NMR STUDY OF SYNTHETIC LECITHIN BILAYERS IN THE VICINITY OF THE GEL-LIQUID-CRYSTAL TRANSITION

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ABSTRACT ^1H, ^2H, and ^{31}P NMR methods have been employed in the study of dimyristoyl lecithin bilayers hydrated with D_2O in the gel ($L\beta'$), intermediate ($P\beta'$) and liquid-crystalline ($L\alpha$) phases. For D_2O/lipid molar ratios, n, in the range $7 \leq n \leq 11$ discontinuities are observed in the deuterium NMR splittings at both main and pretransitions. A partial phase diagram based on NMR and differential scanning calorimetry data is presented. ^1H NMR dipolar splittings are observed for macroscopically oriented samples in all three phases. Changes in the ^1H splittings are correlated with ^2H and ^{31}P data and interpreted to show that the chain tilt in the gel phase undergoes a discontinuous change on transition to the intermediate phase, which brings the chain axes closer to the bilayer normal. An estimate of chain tilt in the gel phase is made on the basis of NMR data and found to be ~23° for a sample with $n = 11$ at 18°C.

INTRODUCTION

It is well known that hydrated lipid bilayers formed from fully saturated synthetic phospholipids of uniform chain length exhibit a well-defined phase transition associated with the melting of the hydrocarbon chains (1–3). In lecithin bilayers above the (hydration dependent) chain-melting temperature a lamellar liquid-crystalline ($L\alpha$) phase is formed in which there is rapid translational diffusion of the molecules in the plane of the bilayer. On cooling to the "gel" phase the hydrocarbon chains of the lipids take up an ordered arrangement which approximates to a two-dimensional hexagonal lattice. The details of the phase structure and molecular conformation of lecithin molecules in the gel phase however remain a subject of controversy.

Differential scanning calorimetry (4, 5) reveals a strongly exothermic peak on cooling through the "main" chain melting transition, and a smaller "pretransition" at lower temperature. The separation of the main and pretransition peaks is a function of both lipid chain length and hydration. On the basis of x-ray evidence (6–13) several structures have been proposed for the phases existing below the main transition. In the gel ($L\beta'$) phase below the pretransition the chains are tilted at an angle to the normal of the planar bilayers, but it is not clear how the chain tilt varies with temperature in the transition region. In the "intermediate" ($P\beta'$) phase between the thermal transitions, low-angle x-ray diffraction results indicate the presence of a long-range periodicity of the structure in the plane of the bilayers. This result is supported by evidence from freeze-fracture electron microscopy (14).

Neutron diffraction evidence (15) indicates that in both gel and liquid-crystalline phases the average orientation of the phosphocholine headgroups is almost parallel to the bilayer surface. Even in the gel phase there is evidence that the lipid molecules exhibit considerable translational mobility (16).

In this work, deuterium magnetic resonance (DMR) has been employed to study the binding of water (D_2O) to lecithin bilayers as a function of temperature and hydration in both gel and liquid-crystalline phases. Similar techniques have been used in a number of previous studies of water binding to phospholipid bilayers (17–22). However most studies have concentrated on the liquid-crystalline phase, with very little data below the chain-melting transition, although Ulmius et al. (21) used the sensitivity of the water (^2H) splittings to bilayer structure in order to map the phase behaviour of dipalmitoyl lecithin bilayers in the vicinity of the phase transitions. In the present study we relate changes in water splittings to changes in bilayer structure between gel, intermediate and liquid-crystalline phases for dimyristoyl lecithin bilayers. The results are correlated with observed changes in proton magnetic resonance (PMR) spectra, in particular resolved ^1H splittings obtained from macroscopically oriented bilayers (23).

THEORY

In the presence of an electric field gradient (EFG), the Zeeman energy levels of a deuteron (spin $I = 1$) are perturbed, giving rise to a doublet NMR spectrum. For a crystalline sample in which the spins are ordered on a rigid lattice and experience an axially symmetric EFG, the splittings are given by (24)

$$\Delta\nu_Q(\alpha) = \frac{3}{2}\left(\frac{e^2qQ}{h}\right)\left|\left(\frac{3\cos^2\alpha - 1}{2}\right)\right| \tag{1}$$

where the quadrupole coupling constant $(e^2qQ/h) \approx 240$ kHz for D_2O and α is the angle between the axis of the EFG and the applied magnetic field $\mathbf{H}_0$. The assumption of axial symmetry is valid for D_2O (asymmetry parameter $\eta \approx 0.1$), the EFG symmetry axis being along the O—D bond (25).

A doublet spectrum is also obtained for the case of an isolated pair of $I = \frac{1}{2}$ nuclei interacting via the magnetic dipolar coupling. In this case the splitting is given by (26)

$$\Delta\nu_D(\beta) = \frac{3\gamma^2h}{\mathbf{r}^3}\left|\left(\frac{3\cos^2\beta - 1}{2}\right)\right| \tag{2}$$

where β is the angle between the interspin vector $\mathbf{r}$ and the magnetic field, and γ is the gyromagnetic ratio of the nuclei. Whereas the quadrupolar splittings are substantially intramolecular in origin, however, magnetic dipolar couplings may comprise contributions from both intermolecular and intramolecular interactions.

In the presence of rapid molecular motion, the splittings are averaged over all orientations through which the molecule is carried by the motion. Rapid isotropic reorientation will therefore result in the collapse of the doublet to a singlet. In the case of a lamellar phase, however, the motion of both lipid molecules and bound water can be expected to be anisotropic, giving rise to a doublet spectrum of reduced splitting relative to the corresponding rigid-lattice value.

For the case of macroscopically oriented bilayer samples in the presence of rapid anisotropic molecular reorientation, the observed water (^{2}H) splittings are given by

$$\Delta\nu_Q(\phi) = \frac{3}{2}\left(\frac{e^2qQ}{h}\right)\left|S_Q\left(\frac{3\cos^2\phi - 1}{2}\right)\right| \tag{3}$$

where ϕ is the orientation of the bilayer normal to the external magnetic field, and S_Q is an "order parameter." The observed DMR spectrum from bound water is the result of rapid exchange of water molecules between different binding environments, giving rise to an average order parameter:

$$S_Q = \sum_i p_i S_{Qi} \tag{4}$$

where p_i is the proportion of water molecules in the ith environment of order parameter

$$S_{Qi} = \tfrac{1}{2}\overline{(3\cos^2\theta_i - 1)} \tag{5}$$

and θ_i is the angle between the EFG symmetry axis and the bilayer normal. For powder samples the splittings of the principal peaks of the resulting 'Pake' spectrum correspond to substituting $\phi = 90°$ in Eq. 3.

It is thought that the lipid molecules themselves may undergo rapid reorientation about the molecular long axis in the gel (13, 22, 27) as well as liquid crystal phases. The axis itself reorients more slowly over a range of angles relative to a preferred direction which in the Lα phase is normal to the lamellae, but in the Lβ' phase subtends a well-defined tilt angle with respect to the bilayer normal. On this assumption, and assuming cylindrical symmetry about the instantaneous molecular long axis, we can write for the ^{1}H dipolar splitting arising from a microdomain in which all the molecules are tilted in the same direction

$$\Delta\nu(\delta) = \frac{3\gamma^2h}{\mathbf{r}^3}\left|S_D\left(\frac{3\cos^2\delta - 1}{2}\right)\right| \tag{6}$$

with

$$S_D = -\frac{1}{2}S_{mol} = -\frac{1}{2}\left(\frac{\overline{3\cos^2\gamma} - 1}{2}\right). \tag{7}$$

Here δ is the angle between the average direction of the molecular axis and the magnetic field, while γ is that between the instantaneous molecular axis and its average direction. S_{mol} is the usual molecular order parameter (28).

MATERIALS AND METHODS

Dimyristoyl lecithin (DML) was obtained from Calbiochem, Los Angeles, Calif., and used without further purification. Powder samples were made by weighing appropriate quantities of lipid and D_2O into a glass tube. The samples were mixed by centrifuging the contents backwards and forwards through a narrow constriction at a temperature corresponding to the liquid-crystalline phase. Oriented samples were made by dissolving the lipid in chloroform and pipetting the solution on to small (20 × 6 mm) glass slides ~0.1 mm thick. The solvent was then allowed to evaporate, and final traces were removed under vacuum. The samples were hydrated by pipetting D_2O on to each slide from a microliter syringe. The

slides were then stacked at a temperature above the chain-melting temperature; each slide was gently rubbed back and forth on the stack to enhance sample orientation. Typical samples consisted of 20–30 such slides. All the samples were kept under dry nitrogen and allowed to equilibrate for at least 2 wk before use. In the case of the oriented samples a small tube of D_2O was suspended in the top of the sample tubes to maintain sample hydration. Hydration of these samples was estimated from the ratio of ^2H to ^1H signal sizes at constant frequency, and checked by subsequent weight analysis. Although the hydrations determined in this way agreed to within ~10%, some variability in hydration for the oriented samples was likely. In particular sample dehydration was detected at temperatures >~40°C for DML. Sample purity was monitored by thin-layer chromatography.

^2H NMR measurements were carried out using a Bruker SXP spectrometer (Bruker Instruments, Inc., Billerica, Mass.) operating a 9.8 MHz. A "quadrupolar echo" technique (29, 30) was used to overcome receiver-recovery problems resulting from the short free-induction decay of the ^2H signals. ^1H and ^{31}P results were obtained on a Bruker CXP 100 spectrometer operating at 90 and 36.4 MHz, respectively. The macroscopically oriented samples were supported in a homemade goniometer which enabled the sample orientation to be adjusted to ±0.1°. ^1H spectra were obtained by Fourier transforming from the peak of the dipolar "solid" echo after a 90°-τ-90°$_{90}$ pulse sequence (31, 32) again in order to overcome receiver-recovery problems.

RESULTS

^2H NMR

Typical DMR spectra from powder samples of DML-$n$$D_2O$ are shown in Fig. 1 for Lα, Pβ' and Lβ' phases. The spectra approximate to the Pake form, broadened by inhomogeneities in

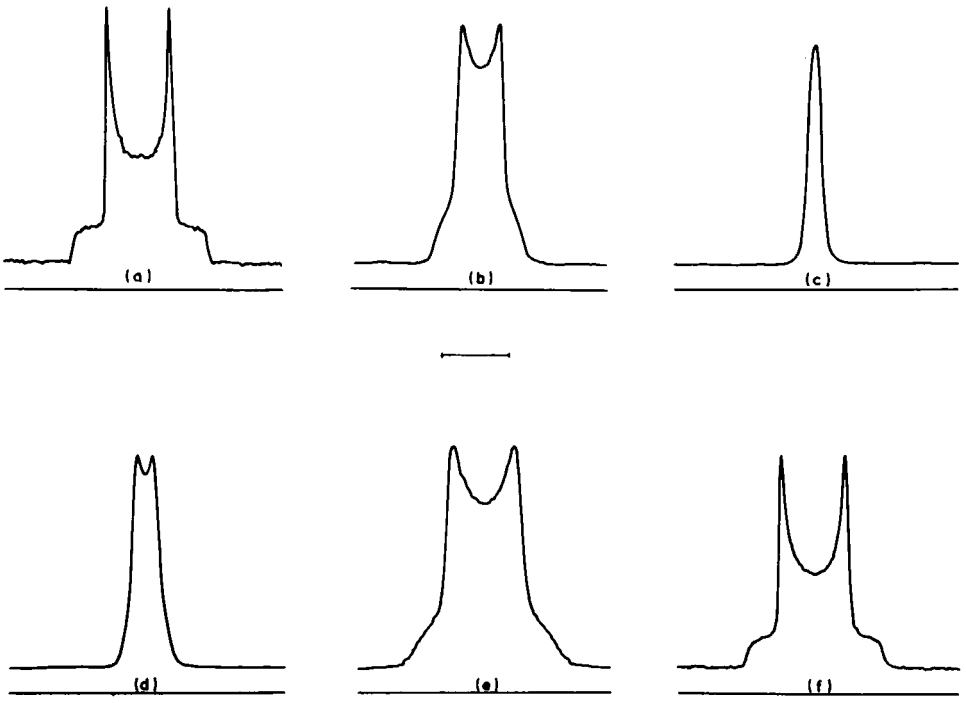

FIGURE 1 Typical DMR spectra from water (D_2O) in DML bilayer dispersions for Lα (*a–c*), Pβ' (*d*) and Lβ' (*e*, *f*) phases. Sample hydrations were n = 8 (spectra *a* and *f*, scale 1 kHz) and n = 11 (spectra *b–e*, scale 500 Hz), spectrometer frequency 9.8 MHz.

the magnetic field $\mathbf{H}_0$. The variation of doublet splitting with temperature for samples with hydration levels $n \approx 5.5$, 8, and 11 mol of D_2O/mol of lipid are shown in Fig. 2. The results of $n \approx 5.5$ were obtained for an oriented sample with the bilayer normals parallel to the applied magnetic field ($\phi = 0$), and half splittings are shown in Fig. 2 for comparison with the powder samples. Thus in both cases the plots reflect the variation of the quantity $\frac{3}{4}(e^2qQ/h)S_Q$. In general good agreement was achieved between results from macroscopically oriented and powder samples.

In the hydration range $7 \leq n \leq 11$ discontinuities occur in the splittings at both the pretransition and the main transition. The transitions themselves are hydration dependent in this hydration range. At the transitions from both gel to intermediate and intermediate to

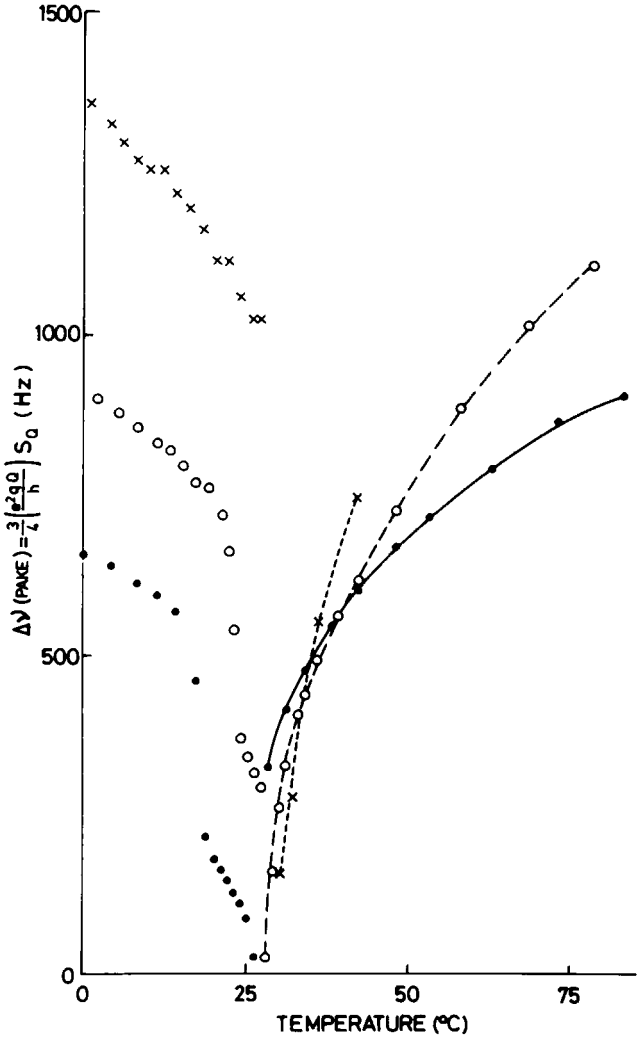

FIGURE 2 Variation of doublet splitting $\Delta\nu$ with temperature for water in DML-nD$_2$O bilayers with hydrations $n = 5.5$ (×), $n = 8$ (○), and $n = 11$ (●).

liquid-crystalline phases the splittings are reduced. For hydrations $n \leq 6$ no pretransition is evident, but a mixed-phase region is observed in the vicinity of the main transition, double splittings corresponding to the coexistence of $L\beta'$ and $L\alpha$ phases being observed over a temperature range of ~2°C in the macroscopically oriented samples. No such mixed-phase regions were observable at the boundaries of the intermediate phase. At hydrations $n \sim 12$ the pretransition is no longer evident from the water splittings, (although its presence can still be detected from differential scanning calorimetry), whereas for $n = 15$ the splittings themselves could no longer be resolved over the transition region. Above the main transition, the water splittings increase rapidly with increasing temperature in the $L\alpha$ phase, the increase being most rapid for low hydration samples. Although the splittings must eventually pass through a maximum when the temperature is high enough (17), we did not investigate this in our study because we wanted to avoid sample dehydration, particularly in the case of the macroscopically oriented samples. There was some evidence of sample hysteresis at the pretransition, superheating (supercooling) by as much as 2°C being necessary to induce a transition to the intermediate (gel) phase. No hysteresis of this magnitude was associated with the main transition as evidenced by both water splittings and lipid ^1H spectra (22). On the basis of the water splitting data it is possible to draw a phase diagram for the DML-D_2O system in the vicinity of the phase transitions (Fig. 3). Results from differential scanning calorimetry have been included in order to extend the diagram to hydrations beyond that for which the water splittings were observable.

^1H and ^{31}P NMR

^1H spectra for a macroscopically oriented sample of DML-nD_2O ($n \approx 11$) with the bilayer normal oriented parallel to the applied field ($\phi = 0$) are shown in Fig. 4, for liquid crystal, intermediate, and gel phases. All exhibit resolved dipolar splittings. The contributions to the observed spectrum from different groups of protons in the lecithin molecule can be assigned on

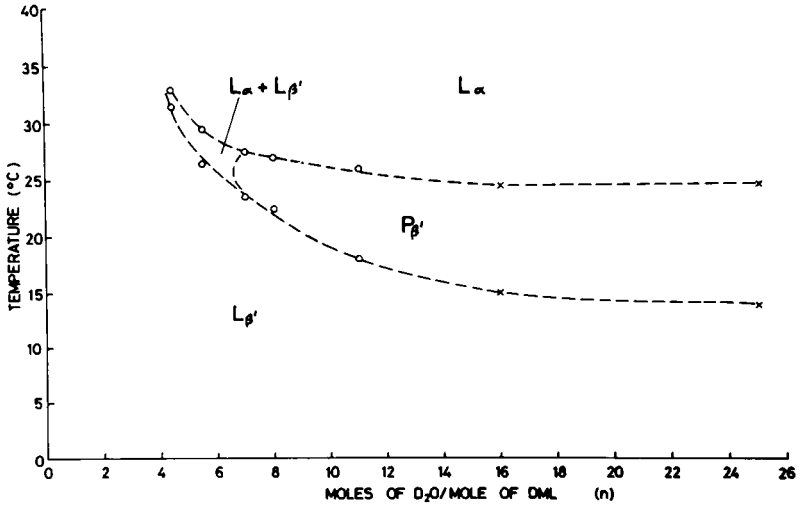

FIGURE 3 Phase diagram for DML-D_2O bilayer dispersions in the phase-transition region from DMR data (O) and differential scanning calorimetry (×).

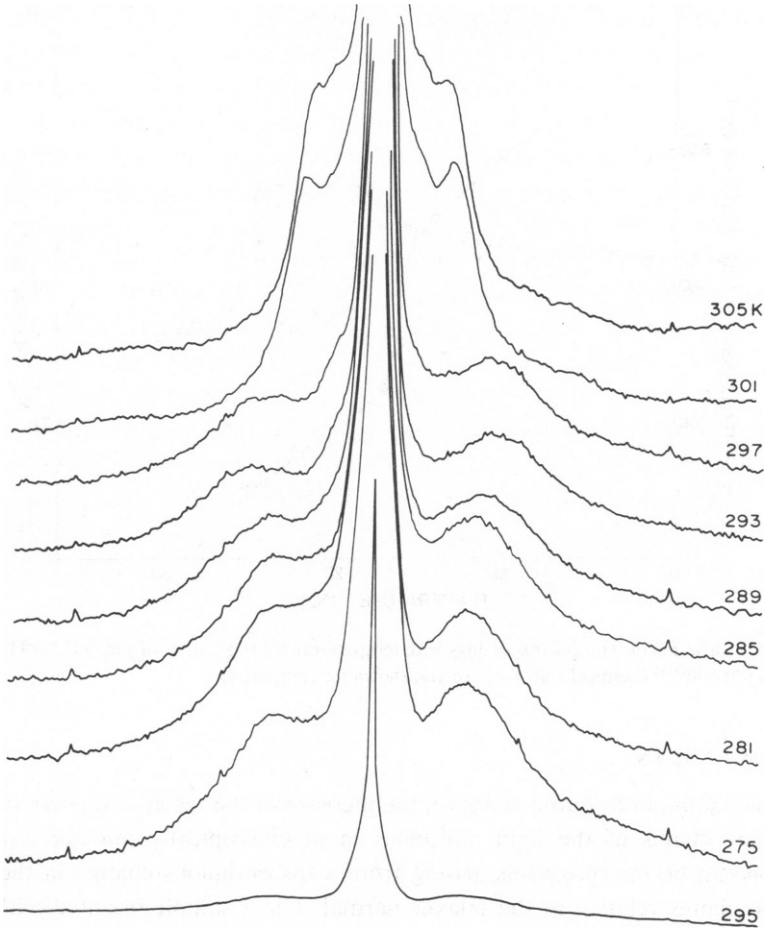

FIGURE 4 ^{1}H NMR spectra (90 MHz) from macroscopically oriented DML-nD$_2$O bilayers as a function of temperature in the phase-transition region, showing the resolved dipolar splittings. The sample, which had a hydration $n \approx 11$, was oriented with the bilayer normals parallel to the magnetic field.

the basis of their chemical shifts relative to the narrowest component that derives from residual protons in the heavy water used to hydrate the lipid. Using a simple data manipulation technique (23) we established that the resolved splittings are derived from methylene protons in the lipid molecules.

The variation of methylene proton dipolar splittings with temperature through the phase transition region is shown in Fig. 5. Also shown are water (^{2}H) half splittings and the chemical shift variation of the ^{31}P NMR signal from the phosphocholine headgroups for the same sample. Sharp discontinuities in both water and proton splittings coincide with both thermal transitions. The proton splittings increase on passing from the gel to intermediate phase for samples oriented at $\phi = 0$. An increase in ^{1}H second moment for the sample as a whole was also observed for this orientation although the second moment for a powder sample fell monotonically over the same temperature range. The ^{31}P NMR spectrum from a powder sample is characteristic of an anisotropic chemical shift.

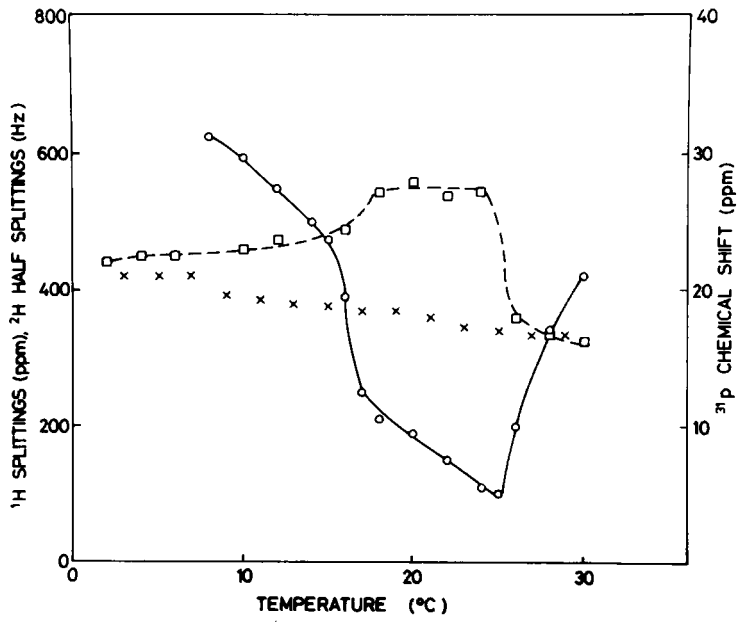

FIGURE 5 Variation of ^{1}H dipolar splittings with temperature for the sample of Fig. 4 (□). ^{2}H NMR half splittings (○) and ^{31}P chemical shift (×) are also shown for comparison.

DISCUSSION

Based on the assumptions outlined above, the increase in the resolved dipolar splittings from the methylene chains of the lipid molecules in macroscopically oriented samples at the pretransition can be interpreted as arising from a discontinuous change in the average tilt angle of the chains relative to the bilayer normal. For a sample oriented with the bilayer normals parallel to the applied field, ($\phi = 0$), the angle δ in Eq. 6 becomes equal to this tilt angle for all domains in the sample. A reduction in tilt angle at the pretransition would bring the molecular axes closer to the bilayer normal, resulting in an increased dipolar splitting $\Delta\nu_D$, without affecting the total second moment M_2 obtained for a powder sample. We believe this result confirms that such a reduction in tilt angle does occur between the gel and intermediate phases (8, 11). The observed ~20% increase in splittings between 10° and 20°C for samples with ~20% water ($n \sim 10$) is consistent with a change in tilt angle between ~35° in the Lβ' phase (12) and ~31° in the Pβ' phase. It should be noted however that the change in angle of tilt derived from such data is sensitive to the magnitude of the tilt chosen for the gel phase; smaller tilt angles require a greater change to explain the observed effects. Thus a 20% change in splittings is also consistent with a change in tilt from 20° to 0 in the intermediate phase, consistent with the suggestion of Rand et al. (8) that the chains are not tilted in this phase. A change of tilt angle of 20° is comparable to that of 29° from x-ray data reported by Brady and Fein (11) for DPL in excess water. Indeed it is thought that the angle of tilt in the gel phase is hydration dependent and increases with water content; a value of 12.5° was obtained for DPL at a hydration (10%) below that for which the intermediate phase exists (13). The reduction in dipolar splittings at the main transition results from greater molecular mobility and hence a

reduced order parameter S_{mol} in the liquid-crystalline phase. The effect of a "ripple" in the bilayer in the intermediate phase has not been included in the above discussion, but can be expected to have only a minor influence on the ^1H splittings provided the amplitude of the ripple is small, or the mean orientations of the molecular long axes are symmetrically disposed about the normal to the average plane of the rippled bilayer.

In an attempt to obtain an estimate of the magnitude of the gel-phase chain-tilt angle θ_T directly from NMR rather than x-ray data, ^1H free-induction decay signals from macroscopically oriented samples were simulated for a range of chain-tilt angles and compared with experimental results. As noted above, for a sample oriented with the bilayer normals parallel to the magnetic field ($\phi = 0$), the directions of the chain axes, which lie on a cone of semivertical angle θ_T, subtend a unique angle $\delta = \theta_T$ with respect to the magnetic field. For other orientations ϕ the direction of a given chain axis is dependent on the variable azimuth ϵ of the tilt with respect to the bilayer normal (i.e., the cone axis) and given by

$$\cos \delta = \cos \theta_T \cos \phi + \sin \theta_T \sin \phi \cos \epsilon. \tag{8}$$

In the liquid crystal phase where the average orientation of the chains is parallel to the bilayer normal ($\theta_T = 0$; $\delta = \phi$), the free-induction (FID) signals have been shown to scale as $(3 \cos^2 \delta - 1)/2$. Thus given the FID signal for one sample orientation, it is possible to predict the result for any other (22). In the gel phase, however, only one sample orientation ($\phi = 0$) corresponds to a unique value of δ. Thus to simulate the FID signal for an arbitrary orientation it is necessary to superimpose FIDs corresponding to each unique value of δ contained within the overall distribution $|\phi - \theta_T| \leq \delta \leq \phi + \theta_T$. This was done for given values of ϕ and θ_T by calculating δ from Eq. 8 for $\epsilon = 0° - 359°$ in 1° steps, scaling the $\phi = 0$ FID by a factor $|(3 \cos^2 \theta_T - 1)/(3 \cos^2 \delta - 1)|$ and summing the 360 resulting FID signals. Calculated and experimental FID signals were then compared for a range of values of chain tilt and two sample orientations, $\phi = 55.7°$ (the 'magic angle') and $\phi = 90°$. Typical results are shown in Fig. 6. The results are consistent with a chain tilt in the $P_{\beta'}$ phase of ~23° for DML-nD$_2$O with $n = 11$ at 18°C.

It should be stressed that only for an all-*trans* hydrocarbon chain undergoing rapid reorientation about the average long axis is it expected that all of the inter- and intrachain dipole-dipole interactions will be projected onto the reorientation axis. This has been shown to occur in aligned phospholipid systems in the Lα phase and in the Lβ' phase of soaps (33). In the present study the observation of the orientation dependence of the dipolar splittings suggests that even in the $P_{\beta'}$ phase (and possibly the higher temperatures of the Lβ' phase), sufficient motion is occurring to produce a similar effect in these crystalline systems. The extent to which the motion is a complete rotation of the chain is not clear from the data. However, we have felt it useful to assume a sufficiently large range of angles is swept out to permit an interpretation in terms of the dipolar interaction vector for all proton pairs being effectively aligned along the rotation axis. Realizing the assumption of rapid chain motion on which this interpretation depends, the model shows a surprisingly good agreement with the data. In the Pβ' phase the fits are quite sensitive to the chain-tilt angle assumed. As the temperature was lowered into the Lβ' phase the predicted free-induction decays became a progressively poorer fit to the experimental curve. This is to be expected as the assumptions on which the model is based break down. However on the basis of the calculated chain-tilt angle

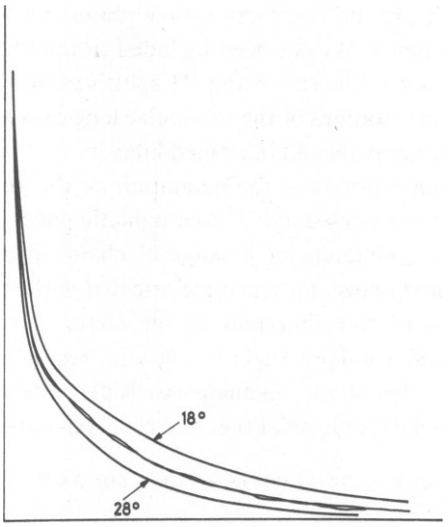

FIGURE 6 Comparison of the measured ^1H free-induction decay for an aligned DML-nD$_2$O sample (n = 11 at 18°C) oriented at the magic angle to the applied field (ϕ = 54.7°) with that predicted from the corresponding ϕ = 0 result, assuming a chain tilt angle θ_T = 23°. Corresponding results for chain-tilt angles of 18° and 28° are also shown.

of 23° for the Pβ' phase and the observed change in ^1H splittings at the pretransition, a chain-tilt angle of 29° can be estimated for the Lβ' phase. Our conclusion therefore is that while the chain-tilt angle is substantially reduced in the Pβ' phase, it does not go to zero at the pretransition.

The variation of ^{31}P chemical shift for a macroscopically oriented sample (Fig. 5), shows no discontinuities at either phase transition. Since a change in the (anisotropic) motion of the phosphocholine head group would be expected to result in a change in the projection of the chemical shift tensor (σ_{zz}) onto the direction of the applied magnetic field $\mathbf{H}_0$, this result suggests that the average conformation of the lipid head groups is not significantly affected by the phase changes that primarily affect the behavior of the hydrocarbon chains. Although this conclusion is consistent with the results of Buldt et al. (15), it remains necessary to explain the sensitivity of the water splittings to phase structure.

Owing to rapid exchange of water molecules between different binding environments, the observed ^2H splittings reflect the average order parameter for all the water in the sample (Eqs. 3–5). In the Lβ' phase, the variation of the observed splittings with water content for hydrations $n \gtrsim 5$ is within experimental error, consistent with the exchange of bound water with free or trapped water of negligible intrinsic order parameter S_{Qi}. Reduction in the splittings with increasing temperature in this phase results from more isotropic reorientation for the bound water, consistent with increased thermal activation. One factor that will certainly influence the binding of water to the hydrophilic groups at the lipid-water interface is the bilayer area per molecule. This in turn will be a function of the angle of chain tilt in the gel phase. However for the changes in chain tilt observed in the present study, the corresponding changes in area per lipid molecule (~5–6%) alone are unable to account for the magnitude of the changes in water splittings observed at the pretransition, particularly since

the development of a ripple in the bilayer would tend to offset this change. Rather it is likely that the change in bilayer area at the pretransition facilitates a reorganization of the bound water in the $L\beta'$ phase. Similarly, increases in bilayer area per molecule above the main transition (12) must facilitate changes in water binding that give rise to increased splittings in the $L\alpha$ phase to temperature $\gtrsim 60°C$ above the transition temperature.

At the main transition itself, the water splittings fall to a value close to zero for all sample hydrations studied. One possible explanation for this effect, which avoids the necessity to assume that all the water is experiencing isotropic reorientation, is that there is rapid exchange of bound water between environments with order parameters S_{Qi} of opposite sign, giving rise to an average order parameter $S_Q = 0$ (Eq. 4).

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