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WATER–LECITHIN BINDING IN LECITHIN–WATER LAMELLAR PHASES AT 20°C

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SUMMARY

^{1}H and ^{31}P continuous wave and spin-echo NMR measurements have been made on lecithin–water mixtures as a function of water content at 20 °C. An analysis of the data demonstrates the existence of two water environments, lecithin bound and free. Conclusions are presented concerning the stoichiometry and kinetics of the binding. The results indicate that six molecules of water are bound to one lecithin molecule and the lifetime of the bound water is $6 \cdot 10^{-5} \pm 3 \cdot 10^{-5}$ s.

INTRODUCTION

The detailed structure of lipid and protein in the biological membrane is still uncertain but recent conclusions[1] tend to support the hypothesis that some regions of the membrane contain lipid bilayers. Any water associated with the lipids or protein of the membrane could play an important role in determining the permeability properties of the membrane. Recent studies on lipid–water systems as a function of water content indicate the presence of two water environments, "bound" and "free"[2,3]. The exact amount and nature of this "bound" water is uncertain.

From water vapor sorption isotherm studies Elworthy[4] determined that 1 g lecithin binds up to 0.48 g water. His studies indicated the presence of different layers or shells of hydration water.

Calorimetric studies by Chapman *et al*[2] also indicate that 1,2-dipalmitoylphosphatidylcholine binds about 20% water: water freezing at 0 °C is only detected after 20% water has been added to the lipid. The amount of this water may well be a measure of the minimum water requirement for structural stability in the biological membrane.

Recent measurements[5] of the diffusion of water in lamellar egg lecithin–water phases at various water contents suggest that the presence of water of hydration plays a role in the diffusion process. To obtain further information about the state of water in these phases we have studied the NMR relaxation of the water proton at different phase water contents.

The measurement of nuclear magnetic resonance relaxation times to study

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the structure and time dependence of molecular environments is well known and theoretically understood. The spin-lattice relaxation rate $1/T_1$ for relaxation between pairs of magnetic dipoles under conditions of molecular motion is given by[6]:

$$\frac{1}{T_1} = K\left[\frac{\tau_c}{1+\omega_0{}^2\tau_c^{\,2}} + \frac{{}^4\tau_c}{1+4\omega_0{}^2\tau_c{}^2}\right]$$

where K is a constant, ω_0 is the angular NMR frequency and τ_c is the correlation time for molecular motion and hence a measure of structural order in the system. The assumption that we are operating under rapid molecular motion conditions where $T_1 = T_2$ in lipid–water systems at room temperatures is supported by our own measurements and those of Chapman on lipid–^2H_2O systems[7] and will be discussed later.

The spin–spin relaxation time T_{2cp} measured from the decay of NMR spin echoes in the Carr and Purcell[8] rf pulse sequence contains information on the natural spin–spin relaxation time $T_2 (= T_1$ for $\omega_0\tau_c < 1)$ and any other process contributing to the random dephasing of spin isochromats. The two processes relevant to the lipid–water system are spin diffusion and the possible chemical exchange of water between different structural environments. By using a rapid pulsing rate the effects of diffusion can be eliminated[8] and by varying the pulsing rate on the order of the exchange rate the details of any kinetic process can be elucidated[9,10]. With this in mind we have undertaken an investigation of NMR relaxation of ^1H and ^{31}P in lecithin–water lamellar phases at 20 °C as a function of water content, ϕ_ω defined as g water per g total phase.

EXPERIMENTAL

Materials

Lecithin was extracted from egg yolk according to the method of Singleton *et al.*[5]. The gels were placed in 5-mm outer diameter NMR sample tubes (approx. 1 ml volume). The precision of ϕ_ω was estimated at $\pm 1\%$.

Methods

The ^1H continuous wave NMR measurements were made on a JEOL C-60H spectrometer operating in external lock mode at 20 °C with a non-spinning line width for pure water of about 5 Hz. The pulse measurements were made on a NMR Specialities PS60 instrument operating at 30 MHz and the field was provided by a Bruker BM 12 electromagnet equipped with a 5-digit Precision Hall Stabilizer and 8-Gradient Electric Current Shim Pole Caps.

The continuous wave measurements were made under slow sweep conditions care being taken to avoid saturation. The T_1 measurements were made using the conventional π–τ–$\pi/2$ pulse sequence[8]. The Carr–Purcell measurements were made using the modification of Meiboom and Gill[12] with the $\pi/2$, π pulse separation, τ, ranging from 2.54 ms to 0.05 ms. The precision on the T_1 values was estimated at $\pm 10\%$ and on the T_{2cp} values $\pm 15\%$. The width of the $\pi/2$ pulse was 9 μs. A Nicolet 1070 signal averager was used for data handling and signal/noise improvement for ^{31}P.

RESULTS

The dependence of the water proton line width, $\Delta\nu$ on ϕ_ω is shown in Fig. 1. A single broad resonance is observed which does not appear to have a simple lorentzian form indicating that it may well be due to more than one nuclear species. A sample of the lecithin–water system was prepared, doped with Mn^{2+} (0.002 and 0.004 M) (Fig. 1) to determine if there exists two or more distinct non-exchanging environments.

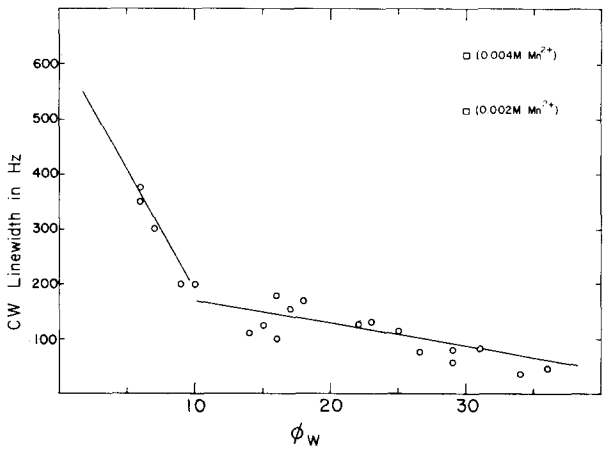

Fig. 1. ○, Water proton continuous wave (CW) line widths for lecithin–water gels as a function of water content (ϕ_ω). □, paramagnetic doped gels (Mn^{2+}).

The technique of paramagnetic doping in systems of this type is well known[13] and in general the line due to any free water which is present and will coordinate readily to Mn^{2+} will be considerably broadened relative to any bound water and hence may be distinguished. In this system the doping resulted in a broadening of the total line and very little shift in the resonance position, no sharp component was distinguished. The conclusion from this is that either there is only one molecular environment for water in the system or different water environments are exchanging rapidly on the time scale of the experiment. In view of the non-lorentzian shape and the conclusions of other investigations we proceed on the assumption of the latter viewpoint. Fig. 1 shows a fairly smooth dependence of $\Delta\nu$ *versus* ϕ_ω though a significant change in slope can be discerned in the region around $\phi_\omega = 10\%$. Similar breaks in the dependence of the diffusion coefficient in this region have been interpreted in terms of the amount of lecithin-bound water in this system.

The values of T_1 and T_{2cp} for various compositions are given in Table I and illustrated graphically in Figs 2 and 3. The dependence of $1/T_{2cp}$ *versus* $1/\tau$ in Fig. 3 indicates that there are contributions to transverse relaxation from either diffusion and/or chemical exchange. Knowing the diffusion coefficients[5] and the pulse separations (τ) used in our study we can estimate the magnetic field gradient, G, required to make a significant diffusion contribution to the relaxation rate from the equation[8]:

$$\frac{1}{T_2} = \frac{1}{T_{2_o}} + 1/3\gamma^2 G^2 D\tau^3$$

TABLE I

WATER PROTON SPIN-LATTICE RELAXATION TIMES (T_1) AND SPIN-SPIN RELAXATION TIMES (T_{2cp}) FOR VARIOUS PULSE INTERVALS (τ) IN THE CARR-PURCELL SEQUENCE AT DIFFERENT COMPOSITIONS

ϕ_ω	T_1 (*ms*)	$\tau(\pi/2\text{-}\pi)$ (*ms*)	T_{2cp} (*ms*)
10	90 ± 15	0.05	16.0 ± 2
		0.09	15.5 ± 0.7
		0.17	12.7
		0.33	8.2
		0.64	7.7
		1.28	6.5
18	120 ± 15	0.06	28.5
		0.13	19.0
		0.25	14.5
		0.51	12.5 ± 1.0
		1.02	8.2
24	170 ± 20	0.05	48.5
		0.09	40.6
		0.17	28.5
		0.33	18.0
		0.65	14.0
		1.28	10.7
		2.54	9.0
29	215 ± 20	0.06	52.0
		0.13	35.0
		0.25	26.5
		0.51	19.0
		1.02	14.0

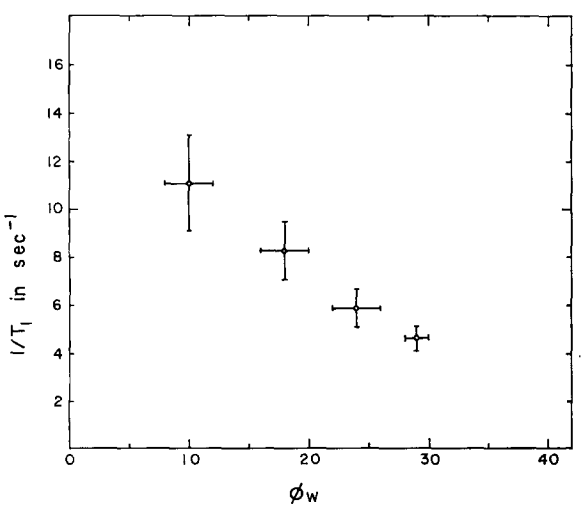

Fig. 2. Proton spin-lattice relaxation rate ($1/T_1$) as a function of water content (ϕ_ω) in lecithin - water gels.

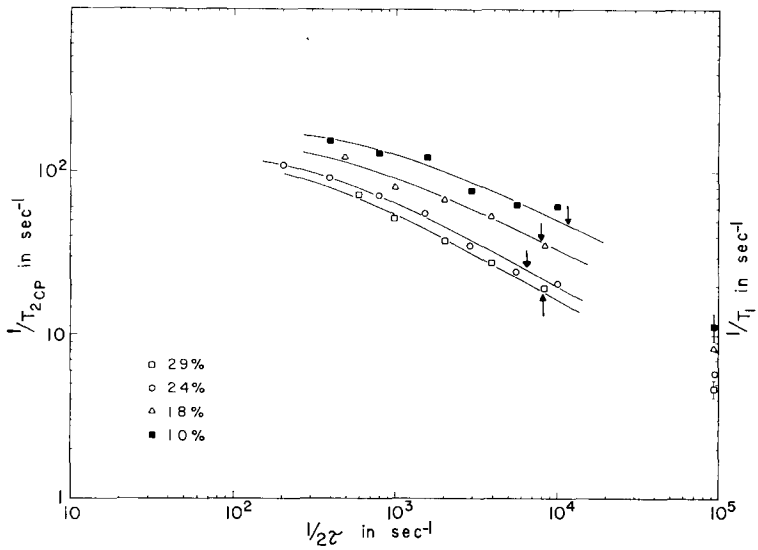

Fig. 3. Proton spin–spin relaxation rates measured by the Carr–Purcell sequence ($1/T_{2_{cp}}$) as a function of pulse frequency ($1/2\tau$) for various compositions. Points on extreme left are proton spin-lattice relaxation rates ($1/T_1$). Arrows indicate $1/\tau_{\frac{1}{2}}$ values (mid-points between slow and fast pulsing).

Based on a range of self-diffusion coefficients from $0.4 \cdot 10^{-6}$ to $1.0 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$ and values of τ used from 0.05 to 2.54 ms we estimate a field gradient of 80 G/cm would be required to make a significant contribution to the relaxation. In our magnet system a value of $G = 0.007$ g/cm is estimated from the free induction decay following a single pulse.

We can therefore conclude that the dependence of $1/T_{2_{cp}}$ *versus* $1/\tau$ arises from a kinetic process on the time scale of $1/\tau_{\frac{1}{2}}$. We can note that in the slow pulsing limit the $1/T_{2_{cp}}$ value tends towards that obtained from the continuous wave linewidth and in the fast pulsing limit the $1/T_{2_{cp}}$ tends toward the value $1/T_1$. Instrumental requirements prevented us from reducing τ below 0.05 ms and so we were unable to directly verify that $T_2 = T_1$. However in view of the consistency of our analysis (see further) and the work by Daycock *et al.*[7] in which they measured the temperature of the T_1 minimum for various concentrations of ^2H_2O in lecithin–^2H_2O we feel that we are in the region of "extreme narrowing" where $T_1 = T_2$ on the high temperature side of the T_1 minimum. The work of Daycock *et al.*[7] indicates that at temperatures about -5 °C and for compositions up to 40% water, the lecithin molecule has a motional correlation time $< 10^{-8}$ s implying that we are in the "extreme narrowing" region for the lecithin-bound water in our experiments at 20 °C.

The curves in Fig. 3 are in agreement with the theoretical behavior of $1/T_{2_{cp}}$ with $1/\tau$ for a nucleus exchanging rapidly between two sites of similar chemical shift. The mid-point of the step in the curves occurs when $1/\tau_{\frac{1}{2}} = 1/2\tau_B$, where τ_B is the lifetime of the bound species[10]. An examination of the curves reveals that τ_B is approximately constant for all the systems studied and a value of $6 \cdot 10^{-5} \pm 3 \cdot 10^{-5}$ s is obtained (Table II). Water is exchanging between the bound and free environments

TABLE II

LIFETIMES AND STOICHIOMETRY OF WATER IN LECITHIN–WATER GELS

ϕ_ω	$1/\tau_1$ (s^{-1})	$\tau_B = 1/2\tau^*$ ($s \times 10^5$)	P_B	n_B
10	$1.3 \cdot 10^4$	3.9	0.86 ± 0.16	4.0 ± 0.7
18	$8 \cdot 10^3$	6.3	0.64 ± 0.10	5.9 ± 0.8
24	$6.3 \cdot 10^3$	7.6	0.45 ± 0.06	6.0 ± 0.6
29	$8 \cdot 10^3$	6.3	0.35 ± 0.03	5.9 ± 0.4

* $\tau_B = 6 \pm 3 \cdot 10^{-5}$ s.

with a rate constant $k = 1.7 \cdot 10^4$ s^{-1}. The number of water molecules bound to a lecithin molecule, n_B, can now be evaluated using the equation[10,14]:

$$\frac{1}{T_2} = \frac{1}{T_1} = \frac{1}{T_{1_A}} + \frac{P_B}{\tau_B + T_{1_B}}$$

where T_{1_A} is the spin-lattice relaxation time of free water (approx. 3.0 s), T_{1_B} is the spin-lattice relaxation time of bound water (this value is obtained from the crystalline system with $\phi_\omega = 3\%$ in which all the water is presumably bound), and P_B is the mole fraction of the total water which is lecithin bound. The lecithin molecular weight is taken as 760.

The values of n_B and P_B for the systems measured are shown in Table II. The analysis is consistent and shows that six molecules of water are bound to a lecithin molecule. The data for the $\phi_\omega = 10\%$ system corresponds to a system below the stoichiometric composition. The stoichiometry corresponds to $\phi_\omega = 12\%$ being the amount necessary for complete binding.

We made ^{31}P measurements on the system with the aid of a signal averager. With one phosphorus atom per lipid molecule the signal to noise ratio is very poor and the errors on our measurements quite large. The results should however compare with our conclusions concerning the bound water. T_2^* measured from the free induction decay following a $\pi/2$ pulse for ^{31}P is approx. 0.3 ms corresponding to a line width of about 1 kHz indicative of a rather rigid molecular environment. The T_1 value obtained was 0.8 ± 0.5 s, fairly long and again indicative of a rigid structure characteristic of the lamellar phase. We plan to make a more precise study of ^{31}P NMR in these systems in an attempt to elucidate the details of the lecithin environment.

CONCLUSIONS

We conclude that the water present in the lecithin–water lamellar phase is bound to the extent of 12%, that is, 6 molecules of water per molecule of lecithin. This result can be compared with the measurements of Elworthy[4] who found that there are two shells of hydration water of lecithin, the first containing 2.5 and the second 5.1 moles water per mole lecithin. Salsbury *et al.*[3] carried out deuteron magnetic resonance studies of the water associated with lecithin in the lamellar

liquid crystalline phase and showed that 4 deuterons per lecithin molecule were in the class denoted as exhibiting "restricted motion". The agreement of our results with these, obtained by different methods, is quite good. We have found in addition that the water of hydration is in a more structured state than pure water and exchanges with excess bulk water in the system with a rate constant of $1.7 \cdot 10^4\ s^{-1}$.

We can compare our results with the X-ray diffraction data of Reiss-Husson[15] on the lamellar egg lecithin–water phase which indicate that at $\phi_\omega = 12\%$ the average area available to one hydrophilic group on the lipid–water interface is 57 Å^2. Assuming for the choline phosphate group a density of $1.32\ g \cdot cm^{-3}$ (ref. 16) and an extended length of 10 Å, the cross-sectional area of the group is approximately 24 Å^2. Assuming the volume of a water molecule to be 30 Å^3, it is then possible to fit eleven water molecules in the free volume around each choline phosphate group. At $\phi_\omega = 12\%$ the thickness of the aqueous layer is about 18 Å (ref. 16) implying that there is a slight overlap of choline phosphate group located on opposite sides of the aqueous layer. This would imply that somewhat fewer than eleven molecules of water can be arranged around each group. In spite of the number of simplifying assumptions made in the above calculation it is clear that there is easily place to fit six bound water molecules around each polar group.

A comparison between the T_1 for pure water and lecithin-bound water (3 and 0.1 s) gives us a measure of the difference in structure between the two forms of water. Knowing that the relaxation rate ($1/T_1$) is a direct measure of τ_c (the correlation time for molecular motion) in the "extreme narrowing" limit[6], and using a value of $3 \cdot 10^{-12}$ s for τ_c for pure water[17] we can estimate the correlation time for lecithin-bound water to be $9 \cdot 10^{-11}$ s. This is a factor of 30 times that for pure water and is indicative of a very ordered water structure of lower density between the lipid leaflets. The value of τ_c is much greater than that corresponding to the structure induced by the presence of a tetraalkyl cation in water (τ_c is approx. 10^{-11})[17] but less than that corresponding to a nucleus bound to a macromolecular species such as hemoglobin in solution (τ_c is approx. $5 \cdot 10^{-10}$)[18].

Comment should be made about the validity of the two-site model for the analysis of the data of Carr and Purcell[8]. It is possible that the "bound" water we refer to is not of a single structural type. The data of Elworthy[4] indicate that a number of layers or structural types of bound water are present. Our data are consistent with a two-site model of "bound" water (T_1 approx. 0.1 s) and free water (T_1 approx. 3.0 s) though we freely admit that this "bound" water may be an average of more than one structural type.

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