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Lipid headgroup hydration studied by ^{2}H-NMR: a link between spectroscopy and thermodynamics

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Abstract

The conformational and dynamic response of the phosphatidylcholine headgroup to hydration has been characterized by ^{2}H-NMR measurements of selectively deuterated DOPC at positions α, β, and γ, and to complement the structural results from diffraction studies. Quadrupole splittings ($\Delta\nu_Q$) and spin–lattice relaxation rates ($1/T_1$) were monitored over the range of water/lipid mole ratios from 4 to 100. Their hydration dependence reveals a concerted change in headgroup conformation together with an increase in mobility. Both spectroscopic parameters ($\Delta\nu_Q$ and $1/T_1$) are found to be linearly correlated with the activity of the interbilayer water, and it is thus concluded that the ^{2}H-NMR hydration curves are equivalent to lipid sorption isotherms. Using this thermodynamic correlation, the repulsive hydration force between apposing bilayers can be calculated from the ^{2}H-NMR data and a decay constant is evaluated for DOPC of around 3.5 water molecules per lipid. The characterization of the headgroup properties by ^{2}H-NMR is complemented by recent investigations on interbilayer ^2H_2O, which demonstrates that the lipid–water interface constitutes a single thermodynamic entity.

Keywords: Lipid hydration; Deuterium NMR; Phosphatidylcholine bilayers; Headgroup conformation; Surface dynamics; Water activity; Hydration force

1. Introduction

The hydration properties of lipids have been characterized by recording sorption isotherms gravimetrically [1–3], by monitoring the bilayer structural parameters using diffraction methods [4–6], and by evaluating the associated hydration forces from the interbilayer pressure [4,6,7–13]. Various NMR (nuclear magnetic resonance) studies have concentrated both on the properties of the interbilayer water molecules [14–17] and on the effects of hydration on the lipid headgroup conformation and dynamics [15,18–20]. It has thus been shown that the lipid mobility increases with the hydration-induced expansion in surface area [9,15,19,20], and that the polar headgroup undergoes a small conformational adjustment upon progressive hydration [18,21]. However, it has not yet been possible to relate the corresponding spectroscopic parameters in a quantitative way to bilayer thermodynamics or to hydration pressure, and any available NMR data is currently limited to a few examples. In this contribution we will

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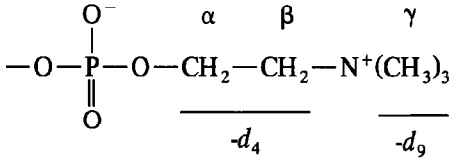
summarize the results of a recent ^{2}H-NMR study on the hydration of DOPC, which is being presented in greater detail elsewhere [22]. First, we will describe the conformational and dynamic response of the DOPC (dioleoylphosphatidylcholine) headgroup to hydration by ^{2}H-NMR, and we will then establish a quantitative correlation between the ^{2}H-NMR method and the thermodynamics of hydration.

In combination with selective deuterium labelling, solid-state ^{2}H-NMR is an ideally suitable and non-perturbing method for the characterization of molecular properties in lipid bilayers [16,18,20,23–27]. As a model system, we have used synthetic DOPC which carries specific deuterium-labels at the three segments α, β and γ in the zwitterionic choline headgroup, and which forms liquid crystalline bilayers at ambient temperatures. For DOPC dispersions consisting of multilamellar liposomes, we have examined several NMR parameters as a function of the water/lipid ratio between 4 H_2O and 100 H_2O per lipid headgroup, and over a range of temperatures. Parallel variations with hydration were found for both the deuterium quadrupole splittings ($\Delta\nu_Q$) and the spin–lattice relaxation rates ($1/T_1$), which are sensitive to headgroup conformation and dynamics, respectively [18,20]. For the interpretation of the ^{2}H-NMR data, DSC was used to monitor the lipid phase transition temperature (T_m) which also depends on the degree of bilayer hydration [8,9,13,28].

Since the present as well as previous NMR experiments have been carried out on gravimetrically prepared samples, any variations in the NMR parameters have so far only been described in terms of the added water volume. In order to gain further insight into the thermodynamics of hydration, it would be interesting to analyze the ^{2}H-NMR response as a function of the osmotic or vapour pressure, since these pressures are directly representative of the thermodynamic activity of the interbilayer water. That way, it might be possible to establish a connection between the spectroscopic and the thermodynamic aspects of bilayer hydration. From this kind of analysis, we find that there exists a quantitative correlation of the ^{2}H-NMR data with the thermodynamic activity of the water, from which it is then possible to calculate the hydration force itself. We will thus demonstrate that the ^{2}H-NMR measurements of gravimetrically prepared samples are sufficient to characterize the hydration force for DOPC, without the need to invoke the osmotic stress technique or any knowledge of sorption isotherms or diffraction data.

2. Materials and methods

The headgroup-deuterated lipids DOPC-d_9 and DOPC-d_4 for the ^{2}H-NMR experiments were synthesized and purified as described before [26,27],



For the DSC measurements, commercial DOPC (Sigma) was used without purification. A series of lipid samples (10 to 50 mg each) was prepared gravimetrically in pre-weighed 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 lipid headgroup [8]. Multilamellar liposomes with different hydration levels, $4 \leqslant n_W < 100$ 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.

The ^{2}H-NMR measurements on the multilamellar liposomes were performed on a home-built 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 were determined from the processed spectra with 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 ^{1}H-decoupling. DSC experiments on samples of commercial DOPC with varying water content were carried out on a Perkin Elmer DSC7 differential scanning calorimeter as described elsewhere [28].

3. Results

Representative ^{2}H-NMR and ^{31}P-NMR spectra of DOPC are shown in fig. 1, with different hydration levels n_W of 4, 14, and 54 water molecules per phospholipid, recorded at 30°C. All spectra have characteristic powder pattern lineshapes, indicating that the bilayers are in the liquid crystalline phase in which the lipid molecules undergo fast long-axial rotation about the bilayer normal [23,25]. The quadrupole splittings of the two superimposed components in the ^{2}H-NMR spectra of DOPC-d_4 (fig. 1, left column) have been assigned to the α and the β segments in the choline headgroup, and the central isotropic peak is due to residual HDO or micellar components in the sample. The distinctive powder spectra from the nine equivalent γ deuterons on the terminal methyl-groups of DOPC-d_9 are shown on an expanded frequency scale (fig. 1, middle column). The ^{31}P-NMR spectra (fig. 1, right column) have a chemical shift anisotropy around 50 ppm, which is typical of fluid bilayers [18,24]. It is seen from the ^{2}H-NMR spectra DOPC-d_4 (fig. 1, left column) that both the α and β quadrupole splittings change dramatically upon going from 4 to 14 H_2O molecules per lipid, while no significant further change occurs between 14 and 54 H_2O. A similar trend is also apparent for DOPC-d_9 (fig. 1, middle column) and for the ^{31}P-NMR spectra (fig. 1, right column), although here the effect is much less pronounced. Fig. 2 summarizes the variations in $\Delta\nu_Q$ as a function of hydration ($4 \leqslant n_W < 100$ H_2O), for the α, β and γ-segments of the choline headgroup in DOPC multilamellar liposomes at 30°C, which correlate well with previous results on POPC [18]. At low hydration, $\Delta\nu_Q$ initially changes continuously with water content and then levels out gradually at a water/lipid ratio n_W of around 14–18, approaching a limiting plateau which corresponds to full hydration of the bilayer.

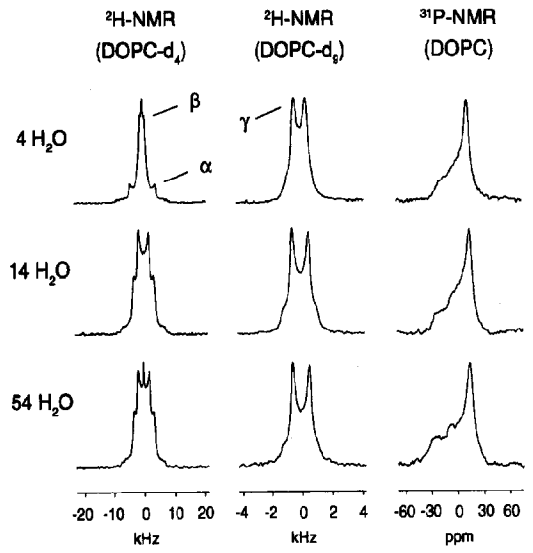


Fig. 1. Representative ^{2}H-NMR and ^{31}P-NMR spectra from liquid crystalline DOPC multibilayers, recorded for different levels of hydration (4 H_2O, 14 H_2O, and 54 H_2O) at 30°C. The choline headgroup was selectively deuterium labelled on the α and β segments (DOPC-d_4) and at the γ position (DOPC-d_9).

In the characterization of molecular structure and dynamics by ^{2}H-NMR, the quadrupole splitting can be used to monitor conformational changes of the labelled segment, since the value of $\Delta\nu_Q$ is highly sensitive to the time-averaged orientation of the deuterium bond-vector within the membrane [18,20,24,26,27]. The observed variations in fig. 2 are therefore attributed to a

conformational change in the DOPC headgroup with progressive hydration. It is known from ^{31}P-NMR [29] and from neutron diffraction studies at several different hydration levels [21] that the choline headgroup is, on avarage, aligned approximately parallel to the bilayer plane. A model for a hydration-induced conformational change has recently been proposed by Bechinger and Seelig [18] from ^{2}H-NMR studies of the POPC headgroup, which applies directly to the results on the 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 [24,26]. It has been suggested that these charges, which are buried in the polar surface region, interact electrostatically with the dipole of the zwitterionic choline headgroup and can induce a tilting of the positively charged trimethylammonium-end closer towards or further away from the bilayer plane [24]. A similar process may be envisaged to occur in the event of lipid dehydration, for which the direction of change (α increasing, β decreasing) would be such that the trimethylammonium-end moves, on average, closer towards

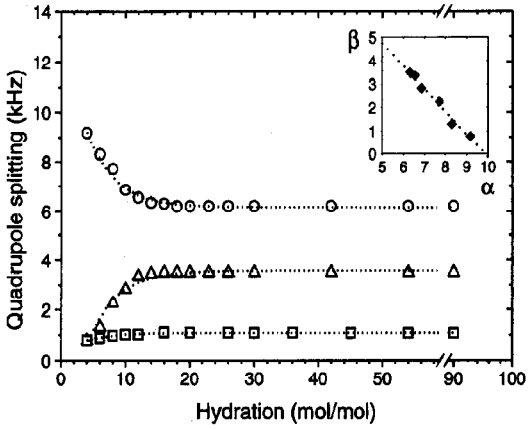


Fig. 2. ^{2}H-NMR quadrupole splittings $\Delta\nu_Q$ recorded as a function of the water/lipid ratio n_W of DOPC multibilayers at 30°C. The data points for the α (○), β (△) and γ (□) segments of the choline headgroup were curve-fitted using eq. (1) (dotted lines). The inset shows the linear correlation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

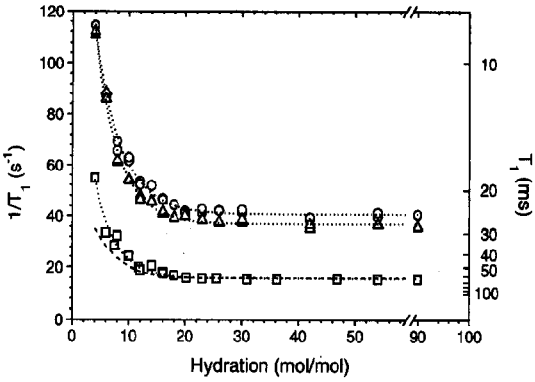


Fig. 3. ^{2}H-NMR spin–lattice relaxation rates, $1/T_1$, recorded as a function of the water/lipid ratio n_W of DOPC multibilayers at 30°C, for the α (○), β (△) and γ (□) segments of the choline headgroup. The isothermally recorded data points were curve-fitted (dotted lines) using eq. (1). The dashed line for the γ segment represents the hypothetical results calculated for a uniformly reduced temperature ($T_{red} = 0.18$), taking into account the effect of the phase transition on the molecular dynamics in the bilayer.

the hydrophobic bilayer interior upon the progressive removal of water from the bilayer surface [18]. 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 tensor [24]. Over the hydration range studied here ($n_W \geq 4$ H_2O), the average realignment relative to the membrane plane would be no greater than 10 deg, which lies within the error margin of corresponding neutron diffraction studies [21].

In ^{2}H-NMR investigations of lipid bilayers, further information about the fast motions of the deuterated segment can be gained from measurements of the spin–lattice relaxation rates $1/T_1$ [25,27]. Fig. 3 summarizes the variations in $1/T_1$ with hydration ($4 \leq n_W < 100$ H_2O), for the α, β, and γ segments of the choline headgroup in DOPC at 30°C [20]. Since all three curves are seen to run in parallel, we conclude that the dynamic response to hydration is the same for all three segments and that the choline headgroup moves more or less as one unit on the timescale of the NMR experiment. An analysis of the spin–lattice relaxation data is relatively straightforward, provided the process can be described by a single motional correlation time τ_c, as shown

for the DOPC headgroup [20,23,25,27]. 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). The value of $1/T_1$ is then 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. 3) 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 [14,15,19,20].

A comparison of the relaxation data in fig. 3 with the quadrupole splittings in fig. 2 shows that these different NMR hydration curves behave essentially the same way as a function of hydration, with plateau values being approached uniformly at a hydration level around 14 to 18 H_2O. This qualitative similarity can be demonstrated in a more quantitative manner by curve-fitting the data to a suitable mathematical function. The experimental hydration curves in figs. 2 and 3 are empirically found to resemble the shape of an exponential decay function. It is thus possible to describe the variation in the NMR parameter ($\Delta\nu_Q$ or $1/T_1$) by the generalized expression $f(n_W)$:

$$f(n_W) = f_S + (f_0 - f_S)\exp(-n_W/\phi), \qquad (1)$$

where f_S is the limiting value for excess hydration, f_0 represents the hypothetical value extrapolated to zero hydration, and the exponential coefficient ϕ describes the characteristic decay constant of the curve.

The dotted lines in figs. 2 and 3 represent the fitted exponential curves (eq. 1) to the experimental ^{2}H-NMR data, from non-linear least-squares analysis. The values of the three fitting parameters f_0, f_S, and ϕ for the quadrupole splittings and the relaxation rates of the α, β and γ segments of DOPC are summarized in table 1. Significantly, it is found that all fitted curves have very similar decay coefficients $\phi \approx 3.6$ to 4.6. This not only indicates that all three segments respond to hydration in a concerted way, but it furthermore confirms that the hydration-induced changes are essentially equivalent for $\Delta\nu_Q$ and for $1/T_1$. 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 remarkable in view of the fact that the respective experimental ^{2}H-NMR parameters have different units, are sensitive to different time scales, and describe different molecular properties.

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. 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

$f(n_W)$	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

Having demonstrated that lipid headgroup conformation and dynamics are intrinsically correlated with one another, it would be interesting to find a common explanation for their hydration-induced changes. The question thus arises whether the observed effects may be simply a manifestation of the shift in the lipid chain-melting phase transition temperature, which is known to exhibit a strong hydration dependence. Therefore, we have recorded thermograms from DOPC samples with varying water content ($4 \leqslant n_W < 100$ H_2O) by differential scanning calorimetry (DSC). The bilayer gel to liquid-crystalline phase transition temperature (T_m) is given in fig. 4 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 temperatures are considerably elevated, as has been reported for a variety of other lipids [8,9,13]. A comparison of the DSC data in fig. 4 with the ^{2}H-NMR results in figs. 2 and 3

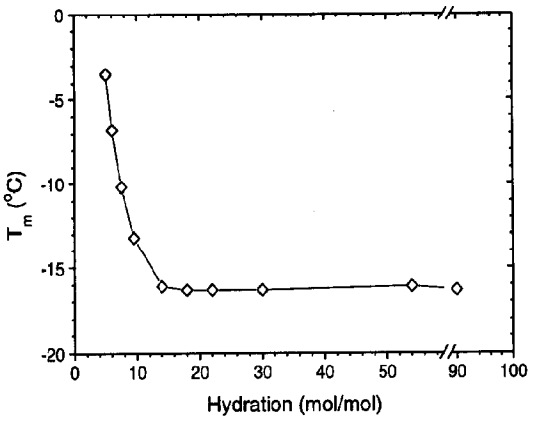


Fig. 4. Gel to liquid-crystalline phase transition temperature T_m of DOPC multibilayers, recorded by differential scanning calorimetry as a function of the water/lipid ratio n_W.

shows that, in contrast to the gradual levelling of $\Delta\nu_Q$ and $1/T_1$, the curve of T_m reaches its limiting value in an almost linear fashion and saturation already appears to be complete at around 14 water molecules per lipid. Therefore, the hydration-dependent shift in the melting temperature of the acyl-chains is unlikely to be the underlying cause for the much more gradual response of the headgroup to hydration that is monitored here by ^{2}H-NMR. Infact, it appears that the DSC data in fig. 4 represents the hydration characteristics of the DOPC bilayers in the gel phase rather than the liquid-crystalline state [28].

As a consequence of the elevated phase transition temperature T_m at low hydration levels, the viscosity of the bilayer is effectively increased and the molecular mobility decreased. Therefore, the concept of a reduced temperature $T_{red} = (T - T_m)/T_m$ has been invoked to estimate the effect of the phase transition on the deuterium relaxation rates that are given in fig. 3. The data have been 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. 4). From the known temperature dependence of $1/T_1$ (see fig. 5 below), the adjusted values of $1/T_1$ can be calculated using the Arrhenius equation (see below). The resulting $1/T_1$-hydration 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. 3. 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 $\frac{1}{2}$ 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.

By measuring the temperature dependence of the spin–lattice relaxation rate, it is possible to gain further information about the energetics of the lipid motion [27]. Activation energies E_A can be evaluated from logarithmic Arrhenius plots of $\ln T_1$ versus $1/T$. Representative results ($n_W = 4$, 14 and 54) are shown in fig. 5, from ^{2}H-NMR measurements at 25, 30, 35, and 40°C. 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 T_1 time scale (10^{-6} s $< \tau_c < 10^{-10}$ s) [27]. 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 [20]. This dynamic effect must now be explained in view of the fact that the height of the activation energy barrier for this motion remains unchanged with hydration. From transition state theory, the rate of motion is known to depend on both the enthalpy $\Delta H^\ddagger$ and the entropy $\Delta S^\ddagger$ of activation which are required for passage of the fluctuating headgroup over the Gibbs free energy barrier. Since $\Delta H^\ddagger$ corresponds to the value of E_A, which is constant, we conclude that the entropy of activation, $\Delta S^\ddagger$, is the dominant thermodynamic parameter which leads to the observed hydration dependence of $1/T_1$, even at reduced temperatures. The energetically favourable entropic impact that accompanies a reorientation of the lipid headgroup becomes amplified with an increasing number of water molecules being accommodated within the

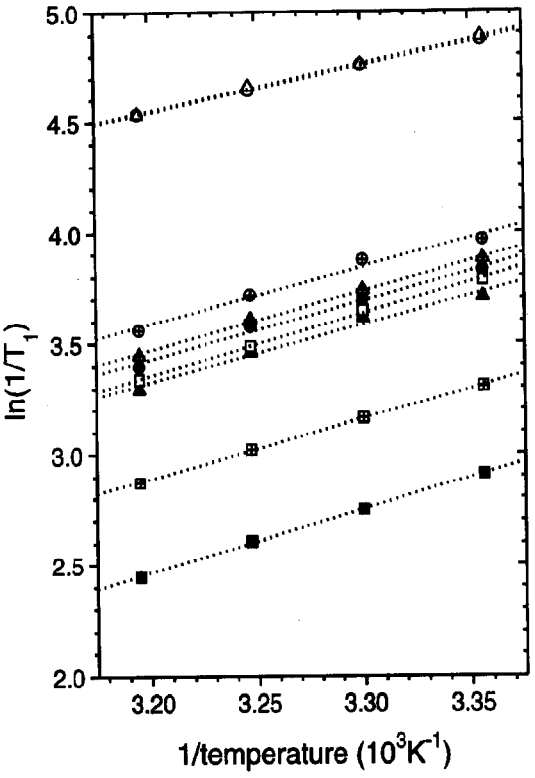


Fig. 5. Arrhenius plots of the logarithmic ^{2}H-NMR spin–lattice relaxation rate $1/T_1$ against the inverse temperature (25°C, 30°C, 35°C, 40°C), for the α (○), β (△) and γ (□) segments of the DOPC headgroup. Three representative water contents are shown, namely $4H_2O$ (open symbols), $14H_2O$ (crossed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

polar surface region of the bilayer, which thus enhances the rate of motion.

4. Discussion

The hydration dependence of the two ^{2}H-NMR parameters, $\Delta\nu_Q$ and $1/T_1$, has been described qualitatively for the α, β and γ position in the DOPC headgroup, from gravimetrically prepared liquid-crystalline multibilayer samples with water/lipid mole ratios of $4 \leqslant n_W < 100$. It is found that for all headgroup segments the relative variations in $\Delta\nu_Q$ and in $1/T_1$ occur in parallel with one another, and the hydration dependence of either of the NMR parameters may be written in a generalized form $f(n_W)$ (eq. (1)) when plotted as a function of the water/lipid ratio n_W. In order to relate the spectroscopic data to the thermodynamics of hydration, complementary information would be required from ^{2}H-NMR experiments carried out with samples prepared by osmotic stress. This approach would reveal the dependence of $f(n_W)$ on the thermodynamic activity a_W of the interbilayer water molecules rather than on n_W. Instead of having to prepare a new set of lipid samples with a range of different water activities, the original gravimetric ^{2}H-NMR results can be analyzed by making use of published sorption isotherms. That way, the water activity a_W, which is equal to the relative vapour pressure (p/p_0), can be related to each hydration level n_W and thus to the corresponding value of the measured NMR parameter $f(n_W)$. We have used the sorption data from Jendrasiak and Hasty [1], which was recorded for DOPC multibilayers at room temperature and is thus compatible with our NMR experiments at 30°C. Fig. 6 shows the dependence of the ^{2}H-NMR spin–lattice relaxation rates $1/T_1$, for the α, β and γ segments on the corresponding water activity a_W of the partially hydrated samples. A striking linear correla-

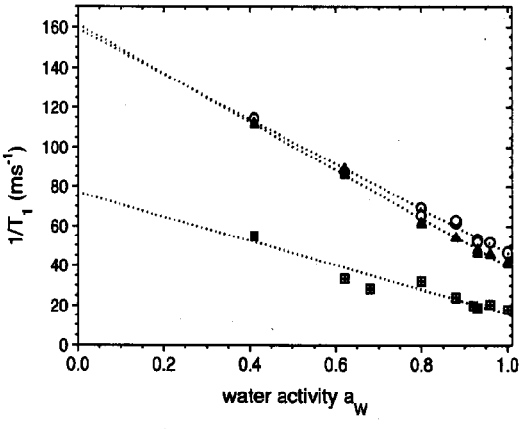


Fig. 6. Plot of the ^{2}H-NMR spin–lattice relaxation rate $1/T_1$ versus the corresponding water activity a_W known from the DOPC sorption isotherm [1]. A linear dependence is found (dotted lines) for all three labelled segments, α (○), β (▲) and γ (⊞).

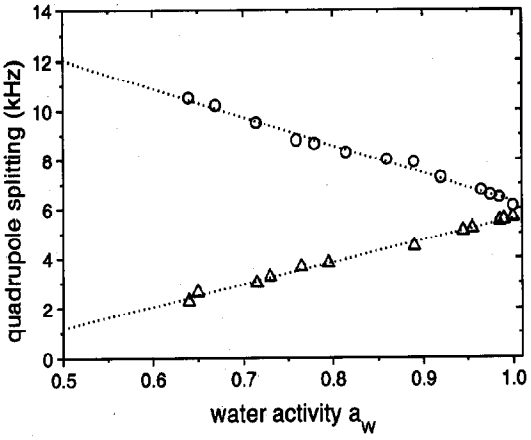


Fig. 7. Plot of the ^{2}H-NMR quadrupole splitting $\Delta\nu_Q$ (data from [18]) versus the corresponding water activity a_W known from the POPC sorption isotherm [2]. The linear correlation is confirmed (dotted lines) for both the α (○) and β (△) segments.

tion between $1/T_1$ and a_W is seen for all three sets of data, which have been fitted by least-squares analysis. The data points in fig. 6 cover the hydration levels from $n_W = 4H_2O$ up to only $16H_2O$, since the published sorption isotherm is confined to this range. It is a well-known phenomenon, commonly referred to as the vapour pressure paradox, that complete saturation of the bilayers with water cannot be reached at 100% humidity, as a result of minute temperature gradients and fluctuations [6].

Similar to the analysis of $1/T_1$ in fig. 6, we also find a linear correlation between the water activity and the ^{2}H-NMR quadrupole splittings $\Delta\nu_Q$, which were also measured for the α, β and γ segments of DOPC (fig. 2). Since the linear relationship between the NMR parameters and the water activity forms an essential argument for the following interpretation of lipid hydration, we also present the quadrupole splittings of POPC published by Bechinger and Seelig [18] for the α and β segments of the choline group as shown in fig. 7, plotted against the water activity a_W that was taken from the corresponding POPC isotherm published by Klose et al. [2]. In this case, both the ^{2}H-NMR data [18] as well as the sorption isotherm [2] have been meticulously well recorded, with a much higher density of data points compared to the sorption isotherm that is available for DOPC [1]. From this high quality experimental data, it is confirmed once again that there exists a linear relationship between the deuterium quadrupole splitting and a_W (fig. 7). The consistently linear correlation with the water activity therefore demonstrates that the variations in both NMR parameters, $1/T_1$ and $\Delta\nu_Q$, reflect directly the changes in the thermodynamic state of the lipid–water system. This empirical observation leads to the important conclusion, that the NMR hydration curve itself, $f(n_W)$ as a function of n_W, represents the *sorption isotherm* of the lipid. The original phosphatidylcholine sorption isotherms, which had been determined independently for DOPC and POPC [1,2], have the same shape as the corresponding ^{2}H-NMR hydration curves (figs. 2 and 3), although note that the x and y axes are interchanged compared to the conventional representation in which n_W would be plotted versus a_W.

The correlation of the lipid headgroup properties, such as conformation and dynamics, with the thermodynamic activity of the interbilayer water implies that the hydrated lipid surface region constitutes a single thermodynamic entity. This conclusion leads on to a related question concerning the ^{2}H-NMR parameters of the water molecules themselves. While earlier studies on ^2H_2O had suggested that hydration occurs over a series of discrete steps [14,16], it has been recently demonstrated that both the deuterium quadrupole splitting and the spin–lattice relaxation rate of the interbilayer water vary smoothly with hydration [17]. For a variety of lipids, these hydration curves of ^2H_2O have been shown to obey an exponential dependence, a finding which correlates well with our present study on the lipid headgroup, although the ^2H_2O analysis involved an averaging of $\Delta\nu_Q$ or $1/T_1$ over the ensemble of all water molecules at the bilayer surface. We have thus re-analyzed the available ^{2}H-NMR data for ^2H_2O in DOPC and in POPC [17] in terms of the water activity, using the same strategy and sorption isotherms as for the lipid headgroup data above. Fig. 7 illustrates the remarkable finding that the deuterium quadrupole splittings of

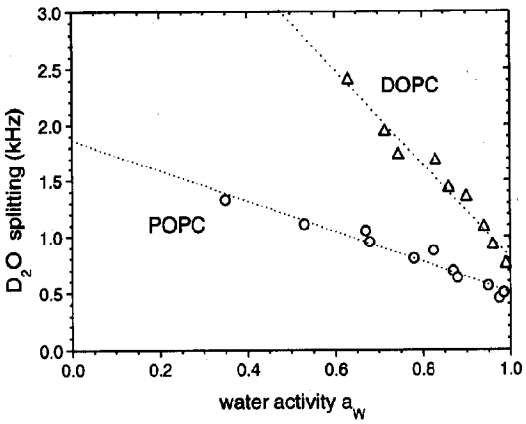


Fig. 8. Plot of the ^{2}H-NMR quadrupole splitting $\Delta\nu_Q$ of the interbilayer ^2H_2O (data from [17]) versus the corresponding water activity a_W known from the lipid sorption isotherms [1,2]. A linear correlation is found (dotted lines) both in DOPC (△) and in POPC (○).

^2H_2O, too, are linearly correlated with the thermodynamic water activity, just like the NMR data from the lipid headgroup, (although the $1/T_1$ data of ^2H_2O does not yet reveal such a clear-cut picture). The molecular properties of the water molecules, in this case their degree of orientational order, are thus directly correlated with the analogous conformational and dynamic properties of the lipid headgroups. The whole region of the lipid–water interface should therefore be regarded as one "interphase" [30], which changes in a uniform manner in response to progressive hydration. The thermodynamic state of this system is directly defined through the water activity, and yet it determines the molecular and spectroscopic properties of both the lipid and water molecules alike.

The straight lines in figs. 6, 7 and 8 can be expressed by the following simple relationship between the water activity a_W and $f(n_W)$, which represents the generalized ^{2}H-NMR parameter, $1/T_1$ or $\Delta\nu_Q$, for the α, β and γ segments or for ^2H_2O;

$$a(n_W) = [f(n_W) - f_0]/(f_S - f_0), \qquad (2)$$

where the two limiting values, f_0 and f_S, represent the extrapolated intercepts with $a_W = 0$ and 1, respectively, and correspond to the hypothetical NMR values at zero and at full hydration. The analogy between the two different ways of representing the same ^{2}H-NMR data $f(n_W)$ as a function of either n_W (eq. (1)) or a_W (eq. (2)) reveals that the limiting values of f_0 and f_S must be the same in the original rounded hydration curves (figs. 2 and 3) as they are in the corresponding straight lines (figs. 6 and 7). The two constants may thus be regarded as scaling factors for the individual experimental ^{2}H-NMR hydration curves, and they have already been found by extrapolation of the original NMR data (eq. 1) and are given in table 1. While the values of f_S are rather accurately determined, the non-linear extrapolation to f_0 from the original ^{2}H-NMR hydration curves (figs. 2 and 3) is less reliable. Therefore, a logarithmic extrapolation procedure is more appropriate, and such improved values for f_0 will be used in the following analysis.

We will now demonstrate that the present ^{2}H-NMR approach can be used as a quantitative measure tool to hydration forces. The repulsive hydration pressure between two apposing bilayers is attributed to the work needed for the removal of water from the two surfaces [3,4,6–13,30]. Its direct experimental verification lies in the empirically derived force law, which relates the hydration pressure P to the interbilayer distance d_W or to the number of water molecules n_W,

$$P = P_0 \exp(-n_W/\nu), \qquad (3)$$

where ν is the characteristic decay constant for the bilayer and P_0 the nominal pressure at zero hydration. Since it has been possible to establish a direct correlation of the NMR parameters $1/T_1$ (fig. 6) and $\Delta\nu_Q$ (fig. 7) with the activity a_W of the interbilayer water, the value of P can now be calculated for any hydration level n_W. From thermodynamics, the effective repulsive pressure P between the bilayers can be defined as [3,6]

$$P = -RT/V_W \ln(a_W), \qquad (4)$$

where $V_W = 18 \times 10^{-6}$ m^3 is the molar volume of water. To express P in terms of the NMR parameter $f(n_W)$, eq. (2) is substituted into eq. (4), giving

$$P = -RT/V_W \ln\{[f(n_W) - f_0]/[f_S - f_0]\}. \qquad (5)$$

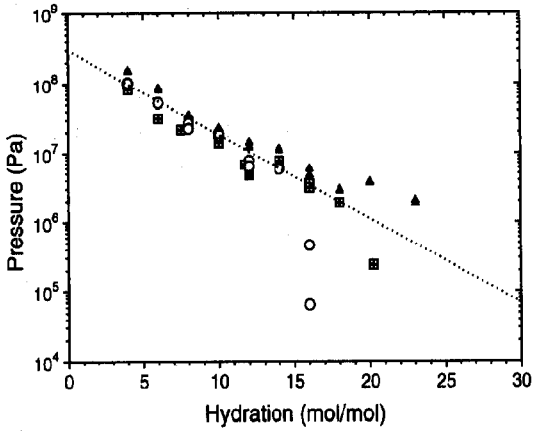


Fig. 9. Logarithmic plot of the hydration pressure versus the amount of interbilayer water n_W. The pressure was calculated (eq. (5)) from the ^{2}H-NMR relaxation rates ($1/T_1$) of gravimetrically prepared samples, for the α (○), β (▲) and γ (⊞) segments of the DOPC headgroup at 30°C. The slope of the linear fit (dotted line) gives a hydration force decay constant of $\nu \approx 3.5$ water molecules per lipid.

This equation (5) links the experimental ^{2}H-NMR observable $f(n_W)$, that is $1/T_1$ or $\Delta\nu_Q$ for the α, β or γ segment of the phosphatidylcholine headgroup, to the thermodynamic hydration pressure P. To calculate the hydration force directly from the ^{2}H-NMR data, only the values of f_S and f_0 need to be known (see table 1), and this analysis does not require the involvement of sorption data or any other information on the lipid and is based on the gravimetric ^{2}H-NMR results alone.

The further strategy to extract the classic hydration force decay number ν from the gravimetrically prepared NMR samples is straightforward and has been applied previously in a similar way to analyze sorption isotherms [3]. Using eq. (5) we have constructed pressure-versus-hydration plots from the experimental $1/T_1$ values of DOPC at 30°C, for the α, β and γ segments. The three sets of data are shown in fig. 9, where the repulsive pressure P is represented on a logarithmic scale in order to reveal the exponential nature of the empirical hydration force law given by eq. (3). Indeed, it is seen that the data points fall on the same straight line, for all three labelled headgroup segments. The corresponding analysis of the quadrupole splittings $\Delta\nu_Q$ of any of the labelled segments in DOPC or POPC gives essentially the same picture (data not shown). The linear behaviour seen in fig. 9 is immediately reminiscent of the characteristic results from the classic hydration force measurements by X-ray diffraction or the direct force apparatus [3,4,6,9, 11–13]. Compared to conventional hydration force measurements, the experimental scatter from our present ^{2}H-NMR data is somewhat larger, but it is nevertheless remarkable that this novel application of ^{2}H-NMR to characterize hydration forces yields such a good agreement. For future investigations, the potential accuracy that can be achieved by ^{2}H-NMR compares favourably with diffraction methods, and the interpretation is, furthermore, model independent and does not require any assumptions about the bilayer dimensions.

From the gradient $1/\nu$, of the linear fit in fig. 9 (dotted line), the hydration decay number is found to be $\nu \approx 3.5 \pm 0.4$ water molecules per lipid. Extrapolation to $n_W = 0$ yields the nominal pressure at zero hydration $P_0 \approx 3.0\ (\pm 1.0) \times 10^8$ Pa. These values are in excellent agreement with the results derived by Marsh [3] from the sorption isotherms ($\nu \approx 3.3$, $P_0 \approx 4.1 \times 10^8$ Pa) and with another literature value ($\nu \approx 4.0$) for DOPC [6]. The same kind of analysis applied to the quadrupole splittings of DOPC yields essentially the same values for ν and P_0 as were found from the $1/T_1$ data. The various ^{2}H-NMR data sets can thus be reduced to one uniform hydration dependence, and this dependence is indeed characterized by the classic type of exponential force law. This observation is yet another manifestation of the fundamental relationship between the molecular response of the lipid headgroup to hydration and the thermodynamics of the lipid–water system.

5. Conclusions

For a thorough understanding of any physical event on a molecular scale it is necessary to have simultaneous information about the structures of the molecules involved, their dynamics, as well as the thermodynamics of the whole system. Con-

cerning the complex interactions between the lipid headgroups and the water molecules at the bilayer surface, we have illustrated how ^{2}H-NMR studies have the potential of providing clues to all three of these fundamental questions. The analysis of two ^{2}H-NMR parameters, i.e. the quadrupole splitting $\Delta\nu_Q$ and the spin–lattice relaxation rate $1/T_1$, has provided a qualitative description of the molecular events at the bilayer surface upon progressive hydration. We suggest that the structural basis for the hydration-induced changes seen in these NMR parameters is an overall three-dimensional 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" [30]. When water molecules are accommodated within this 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 [18]. A three-dimensional 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 described by ^{2}H-NMR the acceleration in the headgroup fluctuations, which may be attributed to two distinct energetic effects. Firstly, the relative temperature of the system becomes effectively increased as a result of the decreasing phase transition temperature of the lipid. And secondly, the favourable entropic impact of a fluctuating headgroup on the surrounding solvent molecules increases with progressive hydration.

The hydration dependence of the general NMR parameter $f(n_W)$ has been expressed not only in terms of the experimentally varied water content n_W but also as a function of the interbilayer water activity a_W. This way, the ^{2}H-NMR data from the gravimetrically prepared samples was found to be linearly related to a_W, which has lead to the conclusion that the underlying physical relationship for the NMR hydration dependence is given by the lipid sorption isotherm. However, our present interpretation still remains limited to an empirical description of the observed phenomena. In particular, the question concerning the physical relationship between the ^{2}H-NMR response monitored for the lipid headgroup and the thermodynamic activity of the interbilayer water would need to be resolved. Nevertheless, we have shown that simple spectroscopic ^{2}H-NMR studies provide quantitative information about the thermodynamics of the lipid–water system. That way, the hydration pressure between apposing bilayers can be characterized from the NMR measurements alone, and the value of the hydration force decay length is accessible, even for non-lamellar systems. The unique potential of this non-perturbing and model-independent ^{2}H-NMR approach lies in the simultaneous description of the structural, dynamic and thermodynamic response of the polar lipid headgroups to hydration.

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