Molecular Response of the Lipid Headgroup to Bilayer Hydration Monitored by ^{2}H-NMR

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ABSTRACT The effect of hydration on the conformation and dynamics of the phosphatidylcholine headgroup has been investigated by ^{2}H-NMR measurements of liquid crystalline dioleoylphosphatidylcholine in multilamellar liposomes. Deuterium quadrupole splittings ($\Delta\nu_Q$) and spin-lattice relaxation rates ($1/T_1$) were recorded for three selectively labeled headgroup segments (α, β, and γ) over the range of water/lipid mole ratios from 4 to 100. The smooth changes in $\Delta\nu_Q$ and $1/T_1$ are found to essentially parallel each other and can be described by a single exponential decay function. Progressive hydration thus induces a concerted change in headgroup conformation together with an increase in its rate of motion (detected by $\Delta\nu_Q$ and $1/T_1$, respectively). The enhanced mobility is partially due to a shift in the lipid phase transition temperature (as monitored by differential scanning calorimetry) and is furthermore attributed to an entropic contribution. It is concluded that the choline dipole becomes slightly raised in its average orientation into the aqueous layer and that the rate is increased at which the headgroup is fluctuating and protruding. The observed molecular changes can thus be accommodated within a model where the effective accessible headgroup volume expands with increasing hydration.

INTRODUCTION

The lipid-water interface may be regarded, more appropriately, as a lipid-water "interphase" in which the properties of the solvent molecules and the headgroups are intrinsically coupled (Cevc, 1991). The interactions within this polar headgroup region give rise to a repulsion between hydrated surfaces over distances of up to 30 Å, which mediates associations between membranes, including fusion or polymorphic transformation and colloid stability. Much insight into the hydration characteristics of lipids has been gained from the structural characterization of multibilayers by diffraction methods and from the associated forces (Cevc, 1992; Cevc and Marsh, 1985, 1987; Gruen and Marcelja, 1983; Israelachvili and Wennerström, 1992; Klose et al., 1988; LeNeveu et al., 1976; Luzatti and Husson, 1962; McIntosh and Magid, 1993; McIntosh et al., 1987; Rand and Parsegian, 1989; Simon et al., 1991), while other studies have concentrated on the properties of the interbilayer water molecules (Borle and Seelig, 1983; Davenport and Fisher, 1975; Finer and Darke, 1974; Fung and McAdams, 1976; Gawrisch et al., 1978, 1985; Volke et al., 1994). At a molecular level, it is generally understood that the lipid mobility increases with the hydration-induced expansion in surface area (Cevc and Marsh, 1987; Fung and McAdams, 1976; Koga and Kanazawa, 1984; Ulrich et al., 1990a; Walter and Hayes, 1971), and that the polar headgroup undergoes a small conformational adjustment upon progressive hydration (Bechinger and Seelig, 1991; Büldt et al., 1979).

Solid-state nuclear magnetic resonance (NMR) is an ideally suitable and nonperturbing method for the characterization of molecular properties in lipid bilayers (Bechinger and Seelig, 1991; Gally et al., 1975; Gawrisch et al., 1978, 1985; Jarrell et al., 1988; Scherer and Seelig, 1989; Seelig and Seelig, 1980; Seelig et al., 1987; Sixl and Watts, 1982; Ulrich et al., 1990a; Volke et al., 1994; Watts and Van Gorkom, 1992). Particularly in the application to specifically deuterium-labeled molecular segments, ^{2}H-NMR measurements of lipid bilayers and methods for the data analysis are well-established (Brown, 1982; Davis, 1983; Seelig, 1977). In studies of hydration, ^{2}H-NMR has primarily been used to investigate the properties of interbilayer ^2H_2O molecules (Borle and Seelig, 1983; Finer and Darke, 1974; Gawrisch et al., 1978, 1985; Volke et al., 1994), while labeled lipids have not yet been examined so extensively (Bechinger and Seelig, 1991; Ulrich et al. 1990a). At present, the available data is limited to a few examples and not yet sufficient for any one lipid species to correlate the various molecular properties with one another and with hydration. Therefore, we have undertaken a further systematic characterization of the conformational and dynamic response of the DOPC headgroup to hydration. This may help understand the quantitative correlation that has been observed between the ^{2}H-NMR parameters and the thermodynamics of hydration (Ulrich and Watts, 1994).

As a model system, we have used synthetic dioleoylphosphatidylcholine (DOPC) which carries specific deuterium labels at the three segments α, β, and γ in the zwitterionic choline headgroup. Anhydrous DOPC bilayers undergo a limited swelling in pure water, with a relatively high hydration limit compared to other lipids, which allows a thorough investigation of the hydration effects over a wide range of water contents. The unsaturation in the chains leads to a low phase transition temperature, at -16.5°C for the fully hydrated lipid or somewhat higher for lower water contents,

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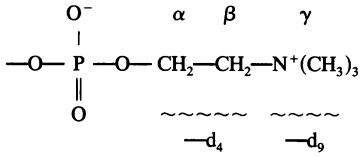
so that the bilayers are in the liquid-crystalline state at ambient temperature. For DOPC dispersions of multilamellar liposomes with water/lipid ratios between 4 and 100 (mol/mol), we have examined the variations of two characteristic ^{2}H-NMR parameters in the range of 25–40°C, to study headgroup conformation and dynamics as a function of hydration (Bechinger and Seelig, 1991; Ulrich et al., 1990a). For the interpretation of the ^{2}H-NMR data, DSC was used to monitor the lipid phase transition temperature (T_m) which varies with the degree of bilayer hydration (Cevc, 1992; Cevc and Marsh, 1985, 1987; Simon et al., 1991; Ulrich et al., 1994).

MATERIALS AND METHODS

Lipid hydration

The headgroup-deuterated lipids DOPC-d_9 and DOPC-d_4 for the ^{2}H-NMR experiments were synthesized and purified as described before (Sixl and Watts, 1982; Watts and Van Gorkom, 1992).



For the DSC measurements, commercial DOPC (Sigma) was used without purification. A series of lipid samples (10–50 mg each) was prepared gravimetrically in preweighed NMR tubes from the lipid dissolved in chloroform, by removing the solvent under a stream of nitrogen and drying overnight under high vacuum. Under these conditions, one water molecule remains bound to the lipids headgroup (Cevc, 1992). Multilamellar liposomes with different hydration levels, $4 \leq n_W < 100$ water molecules per lipid, were prepared by directly adding a well-defined amount of deuterium-depleted water (Aldrich) with a Hamilton microsyringe. The mixture was homogenized with a glass rod, and the tube immediately sealed under nitrogen and centrifuged. The accuracy in the water/lipid ratio (n_W) of the multilamellar dispersions lies within ± 1 H_2O molecule per lipid.

NMR experiments

The ^{2}H-NMR measurements on the multilamellar liposomes were performed on a homebuilt spectrometer operating at a deuterium frequency of 55.3 MHz. Spin-lattice relaxation rates ($1/T_1$) were measured by a inversion recovery pulse sequence, with a $\pi/2$ pulse width of 7 μs, a repetition time of 500 ms, and using a standard phase cycling procedure. All inversion recovery data gave single exponential decays and the T_1 relaxation time constants have typical experimental errors of around 2 ms. The accuracy of $\Delta\nu_Q$ lies within ± 0.3 kHz for α and β, and ± 0.1 kHz in the case of the γ quadrupole splittings. All ^{2}H-NMR hydration curves were recorded isothermally at 30°C, and some additional experiments were carried out at 25, 35, and 40°C. The sample temperature was controlled by a stream of air to within ± 1°C. Additional ^{31}P-NMR experiments were carried out on a Bruker MSL 400 at a phosphorus frequency of 162 MHz and with high power proton-decoupling.

Differential scanning calorimetry

Thermograms of DOPC were recorded for a series of gravimetrically prepared samples with different water/lipid ratios ($4 \leq n_W < 100$ H_2O) on a Perkin Elmer DSC 7 differential scanning calorimeter (Ulrich et al., 1994). Aliquots of 10 μl from the hydrated and equilibrated lipid dispersions were sealed in large-volume aluminium sample pans, and an empty pan was used as a reference. The temperature range from -40 to $+10$°C was scanned at a rate of 2°/min. The lipid transitions gave smooth curves over the entire hydration range, thus confirming that the DOPC samples were well equilibrated and homogeneous.

RESULTS AND DISCUSSION

Headgroup conformation

All the solid-state NMR spectra recorded in this study have characteristic powder pattern lineshapes, which is indicative of DOPC bilayers being in the liquid crystalline phase (Davis, 1983; Seelig, 1977; Seelig and Seelig, 1980). Representative ^{2}H-NMR and ^{31}P-NMR spectra are shown in Fig. 1 for gravimetrically prepared multibilayer samples with different hydration levels n_W of 4, 14, and 54 water molecules per phospholipid, recorded at 30°C. The ^{2}H-NMR spectra of DOPC-d_4 (Fig. 1, left column) contain two superimposed components which correspond to the α and the β segment in the choline headgroup, respectively. Their quadrupole splittings have been assigned (Gally et al., 1975), and the central isotropic peak is due to residual HDO or micellar components in the sample. The nine equivalent γ deuterons on the terminal methyl-groups of DOPC-d_9 also give rise to distinctive powder lineshapes (Fig. 1, middle column), which are shown on an expanded frequency scale. The ^{31}P-NMR spectra (Fig. 1, right column), too, are typical of fluid bilayers, with a chemical shift anisotropy around 50 ppm (Bechinger and Seelig, 1991; Scherer and Seelig, 1989). Therefore, the lipid molecule as a whole is undergoing fast long-axial rotation about the bilayer normal at all water/lipid ratios investigated here ($n_W \geq 4$). Furthermore, the headgroup must be engaged in rapid interconversion between the

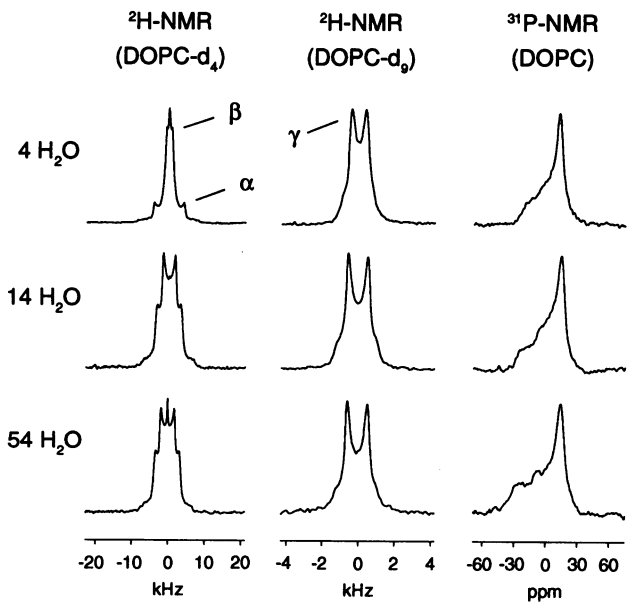


FIGURE 1 Representative ^{2}H-NMR and ^{31}P-NMR spectra from liquid crystalline DOPC multibilayers, recorded for different levels of hydration (4 H_2O, 14 H_2O, and 54 H_2O) at 30°C. The choline headgroup was selectively deuterium-labeled on the α and β segments (DOPC-d_4) and at the γ position (DOPC-d_9).

two preferred *gauche*$^+$ and *gauche*$^-$ conformations on the timescale of the quadrupole interaction ($\tau_c < 10^{-6}$ s), since the two inequivalent α-deuterons are averaged out and appear as a single component in the spectra of DOPC-d_4.

The deuterium NMR quadrupole splitting ($\Delta\nu_Q$) from the lineshape maxima of a powder spectrum is highly sensitive to the time-averaged orientation of a deuterium bond, and it can be used to monitor conformational changes of the labeled segment within the membrane (Bechinger and Seelig, 1991; Gally et al., 1975; Gawrisch et al., 1978; Scherer and Seelig, 1989; Seelig et al., 1987; Sixl and Watts, 1982; Ulrich et al., 1990a; Watts and Van Gorkom, 1992). The representative ^{2}H-NMR spectra of liquid crystalline bilayers of DOPC-d_4 (Fig. 1, left column) at different water/lipid ratios n_w, show that the α and β quadrupole splittings change dramatically upon going from 4 to 14 H_2O molecules per lipid, while no further change occurs between the spectra of 14 and 54 H_2O. A similar trend is also seen for DOPC-d_9 (Fig. 1, middle column) and the ^{31}P-NMR spectra (Fig. 1, right column), although here the effect is much less pronounced. Fig. 2 summarizes the variation in $\Delta\nu_Q$ as a function of hydration ($4 \leq n_w < 100$ H_2O), for the α, β, and γ segments of the choline headgroup in DOPC multilamellar liposomes at 30°C. These results on DOPC correlate well with a previous hydration study on the α and β splittings in POPC bilayers (Bechinger and Seelig, 1991). At low hydration, $\Delta\nu_Q$ initially changes continuously with hydration and then levels out smoothly, approaching a limiting plateau beyond approximately 22 H_2O which corresponds to saturation of the bilayer surface with water.

Empirically it is found that the experimental hydration curves in Fig. 2 resemble closely the smooth shape of an exponential decay function. In order to characterize and compare the variation in the NMR parameter $\Delta\nu_Q$ for the α, β, and γ segments in a quantitative manner, the experimental data points were fitted to the generalized expression $f(n_w)$, as a function of the water/lipid ratio n_w.

$$f(n_w) = f_s + (f_0 - f_s) \cdot \exp(-n_w/\phi) \quad (1)$$

Of the three fitting parameters, f_s is the limiting value for excess hydration and f_0 represents the hypothetical value extrapolated to zero hydration. The exponential coefficient ϕ describes the characteristic decay constant of each curve and is given in units of the amount of added water molecules per lipid. Note that the factor $(f_0 - f_s)$ has a negative sign for asymptotically increasing curves like the ones for the α and γ segments in Fig. 2. The exponential nature of this hydration dependence and any physical implications are discussed elsewhere (Ulrich and Watts, 1994).

The dotted lines in Fig. 2 represent the fitted curves (Eq. 1) to our experimental ^{2}H-NMR data, from a nonlinear least-squares analysis provided in the software package ORIGIN. The values of the fitting parameters f_0, f_s, and ϕ for the quadrupole splittings of the α, β, and γ segments of DOPC are summarized in Table 1, together with the results from the relaxation time measurements (see below). The three fitted curves in Fig. 2 have very similar coefficients $\phi \approx 4.1$ to 4.5 H_2O, which indicates that all three segments respond to hydration in a concerted way, and the whole choline group is thus affected as a single unit. While all three deuterated sites experience the same effect, the quadrupole splittings of α and β are the more sensitive indicators of the hydration-induced changes at the bilayer surface (Bechinger and Seelig, 1991), which is just a consequence of the respective orientations of the deuterium bond vectors within the membrane.

Generally, the deuterium quadrupole splitting is sensitive to both temperature and to conformational changes in the molecule. In order to distinguish these different effects, we have recorded thermograms from DOPC samples with varying water content ($4 \leq n_w < 100$ H_2O) by DSC. The bilayer gel to liquid crystalline transition temperature (T_m) is given in Fig. 3 as a function of n_w. At full hydration, the phase transition of DOPC occurs at around −16.5°C, while for reduced water/lipid ratios the transition temperature is considerably elevated, as has been reported for a variety of other lipids (Cevc, 1992; Cevc and Marsh, 1985, 1987; Simon et al., 1991). A comparison of the DSC data in Fig. 3 with the ^{2}H-NMR results in Fig. 2 shows that, in contrast to the

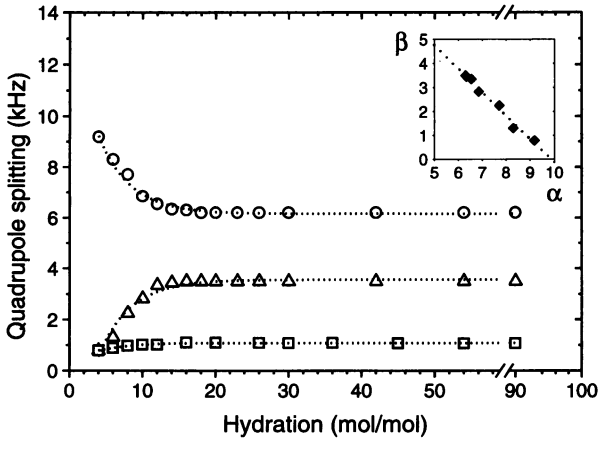


FIGURE 2 ^{2}H-NMR quadrupole splittings $\Delta\nu_Q$ recorded as a function of the water/lipid ratio n_w of DOPC multibilayers at 30°C. The data points for the α (○), β (△), and γ (□) segments of the choline headgroup were curve-fitted using Eq. 1 (*dotted lines*). The inset shows the linear correlation between the α and β splittings, fitted by the straight line which gives a gradient of $m \approx -1$.

TABLE 1 Curve-fitting parameters f_0, f_s, and ϕ, from the empirical function $f(n_w)$ (Eq. 1) that is used to describe the experimental ^{2}H-NMR hydration curves in Figs. 2 and 4.

$f(n_w)$	f_0	f_s	ϕ
$\Delta\nu_Q$ (α)	13.9	6.2	4.5 ± 0.3
$\Delta\nu_Q$ (β)	−4.0	3.6	4.2 ± 0.3
$\Delta\nu_Q$ (γ)	0.3	1.1	4.1 ± 0.5
$1/T_1$ (α)	220	40	4.6 ± 0.1
$1/T_1$ (β)	237	37	4.1 ± 0.1
$1/T_1$ (γ)	133	16	3.6 ± 0.2

The quadrupole splittings ($\Delta\nu_Q$) and the spin-lattice relaxation rates ($1/T_1$) of the three selectively deuterated segments (α, β, and γ) in the DOPC headgroup were measured as a function of the water-lipid ratio n_w, at 30°C.

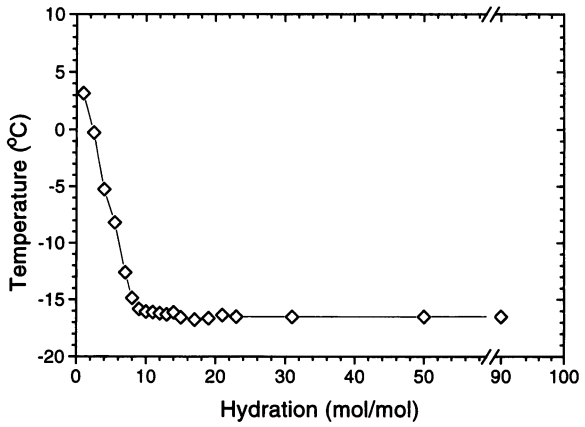


FIGURE 3 Gel to liquid crystalline phase transition temperature T_m of DOPC multibilayers, recorded by differential scanning calorimetry as a function of the water/lipid ratio n_W.

gradually levelling quadrupole splittings, the curve of T_m reaches its limiting value in an almost linear fashion and saturation appears to be complete already at around 10 water molecules per lipid. Therefore, the response of the headgroup to hydration, which is monitored here by ^{2}H-NMR at 30°C, does not reflect directly the hydration-dependent shift in the melting temperature of the acyl-chains. Rather, we have recently demonstrated that the DSC data in Fig. 3 represents the hydration characteristics of the lipid bilayer in the gel phase (Ulrich et al., 1994).

The elevated phase transition temperature of DOPC at low hydration might be expected to have a slight effect on the deuterium quadrupole splittings. However, the intrinsic temperature dependence of this parameter does not explain the counter-directional changes that have been measured for DOPC as a function of hydration (Seelig et al., 1987; Sixl and Watts, 1982). Therefore, the observed variations must be due to a progressive conformational change in the DOPC headgroup with hydration. For a detailed analysis, a description of the lipid headgroup in terms of average torsion angles would be required, which is not available from the deuterium quadrupole splittings. Nevertheless, it is known from ^{31}P-NMR (Yeagle, 1978) and from neutron diffraction studies at several different hydration levels (Büldt et al., 1979) that the choline headgroup is, on average, aligned approximately parallel to the bilayer plane. To illustrate the effect of progressive hydration on the headgroup conformation, two independent suggestions have been put forward which come to the same qualitative conclusion (McIntosh et al., 1987; Bechinger and Seelig, 1991). Based on a simple model for headgroup rotation, it was suggested that the probability of lipid headgroups being momentarily rotated into the fluid space would be progressively reduced with decreasing bilayer separation, for interbilayer distances of less than 5 Å (McIntosh et al., 1987). Dehydration of the lipid bilayers would thus result in the choline group being on average more closely aligned with the surface. While this proposal was originally put forward to illustrate the onset of steric repulsion between two apposing bilayer surfaces, the idea of a highly mobile interface that extends over some distance, which may even involve parts of the acyl-chains, is recently receiving increased attention (Cevc, 1991; Israelachvili and Wennerström, 1992; Wiener and White, 1992).

Another explanation of the headgroup conformational change has been proposed recently by Bechinger and Seelig (1991) from ^{2}H-NMR studies of POPC, which applies directly to the results from DOPC shown in Fig. 2. The hydration-induced counter-directional shift in the quadrupole splittings of the choline α and β segments is similar to the changes observed upon the binding of various charged species to the bilayer surface (Seelig et al., 1987; Scherer and Seelig, 1989; Sixl and Watts, 1982). These charges, which are buried in the polar surface region, interact electrostatically with the dipole of the zwitterionic choline headgroup, which is aligned approximately parallel to the bilayer surface. It has thus been proposed that a conformational change is induced by a rotation of the positively charged trimethylammonium end closer towards or further away from the bilayer plane (Seelig et al., 1987; Scherer and Seelig, 1989), and a similar process may be envisaged to occur in the event of lipid hydration or dehydration. The direction of change in the case of increasing hydration ($\Delta\nu_Q(\alpha)$ decreasing, $\Delta\nu_Q(\beta)$ increasing) corresponds to the effect observed for phosphatidylcholine headgroup interaction with positive charges, which is to move the trimethylammonium end, on average, further away from the hydrophobic bilayer interior (Bechinger and Seelig, 1991). The quantitative response of the lipid headgroup is conveniently characterized by plotting the β splittings against the corresponding α splittings. The strictly linear correlation between the two splittings in DOPC is illustrated in the inset of Fig. 2, where the slope is found to be $m \approx -1.0$. For POPC, on the other hand, a value around $m \approx -0.7$ has been reported for the interaction with water (Bechinger and Seelig, 1991), which is intermediate to the characteristic slopes for negative ($m \approx -1.0$) and positive charge ($m \approx -0.5$) (Scherer and Seelig, 1989). Since the β splitting of the choline headgroup is generally known to be more sensitive to temperature than the α splitting (Gally et al., 1975; Sixl and Watts, 1982), the value of m appears to be affected not only by the direction of the conformational change but also by the relative temperature or by the molecular surface area of the lipid.

The range of angles over which the whole polar group can be re-aligned has been estimated from an analysis of the ^{31}P-CSA (chemical shift anisotrophy) tensor (Scherer and Seelig, 1989), and our NMR data on both the phosphate group and the deuterated segments can be correlated with this model. Over the hydration range studied here ($n_W \geq 4\ H_2O$), the angle by which the headgroup would change its average alignment relative to the membrane plane is no greater than 10°. This order of magnitude is typical for the response seen in other NMR studies (Bechinger and Seelig, 1991; Seelig et al., 1987; Scherer and Seelig, 1989; Sixl and Watts, 1982), while it lies within the error margins of corresponding neutron diffraction measurements (Büldt et al., 1979). This model which is used to analyze the α- and β-quadrupole

splittings thus gives a plausible interpretation of the hydration-induced shifts, in terms of a small change in the average alignment of the headgroup dipole, which may nevertheless give rise to considerable electrostatic effects at the bilayer surface. We note, however, that this model is based on the assumption that the static quadrupole interaction remains unchanged upon the binding of water or of charged species to the bilayer surface, which might be questionable in view of the intense local field gradients created by such interactions (F. Volke, personal communication).

Lipid dynamics

Since the deuterium relaxation is dominated by a quadrupolar mechanism, it is an intramolecular process and thus directly linked to the segmental motions (Brown, 1982; Davis, 1983; Jarrell et al., 1988; Seelig, 1977; Seelig and Seelig, 1980; Watts and Van Gorkom, 1992). Fig. 4 summarizes the spin-lattice relaxation rates $1/T_1$, for the α, β, and γ segments of the choline headgroup in DOPC as a function of hydration ($4 \leq n_W < 100$ H_2O), at 30°C (cf. Ulrich et al. (1990a)). All three curves are seen to run in parallel, and we conclude that the dynamic response to hydration is the same for all three segments. It is also found, once again, that the shape of the $1/T_1$ curves in Fig. 4 resembles that of an exponential decay function, like it had been observed for the quadrupole splittings (see Fig. 2). The experimental $1/T_1$ points have thus been fitted by a least-squares analysis (*dotted lines*) to the same type of empirical exponential decay function described above (Eq. 1). The resulting fitting parameters f_0, f_S, and ϕ are listed in Table 1, and the coefficients ϕ are indeed very similar for all three labeled segments, with values in the range of 3.6–4.6 H_2O.

A comparison of the relaxation data in Fig. 4 with the quadrupole splittings in Fig. 2 shows that these different hydration curves behave essentially the same way as a function

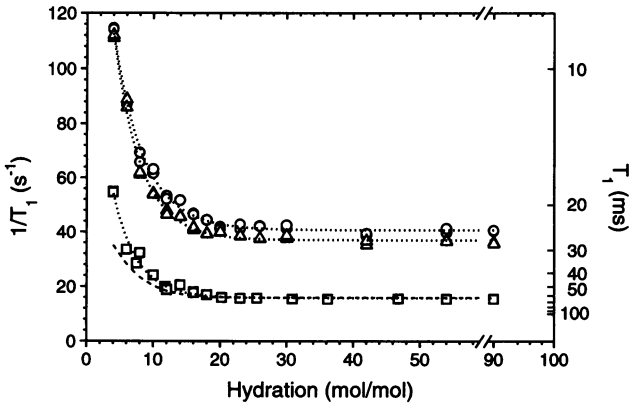


FIGURE 4 ^{2}H-NMR spin-lattice relaxation rates, $1/T_1$, recorded as a function of the water/lipid ratio n_W of DOPC multibilayers at 30°C, for the α (○), β (△), and γ (□) segments of the choline headgroup. The isothermally recorded data points were curve-fitted (*dotted lines*) using Eq. 1. The dashed line for the γ segment represents the hypothetical results calculated for a uniformly reduced temperature ($T_{red} = 0.18$), to take into account the effect of the phase transition on the molecular dynamics in the bilayer.

of n_W. This qualitative observation is supported quantitatively by the close similarity in the values of the exponential decay constants ϕ in Table 1, thus confirming that the hydration-induced changes in $1/T_1$ and $\Delta\nu_Q$ are mathematically essentially equivalent. Therefore, we conclude that the change in headgroup mobility occurs in parallel with the change in its average conformation. The cooperative nature of these different effects of hydration is significant in view of the fact that the respective experimental ^{2}H-NMR parameters have different units, are sensitive to different timescales, and describe different molecular properties. We note that this analysis of the relaxation data was appropriately based on an evaluation of the directly measured spin-lattice relaxation rate $1/T_1$ rather than the value of the relaxation time constant T_1 (Davenport and Fisher, 1975; Volke et al., 1994). The latter, being the inverse of the physically determined parameter, would appear to overemphasize the comparatively minor changes around $18 < n_W < 22$ upon approaching saturation, which is illustrated by means of the nonlinear T_1-scale in Fig. 4. In the representation as T_1, the same relaxation data would thus appear to show a higher hydration level for reaching saturation (Ulrich et al., 1990a), and the curve would no longer resemble an exponential decay function.

Much knowledge about the molecular dynamics in bilayers has come from ^{2}H-NMR investigations of chain deuterated lipids (Brown, 1982; Davis, 1983; Jarrell et al., 1988; Seelig, 1977; Seelig and Seelig, 1980), while comparatively less detail is known about the complex motions of the headgroups (Gally et al., 1975; Sixl and Watts, 1982; Watts and Van Gorkom, 1992). Nevertheless, an analysis of the spin-lattice relaxation data is relatively straightforward, provided the process can be described by a single motional correlation time. Since it had been suggested that, in addition to fast motions, slow motions such as bilayer undulations might contribute to T_1 relaxation (Brown, 1982), we investigated both the angular anisotropy and the field dependence of the deuterium relaxation rates (Jarrell et al., 1988). In experiments with macroscopically oriented multibilayers, no T_1 anisotropy was observed for either the fully or the partially hydrated samples of DOPC-d_4 and DOPC-d_9 (Ulrich et al., 1990b). Similarly, measurements at three different deuterium frequencies (ω_0 = 30.7, 46.1, and 61.4 MHz) gave values of $1/T_1$ that were essentially independent of ω_0 (data not shown). Therefore, it appears that at these comparatively high field strengths, spin-lattice relaxation in the lipid headgroup is not sensitive to any slow motions. We can thus attribute the observed variations in $1/T_1$ with hydration in Fig. 4 to fast headgroup motions alone. From their temperature dependence, all the deuterium relaxation rates recorded here are known to fall into the extreme narrowing limit ($\tau_c \ll 1/\omega_0 \approx 10^{-8}$ s). It is thus possible to analyze the $1/T_1$ values in terms of a single effective motional correlation time τ_c for isotropic tumbling (Brown, 1982; Seelig and Seelig, 1980; Watts and Van Gorkom, 1992).

$$1/T_1 = (3\pi^2/2) \cdot (e^2qQ/h)^2\tau_c \qquad (2)$$

where $(e^2qQ/h) \approx 170$ kHz is the static quadrupole coupling constant for aliphatic deuterons. According to Eq. 2, a relaxation rate around $1/T_1 \approx 40\ s^{-1}$ for the α and β segment at full hydration gives a value for τ_c around 0.1 ns, and about three times higher for the case of very low hydration ($n_W \approx 4$).

Within the assumptions on the deuterium relaxation, the value of $1/T_1$ is directly proportional to the motional correlation time τ_c for the fast segmental reorientation motion that dominates the relaxation process. The decrease in $1/T_1$ with hydration (Fig. 4) thus demonstrates an increase in the rate of headgroup motion upon the progressive addition of water. An enhanced mobility at the bilayer surface of both the headgroups as well as the water molecules is a well-known phenomenon that has been described by various NMR studies and others (Borle and Seelig, 1983; Davenport and Fisher, 1975; Finer and Darke, 1974; Fung and McAdams, 1976; Koga and Kanazawa, 1984; Ulrich et al., 1990a; Walter and Hayes, 1971). The ^{2}H-NMR relaxation time measurements used here are sensitive to fast fluctuations of the headgroup in all three dimensions, which include the rapid interconversion between two preferred enantiomeric conformations, rotational diffusion within the plane of the bilayer, as well as protrusions of the headgroup or even of the whole lipid molecule into the aqueous interbilayer space (Israelachvili and Wennerstrom, 1992; Gally et al., 1975; Seelig and Seelig, 1980; Watts and Van Gorkom, 1992; Yeagle, 1978). It is seen from the similarity in the $1/T_1$ values of the α and β segments in Fig. 4, that their motions are essentially correlated with one another. The curve for the γ deuterons consists of reduced $1/T_1$ values, because relaxation is less effective due to the additional rotation of the terminal methyl rotors. Therefore, it appears from these correlated relaxation rates that the backbone of the choline headgroup moves more or less as one unit of the timescale of the NMR experiment, and that this motion is enhanced by progressive hydration.

The question now arises, whether the change in lipid headgroup dynamics may be simply a manifestation of the shift in the chain-melting transition, since the viscosity of the bilayer is effectively increased at low hydration levels as a consequence of the elevated transition temperature T_m. Therefore, in order to give a physically based interpretation of the molecular mobility, the concept of reduced temperature $T_{red} = (T - T_m)/T_m$ has been invoked (Seelig and Seelig, 1980). The deuterium relaxation rates shown in Fig. 4 were recorded at 30°C, which corresponds to a reduced temperature of $T_{red} = 0.18$ for the fully hydrated lipid (with $T_m = -16.5$°C). For the low hydration levels, the equivalent absolute temperatures are then found from the variation of T_m with n_W (Fig. 3). From the known temperature dependence of $1/T_1$, (see Fig. 5 below) the adjusted values of $1/T_1$ can be calculated (see Eq. 3 below). The resulting curve, which corresponds to the same uniformly reduced temperature ($T_{red} = 0.18$), is shown for the γ segment by the dashed line in Fig. 4. Compared with the isothermally recorded hydration response of the γ segment at 30°C (*dotted line*), it is seen that the molecular mobility is indeed enhanced through the shift in T_m, but the estimated temperature effect accounts for no more than a factor of 1/2 between the two curves. Therefore, even at a reduced temperature, progressive hydration per se leads to a genuine increase in the rate of headgroup motion.

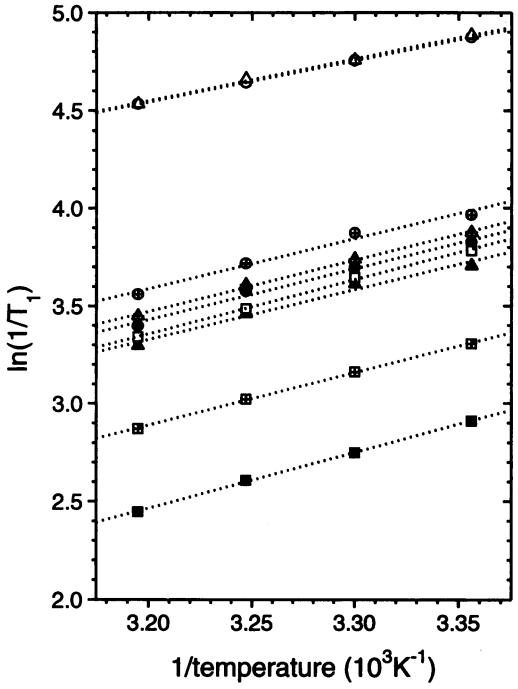


FIGURE 5 Arrhenius plots of the logarithmic ^{2}H-NMR spin-lattice relaxation rate $1/T_1$ against the inverse temperature (25, 30, 35, and 40°C), for the α (○), β (△), and γ (□) segments of the DOPC headgroup. Three representative water contents are shown, namely 4 H_2O (*open symbols*), 14 H_2O (*crossed symbols*), and 54 H_2O (*filled symbols*). The activation energy calculated from the slopes (*dotted lines*) is $E_A = 22 \pm 2$ kJ/mol for all curves.

By ^{2}H-NMR, it is possible to extract some information about the energetics of the lipid motion from the temperature dependence of the relaxation rates $1/T_1$, which were measured at 25, 30, 35, and 40°C. Since the motional correlation time τ_c is assumed to be proportional to $1/T_1$ (Eq. 2), the activation energies E_A can be evaluated.

$$1/T_1 \propto \exp(E_A/RT). \quad (3)$$

Representative Arrhenius plots ($n_W = 4$, 14, and 54) of $\ln(T_1)$ versus $1/T$ are shown in Fig. 5. The data are strictly linear for all three segments α, β, and γ, and at all hydration levels. All lines run parallel to one another, and a uniform value is calculated for E_A around 22 ± 2 kJ/mol. The activation energies are thus identical for the α, β, and γ segments, which confirms that, as suggested above, all three segments move as one unit on the timescale which dominates the T_1 relaxation (10^{-7} s $< \tau_c < 10^{-10}$ s) (Watts and Van Gorkom, 1992). The values of E_A are also independent of the hydration level of the sample, even though the rate of headgroup motion was found to increase with the water content (Ulrich et al., 1990a). This finding must now be explained in view of the fact that the height of the activation energy

barrier for this motion remains unchanged. From transition state theory, the rate of motion is known to depend on both the enthalpy $\Delta H^{\neq}$ and the entropy $\Delta S^{\neq}$ of activation which are required for passage of the fluctuating headgroup over the Gibbs free energy barrier, where $\Delta H^{\neq}$ corresponds to the value of E_A which has been determined above.

$$1/T_1 = C \cdot \exp(\Delta H^{\neq}/RT) \cdot \exp(-\Delta S^{\neq}/R). \quad (4)$$

Both the value of the proportionality factor ($C = h/kT \cdot 3\pi^2/2 \cdot (e^2qQ/h)^2 \approx 67\ \mathrm{ms}^{-1}$, at 30°C) and of the exponential enthalpy term should remain unaffected by hydration, since the temperature dependence of factor C is insignificant and E_A has been shown to be constant. Therefore, we conclude that the entropy of activation, $\Delta S^{\neq}$, is the dominant thermodynamic parameter which leads to the observed hydration dependence of $1/T_1$, even on a reduced temperature scale. An estimation of $\Delta S^{\neq}$ would be affected by the approximations that have been introduced in the expression in Eq. 4, but nevertheless it is evident that the entropy of activation for headgroup motion is positive and that its value increases with progressive hydration. The energetically favorable entropic impact that accompanies a reorientation or protrusion of the lipid headgroup becomes amplified with an increasing number of water molecules being accommodated within the polar surface region of the bilayer, which thus enhances the rate of motion.

Multibilayer assemblies

The spontaneous swelling of an anhydrous lipid in water can be readily monitored gravimetrically or by diffraction (Büldt et al., 1979; Jendrasiak and Hasty, 1974; Klose et al., 1992; Klose et al., 1988; LeNeveu et al., 1976; Luzatti and Husson, 1962; McIntosh and Magid, 1993; McIntosh et al., 1987; Rand and Parsegian, 1989; Simon et al., 1991). As the water molecules partition into the polar region between the bilayers, apposing bilayers move apart until they reach an equilibrium distance where their repulsive and attractive forces balance. Any excess water added to the sample is not accommodated between the bilayers, but rather remains in the lipid dispersion in a bulk state. Since the headgroup is most sensitive to the changes in its immediate environment and becomes continually less so as the bilayer distance increases, the changes in $\Delta\nu_Q$ and $1/T_1$ are most pronounced during the initial incorporation of water molecules into the polar surface region. The ^{2}H-NMR curves (Figs. 2 and 4) level out gradually between 14 to 18 H_2O, and saturation of the lipid appears to be essentially complete above approximately 22 water molecules per lipid. A similar behavior of the water molecules themselves, levelling toward a plateau near 23 H_2O, has been described in a complementary ^{2}H-NMR study of interbilayer ^2H_2O in egg-yolk lecithin (Gawrisch et al., 1985). Similarly, from an analysis of DSC thermograms, we have recently estimated a limiting hydration of around 20 H_2O for liquid crystalline DOPC (Ulrich et al., 1994). Nevertheless, these saturation limits are only relatively approximate estimates in view of the intrinsic smoothness of the data curves. Diffraction studies have indicated a higher swelling capacity for DOPC between 30 and 44 water molecules per lipid (Rand and Parsegian, 1989; MaIntosh and Magid, 1993), although this may be an overestimate due to the presence of water molecules in the amorphous lipid sample, which are trapped between the liposomes and do not contribute to the diffraction signal (Gawrisch et al., 1985; McIntosh and Magid, 1993; Klose et al., 1988). The comparatively low saturation limit of around 16 H_2O that has been found from sorption studies of DOPC (Jendrasiak and Hasty, 1974), on the other hand, is a manifestation of the vapor pressure paradox, which has been explained by minute temperature gradients and fluctuations (Rand and Parsegian, 1989).

The properties of the water molecules, too, have been found to vary smoothly with hydration (Cevc and Marsh, 1985; Volke et al., 1994), with the exception of probably the first few molecules ($n_W < 4$ H_2O) that bind tightly to the phosphate group (Cevc, 1992). Some classic NMR investigations of ^2H_2O in lipid multibilayers had made a distinction in terms of "tightly bound," "weakly bound," "trapped," and "bulk" water (Finer and Darke, 1974; Walter and Hayes, 1971). However, it has recently been shown for a variety of lipids that the hydration curves of ^2H_2O obey a smooth exponential dependence (Volke et al., 1994), a finding which correlates well with our present study on the lipid headgroup. Interestingly, the activation energies for ^2H_2O were also found to be independent of hydration and rather close to our values from the lipid headgroups (Borle and Seelig, 1983; Fung and McAdams, 1976). Similarly, it has been noted that the relaxation rates for the first few water molecules of hydration are very similar to those of the lipid headgroup at low water content (Volke et al., 1994). Therefore, it appears that the local disorder and the dynamics of the lipid headgroups and the water molecules are intrinsically correlated with one another. The polar surface region of the bilayer may thus be regarded as one thermodynamic entity.

From x-ray diffraction studies, it is evident that progressive hydration induces both an increase in the interbilayer water thickness together with a lateral expansion in the molecular surface area and a concomitant thinning of the bilayer (Büldt et al., 1979; Jendrasiak and Hasty, 1974; Klose et al., 1992; Klose et al., 1988; LeNeveu et al., 1976; Luzatti and Husson, 1962; McIntosh and Magid, 1993; McIntosh et al., 1987; Rand and Parsegian, 1989; Simon et al., 1991). However, it is not straightforward to separate these effects, and much depends on the choice of model for the data analysis. From the continuous nature of the ^{2}H-NMR curves over the entire hydration range above $n_W \geq 4$ (Figs. 2 and 4), there is no indication that the lipid headgroup would undergo hydration in discrete stages that might be correlated with either surface area or bilayer thickness. Instead, our results suggest that the structural basis for the hydration-induced changes seen in the various NMR parameters is a smooth, overall expansion in the effective lipid headgroup volume. With this

description, it is necessary to move away from the static picture of a discrete lipid-water interface and to appreciate the dynamic nature of what has been described as the lipid-water "interphase" (Cevc, 1991). Indeed, from a joint refinement of x-ray and neutron diffraction data, it has been recently demonstrated that the polar region of the bilayer is highly inhomogenous and extends far into the interbilayer space due to the thermal motions of the lipids (Wiener and White, 1992). On steric grounds, it is thus reasonable to suppose that the average headgroup conformation and its rate of fluctuation are sensitive to the accessible volume in all three dimensions. When water molecules are accommodated within the polar region of the bilayer surface, this causes a progressive loosening up of the lipid headgroup packing. Since a thermally excited headgroup is not confined to rotate within the bilayer plane, an increase in the accessible headgroup volume would allow the amino-terminal end of the choline group to raise itself slightly, *on average*, away from the hydrophobic plane (Bechinger and Seelig, 1991). An expansion of the bilayer polar region with hydration would also be expected to bring about a decrease in the crowding of the headgroups and thus an increase of their rate of motion. Indeed, we have observed by ^{2}H-NMR an enhancement of the headgroup fluctuations or protrusions, which may be attributed to two distinct energetic effects. First, the relative temperature of the system becomes effectively elevated as a result of the decreasing phase transition temperature. And second, the entropic impact of a fluctuating headgroup on the surrounding solvent molecules increases with progressive hydration.

One of the first hydration models to relate the structural changes at the bilayer surface to the amount of interbilayer water was based on a combination of the mean-field theory with elastomechanics (Cevc and Marsh, 1987). This approach allows the evaluation of the basic bilayer dimensions as a function of water concentration, and the predicted trend for the depth of the polar interphase is in good agreement with our interpretation of the ^{2}H-NMR results. A more detailed description, however, of the headgroup conformational changes or of the lipid dynamics as a function of hydration is not yet available at a molecular level. In recent years, hydration models have focussed on providing an explanation for the repulsive hydration pressure that is experienced by apposing bilayers over distances of up to 30 Å. Originally, this hydration force was attributed to solvent-ordering effects (Cevc and Marsh, 1987; Gruen and Marcelja, 1983), although it has recently been proposed to originate from the effective thickness or the thermal roughness of the bilayer surface (Cevc, 1991; Israelachvili and Wennerström, 1992). The conclusions from these latter two, independent approaches find experimental support from our ^{2}H-NMR investigations of the labeled lipid headgroups. Both models have predicted that the specific characteristics of different surfaces would depend more on the interfacial structure and dynamics than on the solvent properties. Therefore, it appears important to concentrate future studies on the properties of the surface residues, for which ^{2}H-NMR of selectively labeled headgroup is ideally suited, being a sensitive and nonperturbing method that can be applied to a wide range of systems.

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