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Low hydration phase properties of phospholipid mixtures

Evidence for dehydration-induced fluid-fluid separations

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Abstract. An experimental investigation of the low hydration phase properties of phospholipid mixtures is described. ^2H (D_2O) NMR, X-ray diffraction and differential scanning calorimetry have been used to elucidate the phase properties of mixtures of the mixed chain phospholipids palmitoyloleoylphosphatidylcholine (POPC) and palmitoyloleoylphosphatidylethanolamine (POPE). At 10% hydration pure POPE exhibited a H_{II} phase above 330 K, a fluid lamellar phase below 315 K, and a minimally hydrated crystalline phase below 300 K. For the 1:1 mixture, the samples exhibited only gel or fluid phases between 270 K and 360 K for hydrations in the range 15% to 30%. Below 15% hydration the mixture exhibited two fluid phases with different repeat spacings, as predicted previously.

Key words: Phase separations – Membrane dehydration – Phospholipid phases – Lamellar phase – Inverse hexagonal phase – Mixed chain lipids – Deuterium NMR

Introduction

The phase properties of phospholipids have been studied extensively for over twenty years (Luzzati 1968; Chapman 1968). Most of these studies have examined the phase properties of saturated lipids in the presence of excess water. Many biological membranes (particularly those of plants) contain substantial fractions of unsaturated and mixed-chain lipids. Only a small number of unsaturated lipids, and an even smaller number of mixed-chain lipids have been studied to date. Studies of the phase properties at low hydration are also rare: perhaps because dehydration occurs relatively rarely in biological tissue.

There are, however, two areas where membrane behaviour at low hydration is an important consideration for cell survival – anhydrobiology and cryobiology. For instance there are some species of plants and animals which can survive hydrations of several percent by weight at ambient temperature (see Crowe and Clegg 1973; Leopold 1986). The seeds of most plants are similarly tolerant of quite low levels of hydration. It has been shown that one important form of freezing damage in some biological tissues is due to freeze-induced dehydration rather than the direct effects of freezing per se (see Burke et al. 1976; Morris and Clarke 1981; Steponkus 1984). In both cases the loss of semipermeability of the cell membrane at low hydrations is held responsible for tissue damage. The study of the phase properties of membranes at low hydration is therefore important in understanding damage due to dehydration and freezing.

Freeze-fracture electron microscopy studies have suggested that lateral phase separation of membrane components and the formation of the inverse hexagonal phase (hereinafter H_{II})[1] can be correlated with the incidence of dehydration-induced damage in some cells (Crowe and Crowe 1982; Crowe et al. 1983a, b; Steponkus 1984; Gordon-Kamm and Steponkus 1984). The formation of the H_{II} phase in a cell membrane may be lethal, as the geometry of such a phase is unsuitable for maintaining the barrier between the cell and the external solution. The formation of the H_{II} phase is governed by the lipid composition of the membrane. Lipids which naturally form the H_{II} phase revert to the bilayer phase upon the addition of a relatively small fraction of bilayer forming lipids[2]. Thus it is likely that membranes which are com-

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[1] Abbreviations used: NMR – Nuclear magnetic resonance. DSC – Differential scanning calorimetry, PC – Phosphatidylethanolamine, POPC – Palmitoyloleoylphosphatidylcholine, POPE – Palmitoyloleoylphosphatidylethanolamine, DOPC – Dioleoylphosphatidylcholine, DOPE – Dioleoylphosphatidylethanolamine, H_{II} – Inverse hexagonal, L_α – Fluid lamellar, L_β – Gel lamellar

[2] For example the addition of only 5% DOPC to DOPE raises the L_α–H_{II} transition temperature by 6 K (Tate and Gruner 1987; see also Seddon 1990, and references therein)

posed of mainly bilayer forming species will not undergo the transition to the H_{II} phase even under extreme conditions. Crowe et al. (1983a, b) and Gordon-Kamm and Steponkus (1984) argued that lateral separations of membrane components may be a precursor to the formation of the H_{II} phase in these membranes, by concentrating those lipids which are naturally predisposed to H_{II} formation (e.g. phosphatidylethanolamine).

Lateral phase separations of lipids at high hydration can be induced by a number of conditions, including the presence of divalent cations (Tamura-Lis et al. 1986), alcohol (Rowe 1987) or cholesterol (Rand et al. 1980; Knoll et al. 1985). Some phospholipid mixtures can exhibit fluid-fluid separations at high hydrations (Wu and McConnell 1975). However, the induction of phase separations solely as a result of dehydration requires other mechanisms.

Two such mechanisms have been proposed. The first is the gel-fluid phase separation of lipids with different melting points[3] (Crowe and Crowe 1982; Crowe et al. 1983a, b). The chain melting transition temperatures of phospholipids increase as a function of dehydration, causing some lipid chains to freeze during dehydration at constant temperature. Crowe, Crowe and co-workers argued that in a mixture of phosphatidylethanolamine (PE) and phosphatidylcholine (PC), dehydration will induce the chains of the lipid with the higher melting point (usually PE[4]) to freeze, thus concentrating the PE, which would then be free to undergo the transition into the H_{II} phase. There are some problems with this suggestion, however. First, most PEs studied to date do not generally have a direct transition from the gel lamellar to the H_{II} phase, but require an intermediate fluid lamellar phase. Thus the conditions which would allow separation of the PE by freezing are not usually those which encourage H_{II} formation. This is not true for all lipids however[5], so this objection is by no means fatal.

Another problem concerns the nature of the freezing transition in biological membranes. Although the phase diagram of a biological membrane is unknown, the presence of a large range of different lipid headgroups and tailgroups (Lynch and Steponkus 1987) suggests that any domain of lipids which freezes out is likely to contain many different species, and the concentration of PE in such a phase may not be very high.

Another proposed mechanism of separation at low hydration is phase separation of two fluid lamellar phases due to the differing hydration repulsions of two (or more) lipid species (Bryant and Wolfe 1989). When a lipid/water mixture has been dehydrated to the point where the lipid bilayers are of the order of nanometres apart, further dehydration is opposed by strong repulsive forces[6] (LeNeveu et al. 1976; Israelachvili 1977). These forces are approximately exponential, and the strength and decay length of the force are characteristic of the lipid species. We have previously predicted that, under dehydration, two lipids with sufficiently different strong repulsive forces would spontaneously separate into two fluid lamellar phases with different compositions and water separations (Bryant and Wolfe 1989).

In this paper, deuterium NMR, X-ray diffraction and differential scanning calorimetry are used to study the phase properties of mixtures of palmitoyloleoylphosphatidylcholine (POPC) and palmitoyloleoylphosphatidylethanolamine (POPE) as a function of hydration and temperature. These are mixed chain unsaturated lipids which occur in the membranes of some plants (Lynch and Steponkus 1987) and are thus more representative of botanical lipids than most lipids studied to date. It is shown that there is a regime of low hydration where this mixture exhibits fluid-fluid phase separations.

Experimental

NMR

High purity (>99%) phospholipids were purchased from Avanti Polar Lipids (Pelham, Alabama) and were used without further purification. The lipids were stored in chloroform solution until needed. Mixtures were made up using known solution concentrations and flame sealed in glass until use. Enough solution to make up 20–50 mg of lipid was added to a pre-weighed sample tube and dried under a stream of nitrogen gas until most of the liquid was removed. The tube was then placed in a desiccator in the presence of P_2O_5, evacuated to about 0.1 Pa and left overnight to remove all traces of chloroform. The dry samples were then transferred to NMR tubes. Two preparation methods and two sets of experimental apparatus were used.

In the first method, a known volume of D_2O was added to the tube, which was flame-sealed, and the sample was allowed to hydrate for 1–3 days at a temperature where the lipid was in the fluid state, with repeated centrifugation using alternate sample orientations. The experiments were carried out on a Bruker CXP-300 Spectrometer which operates at 46.062 MHz for deuterium. A standard multinuclear probe with a home-made 5 mm solenoid coil was used to give a uniform field and better temperature control. 2000–10000 scans were accumulated for each temperature. A $90°$-τ-$90°_{90}$ quadrupolar echo technique was found to give identical results to a single $90°$ pulse ($\sim$5.6 ms duration) for these linewidths, so the latter was used for most experiments.

In the second method the samples were hydrated to excess in D_2O and then dried slowly in a vacuum oven at

[3] Note that these separations can also occur at high hydrations and sufficiently low temperatures

[4] For lipids with the same chain length PE will freeze at a higher temperature (or hydration) than PC

[5] Some lipids can transit from the gel or crystalline phases directly to the H_{II} phase under some conditions (e.g., Seddon et al. 1984; Quinn and Lis 1986; Seddon 1990)

[6] These forces are generally called "hydration" forces (LeNeveu et al. 1977; reviewed by Bryant and Wolfe 1992). However their origins are disputed (Israelachvili and Wennerström 1990, 1992)

about 300 K. The samples were in standard high resolution NMR tubes, and the same sample was used over several weeks for NMR runs, and dehydrated between runs. The experiments were carried out on a Varian XL-400 spectrometer operating at 61.387 MHz for deuterium, using a single 45° pulse of about 18 ms duration, and 512–2048 scans were accumulated per temperature.

Temperature control for both systems was achieved using the inbuilt controllers, and cooling achieved by passing pre-cooled compressed air or N_2 gas through the probe jacket. During temperature runs, 20 min equilibration time was usually allowed between temperatures. Experiments showed that the phase transitions observed for these lipids occurred in a period of the order of one minute. Samples were tested for purity after all runs with thin layer chromatography using pre-coated TLC plates (Merck, NJ, USA). The solvent mixture used was chloroform:methanol:acetic acid:water (85:15:15:3), and iodine vapour was used as the developer.

X-ray diffraction

Sample preparation was as above. The experiments were carried out on two machines. The first was a Hilger and Watts model Y-33 Microfocus X-ray generator, using the copper K_α line at 1.54 Å. The samples were placed in 1 mm glass capillaries, and the spectra recorded on X-ray sensitive film (Fuji). Temperature was controlled by blowing pre-heated compressed air through the sample chamber, and the variation measured using a max–min thermometer.

The second set-up was a small angle X-ray scattering (SAXS) machine described elsewhere (Aldissi et al. 1988; White et al. 1986) which also used the copper K_α line. The detector was a one-dimensional position sensitive counter (Gabriel 1977) interfaced to a computer and analysed using in-house software (Hayter and Henderson 1988). The sample was placed in a home-made cell enclosed between mylar windows, and placed in a copper block through which was circulated a coolant from a temperature controlled bath.

Differential scanning calorimetry (DSC)

DSC was carried out using either a Dupont differential scanning calorimeter or a Perkin Elmer DSC-7. Calibration was achieved using indium, cyclohexane and millipored water (Milli-Q system, Millipore, Molsheim, France), and the samples were run at a scanning rate of 10 K/min for both cooling and warming runs. The scanning rate was chosen as a compromise – slow scanning rates allow more homogeneous sample temperatures, but give less resolution. High scanning rates broaden the exo/endotherms, but give greater signal to noise ratio and resolution, and also allow more repetitions when availability of the apparatus is limited. We used a relatively high rate in these studies because the exotherm at low, fixed water content is already broadened (see discussion). Samples were taken after NMR and/or X-ray analysis or The onset and peak transition temperatures were measured for each scan, and the results presented are the average of at least 4 runs.

Theory

Phase separations: the theory of dehydration-induced fluid-fluid phase separations was presented by Bryant and Wolfe (1989). The essential elements of that theory are outlined below.

The origins of the large short range repulsive forces observed between lipid bilayers are still debated. Rand, Parsegian and co-workers suggested that the forces are due to the hydration structure near the interface (see Rand and Parsegian 1989 for a recent review). Theoretical models based on this idea have met with some success (see review by Marčelja 1990). However, it has been proposed recently that the addition of the osmotic, electrical and thermal motion forces can explain the observed phenomenon without introducing extra forces (Israelachvili and Wennerström 1990, 1992). The origins of the forces are discussed further in the light of the experimental results.

The large short range repulsive force per unit area[7] P between bilayers can be represented empirically by the form (LeNeveu et al. 1977)

$$P = P_0 \exp(-y/\lambda), \tag{1}$$

where P_0 is the force per unit area at bilayer contact, y is the interbilayer separation and λ is the decay length of the force. In a lamellar phase with a repulsion P between the lamellae, Newton's first law (normal to the bilayer) implies that the pressure in the water is $\mathrm{P} = -P$. The pressure P is negative (i.e. a suction[8]), and Newton's law parallel to the bilayer introduces a compressive stress, and causes the bilayers to be compressed laterally. The integral of this stress over thickness gives the lateral two dimensional stress π (a compressive force per unit length). Newtons's first law in the plane of the bilayer requires that the lateral pressure must equal the water thickness (y) times the pressure:

$$-\pi = y\,\mathrm{P} = -P\,y. \tag{2}$$

In a mixture of two lipids the chemical potential μ (the Gibbs free energy per molecule) for lipid species i may be written in different ways. If one treats the membrane as quasi-two dimensional, then:

$$\mu_i = \mu_i^0 + kT \ln X + U + \frac{\pi a_i}{2} \tag{3}$$

where μ_i^0 is the standard chemical potential, $k \ln X$ is the entropy, U is the total potential energy, π is the lateral stress and a_i is the area per lipid molecule. The final term can be regarded as the two dimensional equivalent of a

[7] Italic P is used for force per unit area, P is reserved for pressure

[8] Note that cavitation is not likely to occur as cavitation nuclei are rare in such small volumes. and the thickness of the aqueous regions is small compared to the critical radius for cavitation except for very large suctions

PV term. Alternatively, one could interpret this term as the work done by one phase on the other (PV) were V is the water volume associated with each lipid molecule = $a\,y/2$. From Eq. (2), PV = $\pi a/2$ so the two interpretations give the same expression. The potential energy U is that due to the strong repulsive force, and is simply one half of the integral of the total force per molecule with respect to the water separation y.

If we assume that only the strong repulsive force is important (true for low hydrations) and that the lipids are an ideal solution in two dimensions, then for a mixture of two lipids with different values for P_0 and the same λ, a phase separation of two phases with different compositions (X of the less repelling lipid and X' of the more repelling lipid) and different water separations can exist when the following conditions are satisfied (see Bryant and Wolfe 1989[9]):

$$\alpha \ln\left(\frac{X}{X'}\right)\left[2\ln\left(\frac{D}{D'}\right)+\frac{\beta}{D}-\frac{\beta}{D'}\right] = \ln\left(\frac{1-X}{1-X'}\right)\left[2\ln\left(\frac{D}{D'}\right)+\frac{1}{D}-\frac{1}{D'}\right]$$

$$P\frac{a_1\lambda}{kT} = \frac{-2\ln\left(\frac{X}{X'}\right)}{2\ln\left(\frac{D}{D'}\right)+\frac{1}{D}-\frac{1}{D'}} \qquad (4)$$

where

$$D = D(X) = \frac{X+\alpha\beta(1-X)}{X+\alpha(1-X)} \quad \text{and} \quad D' = D(X').$$

$$\alpha = \frac{a_2}{a_1},\ \beta = \sqrt{\frac{P_{02}}{P_{01}}}$$

Results and discussion

Differential scanning calorimetry

Figure 1 shows several typical DSC scans during warming for samples of POPE, POPC and POPC:POPE 1:1. The transition temperatures for these samples in excess D_2O, 10% D_2O and unhydrated are given in Table 1. The stated errors are the standard deviations from at least 4 runs. The result in parentheses are from fewer than four experiments. For the 1:1 mixture in excess water, the DSC scans showed some variability, sometimes exhibiting two separate endotherms (peak temperatures shown), and other times exhibiting one broad endotherm with an intermediate peak transition temperature. Because DSC does not measure the sample in equilibrium, the equilibrium transition temperatures will be slightly lower (<2 K) than those measured using this technique for endotherms.

The transitions are broadened for three reasons: first, some of the samples are lipid mixtures which phase separate over a finite range. Second, the constraint of low, fixed water content implies a finite temperature range for the transitions of single lipid-water phases [in a sealed container with a constant water content, the transition does not take place at a constant value of the chemical potential for water (unless there is excess water present), and this causes the transition to broaden (Lynch and Steponkus 1989; Bryant 1991)]. Third, there is the instrumental effect of finite cooling rates of the DSC apparatus (see experimental section). If the sample were known to be always in equilibrium, the onset temperature would be the most reliable measure of the phase transition temperature. However, for many of the samples studied here there are large variations in the value of the onset temperature, caused by metastability of the gel phase (see later). For this reason the peak temperature was the most reproducible measurement for some samples[10].

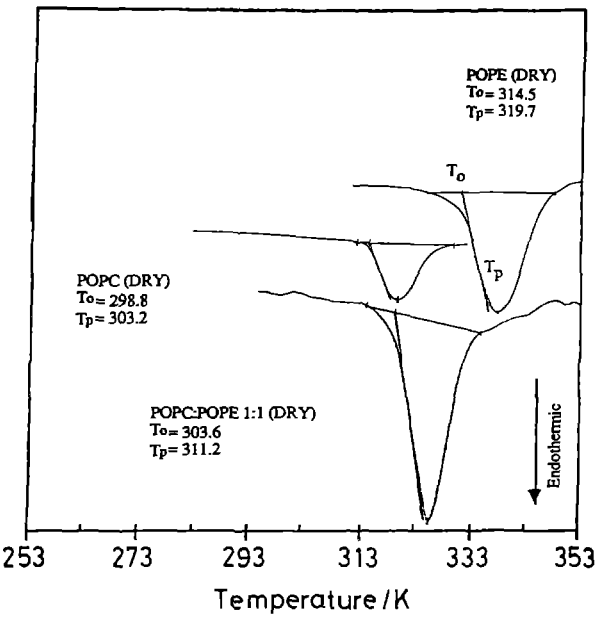

Fig. 1. DSC scans for POPC, POPE and POPC:POPE 1:1 without water. These scans are taken during warming to avoid supercooling. Samples with water also show a water endotherm near 273 K

Table 1

Lipid	Hydration	Onset temperature [K]	Peak temperature [K]
POPE	0 (nominal)	315.52±1.44	320.94±2.36
	10%	308.23±0.34	314.99±1.05
	excess	291.02±1.70	297.28±0.69
POPC	0 (nominal)		(341.15)
	10%	284.21±0.34	293.47±0.19
	excess	271.09±0.77	272.95±0.77
POPC:POPE 1:1	0 (nominal)	303.78±1.55	310.96±0.48
	10%	293.51±1.19	304.94±0.70
	excess		(277.59)
			(283.86)

[9] Note that this differs from equation 12 of Bryant and Wolfe (1989). In that paper, a factor of two in the potential energy (equation 8 of that paper) was omitted

[10] One way to overcome this is to study the transition during cooling. This also causes problems however, due to supercooling. For these samples, supercooling can introduce an error of several degrees

^2H Nuclear magnetic resonance

The theory of deuterium NMR has been reviewed elsewhere (Davis 1983; Seelig 1977). Deuterium NMR of lipids in D_2O has been used to study phase behaviour by a few workers (e.g. Ulmius et al. 1977; Pope et al. 1981; Eriksson et al. 1985). The technique can distinguish between the lamellar and H_{II} phases easily, and the main chain melting transition can be studied in detail.

The orientation of the water molecules is determined both by the interactions between the water and the lipid head groups, and the shape of the lipid-water interface. Analysis of the ^2H NMR spectra therefore reflects the motional averaging of molecules interacting strongly at the interface, and, diffusion of water over the surface of the lipid aggregates, provided the latter occurs rapidly on the NMR timescale (Seelig 1977; Doane 1979). This latter averaging differs substantially for the case of diffusion over cylinders (H_{II} phase), compared with the lamellar phases, resulting in powder splittings reduced by a factor of greater than two for the H_{II} phase.

Figure 2 shows the NMR spectra at a range of temperatures for POPE/10% D_2O and POPC:POPE 1:1/10% D_2O. The spectra for POPC/10% D_2O are not shown. They exhibit simple powder patterns away from the phase transitions (resembling Fig. 2h), and a single narrow resonance close to the phase transition (resembling Fig. 2a). Below this transition the lipids are in a gel lamellar phase, and above it they are in the fluid lamellar phase (see Table 1). The type of gel phase present (L_β or $L_{\beta'}$) was not investigated. In the intermediate region the spectra are narrowed substantially, and at 290 K the splitting disappears. This effect has been observed before (Salsbury et al. 1972; Ulmius et al. 1977; Pope et al. 1981) but has not been adequately explained (Hawton and Doane 1987; Bryant et al. submitted).

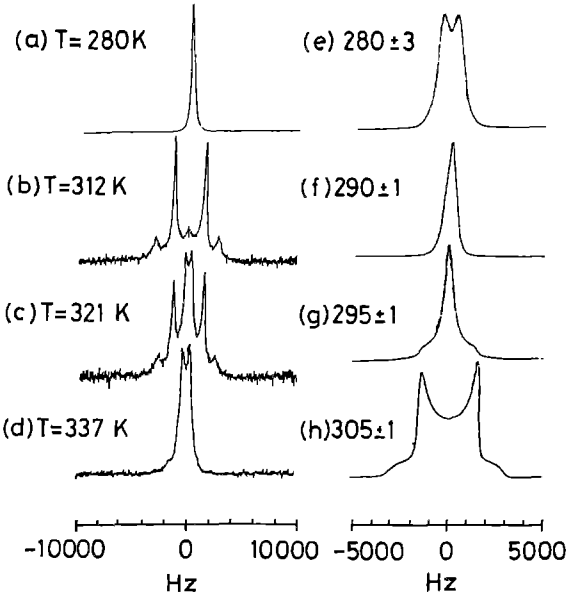

Fig. 2 a–h. ^2H spectra for samples of POPE (**a–d**) and POPC:POPE 1:1 (**e–h**) each with 10% D_2O (by weight) at a range of temperatures near their chain melting temperatures

For POPE the spectra do not exhibit simple powder spectra (in which all orientations are equally likely) – the "shoulders" of the spectra are enhanced, suggesting that the lipid-water interface is preferentially oriented perpendicular to the magnetic field. This phenomenon was not investigated – only the phase information (determined from the splittings) was obtained from these spectra.

Below 307 K the spectra exhibited a single broad resonance whose linewidth at half height varied from 100–800 Hz. This lack of a splitting over the range 275–307 K suggests that the water is not interacting strongly with the lipids. The water is not entirely excluded from the multilayers, however, as this would be represented by a single narrow peak (less than 100 Hz). It seems likely to us that the lipid is in an unhydrated (or weakly hydrated) crystalline phase over this range, and that the water is trapped in small pockets. To account for the observed linewidths, the domains of water must contain on the order of thousands of molecules[11].

The linewidth increases at temperatures above 280 K to a maximum of 2000 Hz at about 300 K. In this regime it is possible that more water is associated with the lipid (possibly indicating a weakly hydrated gel phase just below the fluid transition). For the range $T = 307–328$ K the spectra exhibit two superposed powder patterns with different splittings (and magnitudes). The signal with the narrow splitting is due to the H_{II} phase, and the broad splitting is due to the fluid lamellar phase. As the temperature is increased through this range the contribution from the H_{II} phase increases until, at $T = 332$ K, only the signal due to the H_{II} phase is present[12].

The spectra for the fluid lamellar phase in POPE have a splitting of about 3000 Hz. This splitting is larger than that for POPC[13]. This is at first surprising when one considers the results of interbilayer force measurements (e.g. Rand and Parsegian 1989), which show that the repulsion is, in general, stronger for PCs than for PEs. According to the simplest version of the hydration force explanation of large short range repulsive forces, one would expect that the water polarization should be larger for PC than PE, which should lead to a larger splitting for PC.

The larger splitting observed for PE may be due to the geometry (i.e. size and shape) of the headgroups. The PC headgroup presumably protrudes from the bilayer further than the PE headgroup, allowing water molecules to move into the region between the headgroups. These

[11] An order of magnitude calculation can be carried out here. Consider a spherical group of n water molecules. For $n \gg 1$, the volume fraction of molecules at the surface of such a group is approximately given by $F = 6n^{(-1/3)}$ (assuming that the radius of the sphere is large compared to the diameter of a water molecule). For a substantial broadening, 25% of the molecules (say) must be near the surface, which corresponds to about $n = 10^4$.

[12] The spectral splitting of the signal near the fluid lamellar-H_{II} transition temperature does not exhibit the motional narrowing observed near the fluid-gel transition temperature. This implies that the extreme motional narrowing effect is directly due to the chain melting (this will be discussed in a further paper)

[13] Hawton and Doane (1987) obtained similar results: they found that in excess water the splitting for DMPE was three times greater than that for DMPC

molecules would contribute to the signal by reducing the splitting. The PE has a smaller headgroup, and hence possibly a surface with fewer spaces into which water may move. Thus the water associated with the PE could be more strongly oriented perpendicular to the bilayers, and this would explain the larger splitting. If this hypothesis is true, then the larger force measured for PC could be due to the PC headgroup protruding further from the density weighted average surface. With this interpretation the above results lend some support to the proposal by Israelachvili and Wennerström (1990, 1992) that the short range strong repulsive forces are due to protrusion forces rather than hydration forces.

The mixture POPC:POPE (1:1) shows more complicated behaviour. The spectrum at 300 K exhibits two distinct splittings – a powder spectrum with a splitting of about 1 200 Hz, and an isotropic line with zero splitting. The two groups of water molecules contributing to this spectrum have substantially different average orientations. The unsplit signal is due to the motional narrowing of water associated with lipids near the phase transition. Two superposed signals are seen in the range 290 K – 305 K, which is the phase coexistence region of the gel and fluid phases (e.g. Ulmius et al. 1977). This agrees with the DSC data (see Table 1).

At temperatures above the transition region the spectra exhibited powder patterns with splittings increasing up to 2 000 Hz. This indicates the fluid phase. At temperatures below the transition region, the splittings were approximately half those in the fluid state. This is probably related to the observation that POPE is weakly hydrated below the phase transition temperature. It is therefore unsurprising that the 1:1 mixture has a lower splitting than that for pure POPC in the gel phase.

Between 310 K and 320 K the behaviour of the samples demonstrated a dependence on sample history, represented in Fig. 3, which shows the first temperature run on the left, and the second temperature run on the right. The top two spectra are at 315 K, and the bottom two at 320 K. These spectra are typical of spectra taken using more than 10 samples with several runs each. The pure lamellar phase suggested by (a) was rarely seen. Most of the spectra were similar to (c) showing two unresolved splittings, and some were akin to (b) which shows two distinct splittings. We believe that these splittings represent phase coexistence between two fluid phases with different water separations – the different separations cause the water to be oriented differently (on average) producing different splittings. For spectrum (b) the splittings are 2 950 Hz and 2 400 Hz. The double splittings are seen only at hydrations less than about 15%, and are never seen in samples containing only one lipid. These facts imply that the effect is associated with dehydration, and only occurs in mixtures. The temperatures at which the phenomena occurred are well above the gel phase transition temperature – DSC shows that the onset (warming) temperature is 293.5 K, and the peak temperature is 304.9 K. This will be discussed further in the light of the X-ray results (see below).

In addition to the above studies, several samples of POPC:POPE 1:1 were prepared using the second technique discussed in the experimental section, and NMR experiments were carried out at a number of hydrations. The results were similar to those discussed above, and have been used to construct the phase diagram (see later).

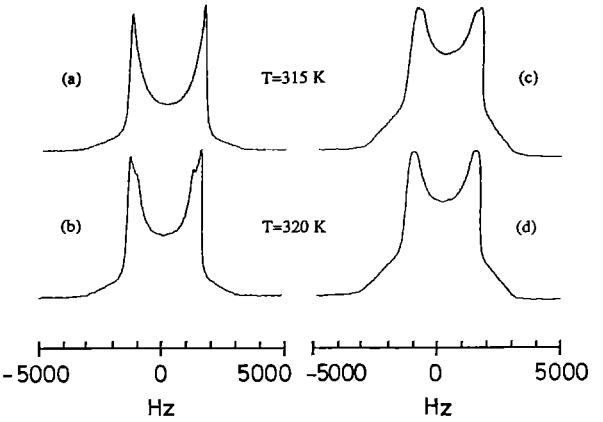

Fig. 3 a–d. Four ^2H NMR spectra for POPC:POPE 1:1 with 10% D_2O. **a**, **c**, at 315 K; **b**, **d** at 320 K. These illustrate the variability of the phase structure depending on sample history

Table 2

Lipid [hydration]	Temperature [K]	Bragg spacing [Å]	Indexed spacing [Å]	Phase/Order
POPE				
0	294±1	53.9±3.0	53.9±3.0	L_c/first
		28.4±0.7	56.8±1.4	L_c/second
		18.6±0.6	55.9±2.0	L_c/third
		4.02±0.14		wide angle
		3.76±0.12		wide angle
		4.68±0.20		wide angle
10±1	294±1	51.4±4.9		L_c/first
		4.35±0.08		wide angle
		4.12±0.15		wide angle
		4.87±0.21		wide angle
		4.60±0.20		wide angle
POPC				
10±1	294±1	50.8±2.5		L_α/first
		4.30±0.3		L_α/w angle
PE:PC (1:1)				
10±1	293±2	55.7±2.5		gel/first
		29.3±1.5	58.6±3.0	gel/second
		18.3±0.60	54.9±1.8	gel/third
		14.3±0.35	57.3±1.4	gel/fourth
		(61.6±6.5)		
		45.0±4.2		L_α/first
		4.22±0.14		L_β/w angle
		4.68±0.18		L_α/w angle
10±1	308±3	50.3±1.7		L_α(PE)
		55.9±2.0		L_α(PC)

X-ray diffraction

The repeat spacings and order assignments are summarized in Table 2. At $T = 294 \pm 1$ K, POPC with 10% D_2O

showed a single first order diffraction reflection at 50.8 ± 2.5 Å, and a very diffuse wide angle reflection centred around 4.30 ± 0.3 Å. The diffuse nature of this reflection suggests that the lipid is in the fluid phase at this temperature (this interpretation agrees with the DSC data). For the fluid phase the reflection is expected to be near 4.5 – 4.6 Å (e.g. Luzzati 1968). The relatively large error here (due to the very diffuse nature of the reflection) makes the determination of an accurate wide angle repeat spacing difficult.

POPE was studied at two hydrations, 0 and 10% D_2O, at 294 ± 1 K. At zero hydration three orders were apparent. The first order was deliberately overexposed to obtain the higher orders, so the second order in this film gave the most accurate repeat spacing (56.8 ± 1.4 Å). The samples also showed three wide angle lines at (in order of strength) 4.02 Å, 3.76 Å and 4.68 Å. The strong order demonstrated by these lines suggests that the lipid is in a crystalline arrangement. These spacings are consistent with those observed for the unhydrated crystalline forms of other phosphatidylethanolamines (Seddon et al. 1984; Caffrey 1985).

POPE with 10% D_2O showed a strong first order reflection at 51.4 ± 4.9 Å (overexposed) and four wide angle reflections at (in order of strength) 4.35 Å, 4.12 Å, 4.87 Å and 4.60 Å. We interpret these reflections as evidence that the lipid was in a crystalline form, either unhydrated or with very low hydration. The outer reflections do not correspond in an obvious way with the reflections seen for the unhydrated sample, so the crystalline arrangement is different, probably indicating a finite but small hydration ($\ll 10\%$).

The samples of POPC:POPE 1:1 with 10% D_2O were studied at two temperatures. At 293 ± 2 K there was evidence of phase coexistence between the gel and fluid lamellar phases with four orders of gel reflections (the most accurate being 57.3 ± 1.4 Å) and one order of the fluid phase (very diffuse and centred around 45 Å). In addition there were two outer reflections at 4.22 ± 0.14 Å (gel) and 4.68 ± 0.18 Å (fluid).

At $T = 308 \pm 3$ K, two first order reflections were detected at 50.3 ± 1.7 Å and 55.9 ± 2.0 Å. There were no wide angle reflections seen (even after an exposure time of 1 week), which was taken to indicate that there was no gel phase lipid present. Thus the two reflections were interpreted as coexisting fluid lamellar phases with different repeat spacings, in agreement with the NMR results (see below).

X-ray diffraction was also carried out on these samples using the SAXS machine described above. Figure 4 shows plots of the intensity of the scattered X-rays as a function of $1/d$ (where d is the repeat spacing) for a sample cycled between 290 K and 310 K. At 295 K the first and fourth orders of the gel phase are clearly visible, with a weak second order reflection. As the temperature is increased, a first order reflection corresponding to the fluid phase appears. At 295 K there is a small percentage of the sample in the fluid phase, and this fraction increases until at 310 K the sample is almost completely in the fluid phase (with possibly a small gel phase component). A second order reflection for the fluid phase is also apparent here. The sample was then cycled between 280 K and 300 K

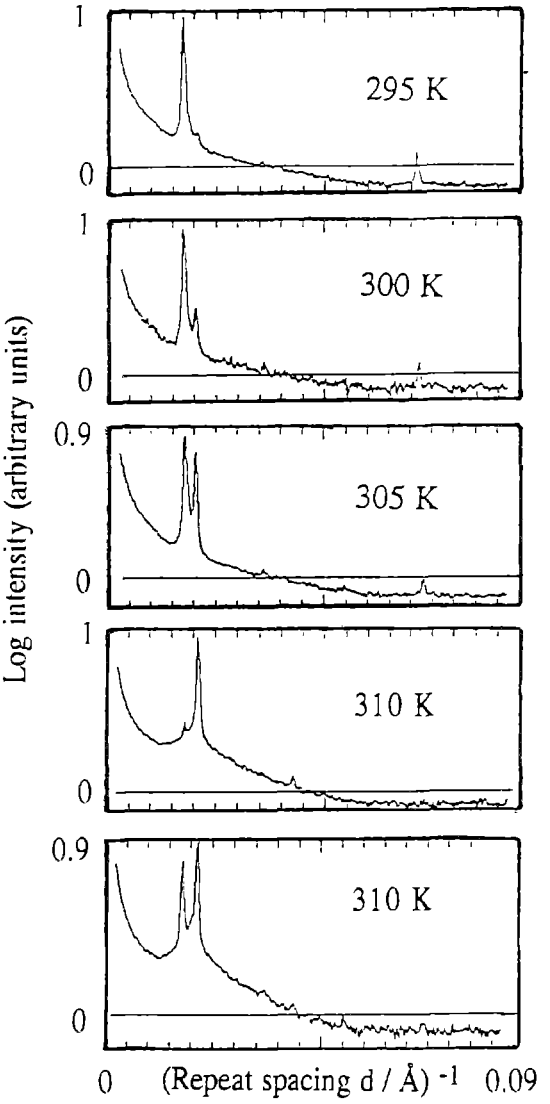

Fig. 4. Plots of intensity of scattered X-rays as a function of $1/d$, where d is the repeat spacing. The first 4 plots show the results as a function of temperature increasing from 295 K to 310 K. The final plot is at 310 K after temperature cycling between 280 K and 310 K several times

twice (showing gel phase with a small amount of fluid) and returned to 310 K. At this temperature there are two first order reflections, and no higher orders. The absence of the fourth order reflection indicates that there is no gel phase present in the sample. The two first order lines correspond to 49 ± 1 Å and 58 ± 1 Å, in reasonable agreement with the results using the fixed anode machine.

These X-ray diffraction results on their own do not unequivocally demonstrate two co-existing lamellar phases, because only first order reflections are present (fluid phases produce only weak higher orders). The NMR results on the other hand, are inconsistent with the possibility of hexagonal or ripple phases, and imply phases with planar interfaces (due to the size of the splitting). These results taken together with the DSC evidence are consistent with the presence of two co-existing fluid lamellar phases.

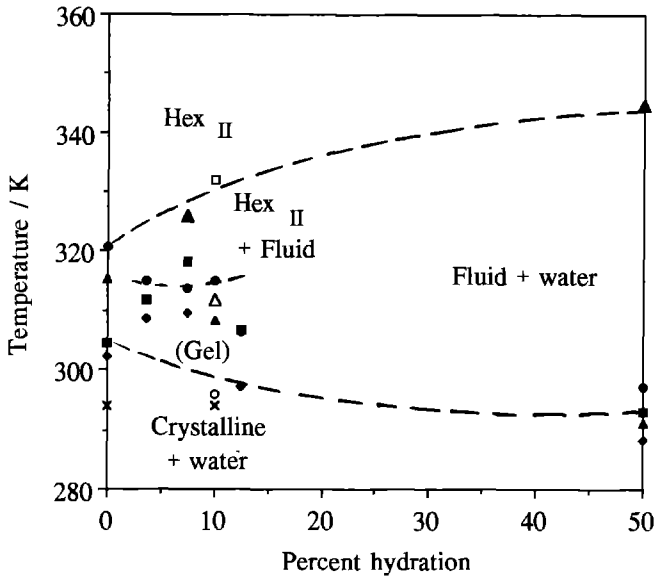

Fig. 5. Phase diagram for POPE as a function of temperature and hydration. The *filled symbols* show DSC data for cooling and warming runs. The *open symbols* indicate NMR data where only a single phase was observed on all runs. Between these temperatures the spectra exhibited phase coexistence over an extended temperature range. The data were confirmed using X-ray diffraction at 0 and 10% hydration (*crosses*)

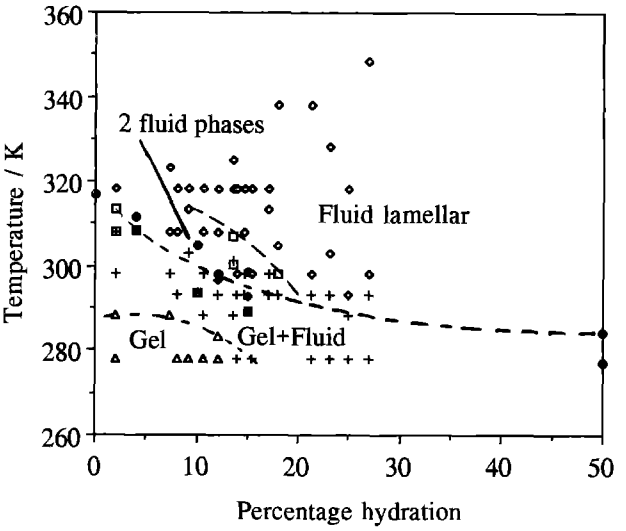

Fig. 6. Phase diagram for POPC:POPE 1:1 as a function of temperature and hydration. Only gel and fluid phases were observed for these samples. NMR data points are shown for fluid lamellar (*diamonds*), gel (*triangles*) and coexisting gel and fluid phases (+). The *open squares* represent points where coexisting fluid lamellar phases were observed. DSC data are also shown (*filled symbols*). The coexisting gel and fluid phases and coexisting fluid phases were confirmed at 10% hydration using X-ray diffraction

The phase behaviour of POPE is summarised in Fig. 5. The filled symbols show the DSC data for cooling and warming runs. At 10% hydration the pure phases (as determined using ^{2}H NMR) are indicated by open symbols. Between the pure phases, the coexistence of two phases was observed. The crystalline phase below about 300 K was confirmed using X-ray diffraction (crosses). Between 15% hydration and excess the phase behaviour was not investigated.

The phase behaviour for POPC:POPE 1:1 is summarised in Fig. 6. The NMR data are open symbols: fluid lamellar (diamonds), gel (triangles), coexisting gel and fluid phases (+) and coexisting fluid lamellar phases (squares). Some DSC data are also shown (filled symbols), indicating the approximate position of the gel-fluid transition. The phase interpretations have been confirmed using X-ray diffraction at 10% hydration between 280 K and 310 K.

The ^{2}H NMR studies have shown that POPC behaves as reported previously for phosphatidylcholines near the gel-fluid phase transition, but that POPE shows markedly different behaviour. It is likely that the apparent direct transition from a fluid to a crystralline phase is responsible for the different behaviour of POPE. For the 1:1 mixture the splittings in the fluid phase is about twice that in the gel phase, which is representative of the decreased water ordering of PE in the gel phase (Bryant et al. submitted). For the 1:1 mixture, both NMR and X-ray diffraction showed that the gel and fluid phases co-exist over a range of temperatures, as has been found for other lipid/water mixtures.

The most interesting feature of the phase diagrams is the coexisting fluid phase region at less than 15% hydration in Fig. 6. As mentioned above, both NMR and X-ray experiments showed that sample history was important in determining whether phase separation occurred, making an exact study of the geometry of the equilibrium phase diagram difficult. The occurrence of this hysteresis may be related to the low enthalpy of the phase separation[14]. The estimated enthalpy of the phase separation is always less than 10^{-21} J/mol (Bryant 1991), which is more than an order of magnitude less than that of the chain melting transition. The two conditions given in Eq. (4) describe two lines where $\mu_1 = \mu_1'$. However, within the region thus bounded there is a large area where the phase separation is stable with respect to the homogeneous phase, and a small region where the reverse is true. Figure 7 shows a theoretical phase diagram[15] depicting this for two hypothetical lipids whose short range repulsions differ by a factor of 400 (from Bryant 1991). There exists a substantial region of the phase space where the difference in free energy between the separation and the homogeneous phase is of the order of 10^{-2} kT per molecule or less. This small free energy difference suggests that hysteresis is likely in this region – the geometry of the phase boundaries are unknown to us, however, so an analysis of the metastability is not possible. The region labelled metastable in Fig. 7 is a region where the homogeneous phase is nominally stable, but where the free

[14] When the transition enthalpy is large, the boundary energy of a finite domain can dominate relatively large domains, and so the critical nucleation size is relatively large

[15] The geometry is complicated: $\Delta\mu$ changes sign 4 times on an isobar at relatively high hydrations, and twice at low hydrations

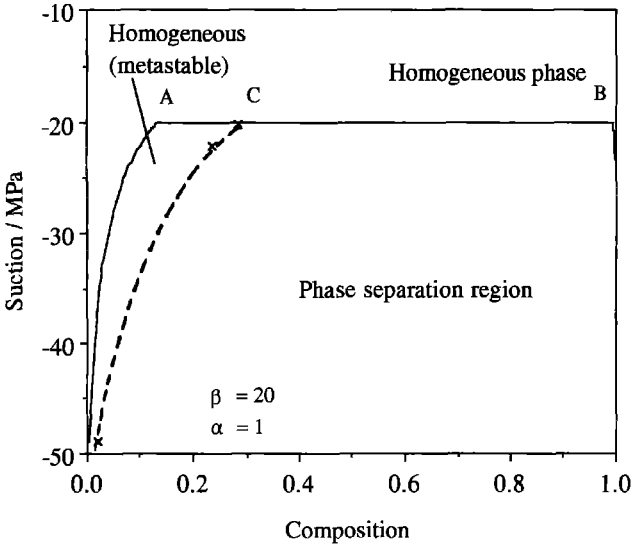

Fig. 7. Calculated phase diagram for a mixture of two hypothetical lipids whose P_0 values differ by a factor of 400. The phase separation region is marked. The region labelled metastable, and the region immediately to the right of it, are areas where the energy difference between the phase separation and the homogeneous mixture is very small

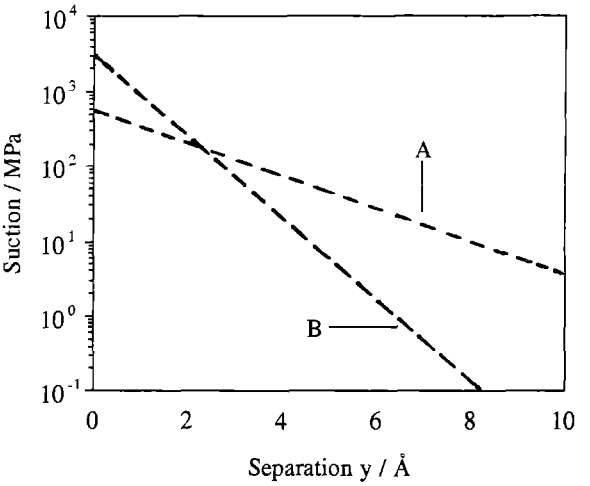

Fig. 8. Plot of the suction against the water separation for two hypothetical lipids whose values for P_0 and λ are different. *Line A* is for a lipid with $\lambda = 2$ Å (0 2 nm) (typical of PCs), and *line B* is for $\lambda = 0.8$ Å (for POPE – data extrapolated from Rand and Parsegian 1989)

energy difference is small. The area immediately to the right is nominally phase separated, but the free energy difference is also exceedingly small.

The model used to construct Fig. 7 is highly simplified, so comparisons between theory and experiment are difficult. For analytical simplicity, the decay lengths were assumed to be the same for the two lipids, which is probably a poor approximation for the two lipids studied here. Nevertheless, the required parameters can be estimated independently, and so an order of magnitude calculation is possible. The values of these parameters for POPE are known ($\lambda = 0.8$ Å, $P_0 = 3\,162$ MPa – Rand and Parsegian 1989). For POPC the analogous parameters are unknown to us, but can be estimated. POPC at 10% hydration corresponds to a suction of about -50 MPa (Lynch and Steponkus 1989), and the water separation at 10% hydration is about 5.1 Å. Assuming that the decay length is similar to that for most PCs (about 2.0 Å – Rand and Parsegian 1989), and using Eq. (1), the value of P_0 is about 570 MPa. Figure 8 sketches plots thus estimated for the force per unit area against the water separation y for these lipids. The dashed line for PC shows that at $y = 5.1$ Å (0.51 nm) the lipid has a larger repulsion than the PE at this hydration. The ratio of the suctions required to obtain this separation of the two lipids is about 10. According to the simple model outlined above such a value will induce a phase separation at suctions of the order of 100 MPa. For the 1:1 mixture, 10% hydration corresponds to about 80 MPa (Bryant 1991). Given the simplicity of the model, we claim this as approximate agreement between theory and experiment.

We known of only one other report of experimental evidence that dehydration induces phase separations. Tamura-Lis et al. (1986) reported that DOPC:DOPE mixtures showed such a separation between 20% and 30% hydration. They did not examine or discuss this in detail, but suggested that the different hydration requirements of the two lipids were responsible for the separation. Oddly, their data show two phases at 20% and 30% hydration, but only a single phase at 10% hydration. The force-distance data for DOPE are unknown to us and this makes calculations difficult. One possible explanation is that the continuum properties inherent in the model break down at very low hydrations. Consider a case where two lipid species have different values of both P_0 and λ, as in Fig. 8. In this case it is possible that at intermediate hydrations (15% say) a phase separation may be stable, but upon further dehydration (as the suctions for the two lipids become similar), the phase separation would become unstable with respect to a single homogeneous phase. This would occur at about $y = 2.3$ Å, or about 4.5% hydration (for these parameter values). At larger water separations, the PC has the stronger repulsion, but at smaller separations, the PE appears to have a stronger repulsion. However, the approximations that the water can be described as a continuum, and that the strong repulsive force is purely exponential, break down at close approach, prohibiting extrapolation of this model.

One example of dehydration induced phase separations may be important. Bryant and Wolfe (1989) argued that the theory of phase separations can be applied quantitatively to proteins as well as lipids. The theoretical phase diagram for a protein-lipid mixture is qualitatively similar to Fig. 7 though separations occur at smaller suctions. This is of course not the only mechanism whereby protein-lipid phase separations may be effected – hydrophobic mismatching of the lipids and proteins has been implicated in causing such separations (e.g. Sperotto et al. 1989). In general, of course, all of the membrane components have strong repulsive forces, and so the separation of some of these components via this mechanism is likely under the right conditions.

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