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NUCLEAR MAGNETIC RESONANCE STUDIES OF THE INTERACTION OF WATER WITH THE POLAR REGION OF PHOSPHATIDYLCHOLINE MICELLES IN BENZENE*

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SUMMARY

1. Both the hydrocarbon chains and the trimethylammonium groups of phosphatidylcholine micelles in benzene have more motional freedom than can be explained by micellar rotation.

2. Addition of water, which is solubilized within the micelles, slows the motion of the hydrocarbon chains but increases the motion of the trimethylammonium groups.

3. The hydrocarbon and the trimethylammonium proton linewidths have a weak temperature dependence, suggesting that the motions of these groups is not an activated process, but may be described by a local viscosity.

4. ^2H_2O is much less efficient than H_2O in influencing the trimethylammonium proton linewidth, suggesting that the interaction between the water and the micelles is dominated by hydrogen bonding rather than by electrostatic interactions.

INTRODUCTION

Phospholipids and the polymolecular structures they form under various conditions have received much attention over the years, especially recently[1-4]. A great deal of the interest, undoubtedly, stems from the belief that a study of phospholipid structures will cast light upon the nature of cell membranes.

The study we report here is concerned primarily with the nature of the interaction of water with the polar part of phosphatidylcholine structures. We have chosen micelles in benzene to study because they are equilibrium structures and because the amount of water and of other additives in the system may be controlled. Phosphatidylcholine micelles in benzene are large enough that the information one gains from them may be presumed to be relevant to the larger structures one finds in biological membranes or in phospholipid dispersions in water. Most of our information on phospholipid micelles in solvents of low dielectric constant comes from the work of ELWORTHY[5-7].

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Only a few NMR studies are available in which phospholipid–water interactions were studied explicitly. CHAPMAN *et al.*[8–10] studied dry, synthetic lecithin and lecithin structures containing various amounts of water. Their work provides information about the gross structure of the sample and the effects they observe are dominated by the bulk viscosity of the system. However, the work did show that the polymethylene chains of the hydrocarbon region show considerable flexibility or fluidity at temperatures comparable to the ones encountered in this work even in the solid state.

CEREBON[11] studied a sonicated aqueous dispersion of ovolecithin, a mixture of lipids which the author reported to consist of 78 % phosphatidylcholine, 18 % phosphatidylethanolamine, and 4 % lyso-derivatives. The author estimated that 192 molecules of water per lecithin molecule were immobilized by the phospholipid and that the effect was partially reversed by $CaCl_2$. HENRIKSON[12], in a more recent article, studied the interaction of water with lecithin micelles in carbon tetrachloride.

EXPERIMENTAL

The egg yolk phosphatidylcholine used in this study was obtained from two sources. The work early in the course of the investigation was done on phosphatidylcholine obtained from General Biochemical Company and designated as Lot GB-1. The chromatographically pure, dry lipid was supplied as individual 100-mg aliquots which were sealed under dry N_2. The aliquots were stored below 0° in a desiccator.

The highest purity phosphatidylcholine commercially available was obtained from Applied Science Laboratories. The lipid was received in benzene solution in sealed ampoules under vacuum (50 mg/ml ampoule). The lipid was shipped under dry ice refrigeration and was refrigerated upon arrival. The samples were stored below −10° and were only allowed to thaw immediately before use. The lots of phosphatidylcholine from this source used in this work were designated 0474 and 0684.

Thin-layer chromatography on inactivated 100-μm silica gel plates (Eastman) was used to demonstrate the chromatographic purity of all phosphatidylcholine utilized. The developing solvent was chloroform–methanol–water (65:25:4, by vol.) and the spots were visualized with iodine vapor.

All organic solvents used in the NMR samples were spectral grade and redistilled over CaH_2.

$[^2H_4]$ Methanol was obtained from Diaprep, and was of 99.5 % purity. The ^2H_2O was from Mallinckrodt and was 99.8 % isotopically pure.

The water used in the hydration studies was double distilled in an all glass system out of an alkaline permanganate solution.

METHODS

Sample preparation

All samples contained 50 mg of phosphatidylcholine in 0.6 ml of benzene.

The lipid sample was transferred from the freshly opened ampoule to a preweighed precision NMR tube, frozen, and placed on a vacuum rack. After any necessary outgassing by the freeze-thaw technique, the frozen sample was freeze-dried overnight under a vacuum of 10^{-6} torr. The sample, which was maintained at 0°, was allowed to come to room temperature the last few hours of the pumping procedure.

No weight loss was noted upon holding a sample of lecithin dried according to the procedure described above for several hours at 95° under high vacuum. This indicates that the results reported below apply to the anhydrous species and not to the monohydrate.

Line width measurements

Line width measurements were performed on a Varian A-60 or XL-100 spectrometer. The normal probe temperature of the A-60 was 40.0 ± 1.0° and that of the XL-100 was 30.0 ± 1.0°.

Temperature dependence studies were performed on the A-60. In this case, the sample was allowed to equilibrate for periods of up to one hour after temperature regulation was begun. Temperature measurements were made by means of inserting a methanol sample before and after each run. The temperature usually agreed within ± 2°.

To obtain a line width, the 500 Hz full scale scan width spectrum was obtained for the sample and at least one 50 Hz full scale scan width spectrum of the trimethylamine peak was superimposed. The line width was measured at half height on the expanded spectrum or spectra. In the case of multiple scans, the average line width was used.

Hydration studies

In order to study the effect of added water a micro syringe (Hamilton Company) of the appropriate size was used to add measured amounts of water to the sample tube. The tube was shaken vigorously and placed in an ultrasonic bath (SonBlaster, Narda, Ultrasonic Corporation) for approx. 30 sec. The sample was then replaced in the spectrometer probe and allowed to equilibrate before its spectrum was recorded. The same procedure was used when solvents other than water were added.

RESULTS

Typical NMR spectra for a sample of phosphatidylcholine, unhydrated and after the addition of 30 % (v/w) water are shown in Fig. 1. Assignments of peak identities were made using the chemical shift value of CHAPMAN AND MORRISON[17]. In general, those features due to protons associated with the polar portion of the molecule show a decrease of their linewidths as a result of hydration and the peaks attributed to those protons in the fatty acid chains are broadened by the addition of water.

The protons most affected by hydration are those of the trimethylammonium group of the choline. A plot of the line width of this peak as a function of the amount of added water shows three discrete linear regions. An example of such a plot is shown in Fig. 2.

The first region, and that of the greatest slope, is attributed to the existence of a first hydration shell of two to three molecules of water per molecule of lecithin; the second region of more gentle slope to a second shell of four to six molecules per lecithin; and finally, a nearly constant region of simple bulk filling of the interior of the micelle. We discuss these hypotheses in more detail later.

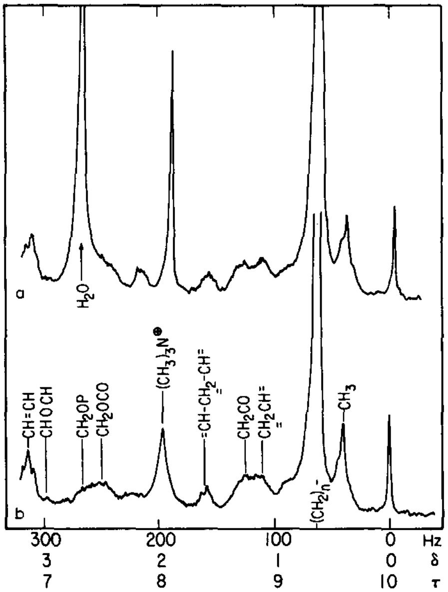


Fig. 1. NMR spectra of (a) dry phosphatidylcholine in benzene and (b) the same sample after addition of 27.5 % (v/w) H_2O.

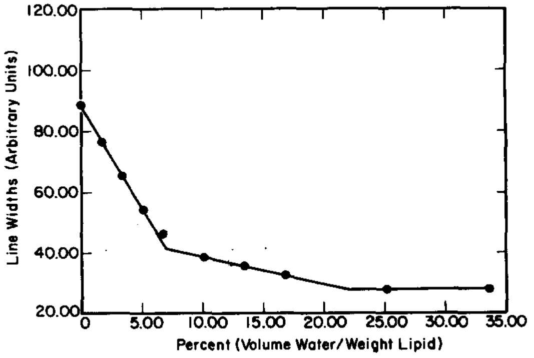


Fig. 2. Dependence of the trimethylammonium proton linewidth on amount of water added to the system.

The linewidth *vs.* percent hydration data were treated by the use of a program which fitted three least square lines through all sequential groupings of data points and selected as the best fit that combination with the least total deviation. The intersections were calculated and a plot prepared. Points on or near the first intersection or break were plotted but not included in the least squares calculations. These results are presented in Table I. The dependence of the trimethylammonium proton linewidth on water added was always as described above, but quantitative variations in the layer structure were evident from lot to lot of phospholipid used. All of the linewidth *vs.* percent hydration data fit well to three linear segments and the limiting widths are similar but the positions of the breaks, which are interpreted as corresponding to the completion of a layer, vary somewhat from lot to lot. The largest such variation by far occurred in the second layer. Even though the highest purity lipid commercially available was used, and thin layer chromatography confirmed the chromatographic homogeneity of each lot, these variations were present. Thus, comparison of values for molecules of water in a layer as a function of some additive must be made only within a given lot. Comparisons within a lot are valid since the number of molecules per layer is reproducible within a lot.

TABLE I

VALUES FOR BREAKS AND INITIAL LINEWIDTHS FOR TRIMETHYLAMINE RESONANCE IN THE HYDRATION OF PHOSPHATIDYLCHOLINE

Numbers in parentheses refer to molecules of H_2O per molecule of lecithin. All measurements on Lot 0684 performed on XL-100 at 30°. All other data were taken on the A-60 at 40.0 ± 1.0°.

Lot	*Sample*	*Breaks in slope (%, v/w)*		*$\lambda°$ (Hz)*
		1st break	*2nd break*	
GB-1	II-4	5.69	16.1	8.74
	III-4	5.95	20.0	6.47
	Av.	5.82 (2.5)	18.1 (7.9)	
0474	V-16	6.99	20.1	8.96
	VI-2	6.61	20.1	8.27
	VI-5	6.55	22.4	8.42
	Av.	6.72 (2.9)	20.9 (9.1)	8.55
0684	XI-11	5.17 (2.3)	14.7 (6.4)	8.96

In two samples the micelles were "hydrated" with ^2H_2O. The results are presented in Table II.

The results for the hydration with H_2O are included for easy comparison.

TABLE II

HYDRATION OF PHOSPHATIDYLCHOLINE WITH ^2H_2O AND H_2O

All samples were from Lot 0684. PC, phosphatidylcholine.

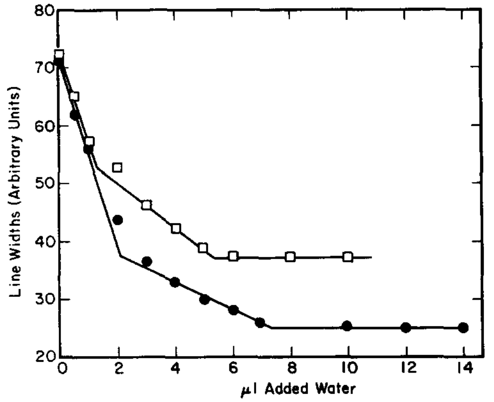
Sample	*Breaks in slope (%, v/w), mole ^2H_2O/mole PC, in parentheses*	
	1st break	*2nd break*
XI-11*	5.17 (2.3)	14.7 (6.4)
XI-17	2.64	10.7
XI-20	3.10	10.4
Av.	2.87 (1.1)	10.5 (4.1)

* Hydrated with H_2O.

Both the first and second layers of the samples hydrated with ^2H_2O are decreased by about 1 molecule of ^2H_2O per molecule of lecithin as compared with the H_2O results.

In addition, the limiting linewidth, that is the linewidth after all hydration-induced narrowing has occurred, is markedly greater than the same sample after H_2O addition. The linewidth behavior is presented in Fig. 3.

Linewidth measurements of the trimethylammonium and polymethylene peaks were made at three temperatures between 10 and 40°. Arrhenius plots of linewidth *vs.* $1/T$ yield activation energies of less than 1 kcal as Fig. 4 shows. Within the limits of experimental error, this energy is the same in the dry micelles and in the hydrated micelles.



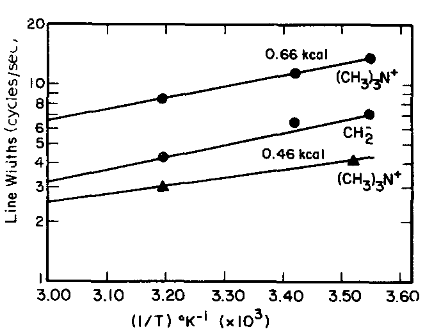


Fig. 3. A comparison of the effect of hydration of the same sample of phosphatidylcholine with H_2O (□) and with ^2H_2O (●).

Fis. 4. The temperature dependence of the linewidths of the polymethylene proton resonance and of the trimethylammonium proton resonance in dry micelles (●), and of the trimethylammonium proton resonance in fully hydrated micelles (▲).

DISCUSSION

We wish to understand the interaction of water with the polar portions of a phosphatidylcholine micelle. The binding displays itself in NMR most directly through changes in the width of the $(CH_3)_3N^+$ resonance, as an examination of Fig. 2 shows. In order to understand the changes in the $(CH_3)_3N^+$ resonance, it is first necessary to examine the mechanisms responsible for the width of the various proton resonances.

The interactions which are most likely to be responsible for the width of the proton resonances are dipole–dipole coupling with the magnetic moments of other nuclei and diamagnetic anisotropy. The latter mode of relaxation is characterized by $1/T_2$ being proportional to H_0^2. Such a field dependence was not observed as can be seen in Table III. None of the other peaks showed any disproportionate broadening either. Therefore, diamagnetic anisotropy is unimportant as a relaxation mechanism. The mechanism for the broadening of the trimethylamine resonance is, thus, probably dipolar coupling with magnetic dipoles in the spin system's vicinity.

In the dry, unhydrated, micelle the nearby dipoles come not only from phosphatidylcholine molecules on the same side of the micelle but also from some molecules on the opposite wall. In the unhydrated state, the micelle is believed to be a flattened sphere, which allows rather close approach of one side to the other[6].

TABLE III

LINEWIDTH OF $(CH_3)_3N^+$ RESONANCE AS A FUNCTION OF H_0

	60 MHz	*100 MHz*
Hydrated	3.0	3.4
Dry	10.1	9.5

Having established the mechanism responsible for relaxation of the various protons of the micelle, we may next inquire into the sources of motional averaging available to a proton. The first type of motion to be considered is the rotation of the micelle as a rigid sphere.

The rotational correlation time for the micelle, τ_m, may be estimated by using the calculated size of the micelle in benzene at 40° reported by ELWORTHY, *et al.*[8-10] and the Debye–Einstein relation:

$$\tau_m = \frac{4\pi\eta a^3}{3kT}$$

The viscosity of benzene at 40° is $\eta = 0.503$ cP, and a is the radius, 50 Å; thus τ_m is approx. $6 \cdot 10^{-8}$ sec. This is slow compared to the tumbling motion of a single small molecule.

The width of a resonance due solely to the motion of the micelle may be estimated from the well-known approximation[13]

$$\delta\omega = \overline{\Delta\omega^2}\tau_m$$

where $\delta\omega$ is the linewidth and $\overline{\Delta\omega^2}$ is the second moment of the resonance in question. This estimate is somewhat crude, especially since the correlation time is not quite in the extreme narrowing region but will serve to yield an order-of-magnitude result. The $\overline{\Delta\omega^2}$ of very similar phospholipids, synthetic diacyl phosphatidylethanolamines, and total phospholipid extract from the white matter of human brain have been measured[11]. From these data we may estimate the $\overline{\Delta\omega^2}$ to be approximately $6 \cdot 10^9$ sec^{-2}. This yields an estimate of the linewidth of a proton due solely to micelle motion of 69 Hz. This is several times greater than the observed linewidth of any proton in the micellar system, so all parts of the micelle must be undergoing some additional motion. The measured values in this work are 4.5 Hz for the polymethylene protons and 8.6 Hz for the trimethylammonium protons in the unhydrated micelle. As water is added, the polymethylene peak broadens and the trimethylammonium peak narrows to values of about 7.5 and 3 Hz respectively. The most interesting part of these data is the fact that hydration frees the trimethylammonium group but inhibits the motion of the chains.

Additional information may be obtained from the temperature dependence of the linewidths of trimethylamine and polymethylene peaks. The small dependence of linewidth on temperature and the fact that there is at most only a small difference between the trimethylamine and polymethylene temperature dependence implies strongly that the motional process involved in the narrowing of the trimethylamine resonance is not an activated one. In other words, there does not appear to be a bound and a free form of the choline separated by a large energy. The results suggest that one may view the motion of the trimethylammonium group as a viscous motion.

Since the observed proton linewidths are so much less than the widths expected from motion of the micelle as a whole, the linewidths must be dominated by the motions of the parts with respect to the micelle. The temperature dependence in Figure 4 must represent, then, the temperature dependence of the local viscosity affecting the various groups.

There are three pieces of information which bear on the time dependence of the interaction between water and lecithin. One is the dependence of trimethylammonium proton linewidth on extent of hydration, shown in Fig. 2. The characteristic features of Fig. 2 are the linear dependence of linewidth on extent of hydration and the clean, sharp breaks in slope. The H_2O proton linewidth, shown in Fig. 5, has the same features. Finally, the trimethylammonium proton line shape remains the same as the line narrows. In particular, there is no evidence for a narrow component and a broad component, which would suggest the presence of two kinds of trimethylammonium group of differing amounts of hydration. It is also true that the H_2O proton linewidth bespeaks a single population of H_2O protons in those regions where the line shape is well defined. At low concentrations of H_2O, however, the H_2O proton resonance is too broad and weak to permit determination of the line shape.

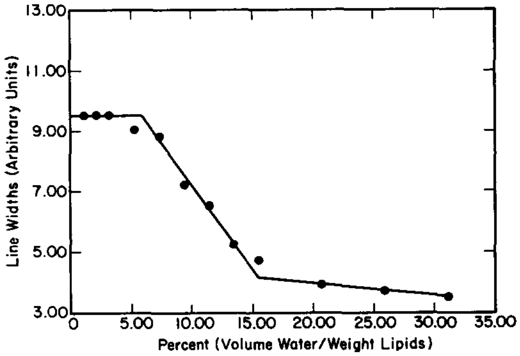


Fig. 5. Dependence of the water linewidth in phosphatidylcholine micelles on the amount of water in the system.

The data, thus, indicate that there is only one population of water and one population of trimethylammonium groups at each concentration of water in the system. This means that all the micelles have the same concentration of water in them, averaged over 10^{-2} sec or so, and that, within a micelle, the water moves rapidly over the various parts of the micelle, and also from one layer of water to another. The uniform distribution of water over the micelles is probably due to the sonication of the system after each addition of water and to the temperature cycling between the room and the NMR probe, and so it does not provide information about the rate of exchange of contents among micelles. If one takes the attitude that the narrowing of the trimethylammonium proton resonance in the first region, of large slope, is the result of a specific interaction between a water molecule and a trimethylammonium group, one concludes that a water molecule must move relatively rapidly from one phosphatidylcholine to another to produce the result, shown in Fig. 2. It is possible, however, that the effect of water is to produce some kind of cooperative change of packing of the micelle rather than a localized interaction, which would vitiate the previous conclusion.

It is apparent that the first layer of water is much more strongly adsorbed than the second, a conclusion based on the breadth of the H_2O proton resonance at low concentrations of H_2O and its sharpening as the second layer forms.

The most obvious explanation of the effect of water in the polar regions would be that it decreases the electrostatic interactions through dielectric shielding, decreasing the binding between a trimethylammonium group and a phosphate. The results using ^2H_2O argue strongly against this explanation. Not only does the ^2H_2O appear to have a different layer structure from that of H_2O, but the overall narrowing of the $(CH_3)_3N^+$ resonance caused by ^2H_2O is substantially less than that caused by H_2O. It appears that this must be ascribed to differences in hydrogen bonding between ^2H_2O and H_2O. There is some evidence for conformational changes of proteins in ^2H_2O due to the differences between hydrogen bonding of H and ^2H (ref. 15) and the temperature of the thermal transition in phospholipid structures in water shows an isotope effect (W. HUBBELL, private communication). It follows that the most significant interaction of water with the hydrophilic part of the micelle is its hydrogen bonding with the phosphate ester. A second conclusion, somewhat less precise, is that the changes in the structure of the polar part of the micelle, causing an increase in the mobility of the choline portion of a lecithin molecule, must be cooperative changes which affect substantially the whole micelle. Otherwise, it is hard to see how a small change in hydrogen bonding could be propagated into a substantial change in the motion of the trimethylammonium group.

The layer hypothesis, which our discussion implies, best explains the linearity of the line segments and is wholly consistent with previously published data. The values we obtain are remarkably close to values of ELWORTHY[16] of 6.12 % and 14 % for the hydration of solid lecithin.

The second break, which represents the filled second layer, has an average value of 20.1 % which is the equivalent of approx. 9 molecules of water per molecule of phosphatidylcholine. This agrees well with the value for the hydration shell of "about 10" reported by CHAPMAN *et al.*[14] from differential scanning calorimetry.

HENRIKSON[12], in a recently reported work, studied the hydration of lecithin micelles in carbon tetrachloride. Since the dielectric constant of the two solvents, benzene and carbon tetrachloride, are nearly identical, one would expect the micellar size to be the same. She did not report the same shape curve for the dependence of the water linewidth on the concentration of water, however. This is due to two factors: Firstly, she did not report the linewidth with less than 7.7 % (w/w) present. Thus, she did not observe the very distinct break corresponding to the beginning of the second layer of hydration. Secondly, the method she used of preparing the large number of samples with varying amounts of water in each is more likely to cause curvature in the neighborhood of the second break due to its demonstrated variability from sample to sample. In any event, the second break is not as pronounced as the first. Henrikson's results are not inconsistent with ours, although they are substantially less complete.

Henrikson has proposed changes in the bulk viscosity as an explanation for the initial narrowing observed. However, the bulk viscosity of lecithin micelle solutions in benzene initially increases up to 5.5 % hydration[8]. This increase would be expected to cause a broadening and not the narrowing observed in this region. Also, a change of linewidth due to changes in the bulk viscosity would be expected to affect all portions of the lecithin molecules in the same way. In fact, as the trimethylamine peak and the other peaks associated with the polar head are narrowing, there is a concomitant trend of broadening of the polymethylene peak as can be seen in Fig. 6. The other peaks associated with the hydrophobic region are also broadened in a like manner. The changes in the linewidths of the two regions are clearly coupled in some way, as may be seen from the fact that the slope discontinuities in each occur at the same water concentrations. However, the two regions are affected in different ways.

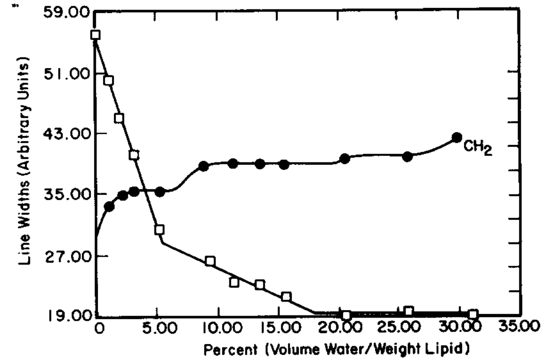


Fig. 6. A comparison of the hydrocarbon proton linewidth and of the trimethylammonium proton linewidth in their dependence on water concentration.

Our data show that water affects the mobility of the polar part of the phosphatidylcholine micelle through hydrogen bonding. The data are explained most readily by postulating a cooperative change in the structure of the micelle due to addition of water, which allows the trimethylammonium groups to move more freely, but which inhibits the motion of the hydrocarbon part of the micelle. We have termed this change, rather loosely, a change in packing.

The data from the various protons of phosphatidylcholine do not reflect any specific bonding, either zwitterionic or iondipole (to the water). On the other hand, the behavior of the water resonance shows that water is bound in the system, in the sense of having its motional freedom restricted. The binding of water, which occurs in layers, is obviously coupled to the changes in structure of the micelle, a fact which is reflected in the breaks or jumps in phosphatidylcholine proton linewidths as layers of water are completed.

ACKNOWLEDGMENTS

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