SPATIAL MODULATION OF WATER ORDERING IN LECITHIN BILAYERS

Evidence for a Ripple-Ripple Phase Transition

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ABSTRACT   Intense motional averaging effects on the ^{2}H nuclear magnetic resonance (NMR) spectrum of ^{2}H$_2$O that occur in aqueous dispersions of dimyristoyl-sn-glycero-3-phosphocholine (Myr$_2$-PtdCho) are explained by a spatial modulation in the orientational order of the water induced by ripplelike structures. The ratio of the amplitude to the periodic length of the ripples, A/λ, at a molar ratio of water/Myr$_2$-PtdCho of 9.5:1, is measured by ^{2}H NMR and found to be consistent with x-ray measurements of this ratio in the P_β phase of dipalmitoyl-sn-glycero-3-phosphocholine (Pam$_2$-PtdCho) bilayers. The sensitivity of ^{2}H NMR allows us to report the presence of two distinct ripple phases mediated with a discontinuous change in the value of A/λ. This result suggests that the two ripple structures observed for several phospholipid systems in excess water by freeze-fracture electron microscopy may be associated with two different phases instead of the same phase as previously assumed.

INTRODUCTION

More than a decade ago it was observed that the ^{2}H nuclear magnetic resonance (NMR) splittings of ^{2}H$_2$O in phosphatidylcholine bilayers undergo additional motional narrowing near the main phase transition (1). This observation was confirmed in several subsequent studies (2, 3) and several different explanations of the data have been provided.

Salsbury et al. (1) suggested that the reduction of the ^{2}H NMR spectral splitting near the main phase transition resulted from a modification of the water structure around the head group, associated with changes in its mobility at the main transition. They attributed the observed increase in splitting with increasing temperature above the main transition to a decreasing repeat distance between bilayers imposing a restraint upon bound water. Pope et al. (3) suggested that the observed spectral splitting of zero at the main transition could result from rapid exchange of bound water between environments with order parameters of opposite sign and equal magnitude. They also suggested that changes in the surface area of the lipid that occur at the pre- and main transitions may also contribute to changes in the ordering of bound water. Ulmius et al. (2) proposed that the smaller splittings observed in the P_β phase could result from diffusion of water molecules along the periodic ripples of the lamellae.

Here we show that this narrowing effect observed in the near the main phase transition of dimyristoyl-sn-glycero-3-phosphocholine (Myr$_2$-PtdCho)/H$_2$O can be explained in terms of a spatial modulation in the orientational order of the water brought on by ripple structures in the lipid bilayer. In addition, we show that at low levels of hydration the ratio of the amplitude to the periodic length of the ripple A/λ, can be measured from the ^{2}H NMR spectral patterns of ^{2}H$_2$O. We find that measured values of this ratio in Myr$_2$-PtdCho are found to be consistent with a value for the same ratio measured by Stamatoff et al. (4) using x-ray diffraction techniques, in the P_β phase of dipalmitoyl-sn-glycero-3-phosphocholine (Pam$_2$-PtdCho) bilayers. A most interesting aspect of this work, however, is an observation in which the values of A/λ, as well as the temperature dependence of this quantity, change discontinuously from one ripple structure to the other. These measurements of A/λ and the observation of two distinct ripple structures were made close to the lowest possible levels of hydration (1 mol Myr$_2$PtdCho/9.5 mol H$_2$O), which exist in the P_β phase, as indicated by the phase diagram (5). There are also large motional averaging effects at higher levels of hydration except that at the higher levels the ^{2}H-NMR spectral lines become narrower and as a result, the uncertainty in the measured values of A/λ is increased. These results, nevertheless, give a convenient method for measuring A/λ and they may give added insight into a confusing result observed in freeze-fracture electron microscopic studies where several investigators

(6–8) have reported the presence of two coexisting structures in phosphatidylcholine samples, which have been quenched from temperatures within the P_β phase.

MATERIALS AND METHODS

Myr$_2$-PtdCho was obtained from Calbiochem-Behring (San Diego, CA) and used without further purification. 1-myristoyl-2-[14,14,14-^{2}H$_3$]myristoyl-sn-glycero-3-phosphocholine was prepared as previously described (9). Powder samples were prepared by sealing weighed quantities of phospholipid and ^{2}H$_2$O (99.8% isotopic purity) in 5-mm outside diameter NMR tubes. Mixing of the two components was accomplished by allowing the sample to equilibrate in the spectrometer at 40°C until the isotropic signal from ^{2}H$_2$O disappeared. ^{2}H NMR spectra were obtained at 30.87 MHz with a 10 cm-bore high-resolution superconducting solenoid (Nalorac, Concord, CA) on a "home-built" four-phase spectrometer. A Nicolet 1080 data system (Nicolet Instrument Corp., Madison, WI) and Biomation 805 digitizer (Biomation Corp., Gould Inc., Instruments Div., Santa Clara, CA) were used to acquire and process the ^{2}H NMR spectra using a 500-kHz effective spectral width and $2 \times 1,024$ data points. The $\pi/2$ pulse width was 4.5 μs. A modified quadrupole-echo pulse sequence was used for data acquisition (9). Typically the value of τ in the quadrupole-echo pulse sequence was 100 μs, and the pulse sequence was repeated five times per second. Spectral patterns were unaltered by τ values up to 10 ms. Spectra were recorded "on-resonance." An adequate signal-to-noise ratio was obtained from 4,000 transients. All observed spectral changes with temperature are both reversible and reproducible.

Sample temperature was controlled with a boron nitride oven, housed in an evacuated brass chamber. The oven was heated by a computer-controlled Neslab RTE-4 circulating water-bath (Neslab Instruments, Inc., Portsmouth, NH). The outer brass jacket was kept at constant temperature with a separate water-flow system. The sample was mounted horizontally in a boron nitride cylinder, which sits inside the boron nitride oven. Temperature could be controlled to 0.1°C. The temperature gradient across the sample (10 mm × 5 mm) was estimated to be <0.1°C by observing the nematic-isotropic phase transition in a thermotropic liquid crystal. After data acquisition, water content in the sample was determined gravimetrically. Lipid purity was checked by thin-layer
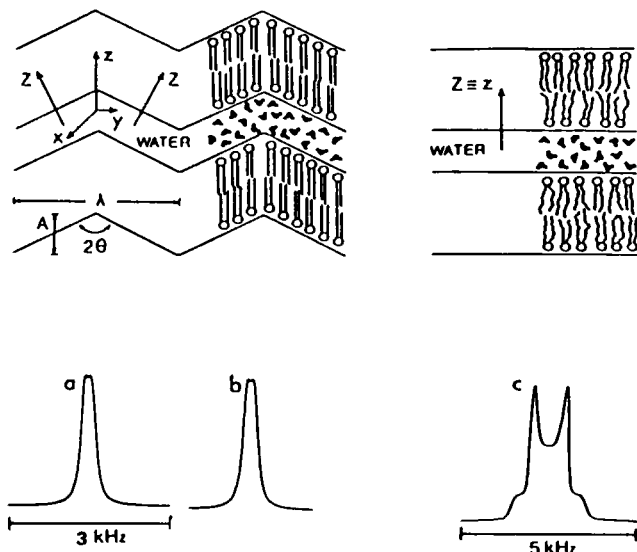

FIGURE 1   Illustration of the ripple and lamellar structures defining the symbols used in the text and showing the corresponding spectral patterns with (a) being the experimental pattern recorded at $T = 28°C$, (b) the corresponding simulated pattern, and (c) the spectral pattern recorded at 45°C.

chromatography (TLC). No results are reported on samples showing any detectable breakdown. In this preliminary study, spectral lineshapes were simulated using as fitting parameters, the time-averaged coupling constant $\bar{v}_Q$, the motionally induced asymmetry parameter $\bar{\eta}$, and a line-broadening parameter σ.

THEORY

A typical model for the ripple structure, which is presented for simplicity as a triangular structure, is shown in Fig. 1. Other models such as a sinusoidal or sawtooth model do not substantially alter the results and will be discussed in a subsequent paper. The purpose of this paper is only to introduce the ripple structure as a mechanism to motionally narrow the ^{2}H NMR spectrum of ^{2}H$_2$O and to demonstrate its potential in structural studies of artificial and biological membranes.

In our analysis we assume the fast-motion regime, in which the ^{2}H$_2$O molecule and hence the deuterated sites have ample time to sample all relevant orientations of the aggregate or structure during the time of the NMR measurement. In this case, one has a spectral pattern with a characteristic shape that depends on the value of the time-averaged coupling constant, $\bar{v}_Q = eQ\bar{V}_{zz}/h$, and the motionally induced asymmetry parameter, $\eta = (\bar{V}_{xx} - \bar{V}_{yy})/\bar{V}_{zz}$. One also needs to add a line-broadening parameter, the source of which is discussed later. Following the usual NMR convention of $V_{xx} \leq V_{yy} < V_{zz}$ for the time-averaged electric field gradient tensor, these compounds can be calculated, using the value V_{ZZ} in the unmodulated lamellar phase, from the usual transformation equations (10)

$$\bar{V}_{xx} = V_{ZZ} \langle \tfrac{3}{2} \sin^2 \theta' \cos^2 \phi' - \tfrac{1}{2} \rangle$$

$$\bar{V}_{yy} = V_{ZZ} \langle \tfrac{3}{2} \sin^2 \theta' \sin^2 \phi' - \tfrac{1}{2} \rangle$$

$$\bar{V}_{zz} = V_{ZZ} \langle 1 - \tfrac{3}{2} \sin^2 \theta' \rangle,$$

where, x, y, z is the principal axes frame of the time-averaged electric field gradient as indicated in Fig. 1 and X, Y, Z is the principal axes frame in the axially symmetric lamellar phase with $V_{XX} = V_{YY} = -V_{ZZ}/2$. The angles θ' and ϕ' give the orientation of Z in the x, y, z frame. V_{ZZ} also results from a time average but V_{xx}, V_{yy}, and V_{zz} are further time averaged by self-diffusion of the deuterated molecule over the rippled surface. The central idea is that, locally, on the membrane surface the principal axis of the quadrupole interaction for ^{2}H$_2$O is normal to the surface, as it is known to be in the lamellar, L_α, and L_β phases. As the water diffuses over the surface of the ripples on the time scale of the ^{2}H NMR measurement, the orientation of the principal axis becomes modulated to further motionally average the quadrupole interaction. The distance, d, a water molecule diffuses can be estimated by the expression, $d \simeq \sqrt{2D\tau}$, where D is the diffusion constant and τ the time scale of the NMR measurement. In bilayer membranes $D \simeq 10^{-6}$ cm^2/s (11) and $\tau \simeq v_Q^{-1} \simeq 10^{-3}$ s where v_Q is the quadrupole coupling constant for water in the L_α phase where no ripples are present. If the length of the periodic ripples is $\simeq 150$ Å (4), then consideration of the estimated value of d ($\simeq 4,000$ Å) indicates that the water molecule can diffuse over many ripples during the NMR measurement.

In this triangular model, diffusion over the surface of the ripples causes an exchange between the two orientations of Z in Fig. 1 to yield a further time averaging of the field gradient tensor of the quadrupole interaction. With x, y, z as the new time-averaged principal axis frame for the ripple phase, we calculate an average of the field gradient tensors (10) to obtain the time-averaged value of the coupling constant, $\bar{v}_Q$, and the motionally induced asymmetry parameter, $\bar{\eta}$ (12, 13, 14)

$$\bar{v}_Q/v_Q = \tfrac{3}{2} \sin^2 \theta - \tfrac{1}{2} \tag{1}$$

$$\bar{\eta} = \frac{\tfrac{3}{2} \cos^2 \theta}{\tfrac{3}{2} \sin^2 \theta - \tfrac{1}{2}}, \tag{2}$$

where the angle θ is confined to the range $0 < \pi/2 - \theta < 35°$. It is seen from Eqs. 1 and 2 that there is a unique relationship between $\bar{\eta}$ and $\bar{v}_Q/v_Q$

whereby

$$\bar{\eta} = \frac{1 - \bar{v}_Q/v_Q}{\bar{v}_Q/v_Q},\qquad(3)$$

which can be used as a test of the model. Eq. 2 together with the geometry of the model as shown in Fig. 1 can be used to determine the ratio of the ripple amplitude to the ripple periodic length. Thus,

$$A/\lambda = 0.5\,[2\bar{\eta}/(\bar{\eta} + 3)]^{1/2}.\qquad(4)$$

Note here that the above model assumes that the direction of the ripple (x axis of Fig. 1) experienced by the diffusing water molecule does not change over the range of diffusion. Since freeze-fracture electron microscopic studies (6–8) show that in some samples the direction of the ripple can change over a dimension of ~5,000 Å it may be necessary to modify Eqs. 2 and 3 to account for this feature. Under this effect these equations must be multiplied by a factor $\langle\cos 2\Delta\alpha\rangle$ where $\Delta\alpha$ reflects the change in direction of a ripple experienced by a diffusing water molecule. The angular brackets indicate a time average as the diffusing water will experience a distribution in angles $\Delta\alpha$. In the samples studied here, $\Delta\alpha \simeq 0$ as will be shown later.

RESULTS

In Fig. 1 we show some typical ^{2}H NMR spectral patterns from a sample of Myr$_2$-PtdCho with ^{2}H$_2$O, at a molar ratio of lipid/water of 1:9.5. These patterns are not unlike those reported by Pope et al. (3). Also in Fig. 1 we show a calculated spectral pattern where the fitted parameters are $\bar{\eta}$ and $\bar{v}_Q$. Substantial line broadening had to be introduced in order to smooth or round the edges of the spectral patterns. The source of this broadening is not understood but is believed to be due to either (a) a nonuniform ripple structure and hence a distribution in the values of A/λ or (b) the ripple amplitude or periodic length being a dynamic phenomenon on the time scale of the ^{2}H NMR measurement. The effect of the broadening was to increase the uncertainties in the fitted values of $\bar{\eta}$ and $\bar{v}_Q/v_Q$, estimated to be ±0.1 and ±0.05, respectively. The line broadening was introduced in a manner used in other liquid crystal systems. (15). A Gaussian broadening factor with a root mean square deviation (σ) was used to broaden the lineshapes in the computer simulations of the experimental powder patterns. This factor has the same temperature dependence as $\bar{v}_Q/v_Q$, with a maximum value of 200 ± 20 Hz at 19 and 45°C and a minimum value of 100 ± 20 Hz at 24–27°C.

In Fig. 2 are shown the measured values of $\bar{\eta}$ and $\bar{v}_Q/v_Q$. The value of v_Q was measured at a temperature of 50°C where the uniaxial appearance of the spectral powder pattern ($\bar{\eta}$ = 0) indicated that there were no ripples present. At 50°C, we measured v_Q = 2.130 kHz. Note that at 15°C, where the value of $\bar{\eta}$ is again zero, we also observe v_Q = 2.130 kHz, which is consistent with our model. In the temperature range between 24° and 28°C we observe two overlapping spectral patterns, which give the appearance of a two phase region in that the intensity of one pattern grows at the expense of the other as the temperature is varied. The values of $\bar{\eta}$ in that region at 25°C are indicated by open squares in Fig. 2.
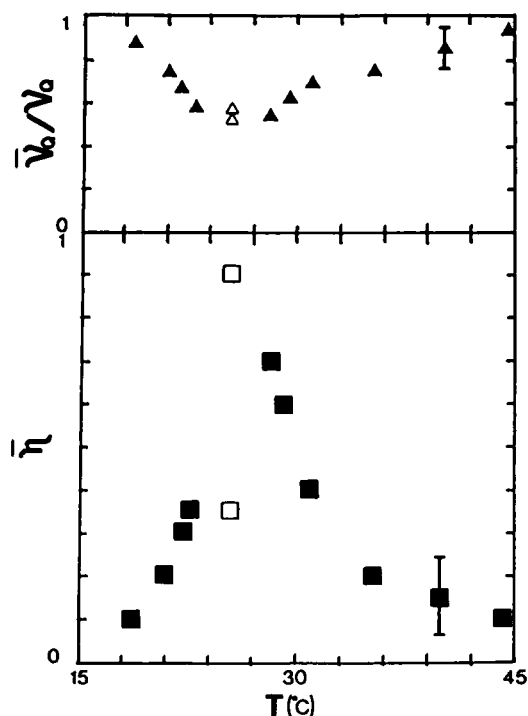

FIGURE 2 Measured values of $\bar{v}_Q/v_Q$ and $\bar{\eta}$ where v_Q = 2.130 kHz as described in the text. Open squares and triangles are from two overlapping patterns in the two-phase region.

Fig. 3 shows a plot of $\bar{v}_Q/v_Q$ vs. $\bar{\eta}$, which is an important check of our model. Even though the error bars are large this figure shows agreement with what the simple triangular model predicts for the interdependence.

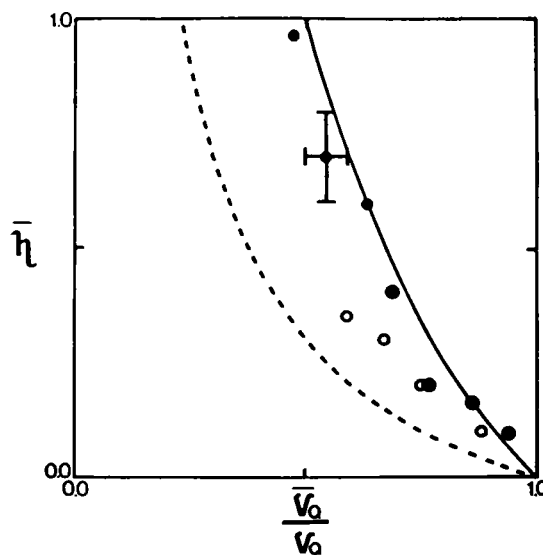Finally, in Fig. 4 we show a plot of A/λ vs. temperature

FIGURE 3 Plot of $\bar{\eta}$ vs. $\bar{v}_Q/v_Q$ with solid circles indicating values at temperatures above the ripple-ripple transition and open circles indicating those below. The solid line is a plot of Eq. 3 and the dashed line shows that expected for the case where changes in the ripple direction further average the quadrupole interaction. The dashed line is plotted for $\langle\cos 2\Delta\alpha\rangle$ = 0.5.
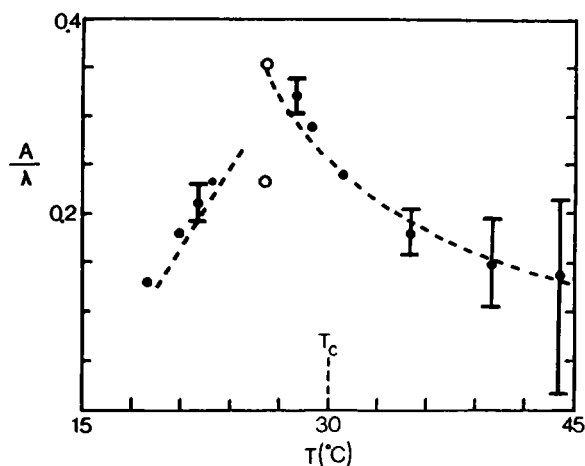
FIGURE 4 Plot of A/λ vs. temperature showing the ripple-ripple transition at 25°C. Open circles obtained from two overlapping spectral patterns in the two phase region. T_c indicates the center of the two-phase chain melting transition for Myr$_2$-PtdCho/water in a mole ratio of 1:9.5, as measured from the ^{2}H NMR spectrum of 1-myristoyl-2-[14,14,14-^{2}H$_3$] myristoyl-sn-glycero-3-phosphocholine/water at approximately the same lipid-to-water ratio.

obtained from the measured values of $\bar\eta$ and Eq. 4. As this figure shows, the error in determining A/λ becomes large for values <0.15. The discontinuity in A/λ and its abrupt change in temperature dependence at 25°C clearly suggest the existence of a two-phase transition at that temperature.

To examine the relationship of this transition to the main lipid gel/liquid-crystalline phase transition, we performed a ^{2}H NMR study of an aqueous dispersion of 1-myristoyl-2-[14,14,14-^{2}H$_3$]myristoyl-sn-glycero-3-phosphocholine, at a water/lipid molar ratio of 9.5:1. In this study we observed a chain-ordering transition with a two-phase region extending from ~25–33°C, which has the appearance of the main (chain melting) transition. The lower value of 25°C is near the temperature at which the ripple transition was observed with the water regions.

DISCUSSION

It is first necessary to establish that our ^{2}H NMR measurements were recorded in the well-defined P_β phase. The water/lipid mole ratio of 9.5:1, corresponding to 19.7 wt % water, places the aqueous Myr$_2$PtdCho dispersion at the leftmost fringe of the phase boundary between the L_β and P_β phases, according to the phase diagram of Janiak et al. (5). Calorimetric measurements of Myr$_2$PtdCho/water dispersions of the same water content as the NMR sample showed between 10 and 20°C a broad transition that we have assigned to the so-called "pretransition," or the transition labeled T_2 by Janiak et al. (5). Since this transition does not occur for water concentrations <20 wt % (5), our NMR spectral data are probably from the P_β rather than the L_β phase.

To confirm this conclusion, we have recorded the ^{2}H

NMR spectra of aqueous Myr$_2$PtdCho dispersions for a water/lipid mole ratio of 11:1, which is certainly within the P_β region of the phase diagram (5). Essentially, the same results were obtained as in the above sample of lower water content. The data for the samples containing less water are reproduced here, however, because it was possible to obtain better spectral fits in these instances than for the samples of higher water content. This is because the spectral lines are broader at the lower water concentrations and the values of $\bar\eta$ and $\bar\nu_Q$ are extracted with less uncertainty. In samples containing excess water, the effect is still present but the technique cannot be used at all. Finally, we have recorded the ^{2}H NMR spectra of an aqueous dispersion of 1-myristoyl-2-[14,14,14-^{2}H$_3$]myristoyl-sn-glycero-3-phosphocholine for a water/lipid mole ratio of 9.5:1. On heating this sample from low-temperature phases, we first observed at 25°C in the NMR spectrum the appearance of a second powder pattern that increased in intensity until near 30°C, when it equaled that of the powder pattern corresponding to the lower-temperature phases. At 33°C only a single powder pattern, corresponding to that observed in the L_α phase, was observed. At this water concentration the sample does not contain excess water and the chain-melting transition occurs over a range of temperatures. Note that the water data suggest that ripples are present over the entire region where the chains are melting.

The agreement between the value of A/λ, determined from x-ray measurements of Stamatoff et al. (4) to be 0.28 for the P_β phase of Pam$_2$-PtdCho, and the corresponding ratio in Fig. 3 for corresponding temperatures, establishes confidence in the above interpretation of the motional narrowing of the ^{2}H NMR spectrum of ^{2}H$_2$O in the Myr$_2$-PtdCho/^{2}H$_2$O system. The fact that a nonzero asymmetry parameter was not recognized earlier as contributing to the shape of the spectral patterns is perhaps due to the large amount of broadening of the deuterium spectral lines, which reduces the characteristic detail of deuterium spectral patterns, thus masking the effect of a finite value of $\bar\eta$. As discussed earlier, this broadening could result from a nonuniformity in the values of A/λ, or A/λ could be time dependent on the time scale of the ^{2}H NMR measurement. It could also be that the above model may be too simple in that the extent of the motional modulation of the water molecules by the rippled surface may be a function of the distance of the water molecule from the surface of the phospholipid. In such a case a diffusing water molecule may have to sample an area with a dimension of the ripple wavelength and intrabilayer distance in order to fully time average the spectrum. Such an area may be too large for the available diffusion time of a water molecule yielding a distribution and an effectively broadened spectrum. A study of the line broadening making use of the quadrupole echo to examine such dynamic effects is currently being undertaken.

A most interesting aspect of this work is the appearance

of a possible ripple-ripple phase transition as suggested by the discontinuity in Fig. 4. The data in Fig. 4 also suggest that the rippling occurs, perhaps as a dynamic process, in the two-phase region where the chains are melting.

Finally, we note that this use of the ^{2}H NMR of ^{2}H$_2$O to examine the modulated structure of lipids is not the first such study, since the technique has recently been used to measure aggregate shapes in soap materials (12, 13). Structures other than ripple structures such as, for example, structures observed in soap could also account for our measured values of $\bar{\eta}$ and $\bar{v}_Q$.

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