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WATER BINDING AND MOBILITY IN THE PHOSPHATIDYLCHOLINE/CHOLESTEROL/WATER LAMELLAR PHASE

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SUMMARY

Measurements of hydration and water self diffusion in lamellar phases of the ternary system phosphatidylcholine/cholesterol/water have been made using pulse NMR relaxation methods Systems containing phosphatidylcholine and cholesterol in a 1 1 mol ratio with varying water contents are studied at 20 5 °C The results indicate that 12 water molecules corresponds to complete hydration of the phosphatidylcholine/cholesterol unit, and in the region of this hydration a 4-fold decrease in water diffusion occurs The nature of the bound water and its relationship to phase stability and overall water mobility in the system are discussed It is concluded that at the stoichiometric composition the diffusion decreases due to the relative immobility of the bound water The implications in terms of permeability regulation in the aqueous channels by water content and hydration are cited

INTRODUCTION

The evidence that the biological membrane contains appreciable amounts of lipids in a bilayer form is now extensive [1] and this fact has prompted a considerable interest in the structure and dynamics of the lipid bilayer This interest has focussed not only on the variety and motional characteristics of the lipid molecules but also on the water which appears to be an intrinsic component of the stable lamellar bilayer phase [2–4] Since in many living systems phospholipids are often found associated with cholesterol, this being particularly true for lecithins, the effect of cholesterol on lecithin/water bilayers has been investigated and the various phases of the ternary system well characterised by X-ray diffraction and calorimetric methods [5, 6]

The purpose of this paper is to extend the investigation of water binding in lecithin (phosphatidylcholine)/water lamellar phases by nuclear magnetic resonance (NMR) techniques [4] to the ternary system phosphatidylcholine/cholesterol/water We have selected systems with phosphatidylcholine/cholesterol in a 1 1 mol ratio, and a water content, φ_w, defined as g water per g total phase, in the range 0 13–0 35

This corresponds to a single lamellar liquid crystalline phase at room temperature (20 5 °C) In this system the crystalline gel → liquid crystal transition is essentially removed A fluid lamellar structure of alternating phosphatidylcholine and cholesterol molecules exists with an increase in the amount of bound water compared to the phosphatidylcholine/water case [6] The structural nature and the dynamics of the water and the relationship to the structural stability of the overall phase will be elucidated and the possible implications in terms of permeability regulation in hydrophilic channels in such a bilayer structure will be discussed

The use of NMR relaxation measurements to probe the structure and time dependence of molecular environments is well-known and theoretically understood The spin-lattice relaxation rate $1/T_1$ in fluid systems involving magnetic dipolar interactions is related to the correlation time for molecular motion (τ_c) and hence, to the structural order at the molecular level The spin-spin relaxation rate $1/T_{2_{cp}}$ measured from the decay of NMR spin echoes in the Carr-Purcell/Gill-Meiboom RF pulse sequence [7] contains information on the natural spin-spin relaxation rate ($1/T_2$) (also related to τ_c) and other processes contributing to the dephasing of nuclear spins such as spin diffusion [8] and the chemical exchange of spins between different structural environments [9] Self diffusion coefficients (D) measured from NMR relaxation [10] yield a direct measurement of molecular mobility In this way, using NMR relaxation measurements of ^1H in the lamellar phases of phosphatidylcholine/cholesterol/water for different water contents, we have characterised the stoichiometry and nature of the water binding in the system in terms of a two site model, and related this to the overall water mobility in the system

EXPERIMENTAL

Materials

Lecithin (phosphatidylcholine) was extracted from egg yolk according to the method of Singleton et al [11], freeze dried, and the purity checked by thin-layer chromatography The cholesterol was obtained from Fisher Scientific The gels were prepared by dissolving phosphatidylcholine and cholesterol in a 1 1 mol ratio in chloroform and the solvent evaporated under reduced pressure, deionized water was then added After manual mixing the mixture was placed in a syringe, evacuated and forced back and forth through a narrow constriction The mixture was then left under N_2 at room temperature for a few days and the existence of the lamellar phase verified by powder X-ray analysis The gels were placed in 5-mm outer diameter NMR sample tubes (approx 1 ml volume) The water content was checked by drying under vacuum at 35 °C to constant weight and the precision of φ_w was estimated at ± 1 %

Methods

The ^1H pulse NMR measurements were made on a NMR Specialties PS60 instrument operating at 30 MHz 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 measurements of self diffusion coefficients were made with the aid of a Bruker pulsed field gradient unit type B-KR 300 Z 18

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 [7] with the $\pi/2$, π pulse separations τ varied over a factor of 10 The precision on the T_1 values was estimated at $\pm 10\,\%$ and on the $T_{2_{cp}}$ values $\pm 15\,\%$ Diffusion measurements were made by observing the attentuation of an echo following the application of a $\pi/2$-τ-π RF pulse sequence when two magnetic field gradient pulses of width δ and amplitude g were applied, one between the RF pulses and the second between the π pulse and the echo Under these conditions where Δ is the field gradient pulse separation the echo amplitude is given by [10]

$$E = E_0 \exp[-\gamma^2 g^2 \delta^2 (\Delta - \delta/3) D] \quad (1)$$

The apparatus was calibrated to obtain g using pure water of known self-diffusion coefficient $D = 2\,51 \pm 0\,01 \quad 10^{-5}\,\mathrm{cm^2\ s^{-1}}$ [12] Also by investigation of the dependence of echo attenuation upon the value of Δ it was possible to determine if restricted diffusion occurs [13, 14] A Nicolet 1070 signal averager was used for data handling and signal/noise improvement

Higher frequency relaxation measurements were made at 60 and 90 MHz on two samples using instrumentation made available by Brandeis University

RESULTS

The values of T_1, $T_{2_{cp}}$ and D of the water protons for various water contents are shown in Tables I and II Fig 1 shows a smooth decrease in proton spin-lattice relaxation rate $1/T_1$ with increasing water content in the range studied These data are

TABLE I

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

φ_w	T_1 (ms)	$\tau(\tau/2\text{-}\tau)$ (ms)	T_{2cp} (ms)
0 136	79 5 ± 8 0	0 12	6 2
		0 18	5 4
		0 24	5 8
		0 36	6 3
		0 48	6 1
0 188	87 7 ± 8 8	0 11	10 2
		0 22	9 2
		0 43	11 9
		0 86	11 9
0 210	101 1 ± 10 1	0 27	19 4
	(60 MHz) 124 0 ± 12 4	0 12	27 1
		0 22	26 5
		0 50	25 8
		1 00	25 6
		1 50	25 9
		2 00	25 6
		5 00	26 6
	(90 MHz) 197 5 ± 19 8		

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φ_w	T_1 (ms)	$\tau(\pi/2\text{-}\pi)$ (ms)	T_{2cp} (ms)
0 227	96 7± 9 7	0 12	15 7
		0 46	15 2
		0 92	15 7
0 240	103 1±10 3	0 11	18 6
		0 22	16 3
		0 31	15 7
		0 44	15 9
		0 88	17 2
0 255	120 9±12 1	0 13	23 0
		0 27	21 7
		0 38	22 3
		0 54	21 2
		1 08	24 8
0 285	135 7±13 6	0 13	24 4
		0 27	24 1
		0 40	22 7
		0 54	24 2
		1 08	26 1
0 305	139 ±14 0	0 14	31 5
		0 27	32 2
		0 40	30 8
		0 54	30 9
		1 08	34 7
0 310	151 8±15 2	0 29	39 9
	(60 MHz) 190 0±19 0	0 12	44 6
		0 22	43 9
		0 50	43 3
		1 00	42 9
		1 50	42 6
		2 00	42 4
		5 00	42 0
	(90 MHz) 241 8±24 2		
0 340	153 4±15 3	0 14	38 8
		0 27	38 3
		0 38	37 7
		0 54	40 1
		1 08	39 1

then analysed using a two site model involving rate exchange between bound water (hydrated to the hydrophilic end groups of the phosphatidylcholine/cholesterol) and free water The number of water molecules bound (n_B) can be evaluated using the equation [9, 15]

$$\frac{1}{T_1} = \frac{P_A}{T_{1A}} + \frac{P_B}{T_{1B}} \tag{2}$$

where T_{1_A} is the spin-lattice relaxation time of free water (approx 2 0 s), P_A and P_B are the mol fraction of total water which is free and bound, respectively (P_A+P_B

TABLE II

WATER SELF DIFFUSION COEFFICIENTS (D_{water}) AT DIFFERENT COMPOSITIONS

φ_w	D_{water} 10^6 (cm^2 s^{-1})
0 146	2 48 ± 0 12
0 188	3 34 ± 0 17
0 227	4 21 ± 0 21
0 240	4 45 ± 0 22
0 255	4 52 ± 0 23
0 265	2 76 ± 0 14
0 285	1 35 ± 0 07
0 305	1 11 ± 0 06
0 340	1 40 ± 0 07

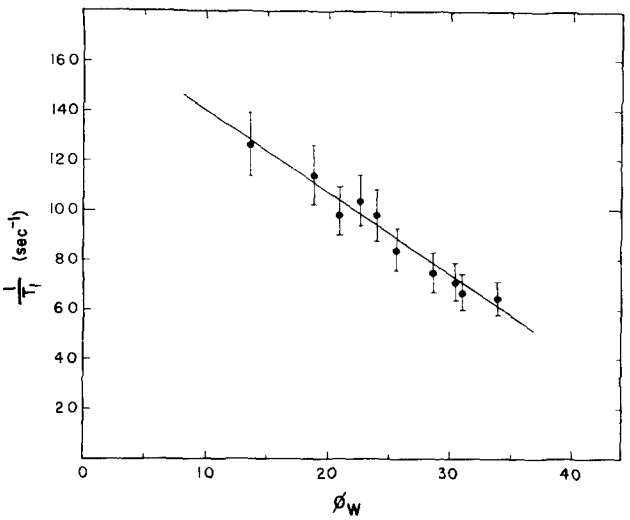

Fig 1 Proton spin-lattice relaxation rate ($1/T_1$) as a function of water content (φ_w) in lecithin/cholesterol/water lamellar phases

= 1), and T_{1_B} is the spin-lattice relaxation time of bound water (a measured value of 61 5 ms was used corresponding to φ_w = 0 105, a low water content where all the water is presumably bound) The values for P_B and n_B obtained from the linear regression analysis are shown in Table III and a consistent value for n_B of approx 12 is obtained after the stoichiometric limit is reached This value is in excellent agreement with estimates from an adsorption isotherm study by Jendrasiak and Hasty [16] and a recent theoretical estimate by Forslind and Kjellander [17]

The dependence of the self diffusion coefficient of water on φ_w is shown in Fig 2 The results reveal a dramatic decrease in the value of the self diffusion by a factor of four at $\varphi_w = 0\ 27 \pm 0\ 01$ Similar decreases in D have been observed in such systems by tracer methods at specific water contents [18] and have been interpreted in terms of a change in conformation of the lipid head group No definitive data, however, has been presented to illustrate the nature or existence of this conformation change It is

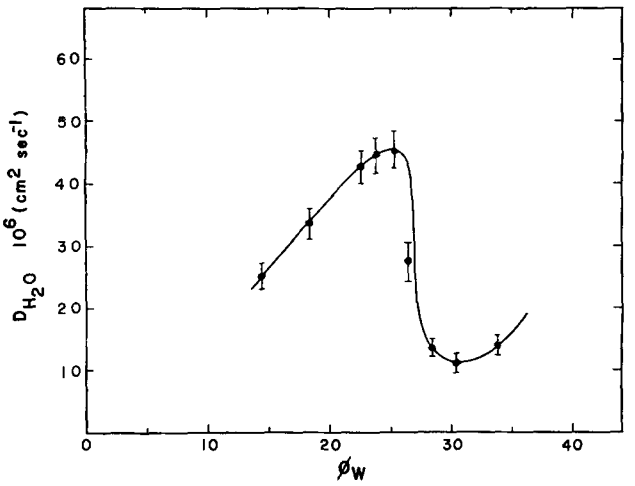

Fig 2 Water self diffusion coefficients as a function of water content (φ_w) in lecithin/cholesterol/water lamellar phases

TABLE III

STOICHIOMETRY OF WATER BINDING

P_B is mol fraction of water bound and n_B is number of water molecules bound

φ_w	P_B	n_B
0 14	0 77±0 11	8 0±0 9
0 16	0 73±0 10	8 9±0 9
0 19	0 67±0 09	10 0±0 9
0 22	0 61±0 09	10 9±0 9
0 24	0 57±0 08	11 4±0 9
0 26	0 52±0 07	11 7±0 8
0 28	0 48±0 07	11 9±0 8
0 30	0 44±0 06	12 0±0 7
0 32	0 40±0 05	11 9±0 6
0 34	0 36±0 05	11 8±0 6

readily apparent, however, from our analysis of the T_1 data that this decrease in the self diffusion occurs at the region of maximum hydration Hence, it is proposed that it is the binding of water molecules to the hydrophilic head groups which controls the overall water mobility in the phase This will be discussed in more detail

At a convenient $\pi/2$-π pulse interval of 40 ms, the echo attenuation was measured as a function of Δ (the field gradient pulse separation), between 10 and 70 ms for a sample with $\varphi_w = 0\ 34$ A continual decrease in echo amplitude with increasing Δ in accordance with Eqn 1 was observed over the entire range of Δ indicating that diffusion is not restricted on the NMR time scale [14] Eqn 1 is valid as long as $(2D\Delta)^{\frac{1}{2}} \ll X$, where X is the dimension characteristic of the region of space to which the spins are confined In this case the value of $(2D\Delta)^{\frac{1}{2}}$ for $\Delta = 70$ ms

gives 5 10^{-4} cm indicating that diffusion is unrestricted over this distance which corresponds to over 100 phosphatidylcholine molecules in the water layer

Structural studies of phosphatidylcholine/water systems were made by Lange and Gary Bobo [18] using polarizing microscopy and freeze fracture electron microscopy They observed perfectly lamellar regions at different water contents that were in the order of 10^{-3} cm in size, and with water channel widths in the order of 100 Å Therefore in the time frame of our diffusion measurements (10-70 ms) it seems likely that the water molecule could diffuse through more than one ordered region Thus, if measurements could be made in a much shorter time scale, we may have seen restricted diffusion Pearson et al [15], in porcine muscle, using the same method of measurement also observed non-restricted water diffusion

It can be seen from Table I that the $T_{2_{cp}}$ values show no dependence on the pulse separation τ, and are over a factor of ten shorter than the corresponding T_1 values They also reveal a progressive increase with water content These results can be interpreted in terms of a fast exchange process in accordance with theory [9 15] In the slow pulsing limit with fast exchange ($\tau_B \quad T_{2_B}$) the relaxation rate is given by

$$\frac{1}{T_2} = \frac{P_A}{T_{2_A}} + \frac{P_B}{T_{2_B}} \tag{3}$$

where A and B refer to the free and bound situations as before The examination of the Carr-Purcell data reveals a linear dependence of $1/T_2$ on P_B in accordance with this equation, except at very low water contents, where the nature of the phase is less defined From this a value for T_{2_B} of 13 ms is obtained A comparison of this value with that for T_{1_B} - 61 5 ms reveals that the bound water is in a rigid environment beyond the extreme narrowing limit defined by the T_1 minimum [19] A calculation for our results on the phosphatidylcholine/water system [4] shows similar behaviour and a T_{2_B} value of 9 ⊥ 2 ms Thus it is apparent that T_{1_B} (phosphatidylcholine/water) $> T_{1_B}$ (phosphatidylcholine/cholesterol/water) and T_{2_B} (phosphatidylcholine/water) $< T_{2_B}$ (phosphatidylcholine/cholesterol/water) showing that we are in the non-extreme narrowing limit, and that the water binding in the phosphatidylcholine/water case is stronger (the water having a longer molecular correlation time τ_c) than in the phosphatidylcholine/cholesterol/water case, in agreement with previous work [6] The reason we observe no dependence on pulse spacing is that the lifetime of the bound species is too short and our instrumental limits prevent us from pulsing fast enough This places a limit on the value of the lifetime of the bound water of $\tau_B \leqq 10^{-5}$ s

Measurements of T_1 at 60 and 90 MHz (Table I) show a frequency-dependent T_1 in accordance with the non-extreme narrowing situation for bound water Analysis by Eqn 2 yields values for T_{1_B} of 82 0 ms (60 MHz) and 119 2 ms (90 MHz) These values represent a verification of the fact that the bound water exists in a rigid environment and an estimate of the rotational correlation time of $\tau_c = 2 \quad 10^{-8}$ s is obtained [19] This value compares favorably with estimates for small molecules bound to macromolecules in ordered systems [3]

There is, of course, evidence that the bound water in these systems is preferentially oriented and undergoes anisotropic motion [3] One component of this motion having a motional frequency less than the observing frequency thus leading to $T_1 > T_2$ The above correlation time then represents an approximation to the exact calcula-

tion based on a distribution of correlation times for the various components of motion.

DISCUSSION

We conclude from the analysis of our data that the water present in the lamellar phase of phosphatidylcholine/cholesterol/water is bound to the extent of 12±1 water molecules per phosphatidylcholine/cholesterol unit and in the region of this hydration a dramatic decrease in water mobility occurs More detailed examination of our results (Table III) reveals a slight peaking of the calculated value of n_B indicative of a maximum stoichiometry at this composition Presumably the phase stability is greatest here and decreases slightly on the addition of further water

In terms of the validity of the two site (bound-free) model used in this analysis we will explore the other possibilities If the water existing in the lamellar phase is of one structural type then our T_1 data could be interpreted as simply an increase in water mobility with increasing φ_w This leads to a progressive increase in T_1 in accordance with the extreme narrowing limit It is difficult to rationalise this explanation, however, with the large difference observed for the values of $T_{2_{cp}}$ compared to T_1 [19] This fact, and the higher frequency T_1 measurements which verify that the bound water is in the non-extreme narrowing situation ($\tau_c = 2\ \ 10^{-8}$ s) where T_1 must decrease with increasing mobility, indicate that T_1 would decrease with increasing water content if all the water was bound This, of course, is in direct opposition to the experimental observations Moreover, the decrease in self diffusion coefficient from $4\ 5\ \ 10^{-6}$ to $1\ 1\ \ 10^{-6}$ cm^2 s^{-1} occurring at $\varphi_w = 0\ 27$ would lead to a change in the intermolecular contribution to T_1 by approximately a factor of 4 Our T_1 results show no corresponding change with water content at $\varphi_w = 0\ 27$ of sufficient magnitude We must conclude then that it is extremely unlikely that our experiments can be interpreted in terms of a single structural species for water There is, however, a distinct possibility that the hydration of phospholipids gives rise to more than one hydration shell and that a number of layers or structural types of bound water are present [3, 16, 20] The bound water described here is characterised by a single overall mobility but may well not be of a single structural type

We can compare our results with the X-ray diffraction data [21] on the phosphatidylcholine/cholesterol/water lamellar phase which indicate that the cross sectional area available to one hydrophilic head in the phosphatidylcholine/cholesterol unit is 76 Å^2 Assuming a density for the choline phosphate group of 1 32 g cm^{-3} [21] and an extended length of 10 Å the cross-sectional area of the group is 24 Å^2 If the volume of the water molecule is 30 Å^3 and the thickness of the aqueous layer is 17 Å (assuming a water density close to unity) it is possible to fit between 13 and 14 water molecules in the free volume around each choline phosphate group This number compares very well with our value for the hydration number of 12

Previously determined decreases in self diffusion coefficients at specific water contents in lamellar phases have been ascribed to the existence of a conformational change of the choline phosphate group [18] However, in view of the fact that in the phosphatidylcholine/cholesterol/water system the decrease in water self diffusion coefficient (D) occurs at the region of maximum hydration it seems likely that in this system, at least, the mobility of the water may also be related to its structural nature

in the phase When the water is bound and the structure is most stable (at φ_w = 0 27–0 33 according to our analysis) the water is relatively immobile Above and below this composition D will increase linearly with water content Below φ_w = 0 27 it is the relative instability of the phase which contributes to the relatively high mobility, above φ_w = 0 27 free water exists and D should once again increase This behaviour could have a considerable effect on the permeability through aqueous channels in bilayer structures especially for ionic species which also interact with water The availability or non-availability of free water for ionic hydration could selectively control ionic permeability based on the hydration number of the ion The ions of primary interest in such bilayer systems are, of course, Na^+ and K^+ due to their significance in the membrane potential Na^+ exists as a hexa-aquo ion and K^+ shows little evidence of hydration We would thus expect K^+ permeability to be insensitive to lipid hydration whereas the availability of free water for $Na(H_2O)_6^+$ formation could selectively regulate the permeability of Na^+ relative to K^+ The situation is obviously more complex than this simple picture and depends on the relative thermodynamic stability of all possible species present but a detailed investigation of ion mobility and its relationship to phospholipid hydration could greatly improve our understanding of membrane permeability

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