Physical Studies of Egg Phosphatidylcholine in Diethyl Ether–Water Solutions†

Pak H. Poon and Michael A. Wells*

ABSTRACT: The binding of water to phosphatidylcholine micelles in diethyl ether has been measured by equilibrium dialysis using acetylated dialysis tubing. As the free water concentration of the solvent increases to about 5 mg/ml, the number of water molecules bound per molecule of lipid increases to about 6–7. This water binding appears to follow a simple association equilibrium. As the free water concentration is increased toward the saturation level of the solvent (~9 mg/ml), there is a marked increase in the number of water molecules bound, approaching a limit of approximately 60 molecules/molecule of lipid. This second phase appears to involve cooperative binding of water. Further support for the interaction of water and phosphatidylcholine was derived from density and refractive index increment measurements. When the results of measurements made at constant chemical potential of the solvent are compared to those obtained at constant composition of the solvent, discrepancies are observed suggesting interaction. The extent of interaction determined from these measurements does not agree with direct measurements by equilibrium dialysis. It is proposed that these differences arise from differences in the properties of water bound to the micelles, as compared to water in the solvent. Analysis of the molecular weight distributions of phosphatidylcholine micelles by sedimentation equilibrium at 25.0° gave the following results: (1) the association of phosphatidylcholine is definite, (2) at each water concentration examined, there is a unique distribution, (3) as the water concentration is increased, the size of the aggregate increases markedly. A model is proposed which assumes that definite aggregation takes place and that there is a continuous phase change with increasing water concentration. Theoretical calculations gave satisfactory agreement with experimental data. The theory allows determination of the aggregation number and the equilibrium constant for micelle formation. In turn these parameters can be used to calculate the free energy of micelle formation and the critical micelle concentration. The values of the latter two parameters depend on the water concentration. Viscosity measurements failed to detect any substantial change in micelle shape with increasing hydration, although there may be small changes. Sedimentation velocity experiments were also carried out. The shape of the boundary and the apparent sedimentation coefficient depend on the water content of the solvent. Near the water saturation level of the solvent there is an abrupt and marked increase in s_c^{25}. On the basis of these data it is proposed that three distinct hydration states exist in ethereal solutions of phosphatidylcholine, the first, which requires about 7 water molecules, a second which requires an additional 25–30 water molecules, and the third, which requires a further 25–30. Analysis of the activity of *Crotalus adamanteus* phospholipase A_2 shows that the enzyme is active only in the second hydration state.

It has been known for several years that snake venom phospholipase A_2 is extremely active in ethereal solutions of phosphatidylcholine (Hanahan, 1952; Long and Penny, 1957). A recent report from this laboratory has characterized the kinetics of the reaction in ethereal solution (Misiorowski and Wells, 1974). In this latter study it was shown that a complex relationship exists between enzymatic activity and the concentration of water and divalent cations present in the ethereal solution of substrate. Based on these studies, we proposed the existence of several distinct substrate species in solution, which had different affinities for the enzyme.

The present study was undertaken in order to gain some insight into the physical properties of phosphatidylcholine in ethereal solutions, and to investigate the effects of water on these physical properties. In this article we shall show that reasonably rigorous treatments of the three-component system, phosphatidylcholine–water–diethyl ether, are possible, and that, within a limited range of solute concentrations, the system can be completely defined in terms of the interactions between phosphatidylcholine and water and the resultant size and shape of the hydrated phosphatidylcholine micelles. We shall also show that one can correlate the activity of phospholipase A_2 with certain parameters related to the physical properties of the substrate micelles.

Materials and Methods

Phosphatidylcholine was purified from hen's egg yolk by the method of Wells and Hanahan (1969). The purified material showed a single spot on thin layers of silica gel G in the solvent system chloroform–methanol–water (95:35:6 (v/v) and gave as typical analyses 4.05% P; 1.82% N; $[\alpha]^{25}_{546}$ +6.4° (*c* 5.0, chloroform–methanol 1:1 (v/v)). The purified material was stored in methanol at −20°.

Preparation of Acetylated Dialysis Tubing. Dialysis tubing, ¾ in. diameter, was obtained from Van Waters and Rogers. The stated average pore value was 24 Å. Several batches of tubing were used with identical results. The tubing was boiled for 1 hr in 0.1 M EDTA (pH 7.0). After cooling, the tubing was washed thoroughly with distilled water and blotted dry. The partially dry tubing was washed with pyridine by soaking for 1 hr. This was repeated two times. The tubing was then suspended in a solution of acetic

† From the Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85724. *Received June 6, 1974.* Supported by a grant from the National Science Foundation (GB 35527).

anhydride in pyridine (25% by volume) and heated at 65° for 3 hr with exclusion of moisture. After cooling, the acetic anhydride–pyridine solution was removed by decanting. The tubing was washed three times with 0.01 M acetic acid, and then allowed to stand in 0.01 M acetic acid overnight. The acetylated tubing was then washed several times with distilled water and stored in distilled water in the cold.

Prior to a dialysis experiment, the acetylated tubing was washed several times with methanol to remove water. The bottom of the tubing was tied, a solution of phosphatidylcholine in ether placed in the bag, and the top of the bag tied. The dialysis bag was then rinsed with several portions of the dialysis solvent. Dialysis was carried out with a 50-fold volume of solvent in a glass-stoppered erlenmeyer flask. The solvent was changed twice at a few hours interval, and the final dialysis was allowed to proceed for 20 hr. At the end of the dialysis period, the bag was placed in a small beaker of diffusate and a small hole punched in the floating bag, and the phosphatidylcholine solution was quickly removed.

^3H_2O (Amersham-Searle) was used to make up the ether–water solvent mixture. After dialysis equilibrium, water binding was estimated from the ^3H_2O content. Aliquots were counted in aquasol (New England Nuclear) using a Beckman scintillation counter. The reproducibility of the water content of the solutions was estimated to ±0.1 mg/ml.

The phosphatidylcholine concentration was determined from the weight of an aliquot before and after drying *in vacuo* over P_2O_5 at room temperature. The volume of the aliquot was calculated from interpolated densities. The accuracy of the phosphatidylcholine concentration measurements was estimated to be ±0.1 mg/ml. The ^3H_2O content of the dried samples was negligible and thus the weight refers to the weight of dry phosphatidylcholine.

Pycnometry and Refractometry. Density measurements were performed in a 10-ml specific gravity pycnometer tube (Ace Glass, Inc., Vineland, N.J.). The capillary was calibrated using quartz-distilled water. Volume measurements were performed in a water bath maintained at 25.00 ± 0.05°. The pycnometers were weighed on a single pan Mettler balance (accuracy ±0.1 mg) and weights were corrected to those *in vacuo*. For each solution, a minimum of two determinations were made at different markings of the pycnometer. The reproducibility of the densities was ±0.00002 g/ml.

Refractive index increments were measured with a Brice-Phoenix differential refractometer (Virtis Co., Gardiner, N.Y.) equipped with a Teflon-stoppered cell. Temperature was controlled by circulating water from a bath maintained at 25.00 ± 0.05° through the instrument. All measurements were made at 546 nm. The reproducibility of Δn was $\pm 0.005 \times 10^{-3}$.

Ultracentrifugations were performed with a Beckman Model E using the Schlieren optical system and monochromatic light at 546 nm. Aluminum centerpieces equipped with polyethylene gaskets were essential for reproducible results. Results obtained using epon centerpieces were very erratic. The use of sapphire windows and maximum compression of the centerpiece (180 in.-lb) eliminated leaks. A two-hole titanium rotor (AN-H) was used, and in most experiments four different solutions were examined simultaneously by using two double sector cells, one of which had a 1°-negative sapphire window. In such cases, the base line was determined from photographs taken immediately after the rotor reached speed. In low-speed sedimentation equilibrium experiments these base lines were not different than those obtained when the diffusate was used as a reference in the double sector cell. Sedimentation equilibrium was usually achieved within 20 hr at 25.0°; beyond this time the schlieren patterns did not change.

Sedimentation velocity experiments were carried out at 40,000 rpm at 25.0° using an initial lipid concentration of 10 mg/ml. Sedimentation coefficients are reported as s_c^{25}, which are the observed values under the experimental conditions.

Viscosity. Viscosity measurements were made in a Cannon-Ubbelohde viscometer attached to an auto-viscometer (Hewlett-Packard). The outflow time of the solvent was approximately 100 sec. Relative viscosities, calculated from the outflow times and densities of the phosphatidylcholine solutions and the diffusate, were reproducible to ±0.1%. Specific viscosity (η_{sp}) and reduced viscosity ($\eta_{sp/c}$) were calculated in the usual manner using the dry weight of phosphatidylcholine.

Enzyme Assays. Phospholipase A_2 assays were carried out essentially as described by Misiorowski and Wells (1974), with the following modifications. Phosphatidylcholine samples (10 mg/ml) were incubated overnight with different amounts of 1 mM $CaCl_2$. Activity was measured after adding 0.5 μl of enzyme (0.1 μg) to 2 ml of the lipid solution.

Results

Water Binding Studies. Binding of water to phosphatidylcholine was studied by equilibrium dialysis using acetylated dialysis tubing. The tubing was soaked in methanol in order to facilitate tying of the knots; however, the final concentration of methanol at equilibrium was less than 0.01%. During a 24-hr dialysis experiment, approximately 5% of the phosphatidylcholine escaped from the bag; however, this did not significantly lower the phosphatidylcholine concentration within the bag. We consider that the small amount of phosphatidylcholine lost from the bag is not important, since the amount of water bound to the phosphatidylcholine was the same at 24 or 48 hr, even though more lipid was lost in the longer experiment. A water-binding parameter, δ, was defined as the grams of water inside the dialysis bag, in excess of that in the equilibrating fluid, per gram of phosphatidylcholine inside the bag. The results of these experiments are present in Table I. For each water concentration, experiments were performed with phosphatidylcholine concentrations ranging from 5 to 30 mg/ml. No significant dependence of δ on the phosphatidylcholine concentration was observed.

A Scatchard plot of the data in Table I did not give a straight line and suggested that cooperative binding of water might be taking place, especially at the higher water concentrations. Cooperative phenomena can be treated by eq 1 (Tanford, 1961), where δ_{max} refers to maximum

$$\delta/(\delta_{max} - \delta) = k_{int}e^{-\phi(\delta)}c \qquad (1)$$

amount of water bound (1.096 g/g), δ refers to the amount bound at any water concentration c, k_{int} is an intrinsic association constant, and $\phi(\delta)$ is an arbitrary function to account for cooperative binding. In logarithmic form eq 1 becomes

$$\log [\delta/(\delta_{max} - \delta)] - \log c = \log k_{int} - 0.434\phi(\delta) \qquad (2)$$

TABLE I: Binding of Water to Phosphatidylcholine in Diethyl Ether.[a]

Free Water (mg/ml)	δ	Log $[\delta/(\delta_m - \delta)] - \log c_w$[b]
2.0	0.057 ± 0.003[c] (8)[d]	−0.31
2.5	0.071 ± 0.005 (4)	−0.30
3.0	0.072 ± 0.006 (10)	−0.38
4.0	0.101 ± 0.004 (8)	−0.34
5.0	0.126 ± 0.008 (8)	−0.33
6.0	0.154 ± 0.005 (6)	−0.31
7.5	0.230 ± 0.012 (6)	−0.19
8.0	0.302 ± 0.007 (5)	−0.07
8.7[e]	0.864 ± 0.023 (6)	+0.57
Saturated[f]	1.096 ± 0.030 (4)	

[a] Free water refers to the concentration of water outside the dialysis bag. δ = g of H_2O bound/g of phosphatidylcholine. δ_m = maximum amount of water bound. [b] c_w = molar concentration of water. [c] Standard deviation. [d] Number of determinations. [e] Approximate value. [f] Approximately 9 mg/ml.

When the water binding data are treated in this fashion, the results shown in the last column of Table I are obtained. For values of δ below approximately 0.15 the left side of eq 2 gives a constant value, suggesting a simple association equilibrium. However, for values of δ above 0.15 there is strong deviation from this constant value, suggesting a positive cooperative binding of water. These data suggest a transition in the nature of the water binding association occurs when approximately 0.15 g of H_2O are bound/g of phosphatidylcholine. This corresponds to approximately 6–7 molecules of water/molecule of phosphatidylcholine.

Further support for the interaction of water with phosphatidylcholine and the suggestion that the nature of water binding changes at 0.15 g of H_2O/g of lipid was obtained from density increment and refractive index increment measurements made in conjunction with the sedimentation analyses which are to follow. The important and extensive analyses of these parameters by Casassa and Eisenberg (1964) clearly indicate that a distinction must be made in the measurements of these parameters by different operations. Following Casassa and Eisenberg's notation, we shall designate $(\partial\rho/\partial c)_\mu$ and $(\partial\Delta n/\partial c)_\mu$ as the density increment and refractive index increment, respectively, measured at constant chemical potential of the solvent components, and $(\partial\rho/\partial c)_m$ and $(\partial\Delta n/\partial c)_m$ as the density increment and refractive index increment, respectively, measured at constant composition of the solvent components. Thus, after dialysis equilibrium, we obtained $(\partial\rho/\partial c)_\mu$ and $(\partial\Delta n/\partial c)_\mu$ of phosphatidylcholine by using the diffusate as the reference; and $(\partial\rho/\partial c)_m$ and $(\partial\Delta n/\partial c)_m$ by dissolving phosphatidylcholine in a solvent of fixed compositions and using the same solvent as the reference.

The results of these experiments are presented in Figure 1. Up to about 50 mg/ml, the density and refractive index increments were independent of the phosphatidylcholine concentration. These results show that the measurements by the two different operations give significantly different results, as much as 30% or more in the density increment and 10% in the refractive index increment. Casassa and

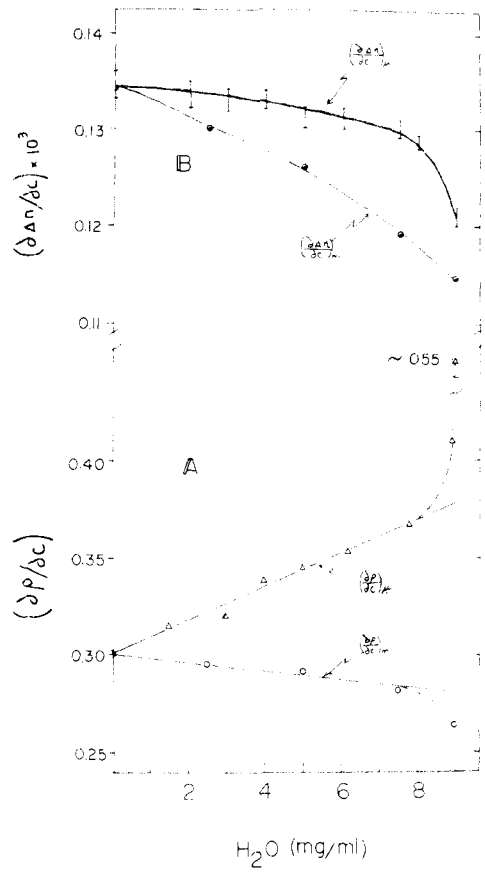

FIGURE 1: (A) Density increments $(\partial\rho/\partial c)$ of phosphatidylcholine in ether as a function of the free water concentration. $(\partial\rho/\partial c)_\mu$ refers to measurements made at constant chemical potential of the solvent. $(\partial\rho/\partial c)_m$ refers to measurements made at constant composition of the solvent. Refer to text for more details. (B) Refractive index increments $(\partial\Delta n/\partial c)$ of phosphatidylcholine in ether as a function of the free water concentration. $(\partial\Delta n/\partial c)_\mu$ refers to measurements made at constant chemical potential of the solvent. $(\partial\Delta n/\partial c)_m$ refers to measurements made at constant composition of the solvent. Refer to the text for details.

Eisenberg show that these features are the result of strong interactions between the solute component and the solvent components. Such interactions have also been noted with DNA in high salt solutions (Cohen and Eisenberg, 1968). This type of interaction can be expressed as

$$(\partial\rho/\partial c)_\mu - (\partial\rho/\partial c)_m \cong (\partial\rho/\partial c_w)_m \xi_w \quad (3)$$

$$(\partial\Delta n/\partial c)_\mu - (\partial\Delta n/\partial c)_m \cong (\partial\Delta n/\partial c_w)\xi_w \quad (4)$$

We have specifically designated the interaction parameter, ξ_w, as occurring between phosphatidylcholine and water. $(\partial\rho/\partial c_w)_m$ and $(\partial\Delta n/\partial c_w)_m$ are the density increment and refractive index increment, respectively, of water in ether, which we have determined to be 0.526 ± 0.017 ml/mg and 0.097 ± 0.001 × 10^{-3}, respectively. The values of ξ_w calculated from the results presented in Figure 1 are shown in Table II along with the corresponding value of δ.

Although all three sets of data clearly indicate interaction occurs between water and phosphatidylcholine, there is rather poor agreement as to the extent of the interaction. We do not feel that this disagreement arises from the errors of measurement. In these calculations we have assumed the density and refractive index increments of the water associated with the phosphatidylcholine are the same as water in ether. There is no *a priori* reason why this should be a valid

TABLE II: Apparent Water Binding Parameters Determined by Different Procedures.

Free water (mg/ml)	g of Water Bound/g of Lipid		
	ξ_w[a]	ξ_w[b]	δ[c]
2.5	0.050	0.023	0.057
5.0	0.099	0.063	0.126
7.5	0.159	0.140	0.230

[a] Calculated from density increment measurements according to eq 3. [b] Calculated from refractive index increments according to eq 4. [c] Measured by equilibrium dialysis.

assumption. If we assume that all measurements should give the same value for the extent of water binding, then we can use eq 3 and 4 and the value of δ to calculate $(\partial\rho/\partial c_w)_m$ and $(\partial\Delta n/\partial c_w)_m$ from the data in Figure 1. The results of these calculations are found in Table III. It is of interest to note that below $\delta = 0.1$, constant values for $(\partial\rho/\partial c_w)_m$ and $(\partial\Delta n/\partial c_w)_m$ are obtained, which are, however, significantly different than the corresponding values obtained with water in ether. Above $\delta = 0.15$ there is a large change in these parameters, again suggesting a change in the nature of the bound water.

Molecular Weight Distributions of Phosphatidylcholine Aggregates from Sedimentation Equilibrium Studies. For a system containing heterogeneous macromolecular species, phosphatidylcholine aggregates in this case, the expression for sedimentation equilibrium is (Casassa and Eisenberg, 1964)

$$\frac{\omega^2}{RT}\left(\frac{\partial\rho}{\partial c}\right)_\mu\left(\frac{dc/dr}{rc}\right)^{-1} = \frac{1}{M_w(r)} + O(c) \qquad (5)$$

where ω, R, T, and r are the angular velocity, gas constant, absolute temperature, and radial distance, respectively; $(\partial\rho/\partial c)_\mu{}^0$ is the limiting value of $(\partial\rho/\partial c)_\mu$ as c approaches zero; $M_w{}^{(r)}$ is the weight average molecular weight at the position r, and $O(c)$ is the expansion of the virial coefficient, which vanishes in an ideal system. In terms of schlieren optics, and assuming ideality, eq 5 becomes

$$M_w{}^{(r)} = \left(\frac{RT}{\omega^2(\partial\rho/\partial c)_\mu{}^0}\right)\left(\frac{y}{r(A_a + A_r)}\right) \qquad (6a)$$

where

$$A_r = \int_a^r y\,dr \qquad (6b)$$

$$A_a = A_0 - \left(\int_a^b A_r dr^2\right)/(b^2 - a^2) \qquad (6c)$$

and

$$A_0 = k(\partial\Delta n/\partial c)_\mu c_0 \qquad (6d)$$

where y is the displacement of the schlieren pattern, a and b the radial distance of the meniscus and cell bottom, respectively, k is the machine constant, and c_0 is the initial solute concentration. It should be noted that, in this formulation, the molecular weight M_w is unambiguously defined in terms of the weight of material as defined by c_0. In our case, all measurements are made in terms of the dried weight of phosphatidylcholine, therefore M_w represents the weight average molecular weight of the phosphatidylcholine portion of the aggregate.

TABLE III: Calculated Values for $(\partial\rho/c_w)_m$ and $(\partial\Delta n/\partial c_w)_m$.

Free Water (mg/ml)	δ	$(\partial\rho/\partial c_w)_m$[a]	$\bar{v}_w$[b]	$(\partial\Delta n/\partial c_w)_m$[c] $\times 10^3$
2.0	0.057	0.439	0.792	0.042
2.5	0.071	0.447	0.781	0.037
3.0	0.072	0.447	0.781	0.039
4.0	0.101	0.438	0.794	0.046
5.0	0.126	0.429	0.806	0.053
6.0	0.154	0.407	0.838	0.056
7.5	0.230	0.305	0.918	0.059
8.0	0.302	0.286	1.008	0.048

[a] Calculated from eq 3 using the measured value of water binding from Table I. [b] Calculated according to $(\partial\rho/\partial c_w)_m = 1 - \bar{v}\rho_0$, where ρ_0 is the density of the solvent. [c] Calculated from eq 4 using the measured values of water binding from Table I.

The molecular weight of phosphatidylcholine aggregates as a function of phosphatidylcholine concentration at several water concentrations are shown in Figure 2. For a single water concentration the reproducibility is not completely satisfactory. This may in part be due to the difficulty of quantitatively transferring the highly volatile solvent. All equilibrium runs were made at or below 10,000 rpm; within this range no significant speed dependence of the molecular weight distribution was observed.

As can be seen from Figure 2, at any given water concentration, M_w increases as the phosphatidylcholine concentration increases up to a certain plateau value, and the value of M_w in this plateau region is a function of the water concentration. At high concentrations of phosphatidylcholine, there is an apparent decrease in M_w, this decrease being more pronounced at higher water concentrations. We consider this decrease to represent nonideal behavior, and shall not be concerned with this aspect of the data in this paper.

Among several possible explanations of these data, it seems most likely that, at any fixed water concentration, the state of aggregation of phosphatidylcholine is discrete, and further, that the aggregation number is a function of the water concentration. Based on this premise, a model can be formulated, which is consistent with our results.

Assume that the system can be described by

$$PC + m'H_2O \underset{}{\overset{k'}{\rightleftharpoons}} PC(H_2O)_{m'}$$

$$nPC(H_2O)_{m'} \overset{k^*}{\rightleftharpoons} PC_n(H_2O)_m \qquad (m = nm')$$

where PC refers to the monomer of anhydrous phosphatidylcholine, $PC(H_2O)_{m'}$ to the hydrated monomer, and $PC_n(H_2O)_m$ to the hydrated aggregate. Furthermore, we assume that $k' \approx \infty$, so that c_1, the concentration of the anhydrous monomer, approaches zero. In effect, then we are dealing only with the monomer-$n$mer equilibrium of the hydrated phosphatidylcholine molecule. Finally it is assumed that a continuous phase transition occurs in the phosphatidylcholine aggregates, as the water concentration increases, and that this phase transition results in a continuous change in the values of m' and n.

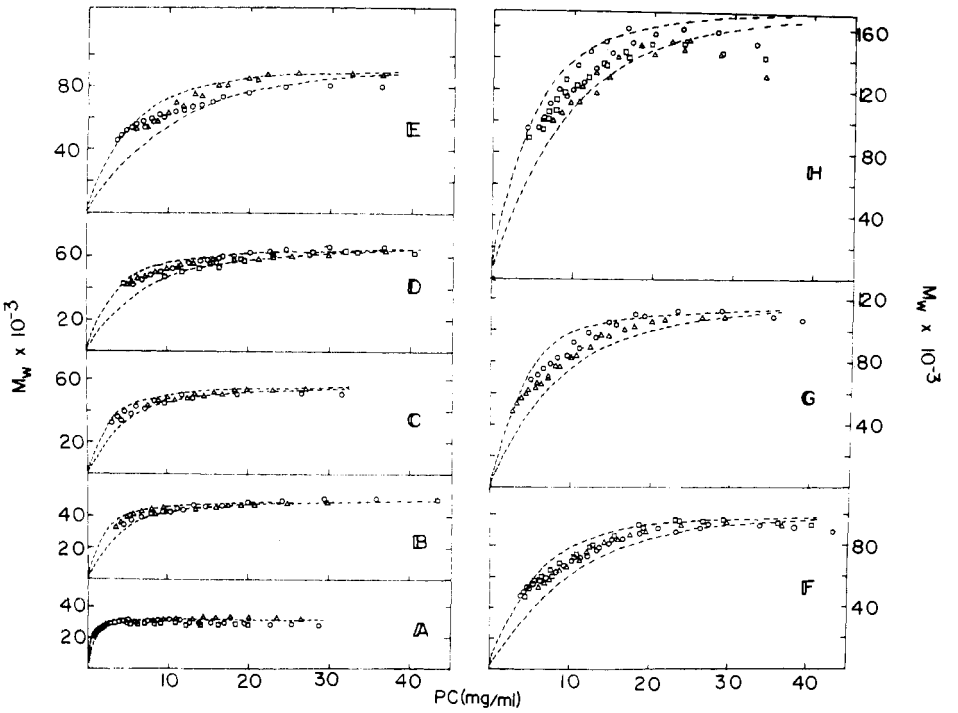

FIGURE 2: Molecular weight distributions of phosphatidylcholine in ether–water solutions. Different symbols represent data from sedimentation equilibrium experiments with solutions prepared on different days. With each preparation three solutions of different initial concentration of phosphatidylcholine were used. The dashed curves represent theoretical curves giving a deviation of ±5% in ΔG^*_m (for thermodynamic parameters refer to Table IV). (A) Free water = 0.0 mg/ml; (B) free water = 2.0 mg/ml; (C) free water = 2.5 mg/ml; (D) free water = 3.0 mg/ml; (E) free water = 4.0 mg ml; (F) free water = 5.0 mg/ml; (G) free water = 6.0 mg/ml; (H) free water = 7.5–8.0 mg/ml.

Based on the analysis of Rao and Kegeles (1958), we can write

$$k^* c^{*(n-1)} = \frac{\frac{1}{n-1}\left[\frac{M_w^*}{M_1^*} - 1\right]}{\left[1 - \frac{1}{n-1}\left(\frac{M_w^*}{M_1^*} - 1\right)\right]^n} \quad (7)$$

where c^* is the total concentration of all hydrated phosphatidylcholine species, in mg/ml, and M_w^* and M_1^* are the weight average molecular weight of hydrated phosphatidylcholine species and the molecular weight of the hydrated phosphatidylcholine monomer, respectively. Since, according to our model, the extent of hydration is fixed at a fixed water concentration, we can write

$$k^* = k_{app}/(1 + \delta)^{n-1} \quad (8a)$$

$$k_{app} c^{n-1} = \frac{\frac{1}{n-1}\left[\frac{M_w}{M_1} - 1\right]}{\left[1 - \frac{1}{n-1}\left(\frac{M_w}{M_1} - 1\right)\right]^n} \quad (8b)$$

where c, M_w, and M_1 are the corresponding quantities for the anhydrous entities, as defined for the hydrated species in eq 7.

Theoretical curves calculated according to eq 8b are represented by the solid curves in Figure 2. At each water concentration the two dashed curves presented represent changes in ln k_{app} and n which alter the value of ΔG^*_m (see below) by ±5%. In Table IV are presented the values for n and ln k_{app} which fit the data best. The theoretical curves were calculated assuming ideality. It is expected that inclusion of nonideal terms will not significantly influence the values of n and ln k_{app}.

Choosing the standard state as the pure monomer and pure micelle, *i.e.*, expressing concentration on a mole fraction basis, we can calculate the standard free energy for micelle formation, ΔG^*_m, per mole of hydrated phosphatidylcholine monomer, and the critical micelle concentration (Shinoda and Hutchinson, 1962).

k_{app} calculated from eq 8b is in terms of mg/ml of reactants, *i.e.*, monomer and aggregate. This equilibrium constant can be transformed into terms of mole fraction of reactants by

$$k_x = k_{app}\left(\frac{M_1^2}{1000\rho}\right)^{n-1}/n^2 \quad (9a)$$

where M_1 = molecular weight of anhydrous monomer, which we assign a value of 800; n = aggregate number, and ρ = density of the solvent. Noting that the molecular weight of the hydrated monomer is given by

$$M_1^* = M_1(1 + \delta) \quad (9b)$$

and the equilibrium constant in terms of the mole fraction of hydrated reactants, k_x^*, is given by

$$k_x^* = k_x\left(\frac{M_1^{*2}}{1000\rho}\right)^{n-1}/n^2 \quad (9c)$$

we have

$$\ln k_x^* = \ln k_x + (n-1)\ln(1 + \delta) \quad (9d)$$

and in terms of the mole fraction of hydrated monomer, k_m^* by

$$\ln k_m^* = \ln k_x^*/(n-1) \quad (9e)$$

The standard free energy for formation of the hydrated micelle is given by

$$-\Delta G_m^* = RT \ln k_m^* \quad (10)$$

TABLE IV: Thermodynamic Parameters for Micelle Formation by Phosphatidylcholine in Ether–Water Solutions.

Parameter	Free Water (mg/ml)							
	0	2.0	2.5	3.0	4.0	5.0	6.0	7.5–8.0
n^a	40	65	70	85	120	130	150	225
ln $k_{app}{}^a$	50	−20	−30	−70	−140	−150	−150	−250
$-\Delta G^*_m$ (cal/mol)^b	4670	3790	3730	3510	3330	3360	3470	3480
cmc* (mg/ml)^c								
Hydrated monomer	0.34	1.64	1.85	2.56	3.85	3.86	3.37	4.07
Anhydrous monomer	0.34	1.48	1.65	2.24	3.21	3.08	2.54	2.52

[a] These values represent the best fit of eq 8b to the data in Figure 2. [b] Calculated from eq 10. [c] Calculated from eq 11b.

TABLE V: Hydrodynamic Parameters of Phosphatidylcholine Micelles Derived from Viscosity Measurements.

	Free Water (mg/ml)				
	0	3.0	5.0	6.0	7.5
$\bar{v}_2{}^\circ$ (ml/g)	0.990	0.996	0.999	1.000	1.011
$[\eta]$ (ml/g)^a	3.94	4.00	4.05	4.45	4.60
ν^b	3.94	4.02	4.05	4.45	4.55
ν^c	3.93	3.80	3.68	3.93	3.76
$[\eta]_m$ (ml/g)^d	3.90	4.40	4.80	5.40	6.00
$\nu_m{}^b$	3.94	4.21	4.43	4.89	5.15
$\nu_m{}^c$	3.94	4.18	4.36	4.78	4.91

[a] From the intercepts in Figure 3. [b] Calculated assuming $\bar{v}_w = 0.670$ and using eq 12a. [c] Calculated using $\bar{v}_w$ from Table III and eq 12a. [d] From the intercepts in Figure 4.

The critical micelle concentration, on a mole fraction basis $(cmc)_x^*$, is given by

$$(cmc)_x^* = e^{-\ln k_m^*} \tag{11a}$$

and on a mg/ml basis by

$$(cmc)^*_{mg/ml} = (cmc)_x^*(M_1^*)^2/1000\rho \tag{11b}$$

The values for ΔG^*_m and $(cmc)^*_{mg/ml}$ are found in Table IV. It should be noted that both ΔG^*_m and $(cmc)^*_{mg/ml}$ change from the anhydrous state until the free water concentration reaches approximately 4 mg/ml or $\delta = 0.10$. Thereafter the values remain more or less constant. The point at which this break occurs corresponds to the break noted above from water binding studies.

Viscosity Measurements. The results of viscosity measurements are presented in Figure 3 as plots of $\eta_{sp/c}$ *vs.* c, where c refers to the dry weight of phosphatidylcholine used to prepare the solutions by equilibrium dialysis. The viscosity number, ν, can be calculated from the intrinsic viscosity, $[\eta]$, by

$$[\eta] = \nu(\bar{v}_2{}^0 + \delta\bar{v}_w) \tag{12a}$$

where $\bar{v}_2{}^0$ refers to the partial specific volume of the phosphatidylcholine portion of the aggregates and is calculated from

$$(\partial\rho/\partial c)_m = 1 - \bar{v}_2{}^0\rho_0 \tag{12b}$$

where ρ_0 is the density of solvent; and $\bar{v}_w$ refers to the partial specific volume of water. The value for $\bar{v}_w$ obtained by direct measurement is 0.670, but as noted above the value in the presence of the lipid appears to be different. Table V contains ν calculated using $\bar{v}_w = 0.670$, as well as the value derived from the data in Table III. The use of either value leads to the conclusion that the shape of the aggregate is not markedly influenced by hydration.

It has been suggested by Ekwall *et al.* (1971) that the viscosity of micellar solutions should be determined according to

$$\left[\frac{\eta_{exp}}{\eta_{cmc}} - 1\right]/c_m = [\eta] + a_2c_m \tag{13}$$

where η_{exp} is the measured relative viscosity, η_{cmc} is the relative viscosity at the cmc, c_m = concentration of micelles ($c_m = c_{total} - cmc$), and a_2 is a constant. Using the cmc values from Table IV, the viscosity data now appear as shown in Figure 4. The calculated viscosity numbers are in Table V. Treatment of the viscosity data by this procedure leads to the conclusion that hydration does influence the shape of the aggregate. At the present time we cannot comment on the possible significance of these different results in the absence of an independent measurement of the shape of the micelles.

Effect of Calcium on the State of Aggregation. Misiorowski and Wells (1974) noted that Ca^{2+} inhibited phospholipase A_2 at low water concentration. Physical studies on the effects of calcium on the state of aggregation of

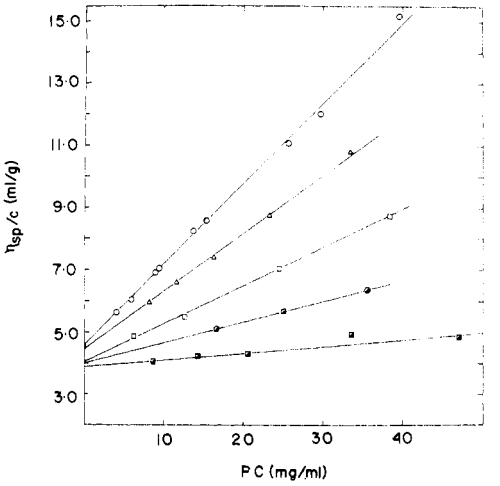

FIGURE 3: η_{sp}/c as a function of the total phosphatidylcholine concentration at various levels of free water. (◪) free water = 0.0 mg/ml; (◑) free water = 3.0 mg/ml; (□) free water = 5.0 mg/ml; (△) free water = 6.0 mg/ml; (○) free water = 7.5 mg/ml.

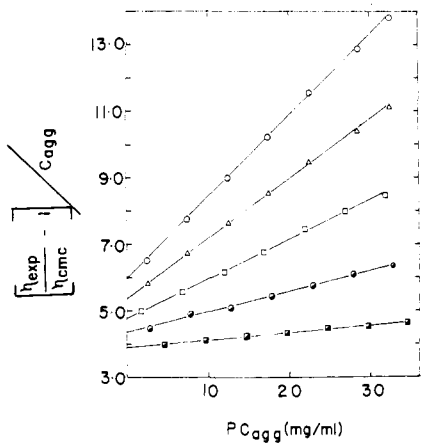

FIGURE 4: Viscosity of phosphatidylcholine aggregates as a function of the concentration of micelles at various levels of free water. The specific viscosity was calculated according to eq 13 of the text. PC_{agg} = total concentration of phosphatidylcholine − cmc. The symbols refer to the same water concentrations as in Figure 3.

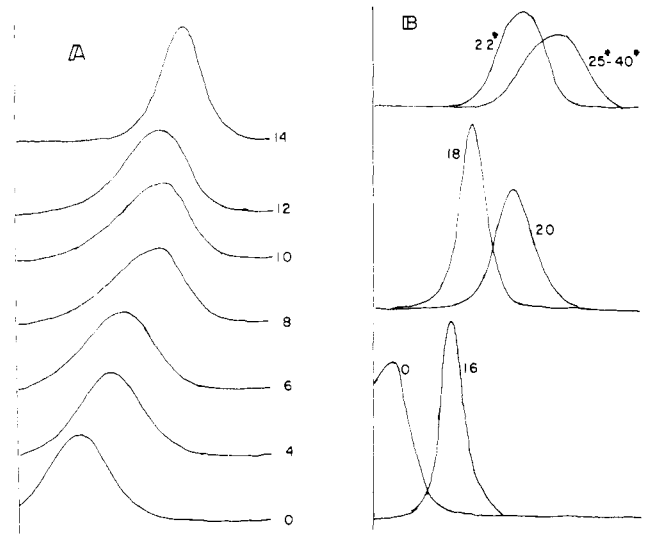

FIGURE 5: Sedimentation velocity patterns of phosphatidylcholine micelles in ether at various concentrations of total water. The solutions all contained 10 mg/ml of lipid. The numbers next to each pattern are the total water concentration in mg/ml of the solution. * means that water droplets were present in the solution. All experiments were carried out at 25° and 40,000 rpm. In panel A the photograph was taken after 22 min, in panel B after 10 min.

phosphatidylcholine in the ether–water system are difficult to carry out due to the solubility behavior of $CaCl_2$ in the system. Calcium chloride is virtually insoluble in ether–water, and its solubility in the presence of phosphatidylcholine is time dependent. We were not able, therefore, to carry out equilibrium experiments with $CaCl_2$ containing solutions in the equilibrating solvent.

However, when phosphatidylcholine solutions (10 mg/ml) were allowed to stand overnight with $CaCl_2$ solutions ($CaCl_2$ = 0.01 mM, and 0.1 mM based on total volume) and water concentration ranging from 1.5 to 7.5 mg/ml, no detectable effect of $CaCl_2$ was observed on the sedimentation equilibrium pattern. These concentration ranges encompass those in which marked inhibition of phospholipase A_2 by $CaCl_2$ is noted.

Sedimentation Velocity Studies. A rigorous treatment of sedimentation velocity experiments is outside the scope of this paper, since it is expected that nonnegligible pressure effects are encountered. We have, however, included some velocity run results in Figure 5 and Table VI, since they illustrate the substantial changes in aggregation state attendant upon water binding.

As the free water concentration in the solution increases from 0 to about 8.2 mg/ml, there is a gradual increase in the apparent sedimentation coefficient. At low water concentrations the bands appear to be skewed toward the low molecular weight side. As the free water increases to 8.2 mg/ml the band becomes nearly symmetrical. When the free water concentration increases from 8.2 to the water saturation level of the solution (~9 mg/ml), there is a large increase in the apparent sedimentation coefficient; however, the bands remain reasonably symmetrical. When the total water content of the solution exceeds 20 mg/ml, we observe droplets, which appear to be water, since all the phospholipid remains in solution (Table VII). In this region of water excess there is a further increase in the apparent sedimentation coefficient, and the band is markedly asymmetrical.

Although it is rather difficult to assign a value of s_c^{25} to any specific hydration state, it is important to note the abrupt change which occurs near a free water concentration of 8.4 mg/ml (Figure 6). We assume that this abrupt change represents a phase transition.

Phospholipase A_2 Activity. Misiorowski and Wells (1974) conducted enzyme assays in solutions which contained 5% methanol in order to circumvent certain solubility problems. The presence of methanol in the solvent introduces complications in physical studies, so we have conducted all of our experiments in the absence of methanol. In order to compare the results of the physical studies with the enzyme activity data, we have carried out some experiments in ether–water mixtures. In Figure 6 we compare the effect of the free water concentration on the apparent sedimentation coefficient and on the specific activity of phospholipase A_2. Also shown in the inset in Figure 6 is a comparison of the activity of the enzyme with the water binding parameter, defined in eq 1. The onset of enzymatic activity occurs at about 5 mg/ml of free water. The increase in activity correlates well with the increased extent of cooperative water binding as defined by eq 1. There is a marked de-

TABLE VI: Apparent Sedimentation Coefficients for Phosphatidylcholine Micelles in Ether–Water Solutions.[a]

Total water (mg/ml)	0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	22.0[b]	25.0[b]	30.0[b]
Free water (mg/ml)	0	3.2	5.0	6.4	7.6	8.2	8.4	8.6	8.8	~9.0	~9.0	~9.0	~9.0
s_c^{25}	13.3	17.8	21.3	26.4	30.7	30.8	34.5	45.5	57.2	85.2	98.9	115.5	118.0

[a] The sedimentation velocity was carried out at 25° and 40,000 rpm at an initial lipid concentration of 10 mg/ml. These values are the measured sedimentation coefficients at this temperature and concentration. [b] Exceeds water saturation level of the solution.

TABLE VII: Solubility of Phosphatidylcholine in Diethyl Ether Solutions Saturated with Water.[a]

Total water (mg/ml)	10	20[b]	25[b]	30[b]	40[b]
Soluble phosphatidylcholine (mg/ml)	10.40	9.49	10.09	10.12	10.12

[a] Various amounts of water were added to ether solutions containing 10.4 mg/ml of phosphatidylcholine and 10 mg/ml of water. After mixing and centrifugation at 1500*g* for 15 min, the supernatant solutions were analyzed for phosphatidylcholine. [b] Visible precipitate.

crease in enzymatic activity when the free water concentration exceeds 8.6 mg/ml. This change correlates well with the abrupt change in s_c^{25} discussed above.

Discussion

The important conclusions to be drawn from this study are (1) the association of phosphatidylcholine molecules into aggregates in ether-water solutions is definite, *i.e.*, above a certain concentration of lipid, the size of the micelle does not change; (2) the size and shape of the micelle are determined primarily, if not solely, by the amount of water bound to the micelle; (3) the properties of the water bound to the micelle depend on the amount of water bound to the micelle; (4) there are at least three distinguishable hydration states, only one of which serves as a substrate for phospholipase A_2.

The suggestion of definite aggregation is supported by low speed sedimentation equilibrium data, and the reasonable fit of the theoretical data to experimental data, since the theory assumes definite aggregation. The rather symmetrical shapes of the sedimentation velocity bands would also suggest a limited range of molecular weight species. There may be additional complications in these latter experiments, which must be considered before a firm conclusion can be drawn. Elworthy (1959a,b), studying the aggregation of phosphatidylcholine in benzene, concluded that aggregation was definite. This point is important in consideration of possible models for the activity of phospholipase A_2 within the micelles (Misiorowski and Wells, 1974).

Several lines of evidence support the suggestion that the size and shape of the micelles are determined by the amount of water bound. At each water concentration a different value of n and ln k_{app} are required to fit the data. Viscosity data also support the suggestion that water influences the shape of the micelle, although there is some ambiguity concerning the correct shape factor, depending on which approach is taken. The values of the sedimentation coefficient also change at different water concentrations. A combination of intrinsic viscosity and sedimentation data could provide information concerning the shape of the micelles; however, such calculations must await further theoretical considerations concerning which treatment of viscosity data is appropriate and how to determine the sedimentation coefficient unambiguously. We note that Ekwall (1969) has reported the importance of water in controlling micelle formation in the ternary system of an alkalai metal soap, water, and a liquid fatty acid or alcohol.

Within certain limits we can conclude that the primary effect of water binding is to greatly increase the size of the micelle without appreciably altering shape. It is of interest

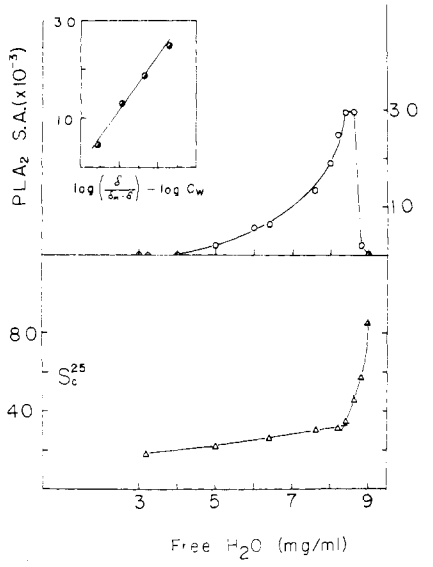

FIGURE 6: Correlation of the apparent sedimentation coefficient, s_c^{25}, of phosphatidylcholine micelles in ether, and the activity of phospholipase A_2 in phosphatidylcholine micelles in diethyl ether with the free water concentration. Lower panel: Dependence of s_c^{25} on the free water concentration. Upper panel: Dependence of the specific activity of phospholipase A_2 (right-hand legend) on the free water concentration. Inset: Correlation of the specific activity of phospholipase A_2 (left-hand legend) with the degree of cooperative water binding log $(\delta/(\delta_m - \delta)) - \log c_w$ (eq 2 in the text).

to compare this conclusion to those of Elworthy (1959a,b) and Elworthy and McIntosh (1964), who studied the aggregation of phosphatidylcholine in benzene solution. In this latter solvent water binding leads primarily to a shape change, the micelles becoming more spherical, with little change in the size of the aggregate. There are a number of differences between the micellization processes, and the effect of water, in ether and benzene. (1) The amount of water that can be solubilized in ether (1.1 g/g of lipid) is considerably higher than in benzene (0.33 g/g of lipid). (2) The critical micelle concentration is higher in ether (0.34 mg/ml) than in benzene (0.033 mg/ml). (3) Water increases the cmc in ether; no data are available in benzene. (4) In benzene the aggregation number is 65–70, and is not affected by water. In ether the aggregation number ranges from 40 to greater than 250 depending on the water concentration. (5) The interaction of water and cations with the micelle is different in the two solvents (Misiorowski and Wells, 1973). (6) We have been unable to devise any conditions under which phospholipase A_2 is active in benzene solutions. In light of the above, it is unwise to generalize about the micellization process in different solvents, or to extrapolate the apparent physical state within the micelle from one solvent to another.

There are at least three distinguishable hydration states of the micelles in ethereal solutions. The first, which requires about 7 mol of H_2O/mol of lipid, is characterized by (1) an apparently simple water association equilibrium, (2) rather constant values for the apparent density and refractive index increments of the bound water, (3) larger values of $-\Delta G^*_m$, and (4) lack of activity toward phospholipase A_2. The second hydration state requires an additional 25–30 mol of water/mol of lipid and shows (1) what appears to be cooperative water binding, (2) a changing value for the apparent density and refractive index increments of the bound water, (3) somewhat smaller values of $-\Delta G^*_m$, and

(4) activity toward phospholipase A_2. The third hydration state, which requires an additional 20–25 mol of water/mol of lipid, is characterized by (1) extremely large size, (2) an abrupt phase transition from the previous state, as reflected in the value of s_c^{25}, and (3) lack of activity toward phospholipase A_2.

In addition to the data discussed above, the accompanying paper (Wells, 1974) provides additional evidence that the properties of the bound water depend on the amount of water bound to the micelle. It has also been noted by Ekwall (1969) that the properties of water in inverted micelles depend on the amount of water bound. In the present case, and as reported by Ekwall, the second hydration state seems to reflect a change in the properties of the bound water toward those of pure water.

There appears to be a discrepancy between the results of the water binding data, as treated by eq 1, and the assumption made in our model that the concentration of the anhydrous monomer is zero. The value of $\log k_{int} - 0.434\phi(\delta)$ is about −0.3. If we assume that $\phi(\delta)$ is zero at low water concentrations this gives a value of $k_{int} = 0.5\ M^{-1}$. Such a value would not be large enough to satisfy the assumption that the concentration of the anhydrous monomer is near zero. Another possibility is that $\phi(\delta)$ is large and positive, but remains constant at low water concentrations. This situation would require k_{int} to be large, and, therefore, the concentration of the anhydrous monomer to be near zero. The large positive value of $\phi(\delta)$ implies that aggregation has a negative cooperative effect on hydration. In other words it is easier to hydrate the monomer of the lipid than it is to hydrate the lipid within the micelle. Such a situation may explain why the cmc is higher in the presence of water. Since the monomer is more readily hydrated than the aggregate, the micellar equilibrium is shifted toward monomer in the presence of water. At a free water concentration of 6 mg/ml there is a change in $\phi(\delta)$, which appears to reflect cooperative water binding. We suggest that this actually is a change in $\phi(\delta)$ to a less positive value, thereby reducing the negative effect on water binding by the aggregate. The decrease in the cmc above a free water concentration of 4–5 mg/ml would be consistent with this suggestion.

It is of interest to speculate how the available data on the effects of water on the physical properties of phosphatidylcholine micelles can be used to understand the activity of phospholipase A_2. Based on hydrodynamic properties of the enzyme (Wells and Hanahan, 1969) and assuming a hydration of 0.2 g of water/g of protein, it requires approximately 350 molecules of water to hydrate the enzyme. The radius of the hydrated enzyme is 20 Å. If we assume a spherical micelle, which is only a rough approximation, it requires approximately 1100 water molecules to produce a core of radius 20 Å. It is of interest to note that the onset of enzyme activity occurs at a free water concentration of 6 mg/ml. The amount of water bound to a micelle is 1065 molecules. At a free water concentration of 7.5 mg/ml there are 2990 molecules of water bound, which gives a hydrated core of radius 28 Å. This latter hydration level gives about half-maximal enzymatic activity. It thus appears that the enzyme is only active when the hydrated core is large enough to accommodate the enzyme. There is also ample water to hydrate the protein.

The data in this paper provide a reasonably complete description of the effects of water on the physical properties of phosphatidylcholine micelles in diethyl ether. We can characterize the physical state of the micelle which is a prerequisite for activity of phospholipase A_2, and provide a plausible explanation as to why the enzyme is active in this type of micelle. We cannot, as yet, describe the process of enzyme solubilization into the micelle, the properties of the interior of the micelle after addition of the protein, nor the state of the protein in micelles which contain insufficient quantities of water to support enzymatic activity.

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