experimental results from both calorimetry and ^{2}H NMR measurements indicate strongly that the *lo* phase is probably a universal phase of both saturated and monounsaturated PC molecules with cholesterol at cholesterol concentrations greater than ~25 mol%. This is the physiological range of concentrations for the plasma membranes of eukaryotic cells. The main features of the PC:cholesterol phase diagram that we are able to comment on in a definitive manner here are that: (*a*) at temperatures below the gel-to-liquid crystalline phase transition temperature (T_m) in the pure lipid system, the addition of cholesterol leads first to a miscibility gap involving *so* and *lo* phases and then to a homogeneous *lo* phase at higher concentrations; (*b*) as one goes from temperatures $T \ll T_m$ to $T \gg T_m$ in the *lo* phase there is no evidence for a phase transition. The orientational order of the acyl chains just decreases gradually as the temperature is increased over a wide range. We believe that these are the most important features of the phase diagram of Fig. 1 *a*.

We are not able to comment on the proposed miscibility gap involving the *ld* and *lo* phases in Fig. 1 *a*. This is, in fact, the most poorly documented part of the phase diagram. As mentioned in the Vist and Davis (1990) study, the ^{2}H NMR spectra cannot be represented as a superposition of spectra associated with the two liquid phases, presumably because of rapid molecular exchange between the two phases on the ^{2}H NMR time scale (Bloom et al., 1991). The Vist and Davis assignments for the phase boundaries of the *lo* + *ld* coexistence region were based on the end of the broad transition from calorimetry and on the widths of ^{2}H NMR peaks. Other information from the large literature on DPPC:cholesterol membranes, stemming from a variety of physical techniques, gave confidence in the assignment of these phase boundaries. The literature on unsaturated PCs with cholesterol is more sparse and hence we have chosen not to attempt to define this region of the phase diagram.

MATERIALS AND METHODS

POPC-d_{31} and PPetPC-d_{31} (both having perdeuterated palmitoyl chains) were obtained by custom synthesis from Avanti Polar Lipids Inc. (Birmingham, AL). Both phospholipids contained ~15% of the lipid with chains interchanged (OPPC-d_{31} and PetPPC-d_{31}) as byproducts of the synthesis. Cholesterol and deuterium depleted water were obtained from Sigma Chemical Co. (St. Louis, MO). To prepare the multilamellar lipid dispersions, aliquots of $CHCl_3$ stock solutions of phospholipid and cholesterol were mixed in the appropriate quantities. The $CHCl_3$ was evaporated under a thin stream of N_2 and the samples placed under high vacuum overnight. The resulting films were dissolved in benzene:methanol (95:5 vol:vol) and then lyophilized overnight. Samples were hydrated with a buffer of 20 mM Hepes, 300 mM NaCl, pH 7.4, made using deuterium depleted water, and mixed thoroughly.

^{2}H NMR spectra were obtained using the quadrupolar echo technique (Davis et al., 1976) with a home-built spectrometer (Davis, 1979; Sternin, 1985) operating at 46 MHz. The typical spectrum resulted from 10,000 repetitions of the two-pulse sequence with 90° pulse length 4 μs, inter-pulse spacing $\tau = 40$ μs and dwell time 2 μs. The delay between acquisitions was 300 ms, and data were collected in quadrature with Cyclops 8-cycle phase cycling.

^{2}H NMR spectral subtractions were carried out on area-normalized spectra. Occasionally the spectra were symmetrized by zeroing the out-of-phase channel to improve signal:noise before subtraction. The temperature dependence of the average inverse time constant, T_{2e}^{-1}, of the decay of the quadrupolar echo as a function of 2τ, was measured for the 5 and 30% cholesterol samples. T_{2e}^{-1} was calculated from the initial linear portion of the ln (echo height) vs. 2τ plot. This linear region extended to at least 200 μs, usually to 400 μs.

DSC measurements were made on a Microcal 2 calorimeter (Northampton, MA) with a Microcal 1 control unit which was interfaced via Optomux (Huntington Beach, CA) digitizer modules to an IBM PS2 computer. 25 mg of phospholipid in a total sample volume of 0.7 ml was used for each run, at a heating rate of 9° per hour.

RESULTS AND DISCUSSION

POPC-d_{31}:cholesterol and PPetPC-d_{31}:cholesterol multilamellar dispersions were prepared for cholesterol concentrations of 0, 5, 10, 15, 20, and 30 mol%. The temperature dependence of the ^{2}H NMR spectrum was measured from −15°C to 40°C for samples containing POPC-d_{31} and from −10°C to 40°C for samples containing PPetPC-d_{31}. In addition, PPetPC:cholesterol dispersions were prepared for DSC for cholesterol concentrations of 0, 3, 5, 8, 10, 12, 15, 18, 20, 25, and 30 mol%.

The theoretical DPPC:cholesterol phase diagram published by Ipsen et al. (1989) is shown in Fig. 1 *a* to facilitate comparison with our experimental findings about monounsaturated PC:cholesterol phase diagrams. At low concentrations of cholesterol the membrane undergoes a transition at T_m from a *so* (corresponding, for DPPC, to the ripple phase) to a *ld* state. At cholesterol concentrations of ~5 to 25 mol % there is coexistence of *so* and *lo* phases below T_m, and for cholesterol concentrations of ~10 to 25 mol% there is coexistence of *ld* and *lo* phases above T_m. T_m varies only slightly with cholesterol concentration below the eutectic point and is constant for higher cholesterol contents. At cholesterol concentrations greater than ~38 mol% the system is in the *lo* state at all temperatures shown.

Partial phase diagrams of PPetPC-d_{31}:cholesterol and POPC-d_{31}:cholesterol are shown in Figs. 1 *b* and *c*. In contrast to pure DPPC, whose main transition occurs over a temperature range of only half a degree, the main transition in both POPC-d_{31} and PPetPC-d_{31} spans two degrees. This is due to lipid heterogeneity: the presence of 15% of the lipid having transmigrated acyl chains causes the broadening. This heterogeneity adds an unwelcome complexity to the determination of the PC:cholesterol phase diagrams, since the three-phase line observed in the DPPC:cholesterol membrane at cholesterol concentrations of 8 to 20 mol% becomes a three-phase band 2–3° wide in our study. The lower boundary of this band is indicated in Fig. 1, *b* and *c*. Another consequence of this heterogeneity was that we were unable to observe the eutectic behaviour at low cholesterol concentrations that was reported by Vist and Davis (1990) for the DPPC:cholesterol system. In addition, it was not possible for us to distinguish with any certainty the boundaries of the liquid–liquid (*lo* + *ld*) coexistence region and they are not plotted. However, despite these complications, the phase diagrams of both POPC-d_{31}:cholesterol (Fig. 1 *c*) and PPetPC-d_{31}:cholesterol (Fig. 1 *b*) have several important features in common with the DPPC:cholesterol phase diagram.

We first discuss the PPetPC-d_{31}:cholesterol phase diagram, Fig. 1 *b*, determined using the complementary techniques of ^{2}H NMR and DSC. The DSC T_m's were obtained for nondeuterated PPetPC except for the cholesterol-free sample whose DSC scan was measured for both PPetPC and PPetPC-d_{31}. (No pretransition between the $L_{\beta'}$ and $P_{\beta'}$ phases, of the type found for DPPC and other diacylphosphocholine systems, was observed for these lipids. Presumably the acyl chain heterogeneity of the *cis*- monounsaturated phosphatidylcholines precludes the formation of the $P_{\beta'}$ phase (Tardieu et al., 1973).) The difference between the T_m's of the deuterated and nondeuterated lipid (1.8°) was then subtracted from all measured PPetPC:cholesterol T_m's before plotting. When this readjustment is made, the DSC T_m's correlate well with the temperatures at which the ^{2}H NMR spectra first display some *ld* phase. T_m decreases by nearly three degrees for cholesterol concentrations of 0 to 10%, then by only a little more than one degree for cholesterol concentrations of 10 to 20%. Up to 15% cholesterol the DSC scans show superpositions of both narrow and broad transitions as has often been observed with PC:cholesterol bilayers (e.g., Mabrey et al., 1978; Vist and Davis, 1990). For 18 and 20% cholesterol the narrow and broad transitions are no longer resolvable and for 30% cholesterol no transition peak is observed. At temperatures below T_m the ^{2}H NMR spectra at 10, 15, and 20% cholesterol are characteristic of mixed phases *so* and *lo*. Phospholipid molecules in these phases are assumed to exchange slowly enough that the ^{2}H NMR spectrum is a superposition of *so* and *lo* spectra. The two spectral components are weighted according to the proportion of phospholipid in each phase, which is calculated using the lever rule.

The edges of the two-phase region can be determined using ^{2}H NMR spectral subtraction (illustrated in Fig. 2 for POPC-d_{31}:cholesterol and described in detail by Vist and Davis, 1990). For PPetPC-d_{31}:cholesterol, pair-wise subtractions of the three mixed-phase samples' ^{2}H NMR spectra (10, 15, and 20% cholesterol) were carried out between 0° and 12°C. By subtracting a fraction K of the normalized spectrum of the sample richer in cholesterol from that of the low cholesterol sample it is possible to obtain a pure *so* spectrum. The criterion for determining the value of K was to maximize the 'featurelessness' of the resulting spectrum, e.g., removing the sharp methyl peaks and vertical plateau 'edges' found in spectra of fluid membranes. This *so* end-point spectrum is the spectrum which would be obtained from a sample hav-

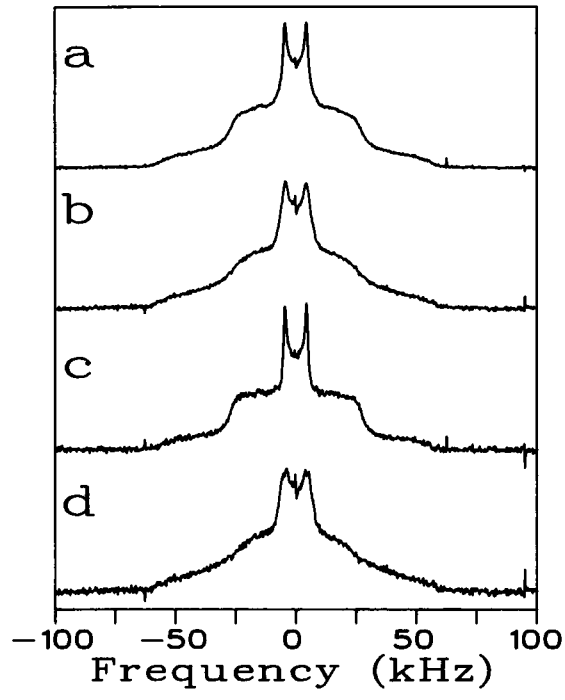

FIGURE 2 (*a*) POPC-d_{31}:cholesterol (85:15 mole ratio), $T = -9°C$; (*b*) POPC-d_{31}:cholesterol (90:10 mole ratio), $T = -9°C$; (*c*) Spectrum *a* minus 0.50 times spectrum *b*, yielding the 'liquid ordered' phase end-point spectrum, apparent $x_f = 0.2$; (*d*) Spectrum *b* minus 0.37 times spectrum *a*, yielding the gel phase end-point spectrum, apparent $x_s = 0.07$.

ing a cholesterol concentration matching the cholesterol concentration, x_s, of the 'solidus' or *so*/*so* + *lo* phase boundary. (Subtracting too large a proportion of the more fluid spectrum resulted in unphysical, narrow features protruding into the smooth gel spectrum.) The opposite procedure, subtracting a fraction K' of the low cholesterol spectrum from the high cholesterol sample's spectrum yields a pure *lo* spectrum characteristic of a sample having the cholesterol concentration x_f of the 'liquidus' or *so* + *lo*/*lo* phase boundary. K is the ratio of *lo* phase phospholipid fractions in the two samples and K' is the ratio of the *so* phase phospholipid fractions. The end-point cholesterol concentrations x_s and x_f can be calculated using the experimentally determined values of K and K'. Spectral subtractions using the 10 and 15% cholesterol samples and those using the 10 and 20% cholesterol samples gave consistent x_s and x_f values, which are plotted in Fig. 1 *b*. The subtractions using the 15 and 20% cholesterol samples gave x_s values that were unreasonably high (phase boundary at 11%) and were therefore not included in the average value of x_s which is plotted. A similar problem was observed recently (Morrow et al., 1991) for a mixture of PCs of different acyl chain lengths. A likely explanation for the systematic errors incurred in determining the solidus concentration from two samples that are mainly fluid is that the assumption of no exchange between the solid and fluid phases breaks down. Another possibility is that in the mainly fluid mixtures the *so* domains are small enough that the *so* ^2H NMR spectrum is dominated by the signal from lipid molecules near the boundaries of the domains, which conceivably give a different spectral shape. These ideas are currently under investigation, but it should be emphasized that the details mentioned here in no way change the topology of the phase diagram.

Another consideration involving the ^2H NMR subtraction method is that it assumes that both phases have the same relaxation time, which is not generally true. Again, this does not fundamentally alter the form of the phase diagram, but only the positions of the *so* + *lo* phase boundaries. We measured T_{2e} as a function of temperature for a sample containing 5 mol% cholesterol, and, therefore, in a homogeneous *so* phase for temperatures $T < T_m$, and for one homogeneous *lo* phase sample containing 30 mol% cholesterol. We then corrected the 'raw' K and K' values by using the different relaxation times obtained for the *so* and *lo* phases to calculate new fluid PPetPC-d_{31} fractions in the two samples and hence new K and K' values, which gave the corrected x_s and x_f values plotted in Fig. 1. The correction to x_s was less than 0.01 in all cases and the correction to x_f was at most 0.067. These corrections caused both x_s and x_f to increase, since for PPetPC-d_{31}:cholesterol the relaxation time of the *so* phase was always shorter than that of the *lo* phase, so that a given mixed-phase spectrum would appear to contain less *so* phospholipid than was actually the case. Note that when making the T_{2e} corrections we made no allowance for variation of T_{2e} with position in the spectrum (Nezil et al., 1991), but based the corrections solely on the average T_{2e}.

The POPC-d_{31}:cholesterol partial phase diagram is shown in Fig. 1 *c*. Pure POPC-d_{31} had a T_m of −6°C. Upon adding 5 to 20 mol % cholesterol to POPC-d_{31}, T_m is reduced by one degree and is constant over this range of cholesterol concentrations, in direct agreement with the DPPC-d_{62}:cholesterol phase diagram which displays $T_m = 37°C$ over a similar range of cholesterol concentrations. Below −7°C the POPC-d_{31}:cholesterol (5 to 20 mol%) system is a mixture of *so* and *lo* domains, giving a mixed-phase ^2H NMR spectrum amenable to the subtraction techniques discussed above. The range of temperatures over which the subtractions could be performed was limited since at temperatures below −12°C the *lo* phase spectrum became distorted, presumably due to the sluggish rotation of the lipid acyl chains. This means that the correlation time for molecular rotation becomes of order or larger than 10^{-5}s at temperatures below −12°C. It would be interesting to check, via micromechanical measurements, below what temperature the macroscopic fluidity becomes manifestly sluggish. The distorted *lo* spectrum was too similar in appearance to the *so* spectrum to allow reliable subtractions. In fact

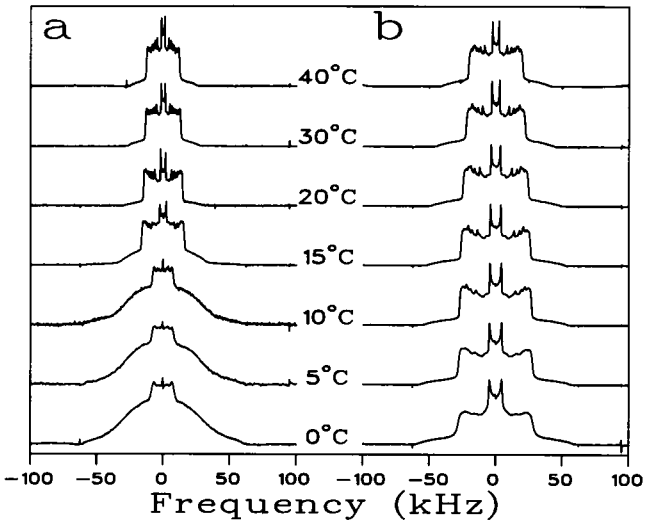

FIGURE 3 (*a*) ^{2}H NMR spectra of PPetPC-d_{31} as a function of temperature; (*b*) ^{2}H NMR spectra of PPetPC-d_{31}:cholesterol 70:30 as a function of temperature.

it is likely that the slope of the liquidus to higher cholesterol concentrations and lower temperatures results largely from difficulties with the x_f determination at −12°C. Fig. 2 illustrates the ^{2}H NMR spectral subtraction technique at −9°C.

The x_s's and x_f's determined for PPetPC-d_{31}:cholesterol are fairly constant over the temperature range studied: $x_s = 0.091 \pm 0.002$ and $x_f = 0.285 \pm 0.006$. The T_{2e}-corrected POPC-d_{31}:cholesterol x_s and x_f values were also found to be roughly constant at 0.068 ± 0.003 and 0.253 ± 0.004, respectively. These phase boundaries are closely comparable to the DPPC-d_{62}:cholesterol solidus of 0.075 and liquidus 0.22 (these values were not T_{2e} corrected). The agreement between the three PC:cholesterol partial phase diagrams is remarkable considering that the three PCs involved have very different T_m's and also differ in their degree and position of acyl chain unsaturation and the length of the *sn*-2 chain. Additional data regarding possible liquid–liquid coexistence in these systems would be most welcome.

At cholesterol concentrations greater than the liquidus value (i.e., 30 mol% cholesterol for our data) all three PC:cholesterol systems are in a homogeneous *lo* phase over the range of temperatures studied. This phase is illustrated in Fig. 3, which compares the thermotropic changes in the ^{2}H NMR spectrum of PPetPC-d_{31} and PPetPC-d_{31}:30 mol% cholesterol. Fig. 3 *a* shows that the pure lipid system is in the *so* phase below 15°C, in a mixed *so*/*ld* state at 15°C and in the *ld* phase at higher temperatures. Fig. 3 *b* shows the spectrum of the *lo* phase from 0 to 40°C. Comparing the *lo* and *ld* phase spectra it is apparent that they are both superpositions of Pake doublets, characteristic of the rapidly reorienting acyl chains in a fluid membrane. The *lo* phase spectral peak separations are much larger than those in the *ld* phase, however. For example at 40°C the spectral width observed for the 30 mol% cholesterol membrane is 1.7 times that of the pure PPetPC-d_{31} membrane.

CONCLUSIONS

These observations indicate that the action of cholesterol in generating the liquid ordered phase in phosphatidylcholine model membranes is quite general. Since the *lo* phase exists for a wide range of different lipids for ≥25 mol% cholesterol concentrations, the phase behavior of even a mixture of very many different types of lipids in a bilayer system with sufficient cholesterol is bound to be simple, namely a single homogeneous *lo* phase over a wide range of temperatures. It is probable therefore that the liquid ordered phase is found in most biological membranes containing more than 25 mol% cholesterol as a fraction of total lipid. Indeed, the capacity of lipids to form the *lo* phase may have been a prerequisite for the development of many plasma membrane functions in the evolution of eukaryotic cells (Bloom and Mouritsen, 1988).

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