PRETRANSITIONAL PHENOMENA IN PHOSPHOLIPID/WATER MULTILAYERS

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ABSTRACT We have measured the water order in monodomain phospholipid samples using ^2H nuclear magnetic resonance (NMR) and analyzed the splittings in terms of critical exponents. Our data and the model developed to interpret them in terms of fluctuations provide an explanation of the puzzling sharp reduction of water order near the chain-ordering phase transition. The temperature range of the fluctuations is approximately the same as that observed for increased $^{22}Na^+$ efflux from phospholipid vesicles.

INTRODUCTION

Hydrated phospholipids show dramatic effects near their L_β to L_α phase transition. Very strong motional narrowing in deuterium spectra of ^2H_2O was first observed in 1972 (1) for a variety of phospholipids, and similar effects have since been studied in pure lipids (2, 3). The reason for this reduction in water order has eluded explanation for more than a decade. A peak in the permeability to ions suggesting a divergence is also observed (4) at the phase transition temperature, T_c, and has been attributed to enhanced lateral density fluctuations by some authors (5–7). For dipalmitoylphosphatidylcholine the permeability reaches an asymptotic value 15–20°C above the chain melting temperature, as does the nuclear magnetic resonance (NMR) data, suggesting that the same physical mechanism may underlie both processes.

We have measured the time-averaged electric field gradient tensor of the deuterium quadrupole interaction for monodomain samples of a variety of phospholipids for the first time. The splittings were found to be proportional to $|(T/T^*) - 1|^\alpha$ with $\alpha \simeq 1/2$ for data taken in the L_α phase. We provide a theoretical interpretation of this data by relating the splitting to order parameter fluctuations giving a critical exponent of $1/2$ in a mean-field approximation. The analysis strongly suggests that the narrowing of deuterium spectra in the L_α, P_β, and L_β or $L_{\beta'}$ phases is due to pretransitional phenomena. The pseudocritical temperatures, T^* for fluctuations in the L_α phase and T^{**} for $L_{\beta'}$ or P_β phase fluctuations, can be estimated from the splitting data. The results indicate that pretransitional phenomena, which are relevant to ion permeability and other biologically important effects, can be studied quantitatively in phospholipids hydrated with ^2H_2O.

MATERIALS AND METHODS

Monodomain phospholipid samples were prepared by evaporating a lipid/chloroform solution (with a drop of methanol if required) to leave a 20-μm lipid layer on a mylar sheet. The sample was then hydrated with 50 wt % ^2H_2O, cut and stacked to give a 50-mg sample of appropriate dimensions, annealed at 80°C and 100% relative ^2H_2O humidity for 24 h, and equilibrated another 24 h in the L_α phase at 100% humidity. Samples were then sealed in NMR tubes and stored at −20°C. Deuterium NMR spectra were obtained as described in reference 3.

Most samples contained excess water and spectra showed a central isotropic peak. With the external magnetic field, **B**, perpendicular to the stacked bilayers, a pair of peaks with splitting $\Delta\nu$ was observed while, with the normal to the stack at the magic angle of 54.74°, all spectra were reduced to a single central peak of width ~100 Hz. A useful feature of spectra from monodomain samples is that the angular dependence completely rules out the presence of an asymmetry parameter in the averaging process of the deuterium quadrupole interaction. For **B** perpendicular to the stack the splitting is thus $\Delta\nu = 3/2\ \nu_Q \langle 3/2 \cos^2\theta - 1/2 \rangle$, where ν_Q is the quadrupole coupling constant (240 kHz) (2) and θ is the angle between a circular dichroic (CD) bond and the axis of symmetry of the liquid crystal. Thus $\Delta\nu$ is proportional to the time-averaged orientational order parameter of the water molecules.

The lipids studied were dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), and dimyristoylphosphatidylethanolamine (DMPE), allowing for variation of chain length and head group. Cholines have a P_β or ripple phase between their low temperature tilted $L_{\beta'}$ phase, and their high temperature L_α phase while ethanolamines go directly from L_β (untilted chains) to L_α. Fig. 1 shows the normalized splittings, $\Delta\nu/\Delta\nu_0$, plotted as a function of $T - T_c$ where T_c is the temperature of the first order phase transition to the L_α phase and $\Delta\nu_0$ is the splitting far from T_c. $\Delta\nu_0$ was based on the DPPC asymptote for DMPC since it has the same head group and hence water is in the same environment in the absence of fluctuations. To evaluate the critical exponents the quantity

$$S = \sum_{i=1}^{n} [\Delta\nu_i/\Delta\nu_0 - A|(T_i/T^*) - 1|^\alpha]^2 \qquad (1)$$

was minimized with respect to the parameters α, T^*, and A using the simplex algorithm. The sum is over the experimental points for a particular sample in a single phase. In Fig. 2 $\log(\Delta\nu/\Delta\nu_0)$ is plotted versus $\log|(T/T^*) - 1|$ where T^* is a pseudocritical temperature. The points for lipids in the L_α and $L_{\beta'}$ phases lie close to a straight line with slope one-half within experimental accuracy. The best fit constants obtained

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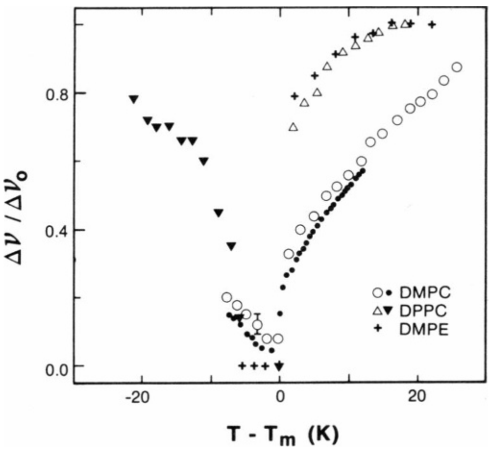

FIGURE 1 Normalized frequency versus $T - T_c$ for DMPC (● and ○), DPPC (△ for $T > T_c$, and ▼ for $T < T_c$) and DMPE (+). The zero fluctuation splittings, $\Delta\nu_0$, are 1.22 kHz for DMPC and DPPC and 4.40 kHz for DMPE. T_c was taken as 23°C for DMPC, 41°C for DPPC, and 50°C for DMPE.

from the simplex algorithm are given in Table I. It was not possible to expand the range of the measurements since small splittings were superimposed on the central peak and could not be measured. Data for DMPC samples in the L_α phase were found to be stable under range shrinking within the stated error limits. The second set of DMPC data were obtained after the sample had been frozen for 3 wk and the spectra indicated that it contained less water, thus accounting for a slightly larger splitting. No splitting was observed in the L_β phase of DMPE, probably due to the small number of waters of hydration in this phase. The DMPC data of Pope et al. (2) for 5.5 to 11 ^2H_2O molecules per phospholipid were also analyzed.

Area Fluctuation Model

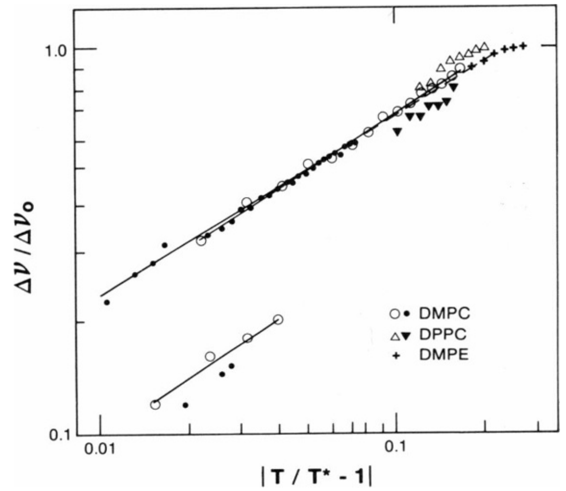

FIGURE 2 Log-log plot of normalized splittings versus reduced temperature, $|(T/T^*) - 1|$. The lipid type is indicated as in Fig. 1 and the same $\Delta\nu_0$ values were used. Values of the fit parameters T^* (or T^{**}), α, and A obtained from use of the simplex algorithm are given in Table I. The lines are drawn according to these parameters for the DMPC samples.

TABLE I
PARAMETERS OBTAINED BY MINIMIZATION OF S IN EQ. 1

Lipid		Water per lipid	α	T^* or T^{**}	A
				°C	
DMPC (●)	L_α	‡	0.48 ± 0.1	21.2 ± 0.5	2.6 ± 0.3
	P_β	‡	0.66 ± 0.2	22 ± 3	2.1 ± 0.5
DMPC (○)	L_α		0.56 ± 0.05	19.3 ± 1	3.1 ± 0.3
	P_β		0.58 ± 0.2	22.6 ± 1	1.6 ± 0.5
DPPC (△)	L_α	‡	0.52 ± 0.1	22 ± 5	3.0 ± 0.5
(▼)	$L_{\beta'}$	‡	0.54 ± 0.1	51 ± 3	2.7 ± 0.5
DMPE (+)	L_α	‡	0.36 ± 0.2	21 ± 5	8.8 ± 0.5
DMPC§	L_α	11	0.34 ± 0.02	26.0 ± 0.2	3.2 ± 0.2
	P_β		0.44 ± 0.05	26.1 ± 0.5	2.0 ± 0.5
	$L_{\beta'}$		0.23 ± 0.05	29 ± 5	2.3 ± 1
	L_α	8	0.46 ± 0.02	27.8 ± 0.2	5.0 ± 0.3
	$L_{\beta'}$		0.20 ± 0.05	31 ± 5	2.8 ± 1
	L_α	5.5	0.48 ± 0.02	30.6 ± 0.2	7.2 ± 0.5
	$L_{\beta'}$		0.23 ± 0.05	36 ± 5	4.4 ± 1

‡Contained some excess water.
§Data taken from reference 2.

We present here a new model in the spirit of Nagle and Scott's (6) ion permeability theory whereby lateral density fluctuations result in short-lived cavities in the head group region into which water molecules can enter. A study of the angular dependence of NMR relaxation times (8) confirms the presence of collective fluctuations in the density of lipid packing, while suggesting that collective fluctuations of the nematic order parameter do not make a significant contribution. In many studies, for example in reference 2, it has been observed that the head groups are little affected by the L_β to L_α transition. It seems likely that density fluctuations are the predominant pretransitional effect near this phase transition. Parsegian et al. (9, 10) measured the pressure required to compress a series of phospholipid bilayers and concluded that the energy needed to change the area of a lipid molecule by 15 Å^2 is $<k_BT$, suggesting that area fluctuations of the required size do indeed occur.

We postulate that the orientational order of the water molecules averages to zero in regions where area fluctuations exceed some size, ΔA_0, corresponding to the size of a water molecule or a small group of molecules. This reduction of orientational order to zero might result from tumbling of the water molecules in the cavity caused by the fluctuating molecular area. It can alternatively be pictured as resulting from a soft interface which does not impose a boundary condition on water order as does the hard surface which exists in the absence of significant fluctuations. The present model only requires that the order parameter be zero in regions where fluctuations are sufficiently large.

$\eta = A - A_\alpha$ (where A is molecular area and A_α is the average area in the L_α phase) is a reasonable order parameter to use in describing the $L_\beta - L_\alpha$ phase transition (11), although it is possibly secondary to the orientational order parameter (12). It is analogous to the use of density difference to describe the liquid-gas phase transition. Since the first derivative of free energy is zero at the equilibrium value of η and the second derivative approaches zero at T^*, the deviation of Landau free energy per molecule from its equilibrium value in the vicinity of the phase transition can be written following Jaehnig (12), but using the order parameter η in place of the orientational order parameter as:

$$\delta F(q, \delta\eta_0, T) = \frac{1}{2} a|T - T^*|(1 + \xi^2 q^2)\delta\eta_0^2, \qquad (2)$$

where q is the wave number of the mode and $\delta\eta_0$ is the amplitude of the deviation of the order parameter, η, from its equilibrium value. ξ is a parameter, called the correlation length, which we will neglect in our analysis. T^* should be replaced by T^{**} in the low temperature phase. If one does not come too close to T^* or T^{**}, as is probably the case since the approach to the hypothetical second order transition is interrupted by a first order transition at T_c, then $\xi^2 q^2$ can be neglected in Eq. 2. This gives a free energy similar to that used by Nagle and Scott to describe ion permeability.

The time-averaged splitting is $\Delta\nu = \Sigma P_i \Delta\nu_i$, where P_i is the probability of finding a molecule in environment i with splitting $\Delta\nu_i$. It has been assumed that regions with area fluctuations less than ΔA_0 contribute a splitting $\Delta\nu_0$ and regions with area fluctuations greater than ΔA_0 contribute zero splitting. Thus the normalized time averaged splitting is just equal to the probability that area fluctuations are smaller than ΔA_0 and $\Delta\nu/\Delta\nu_0 = P(\delta\eta < \Delta A_0)$. For a mode with energy given by $\delta F = 1/2 k_B T$, the amplitude of the area fluctuations is

$$\delta\eta_0 = (k_B T/a)^{1/2} |T - T^*|^{-1/2}. \tag{3}$$

Since $\delta\eta = \delta\eta_0 \sin(q_x x) \sin(q_y y)$ describes a mode with wave vector $\mathbf{q} = q_x \hat{x} + q_y \hat{y}$, the fraction of the surface area whose fluctuations are smaller than ΔA_0 is determined by the range of x and y values for which $\delta\eta < \Delta A_0$. If the energy of an area fluctuation required to accommodate a water molecule is small compared with $k_B T$, then $\sin(qx)$ can be approximated by qx giving:

$$\Delta\nu/\Delta\nu_0 = \Delta A_0 (2/\pi)^2 (a/k_B T)^{1/2} (T - T^*)^{1/2}. \tag{4}$$

The splitting is thus proportional to $|T/T^* - 1|^{1/2}$ and hence $\alpha = 1/2$ as observed in the L_α phase.

DISCUSSION

In Fig. 2 the normalized splittings fall close to a single line except for those points obtained for lipids in the P_β phase. P_β splittings were measured at a significant number of temperatures only for DMPC, where the results suggest that order is reduced by a factor additional to fluctuations. These points are multiplied by 0.53 as compared with those corresponding to temperatures in the $L_{\beta'}$ and L_α phases. This factor can be accounted for by invoking the model developed in reference 3 where the effect of ripple geometry on the splittings is calculated. For lyotropic liquid crystals ^2H_2O order can be a sensitive probe of surface geometry (13). The ripple angle obtained by calculating θ from $(3\cos^2\theta - 1)/2 = 0.53$ is 34°, in good agreement with x-ray data (14). The present results for well-aligned samples require that the water molecules sample ripples in different directions on the time scale of the NMR measurement resulting in a zero asymmetry parameter. Strenk et al. (3) estimated that water molecules diffuse ~4,000 Å in this time. Although the data presented here rule out the analysis in reference 3 based on a nonzero asymmetry parameter, the reduction in order produced by the ripples themselves is consistent with the model. Constancy of the multiplicative factor A indicates that θ and hence the amplitude-to-wavelength ratio of the ripples is independent of temperature while the material is in the ripple phase, in agreement with other observations (15).

The data plotted in Figs. 1 and 2 suggest an interesting and consistent picture of the phospholipid bilayer. The zero fluctuation splitting, $\Delta\nu_0$, is similar for both the cholines, DMPC and DPPC, a reasonable outcome considering the fact that the head group environment seen by the water molecules is very similar, if not identical, for these two lipids. The T^* values suggest that the mechanism responsible for the reduction in water order is insensitive to head group type and hydrocarbon chain length. T^{**} is 9% larger for the 14% longer chain DPPC than it is for DMPC due to the greater interaction energy of the longer chains in the crystalline phase.

For fully hydrated DMPC in the P_β phase T^{**} is lower than the phase transition temperature (of ~23°C). Also, for the DMPC data in reference 2, the transition looks second order where it probably is not and, for lower water concentrations, splittings in the L_α phase approach zero at T^* above the discontinuity in $\Delta\nu$. These results cannot be explained in terms of a first-order phase transition with a single coexistence curve between the L_α and P_β and the P_β and $L_{\beta'}$ phases. Experimental and theoretical phase diagrams (16) show that two-phase regions exist between each pair of phases, as would be expected in the concentration/temperature plane. These two-phase regions are eliminated only if measurements are performed at constant chemical potential. If the two-phase regions are collapsed, a discontinuity in the splitting should be obtained for both coexisting phases. Many of the data have been taken at low water concentrations where the splittings are largest, however, the width of the two-phase region is probably large here. A possible phase diagram is sketched in Fig. 3. Two-phase regions are drawn between each pair of phases in accord with the theoretical and experimental results of reference 16. It is consistent with the results of reference 2 if allowance is made for the fact that separate line splittings will be observed only if a single phase is sampled on the NMR time scale. The boundaries of the phases are only indicated for conceptual clarity, since we do not know their precise shapes.

Hydrated lipids are two-component systems: concentration fluctuations as well as area fluctuations may occur. These two order parameters will be coupled since an increase in area per lipid molecule favors a larger number of water molecules per lipid, that is, an increase in water

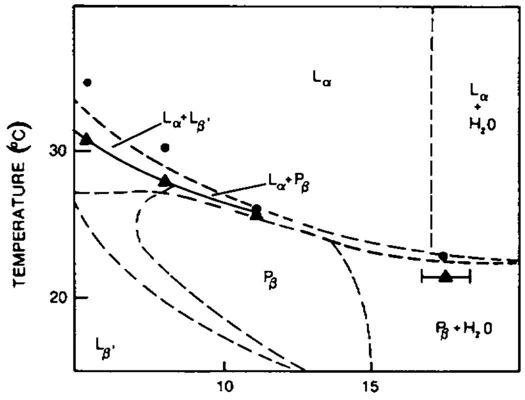

FIGURE 3 Pseudospinodal curve of DMPC. Triangles represent T^* values while circles were used for T^{**}. Possible two-phase regions are defined by dashed lines and the pseudospinodal curve is indicated by a solid line.

concentration. Fluctuations in two-component systems diverge at the pseudospinodal curve which occurs in the two-phase region. The prefix pseudo was added to spinodal (17) to allow for the possibility that the temperature at which fluctuations appear to diverge is not the maximum superheating or minimum supercooling temperature. The pseudospinodal and superheating/supercooling temperatures will not in general coincide, especially in a two-phase region.

The temperature range of the fluctuations obtained from analysis of NMR data is larger than that observed for DPPC using ultrasonics (18) and the separation between the critical temperature and the first-order phase transition temperature is larger. These authors observed three-dimensional density fluctuations rather than the lateral area variations postulated here to reduce water order and suggested by previous authors as an explanation for enhanced permeability. While we cannot explain the disagreement in T^* values obtained by ultrasonics and by the present analysis, the range of $|(T/T^*) - 1|$ values considered here is a bit more than a decade and the range of the ultrasonic measurements is even smaller. If pretransitional phenomena are responsible for enhanced membrane permeability, the coincidence of the temperature ranges suggests that it results from a mechanism similar to that responsible for the reduced water order studied here.

The idea that fluctuation effects are responsible for enhanced permeability of hydrated phospholipids to ions was first introduced by Doniach (5). He proposed that the lipid chain melting phase transition might occur near a critical point. Our results suggest that the lipids do not form a sequence with the long-chain high-melting temperature lipids closer to the critical temperature. This is not unreasonable since T_c is determined by hydrocarbon chain melting, whereas it is proposed here that density and/or concentration fluctuations produce the $|(T/T^*) - 1|$ dependence. However, for a particular lipid, the critical temperature is indeed very close, especially in the case of DMPC. Our T^{**} of 51°C can be compared with his critical temperature of 80°C for DPPC.

The present study of monodomain samples proves that the NMR splitting does go to zero in the vicinity of the chain ordering phase transition. This reduction in water order is a conspicuous and interesting feature of all ^{2}H NMR studies of phospholipids hydrated with heavy water. The hypothesis that this is the result of pretransitional fluctuations can explain the universal nature of these observations and is the only quantitative interpretation of this reduced splitting to date.

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