HYDRATION OF PHOSPHATIDYLCHOLINE

ADSORPTION ISOTHERM AND PROTON NUCLEAR MAGNETIC RESONANCE STUDIES

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ABSTRACT Adsorption-desorption isotherms were obtained for water binding by 1,2-dimyristoylphosphatidylcholine in the temperature range 15°–35°C. The isotherms were analyzed by Brunauer et al.'s (BET) theory and also a polarization theory, the latter being more successful in fitting the data. There was some evidence for a change in the surface field of the lipid bilayer around 25°C.

Proton T_1 and T_2 measurements were used to obtain a log-normal molecular correlation time distribution for water protons in these systems. This distribution was compared with the isotherm data to effect a description of several classes of water molecules.

INTRODUCTION

Water of hydration has an important role in the structure and function of macromolecules and macromolecular assemblies such as cell membranes. Calorimetric, spectroscopic, and thermodynamic studies have all demonstrated the existence of this type of water with properties altered from the bulk state (1–3). In this paper, we present the results of two types of experiments on hydration of phosphatidylcholine: proton spin relaxation measurements and isothermal water adsorption-desorption studies.

A homogeneous phosphatidylcholine, 1,2-dimyristoyl-*Sn* glycero-3-phosphorylcholine (DMPC), has been chosen for study because the bilayers formed on dispersion of this substance in H_2O undergo a thermal phase transition at 24°C for the fully hydrated bilayer and somewhat higher for bilayers with less water (4). This thermal transition, although involving primarily the esterified hydrocarbon chains of phosphatidylcholine, also involves surface expansion and possibly headgroup reorientation. Changes in the properties of hydration waters should reflect any alteration in bilayer surface characteristics above and below this temperature.

The relation of proton spin relaxation to molecular properties is a subtle one. Proton spin relaxation actually occurs via fluctuations in local magnetic fields occur-

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ring with sufficient strength and at appropriate time scales to cause transitions between, or alterations in, nuclear spin energy levels. Variation in magnetic interactions of water protons in lipid bilayer systems such as those studied here can in principle come from a very complex set of interactions, including both inter- and intramolecular ones. In bulk water, however, intramolecular interactions account for approximately two-thirds of all relaxation (5). Moreover, it is likely that in more complex systems, changes in the remaining intermolecular contributions parallel those in intramolecular contributions. Therefore, where only a qualitative characterization of water species is anticipated, the major interaction, the dipole-dipole interaction between geminal protons, will be assumed to be the only one. The potential magnitude of the variation is then fixed by molecular geometry and measured spin-spin and spin-lattice relaxation times, T_2 and T_1, can be related to the time scales of these variations or mobility of various water species. These time scales are conveniently given in terms of correlation times for the decay of an autocorrelation function expressed in terms of second-order spherical harmonics.

Several analyses of spin relaxation in lipid bilayer systems have been presented previously (6–9). Most have used deuterium oxide in place of water and have observed the resulting deuterium resonance. Attention has been directed at a detailed description of the motional properties of bound water. The data given here, employing proton magnetic resonance, are intended more to complement the adsorption isotherm data with a qualitative description of the mobility of identifiable bound species than to provide an independent detailed description of motional characteristics.

Adsorption isotherm data provide a direct measure of the number of grams of water adsorbed per mole of phosphatidylcholine at various water vapor pressures. They can in principle give a measure of the free energy of hydration at different hydration levels. Adsorption isotherm data on lipid bilayer systems have also been presented previously (10, 11), but they have almost universally been interpreted in terms of a single theory, Brunauer et al.'s (BET) theory (12). In this paper, we also employ a second theory, that developed by Bradley (13). Bradley's theory has been used to gain an understanding of the structure and properties of cellular water (14). Its successful application to the model system studied here and its correlation with NMR data suggest that the model on which it is based may provide a reasonable description of other membrane surfaces.

METHODS

Techniques for obtaining the proton magnetic resonance data have been described elsewhere (15). The adsorption isotherms were measured with an automated device described previously (16). Samples of 1,2-dimyristoylphosphatidylcholine were obtained from Calbiochem (San Diego, Calif.) and used without further purification. For the adsorption measurements, from 500 to 600 mg of sample was dissolved in chloroform and then applied to Pyrex wool sheets for use in the sample chamber. Equilibration times for the DMPC samples were approximately the same as those previously reported for egg lecithin (4–5 h). The error in the present isotherms should also be comparable to that reported in the tests of the automated device (± 5%). The NMR samples being fully hydrated, and the isotherm samples at relative vapor

pressures greater than 0.15 are expected to be in lamellar phases (4). After experiments were complete, representative samples were analyzed by thin-layer chromatography (TLC) for decomposition. Silica gel G plates loaded with ~20 μg lipid, developed with chloroform:methanol: water (65:25:4), and visualized with iodine vapor showed negligible amounts of lysolipid.

RESULTS

The transverse and longitudinal relaxation times of protons in 50% DMPC (39 mol water/mol phosphatidylcholine) and in 67% DMPC (19 mol water/mol phosphatidylcholine) suspensions at 15 and 20°C are shown in Table I. There was no measurable effect on T_2 at these temperatures of varying the spacing between successive 180° pulses in the Carr-Purcell experiment from 37 to 117 ms. This is in contrast to the marked effect observed at temperatures above 25°C, where a chemical exchange occurs with a time constant of a few milliseconds (15). Presumably, at the temperatures in Table I, the exchange is either too fast (rate $\geq 10^3$ s^{-1}) or too slow (rate constant ≤ 1 s^{-1}) to be measured in this way. The latter possibility seems unlikely, as the spin-echo decays show no large departure from the single exponential, expected for rapid exchange among different environments.

It is quite significant that the T_2's are considerably shorter than the T_1's. This situation can arise for isotropic motion when the motional correlation time characterizing water reorientation becomes greater than $1/\omega_0$, where ω_0 is the Larmor precession frequency. It can arise even in fast anisotropic motion if one correlation time is greater than $1/\omega_0$. Or it can arise from chemical exchange effects. Calculated chemical exchange contributions to T_2 for processes faster than 10^3 s^{-1} with reasonable choices for populations and chemical shift differences (15) will be only a few percent. Hence we can assume the difference in T_1 and T_2 to arise from the existence of some motionally restricted process. Assignment of long correlation times to particular motionally restricted processes is difficult because it has now been demonstrated that several bound water states exist and that reorientation of the water molecule in these states is highly anisotropic (8,9). A complete analysis would require specification of a number of correlation times inconsistent with the number of data points given here.

On the other hand, we can demonstrate that most common two-parameter descrip-

TABLE I
PROTON RELAXATION TIMES IN CONCENTRATED PHOSPHATIDYL CHOLINE SUSPENSIONS

Temp	Concn.	T_1	T_2
°C	% wt/wt	ms	ms
15	50	470	315
	67	240	100
20	50	540	330
	67	295	115

tions provide an inadequate fit to the present data. If the two parameters are taken to be the two correlation times describing anisotropic motion of a single species, along the lines of the theory developed by Woessner (17), calculations assuming rapid reorientation about either the OH bond or the H—O—H bisector lead to T_1 or T_2 values that deviate from measurement by 40% or more. If the two parameters taken are a fraction of water bound and a correlation time for isotropic motion of the bound species, an usually low number of bound waters is obtained (about 1 mol/mol of phosphatidylcholine.

A less common approach that provides a reasonable compromise and avoids assigning degrees of anisotropy and populations to larger numbers of classes of bound water has been successfully applied to the characterization of water associated with other biological surfaces (18, 19). T_1 and T_2 relaxation times for any state are assumed to be the result of isotropic motion of correlation time τ_c, in which case

$$T_1^{-1} = \frac{3}{10}\frac{\hbar^2\gamma^4}{r^6}\left(\tau_c/(1 + \omega_0^2\tau_c^2) + 4\tau_c/(1 + 4\omega_0^2\tau_c^2)\right), \quad (1)$$

$$T_2^{-1} = \frac{3}{20}\frac{\hbar^2\gamma^4}{r^6}\left(3\tau_c + 5\tau_c/(1 + \omega_0^2\tau_c^2) + 2\tau_c/(1 + 4\omega_0^2\tau_c^2)\right), \quad (2)$$

where $2\pi\hbar$ is Planck's constant, γ is the magnetogyric ratio, and r is the intramolecular proton separation, 1.54 Å (20, 21). Rapid exchange between a multitude of such sites is assumed, so that the observed T_1's and T_2's are given by

$$T_1^{-1} = \int_0^\infty G(\tau_c) T_{1_i}^{-1} d\tau_c, \quad (3)$$

$$T_2^{-1} = \int_0^\infty G(\tau_c) T_{2_i}^{-1} d\tau_c, \quad (4)$$

where $G(\tau_c)$ is a log-normal distribution function. This distribution, equivalent to assuming a Gaussian distribution of activation energies for rotation of water molecules, has been used successfully in treatments of dielectric relaxation (22) and NMR relaxation (18, 19). The distribution is characterized by two parameters: τ_0 is the center of the distribution, and β is a width parameter. Values of β and τ_0 for each concentration at each temperature could be determined by trial and error fits of the resultant T_1 and T_2 expressions to the data. The values found predict T_1 and T_2 values to within experimental error ($\sim$5%) for each measurement. The best values for τ_0 were found to be 3.8×10^{-12} s and 8.3×10^{-12} s for 50% and 67% DMPC, respectively. (The corresponding values of β were 3.4 and 3.8.) Although independent fits were made at 15 and 20°C, no significant variation in τ_0 or β was noted. The distributions at 20°C are plotted in Figs. 1 and 2. The model is unrealistic in the sense that the symmetrical form of the distribution function implies that significant amounts of water have correlation times shorter than that of bulk water. This unrealistic aspect of the model is, however, of small importance in determining the actual relaxa-

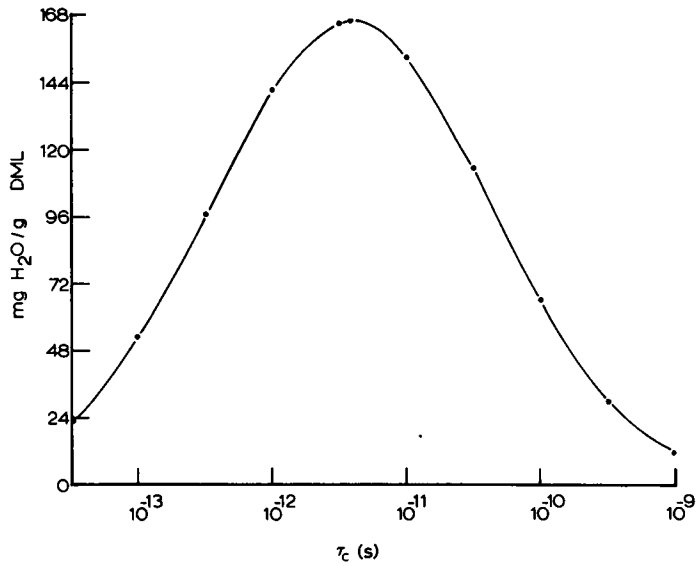

FIGURE 1 Log-normal distribution of water proton correlation times (τ_c) for a 50% DMPC suspension.

tion time, since more than 90% of any given T_1 value is due to protons with correlation times longer than that of bulk water. Hence, with very little error, one can simply consider the effect of correlation times less than 2.7×10^{-12} s as if they were equal to 2.7×10^{-12} s. This has the effect of making the distribution bimodal: a delta function to describe the bulk water component and the remaining part of the curve to describe immobilized water. One then gets values of 44.4% and 32.8% for the fraction of water behaving as bulk water for the 50% and 67% samples, respectively. That is,

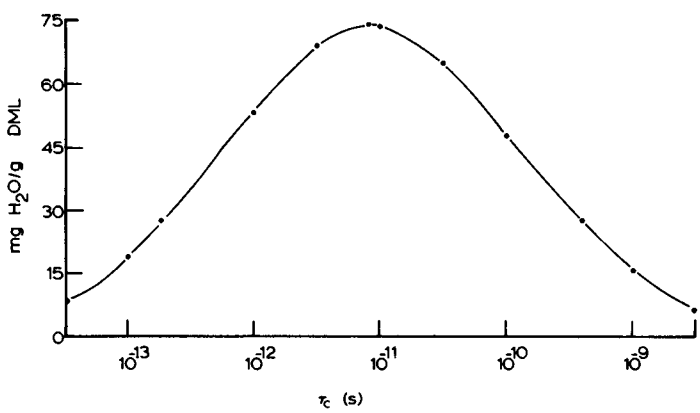

FIGURE 2 Log-normal distribution of water proton correlation times (τ_c) for a 67% DMPC suspension.

13–22 molecules of water per phosphatidylcholine molecule are immobilized relative to bulk water. Their mobilities vary in accord with the distributions of Figs. 1 and 2.

Isotherms

The sorption-desorption isotherms of DMPC at various temperatures are shown in Fig. 3. Hydration, a, in grams of water per 100 g of DMPC, is plotted against the relative vapor pressure, X. Any hysteresis present was less than the estimated error in the measurement, and so both sorption and desorption data points are used for the same isotherm.

The isotherm data were analyzed first according to the theory of Brunauer et al. (12), viz.

$$X/a(1 - X) = 1/a_m C + (C - 1)X/a_m C, \tag{5}$$

where a_m is the monolayer adsorption quantity and C is a measure of how tightly the first layer is bound. When the data were plotted according to Eq. 5, the fit was good up to relative vapor pressures of about 0.35. No real improvement was obtained by using BET theory for a finite number of adsorbed layers.

Values of a_m determined in this way were found to range from 5.5 to 6.6 (2.2–2.6 mol water/mol DMPC), and of C from 5.5 to 9.8. These values are similar to other results in the literature for phospholipids (1, 10, 11). In addition, the amount adsorbed at saturation was determined by plotting a/X vs. X and extrapolating to $X = 1$. This quantity was found to be 25 g water/100 g phosphatidylcholine (10 mol water/mol DMPC) below 25°C, and 35 g water/100 g DMPC (14 mol water/mol

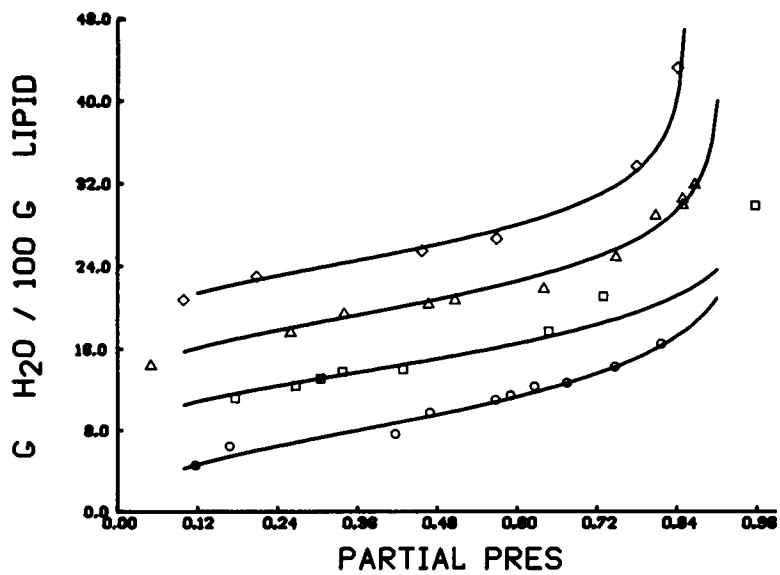

FIGURE 3 Desorption-sorption isotherms of DMPC at 15(0), 20(□), 25%(△), and 35°(◇). The ordinate has been shifted 6.3 units with each successive temperature.

DMPC) at 35°C. These results were checked by exposing weighed amounts of dry DMPC to water vapor at the appropriate temperatures and weighing the resulting saturated lipid several hours later.

The isotherm data were also analyzed according to the polarization theory of Bradley (13). His equation for adsorbates having a permanent dipole moment is

$$\ln X = K_1 K_3^a + K_4, \tag{6}$$

where X and a are as defined previously. The three constants, K_1, K_3, and K_4 are complex functions of properties of both the adsorbent and the adsorbate. K_1 may be considered as the product of two terms, one depending on the properties of water and the other on the solid adsorbent.

$$K_1 = (NF^2/T) \times \text{Function}(\alpha, \mu, a_1), \tag{7}$$

where N is Avogadro's number, F the surface field of the adsorbent, α the polarizability of water, μ the dipole moment of water, and a_1 the spacing between adjacent water dipoles.

$$K_3 = G^{2m}, \tag{8}$$

where m is the number of adsorbed layers and G is another function of the same variables as above—α, μ, a_1.

K_4 accounts for the difference between the heat of vaporization from the polarized surface and from the bulk liquid. In practice this is often set equal to zero (23).

Both K_1 and K_4 depend explicitly on temperature, but all three constants contain temperature-dependent quantities (α, μ, F, m). However, in the temperature range of interest here, both α and μ are smooth functions of temperature, since they are properties of water, whereas F, a_1, and m may show an unusual temperature dependence as they depend on the physical state of the lipid or the whole lipid-water matrix. Hence, any apparent discontinuity in the temperature behavior of the Bradley constants must be due to abrupt changes in these three parameters.

The Bradley isotherm was found to fit all the adsorption data from $X = 0.1$ to $X = 0.8$ with good agreement. The best theoretical curves, as generated by a multiple parameter variation program using chi-squared of adsorbtion as a criterion for fit, are presented in Fig. 3. The constants determined for DMPC at temperatures both above and below the gel-to-liquid-crystalline phase transition are presented in Table II.

The most notable change is that for K_1, which decreases sharply as temperatures are raised above the phase transition. Constants extracted from data by Jendrasiak et al. are presented for comparison. It is interesting to note that in comparing dipalmitoylphosphatidylcholine (DPPC), which is below its phase transition at 22°C, and dioleoylphosphatidylcholine (DOPC), which is above its phase transition at 22°C, the same trend toward a decrease in K_1 is noted.

Changes in K_4 seem to go on through the phase transition, but because of the small size and sensitivity of these values to the few points above 0.8 X where errors are largest, we chose not to attach any great significance to this change.

TABLE II

BRADLEY COEFFICIENTS FOR VARIOUS PHOSPHATIDYLCHOLINES

Lipid	T	K_1	K_3	K_4
	°C			
DMPC	15	5.8	0.80	0.05
DMPC	20	6.4	0.77	0.03
DMPC	25	4.4	0.78	0.10
DMPC	35	3.5	0.77	0.16
DPPC	22	4.5	0.70	0.04
DOPC	22	3.8	0.85	0.04

It is significant, however, that K_3 undergoes very small changes with no evidence of a consistent trend in a region where changes in K_1 are large. The change in K_1 is most probably due to either a decrease in F or an increase in a_1. Since K_3, which also depends on a_1, does not vary with temperature, one might be tempted to attribute the K_1 change entirely to variation in the surface field of the lipid. However, if m were to decrease as a_1 increased, it is also possible that K_3 would remain constant. These possibilities will be discussed.

DISCUSSION

Water in the Lipid Gel Phase

The shape of the isotherms presented here is typical of multiple site adsorption of water, implying that there are many different environments for water molecules, a point of view consistent with a distribution of molecular correlation times. One of the environments corresponds to the BET monolayer and numbers 2–2.5 water molecules per lipid molecule. The monolayer is the most tightly bound layer of the adsorbed water and very likely has the longest correlation times (is the most restricted in an NMR sense). By suitable integration of the distribution curves seen previously, one can assign correlation times longer than 1.8×10^{-10} s to this group. Although they are thus considerably less mobile than bulk water, it is clear that they need not be considered "ice-like," since the correlation time of protons in ice is on the order of 10^{-5} s.

The existence of a second class of water molecule is apparent from the isotherm curves. These seven or eight waters per lipid represent multilayer adsorption and correspond to the water adsorbed between the monolayer amount and the saturation amount. It is most likely that this class is very heterogeneous. That is, the inability of BET theory to describe this group may indicate that the second, third, and so on layers of adsorbed water do not all have the same average energy. Each is predicted to have a distinct energy by the Bradley theory. Since they presumably represent those molecules with NMR correlation times shorter than the first class described, one can integrate the distribution of correlation times to show that they could be described as having correlation times between 1.3×10^{-11} and 1.8×10^{-10} s. Obviously, in

regard to rotational freedom, this class is more like bulk water. It is not surprising, of course, that such a group exists since its component members are probably more distant from the polar headgroup of the lecithin than is the monolayer and so the molecules of this class interact with other water molecules rather than with the lipid directly. If one accepts the applicability of the Bradley isotherm, then this class is affected by phosphatidylcholine through a polarization mechanism.

The two main classes described thus far do not represent all of the water with correlation times longer than that of bulk water according to the log-normal distribution of correlation times. There remains a class (classes) in the NMR samples with correlation times between 2.7×10^{-12} and 1.3×10^{-11} s and numbering from 3 to 10 waters/lipid. Since they exceed the saturation points of the adsorption studies, they quite possibly do not represent water molecules involved in the swelling of the lipid multilayer, i.e. water between adjacent bilayers. Instead, they may be only weakly associated with the multilayer structure, perhaps on its periphery or trapped within the multilayer structure.

The notion of several stages in hydration of lipids is not a new one. Early calorimetric studies by Ladbrooke and Chapman had identified two types of water which did not freeze at temperatures of −50°C, and more recently, adsorption isotherm studies such as those of Jendrasiak and Hasty have divided waters of hydration observed below saturation into three types (1). Our low-temperature data in fact agree well with Jendrasiak and Hasty's data on dipalmitoyl-phosphatidylcholine at 22°C. We emphasize that we have made no effort to define classes of water between first hydration and saturation. The heterogeneity of water adsorbed in this region is accomodated by Bradley's theory and a log-normal distribution of motional correlation times. All of this water is immobilized to some extent and will closely reflect headgroup and hydrocarbon mobility (3, 24).

Effect of the Phase Transition

Two major effects of the lipid phase transition are apparent in this study. First, there is a substantial increase in saturation water binding (from 35 to 40%) above the transition. Such an increase is not unexpected in light of the increased surface area of the bilayers above the transition (25), something that would permit more water molecules to fit into the space between successive bilayers in the multilayer structure. However, the change in surface area is approximately 20–25% (25) and it would seem therefore that either the water molecules are, on the average, closer together above the transition, or that successive bilayers are further apart, allowing for more water layers between them. While data on the thickness of the water layer in DMPC multilayer systems are not available, there is evidence in dipalmitoylphosphatidylcholine liposomes that the water layer either does not change or perhaps even decreases in thickness through the transition (26). It should be noted also that in the analysis of the Bradley isotherm, the observed temperature effects on the Bradley coefficients were consistent with a decrease in m (number of water layers) and increase in a_1 (separation of water dipoles). The decrease in m would seem reasonable, then, in view of the

above, but it seems unlikely that the combination of fewer water layers with greater separation between water molecules could give rise to an increased hydration. What is left from the Bradley theory, then, is a decrease in the surface field strength of the lipid at or near the phase transition. A likely source of such a change is reorientation of the polar headgroup of the phospholipid. A recent ^{31}P-NMR study of dipalmitoyl phosphatidylcholine liposomes has demonstrated that changes in the headgroup occur near the phase transition (27). The precise nature of these changes is uncertain as yet, but there may be a connection between the changes in motional properties of the headgroup as detected by NMR spectroscopy and the alteration in the surface of the bilayer as reported here.

Such a change in the lipid also would be consistent with the increased water mobility above the transition as seen in longer relaxation times for water protons (15). The larger values of T_1 and T_2 could be due to an increase in mobility of any one or more of the water species described earlier. It does not entail, however, an increase in the bulk water fraction, as we have already noted an increase in water binding above the lipid phase transition. In terms of the distribution of correlation times, this would mean either a shift in the center of the distribution towards shorter times or a decrease in the width of the distribution. There does not appear to be any way of distinguishing between these two possibilities on the basis of evidence here. A decreased lipid surface field would lead to a reduced polarization of the water layers and presumably an overall loosening of the structure of the water "lattice" between lipid bilayers.

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