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Motional narrowing of the ^2H NMR spectra near the chain melting transition of phospholipid/D_2O mixtures

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Abstract. The reduction in spectral splitting, or motional narrowing, of the deuterium spectra of D_2O/phospholipid mixtures near the main chain melting phase transition was studied for palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE) and equimolar mixtures of the two at 10% hydration. For POPC the splitting was about 1700 Hz in both the fluid and gel phases, dropping to zero near the phase transition (as reported previously). For POPE the splitting remained approximately constant above the phase transition. Below the phase transition the spectrum showed a single broad line whose linewidth varied between 100 Hz and 800 Hz. This was interpreted as being due to small domains of water within a weakly hydrated crystal. POPC:POPE (1:1) samples exhibited motional narrowing behaviour similar to that for POPC except that the splitting above the phase transition was approximately twice that below the transition. The relatively broad temperature range ($\sim$20 K) of the transition is explained using a simple physical model involving lipid fluctuations near the phase transition.

Key words: ^2H NMR – D_2O – Motional narrowing – Gel-fluid phase transition – Phospholipids – Low hydration

Introduction

The chain melting transition of lamellar phases of pure phospholipids at high hydration occurs over a temperature range of less than a few degrees. Studies of the transition using deuterium NMR of D_2O/lipid mixtures have revealed an effect which occurs over a much broader temperature range ($\sim$20 K). The spectrum of a powder sample of D_2O and phosphatidylcholine (PC) usually consists of two peaks (known as a powder or "Pake" pattern), and the spectrum can be characterized by the width of the spectral splitting. As the temperature is increased through the transition, the splitting is reduced until, near the transition, the splitting is zero (i.e. there is only 1 peak). As the temperature is raised further, the splitting increases to about the same level as below the transition.

This additional motional narrowing of the spectra near the chain melting phase transition of phospholipids was first reported by Salsbury et al. (1972), and has since been studied by several authors (Ulmius et al. 1977; Pope et al. 1981; Strenk et al. 1985; Hawton and Doane 1987). Similar effects have been seen in the gel-fluid phase transition of bilayer forming soaps (Abdolall et al. 1977, 1978).

The effect is interesting because it has a characteristic temperature range of tens of Kelvin, which is unusual for a physical property associated with a first order phase transition. The splitting of a ^2H NMR spectrum is interpreted as being due to the time-averaged anisotropy of the labelled molecules (in this case water), and so its disappearance is taken to imply an isotropic average environment for water, at least over the time scale of the measurement.

The suggestions proposed to account for these observations all have some limitations. Salsbury et al. (1972) suggested that changes in the water structure around the headgroup caused changes in the water mobility, without detailing what changes were responsible or why it occurred over a wide temperature range. Ulmius et al. (1977) suggested that the effect may be due to diffusion along the ripples in the ripple phase which is intermediate between the gel and fluid phases in PC. Strenk et al. (1985) used this idea to make estimates of the ripple periods from their measurements, using a non-zero asymmetry parameter to model the data. Although the data agreed with X-ray measurements, the fitting of the spectra is not unique, and more importantly this theory does not ex-

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Abbreviations: NMR – Nuclear Magnetic Resonance, PC – phosphatidylcholine, PE – phosphatidylethanolamine, POPC – Palmitoyloleoylphosphatidylcholine, POPE – Palmitoyloleoylphosphatidylethanolamine, Inverse hexagonal phase – H_{II}

plain why the motional narrowing is also seen in some phosphatidylethanolamines (PE) which do not exhibit the ripple phase. In addition, Hawton and Doane (1987) have shown that the spectra for PC do not have a non-zero asymmetry parameter. Pope et al. (1981) explained the effect by suggesting that the water molecules undergo rapid exchange between two environments with order parameters of equal magnitude and opposite sign. This explanation poses the question: why should such a special pair of environments appear over tens of degrees about the transition?

Hawton and Doane (1987) applied an order parameter model based on area fluctuations near the phase transition to explain the results. They showed that the experimental narrowing showed a temperature dependence proportional to $\sqrt{|T-T^*|}$ where T^* was called the "pseudocritical" temperature. Other researchers have proposed that the elevated lateral compressibility of the bilayer in the vicinity of the phase transition allows gaps between the lipids into which water or solute molecules could move. Nagle and Scott (1978), for instance, cited this mechanism to explain changes in membrane permeability near the phase transition region (e.g. Papahadjopoulos et al. 1973). In the case of water, this would lead to a reduction in the splitting near the transition temperature. However there is no quantitative justification for the mechanism, nor an explanation of why the effect should be so large.

In this paper, we will present the results for experiments carried out on PC, PE and equimolar mixtures of the two. A simple physical model, also based on lipid fluctuations near the phase transition, will be used to interpret the results.

Material and methods

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 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. 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 than was achievable with the standard insert. 2 000–10 000 scans were accumulated for each temperature. A $90°-\tau-90°_{90}$ quadrupolar

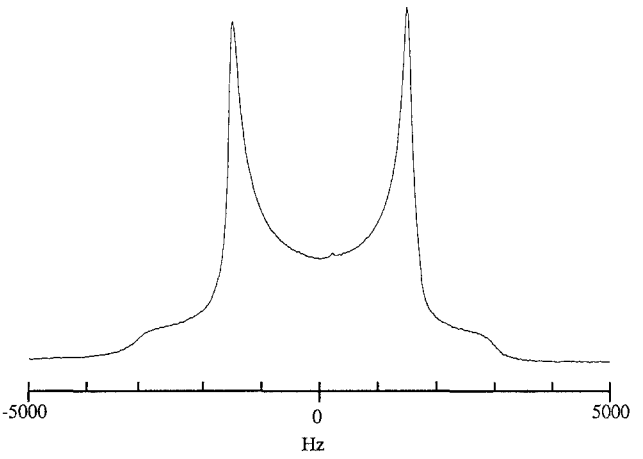

Fig. 1. A typical ^2H NMR powder spectrum of a D_2O/phospholipid mixture in the fluid lamellar phase

echo technique was found to give identical results to a single 90° pulse (~5.6 ms duration) for these linewidths, so the latter was used for most experiments.

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

Results

Figure 1 shows a typical ^2H NMR powder spectrum for a phospholipid in D_2O. The splitting is defined as the distance between the two peaks, and is an indication of the amount of ordering experienced by the water. Experiments were carried out on samples of POPC, POPE and POPC:POPE (1:1) each with 10% D_2O. Representative spectra, and phase diagrams were presented in Bryant et al. (1992).

The variation in the splitting as a function of temperature is shown in Fig. 2 for all three systems (open symbols). The linewidths (i.e. full width at half height) of the spectra with zero splitting are also shown (filled symbols). The transition onset and peak temperatures, as determined using DSC, are shown for comparison (from Bryant et al. 1992).

Figure 2a shows data for POPC – they are qualitatively similar to those previously obtained for other PCs (Pope et al. 1981; Strenk et al. 1985; Hawton and Doane 1987), with the splitting approximately the same magnitude in both the gel and fluid lamellar phases, and zero splitting near the phase transition.

For POPE the behaviour is noticeably different, as seen in Fig. 2b. At temperatures below about 300 K, the

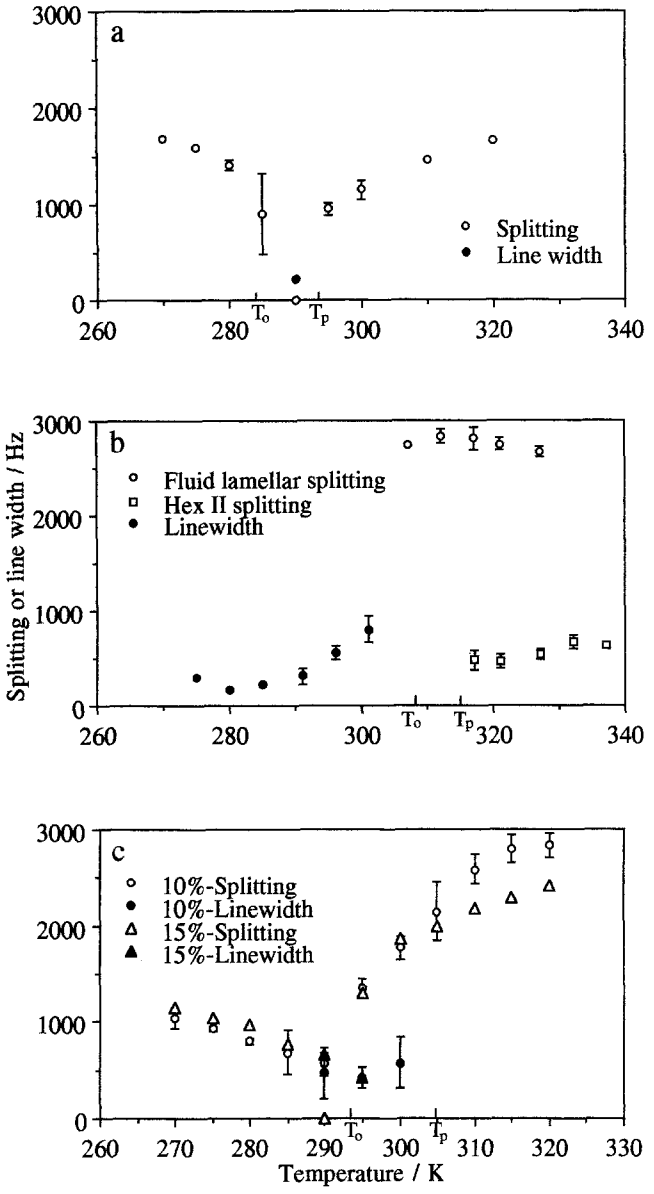

Fig. 2. Plots of splitting and linewidth against temperature for **a** POPC, **b** POPE and **c** POPE:POPC (1:1) each with 10% D_2O. (**c**) also shows data for samples with 15% hydration. The open symbols represent splitting data, and the filled symbols are the linewidths. The transition temperatures determined from DSC (Bryant et al. 1992) are also shown for the 10% hydration samples. The vertical error bars are standard deviations of 2–5 runs. In **a** and **b** the points without error bars are either from a single run, or the plot symbols are larger than the errors. In **c** the errors have been omitted from the 15% hydration sample for clarity, although they are of the same order as the 10% sample. The horizontal errors (usually ± 1 K) are never larger than the plot symbols

spectra exhibit a single resonance whose linewidth (full width at half height) varies between 200 and 800 Hz. Between about 303 K and 315 K the spectra exhibit a splitting of nearly 3000 Hz. Above this temperature the sample undergoes a transition into the inverse hexagonal (H_{II}) phase, and the spectra exhibit two superposed splittings, with the H_{II} splitting being less than half that of the lamellar phase. The contribution from the fluid lamellar phase decreases with increasing temperature until only the H_{II} phase remains at 332 K (see Bryant et al. 1992). The splittings for the fluid lamellar and H_{II} phases do not exhibit any motional narrowing during this transition. This has also been observed for DOPC:DOPE mixtures undergoing the fluid lamellar to H_{II} transition (Bryant 1991). These results show that the motional narrowing effect is a direct function of chain freezing, and is not an effect related to simple conformational changes.

Figure 2c shows the variation in the splittings and linewidths for POPC:POPE (1:1) at both 10% and 15% hydration. The 15% hydration sample has a slightly lower transition temperature (as would be expected), but is qualitatively similar to the 10% sample. The behaviour differs from that for the two pure lipids. In particular, the splitting in the gel phase is significantly lower than in the fluid lamellar phase (see discussion).

Discussion

For the POPE samples, no splitting is observed below the transition. This is due to the fact that the PE is in a weakly hydrated crystalline phase below the transition (Bryant et al. 1992). However, it appears that the water does not exist in a single excess phase, as this would give a signal with a narrow linewidth (~ 100 Hz). It may be that the water is contained in small domains within the sample. Consider a roughly spherical group of n water molecules in a volume v. As the water molecules diffuse through the region, they will sample all orientations, and so the signal will have zero splitting. However, the linewidth of the signal is determined by the molecular environment, so those molecules near the surface of the sphere will experience stronger interactions and will contribute a wider (unsplit) component. As the water molecules can sample all orientations in the time frame of the experiment, the resulting signal will be broadened. For $n \ll 1$ the volume fraction of water molecules near the surface of the sphere is on the order of

$$F = \frac{4\pi r^2 d_w}{\frac{4}{3}\pi r^3} \tag{1}$$

where r is the radius of the sphere and d_w is the diameter of the water molecule. The expression is valid for $r \gg d_w$ and using v_w (the volume of 1 water molecule) $= v/n$, this becomes:

$$F = \frac{6}{\sqrt[3]{n}} \tag{2}$$

For the NMR signal to be substantially broadened by this effect, the fraction of molecules on the surface must be greater than, say 10%, which corresponds to a volume of about 6000 nm^3, or about 2×10^5 molecules. To account for the observations of line broadenings of several hundred Hz the groups of molecules must be even smaller.

This model suggests that the splitting of the signal may be dependent on sample history, with hysteresis likely. In addition, it may be possible to anneal these sample, thus altering the size of the domains of water molecules, and consequently altering the observed splitting. Although

there was some variation observed for the POPE samples, this effect was not investigated in detail.

For the POPC:POPE 1:1 mixtures in the fluid phase, the splittings away from the transition are about 2 700 Hz for the sample with 10% D_2O and about 2 300 Hz for the sample with 15% D_2O. The larger splitting for the 10% mixture is attributed to the stronger (average) interaction experienced by the water molecules in the lower hydration sample.

Below the transition the splittings are similar, to within the accuracy of the experiments. However in the phase transition region these samples do not exhibit a single pure Lorentzian line (unlike the pure PC). This is not surprising as the phase separation from the gel to the fluid phase occurs over a broad temperature range between the fluidus and solidus lines. Thus for mixtures, parameters associated with the phase transition (such as membrane compressibility) are not expected to exhibit a singularity (Marčelja and Wolfe 1979). It is instructive to note the difference between the behaviours of lipids at full hydration and those with less than excess water. For a single lipid in excess water, the amount of water incorporated by the lipid changes as the lipid undergoes the phase transition to maintain a constant chemical potential of water. In principle, such transitions are singularities. For a lipid with less than excess water, however, the water volume per lipid is constant during the phase transition, and thus the chemical potential of water must change (as the interaction between water and the lipids is different for the gel and fluid phases). The effect of this is to broaden the transition. A system with 2 lipid components will of course exhibit a broad transition even in excess water.

Thermal fluctuations

Consider a lipid-water mixture at a temperature T near the main chain melting transition temperature T_m (note that the following analysis is equally valid in the fluid phase (Tslightly above T_m) or in the gel phase (T slightly below T_m)). If the temperature difference $|T_m - T|$ is small, thermal fluctuations will cause the chains of small groups of lipids spontaneously to "freeze" or to "melt". These short lived microphases would form and decay rapidly, and should not be considered as stable domains.

For PC the difference in headgroup area between the gel and fluid phases is about $\Delta a \sim 0.2$ nm^2 per lipid (Lis et al. 1982). If small groups of n lipids are alternately "freezing" and "melting" at a rapid rate, the transitory gaps in the membrane will be formed with areas about n^* 0.2 nm^2. (This is of course a gross simplification of the physical situation – if such "pores" exist they are unlikely to be so well defined.) Thus even small groups of participating lipids could cause large area changes, resulting in transient pores and dimples in the membrane. Note that the formation of such transient domains is associated with large increases in the lateral compressibility of the membrane which has been used to explain the large increases in membrane permeability (Nagle and Scott 1978; Bates and Wolfe 1980 and references therein).

A rapidly moving water molecule diffuses a distance of typically 450 nm during an NMR measurement (the sampling distance is about $\sqrt{2D\tau}$ where D is the diffusion constant (about 10^{-10} m^2 s^{-1}) and τ is the time scale of the experiment (about 1 ms) – Strenk et al. 1985). The NMR signal is the average of the signals from the water molecules in different environments, and so the effect of having water molecules sampling the space between the lipids will be to reduce the splitting. The following is an order of magnitude calculation to determine if such fluctuations can explain the observed water isotropy.

The latent heat of a transition for one molecule is given by $h = T_m \Delta s$ where h is the latent heat, Δs is the change in entropy and T_m is the equilibrium transition temperature. For fluctuations between two phases at $T \neq T_m$, the fluctuation energy per molecule Δg is $\Delta g = h - T\Delta s$. Thus the change in the Gibbs free energy associated with a fluctuation of a group of n molecules is:

$$\Delta G = |nh - nT\Delta s| \tag{3}$$

Exactly at the transition, $T = T_m$, $\Delta G = 0$ and therefore $\Delta s = h/T_m$. Substituting in (3) and defining $\Delta T = |T_m - T|$ gives:

$$\Delta G = \frac{nh}{T_m}\Delta T \tag{4}$$

The probability p that a thermal fluctuation occurs which is large enough to cause such a phase transition is proportional to the Boltzmann energy distribution:

$$p \propto \exp\left(\frac{-\Delta G}{kT}\right) = \exp\left(\frac{-nh\,\Delta T}{kT\,T_m}\right) \approx \exp\left(\frac{-nh}{kT_m^2}\Delta T\right) \tag{5}$$

where the approximation is valid if $\frac{\Delta T}{T} \ll 1$. (At room temperature this condition is satisfied).

The probability that a group of n lipid molecules can undergo a transition between the gel and fluid states solely as a result of thermal fluctuations can be estimated if the parameters h and T_m are known. From DSC experiments (Bryant 1991) for POPC at 10% hydration $h = (1.7 \pm 0.1) \times 10^{-20}$ J/molecule and $T_m = 293.5 \pm 0.3$ K. Substituting into (5), this becomes:

$$p \sim \exp\left(\frac{-n\Delta T}{\tau}\right) \tag{6}$$

where $\tau = 70$ K for these parameters.

Figure 3 shows a plot of the value n as a function of the temperature difference ΔT for different values of the Boltzmann probability p. From these plots it is clear that 10 K from the transition temperature, groups of more than a few molecules are unlikely to undergo the fluctuation collectively. Close to the transition (1 K say), the probability is high that groups of 20 molecules or more will undergo the fluctuation. Exactly at the transition temperature, all the lipid molecules are involved in fluctuations, and thus transient gaps in the bilayers are continually being created. It is possible therefore that water molecules which enter these gaps are free to tumble in all orientations (on average) leading to the observed zero splitting.

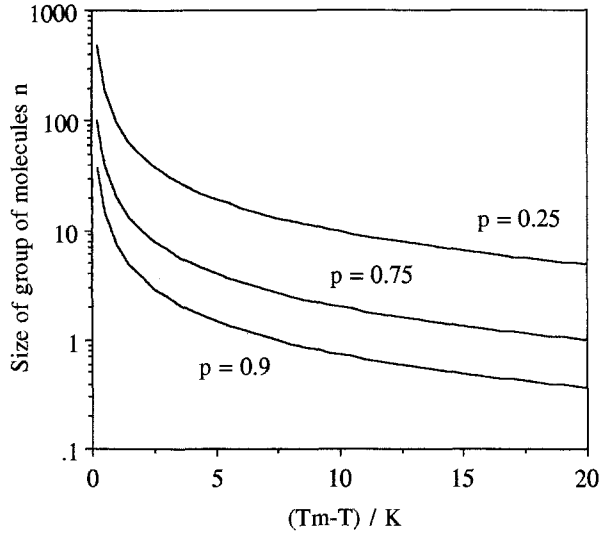

Fig. 3. A logarithmic plot of the number (n) of lipid molecules in a short lived domain as a function of the temperature difference ΔT from the transition temperature, as predicted from equation 6. The 3 lines have Boltzmann probability factors (p) of 0.9, 0.75 and 0.25 (see text for discussion)

The range of the motional narrowing effect seen experimentally is about $\pm 15-20$ K, although most of the narrowing occurs less than 10 K from the transition. From Fig. 3, it can be seen that the probability of groups of molecules fluctuating between the fluid and gel phases is extremely small that far from the transition (in either the gel or fluid phase), and so there will be little or no contribution to narrowing.

To estimate the size of the effect, consider a small group of molecules ($n=4$). The area in the plane of the interface associated with this group undergoing a transition would change by between 0.14 and 0.20 nm^2 per lipid (Lis et al. 1982; Seddon et al. 1984) and the length of the lipid molecule is about 2 nm (Rand and Parsegian 1989). Thus for a fluctuation involving $n=4$ lipids going from gel to fluid configuration, and not otherwise affecting the global geometry, a volume of about 1.5 nm^3 would appear in the hydrophobic region of the bilayer. This equals the volume of about 50 water molecules – as the cavity appears within the bilayer, the molecules taking up the gel configuration would protrude into the interbilayer region and displace water from that region. At 10% hydration the water separation between multilayers is about 0.5 nm, and the lipid area is about 0.55 nm^2 (Bryant 1991), so the volume of water associated with each lipid molecule is about 0.14 nm^3. Thus the fluctuations of a small group of lipid molecules can create a change in volume which can be sampled by the water molecules associated with about 10 lipid molecules.

If a fluctuation of a few molecules occurs every 10 lipids or so during the time of the NMR experiment, then the signal will be severely motionally narrowed. If the fluctuations occur only every 20 molecules or so, then the splitting will be reduced to about half its value a long way from the transition.

The interpretation of motional narrowing of D_2O NMR spectra presented here provides an explanation in terms of a simple physical model of lipid phase fluctuations. Consideration of the suggested physical mechanism can aid in the understanding of both lipid phase transitions and the associated NMR spectra, and complements the more mathematical interpretations of previous authors.

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References

Abdolall K, MacKay AL, Bloom M (1978) The lipid-water interaction in sodium laurate. J Mag Res 29:309–317

Abdolall K, Burnell EE, Valic MI (1977) The temperature dependence of water and counter ion order in soap-water mesophases. A deuterium and sodium NMR study. Chem Phys L 20:115–129

Bates EH, Wolfe J (1980) The elasticity of protein-lipid bilayer membranes and its physiological significance. In: Bata L (ed.) Advances in Liquid Crystal Research and Applications. Pergamon Press, Oxford

Bryant G (1991) Phase behaviour and hydration characteristics of phospholipids at low water content. PhD thesis, University of New South Wales, Sydney

Bryant G, Pope JM, Wolfe J (1992) Low hydration phase properties of phospholipid mixtures: evidence for dehydration-induced fluid-fluid separations. Eur Biophys J 21:223–232

Hawton MH, Doane JW (1987) Pretransitional phenomena in phospholipid/water multilayers. Biophys J 52:401–404

Lis LJ, McAlister M, Fuller N, Rand RP, Parsegian VA (1982) Measurement of the lateral compressibility of several phospholipid bilayers. Biophys J 37:667–672

Marčelja S, Wolfe J (1979) Properties of bilayer membranes in the phase transition or separation region. Biochem Biophys Acta 557:24–31

Nagle JF, Scott HL (1978) Lateral compressibility of lipid mono- and bilayers: Theory of membrane permeability. Biochim Biophys Acta 513:236–243

Papahadjopoulos S, Jacobson K, Nir S, Isak T (1973) Phase transitions in phospholipid vesicles. Fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. Biochim Biophys Acta 311:330–348

Pope JM, Walker L, Cornell BA, Francis GW (1981) NMR study of synthetic lecethin bilayers in the vicinity of the gel-liquid crystal transition. Biophys J 35:509–520

Rand RP, Parsegian VA (1989) Hydration forces between phospholipid bilayers. Biochim Biophys Acta 988:351–376

Salsbury NJ, Darke A, Chapman D (1972) Deuteron magnetic resonance studies of water associated with phospholipids. Chem Phys Lipids 8:142–151

Seddon JM, Cevc G, Kaye RD, Marsh D (1984) X-ray diffraction study of the polymorphism of hydrated diacyl- and dialkylphosphatidylethanolamines. Biochemistry 23:2634–2644

Strenk LM, Westerman PW, Vaz NAP, Doane JW (1985) Spatial modulation of water ordering in lecithin bilayers. Biophys J 48:355–359

Ulmius J, Wennerström H, Lindblom G, Arvidson G (1977) Deuteron nuclear magnetic resonance studies of phase equilibria in a lecithin-water system. Biochemistry 16:5742–5745