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Thermodynamic Model for Micelle Formation by Phosphatidylcholines Containing Short-Chain Fatty Acids. Correlations with Physical–Chemical Data and the Effects of Concentration on the Activity of Phospholipase A_2[†]

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ABSTRACT: A theoretical model of short-chain (C_6–C_8 fatty acids) lecithin aggregation was developed to determine if a change in form or concentration of aggregate species would be predicted which could explain the parabolic kinetic behavior observed near the critical micelle concentration with phospholipase A_2 [Wells, M. A. (1974) *Biochemistry 13*, 2248]. In order to satisfactorily predict the physical properties of the micelles (critical micelle concentration, aggregation number, and molecular weight heterogeneity) as reported by Tausk et al. [Tausk, R. M. J., Kaomiggelt, J., Oudschoorn, C., & Overbeek, J. Th. G. (1974) *Biophys. Chem. 1*, 175; Tausk, R. M. J., Van Esch, J., Karmiggelt, J., Voordouw, G., & Overbeek, J. Th. G. (1974) *Biophys. Chem. 1*, 184; Tausk, R. M. J., Oudshoorn, C., & Overbeek, J. Th. G. (1974) *Biophys. Chem. 2*, 53], the ^{13}C NMR data of Schmidt et al. [Schmidt, C. F., Barenholz, Y., Huang, C., & Thompson, T. E. (1977) *Biochemistry 16*, 3948], and the kinetic data, we developed a two micelle model. In this formulation the first micelle is formed at lecithin concentrations near the critical micelle concentration and the second micelle arises from the first at higher concentrations of lecithin. It was shown that a satisfactory fit to the kinetic data as well as ^{13}C NMR data was achieved, assuming that the second micelle is the form of the substrate responsible for the large rate enhancement observed above the critical micelle concentration as well as the substrate form responsible for the majority of the ^{13}C NMR shift above the critical micelle concentration. It was suggested that dehydration of the carbonyl groups of the substrate could account for both the ^{13}C NMR shifts and the enhanced activity as a substrate for phospholipase A_2.

The kinetics of hydrolysis of lecithins containing short-chain fatty acids by phospholipases often show anomalous regions in velocity vs. substrate concentration plots. In these regions the plots are parabolic rather than hyperbolic (Allgyer and Wells, unpublished experiments). In the case of phospholipase A_2, this region occurs near the critical micelle concentration (cmc)[1] (Wells, 1974a), but it occurs well below the cmc in the case of phospholipase D (Allgyer and Wells, unpublished experiments). It has been proposed that this parabolic region represents a range of concentration of substrate over which the properties of the micelle are changing (Wells, 1974a), although the nature of the change has not been defined. In order to more completely understand these kinetic "anomalies" it is necessary to know the properties and concentration of the various species which may exist in solution.

A theoretical model of lecithin aggregation was developed to determine if a change in form or concentration of aggregate species would be predicted which could explain the parabolic kinetic behavior observed near the cmc with phospholipase A_2.

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[1] Abbreviations used: cmc, critical micelle concentration; DiC_4, 1,2-dibutyryl-*sn*-glycero-3-phosphorylcholine; DiC_5, 1,2-divaleryl-*sn*-glycero-3-phosphorylcholine; DiC_6, 1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine; DiC_7, 1,2-diheptanoyl-*sn*-glycero-3-phosphorylcholine; DiC_8, 1,2-dioctanoyl-*sn*-glycero-3-phosphorylcholine; rmse, root mean square error.

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We have combined thermodynamic theories of micelle formation and the results of physical studies on short-chain lecithins in order to obtain a set of equations which could be used to predict the concentration of various substrate species. We then used these results to interpret the kinetics of hydrolysis of dihexanoyllecithin by phospholipase A_2.

We shall limit ourselves to consideration of short-chain lecithins since fairly extensive data have been reported for the cmc and micellar weights of dihexanoylphosphatidylcholine (DiC_6), diheptanoylphosphatidylcholine (DiC_7), and dioctanoylphosphatidylcholine (DiC_8). An apparent micellar weight for DiC_6 was calculated from sedimentation and diffusion data, and the cmc was determined by refractive index measurements by Roholt & Schlamowitz (1961). Pieterson (1973) has reported cmc's for divaleroylphosphatidylcholine (DiC_5), DiC_6, and DiC_7 determined by a spectrophotometric dye solubilization technique. Wells (1974a) reported cmc's for dibutyrylphosphatidylcholine (DiC_4), DiC_6, and DiC_8 determined by a fluorescent dye incorporation technique. Tausk and co-workers have published a series of articles on the physical properties of the short-chain lecithins: cmc's of the C_6, C_7, C_8, and C_9 homologues were determined by surface tension measurements (Tausk et al., (1974a); micellar weights of DiC_6 and DiC_7 were determined by light scattering and ultracentrifugation (Tausk et al., 1974b); micellar weights of DiC_8 were determined by light scattering (Tausk et al., 1974c). In addition, we shall make use of the results of ^1H (Hershberg et al., 1976) and ^{13}C (Schmidt et al., 1977) nuclear magnetic resonance (NMR) studies on short-chain lecithins.

Kinetic data at 25 °C, pH 8.0, and 1 mM Ca^{2+} for DiC_6 were obtained as previously described (Wells, 1972, 1974a). Theoretical calculations and curve fitting were carried out on a Wang 600 with an extended memory module and flat-bed plotter using programs written in this laboratory. The root mean square deviation of calculated and experimental values was used in parameter evaluation.

For clarity of presentation we present a qualitative description of the model first and then the thermodynamic model will be described. We then show that NMR studies on short-chain lecithins provide strong evidence for the existence of two types of micelles and that the ^{13}C NMR studies on DiC_6 can be analyzed to provide an expression describing the concentration of each of these types of micelles at any total concentration of the lipid. Using this result, we will then show that it is possible to completely describe the velocity vs. substrate concentration curve for the phospholipase A_2 catalyzed hydrolysis of DiC_6. Finally, we discuss briefly earlier forms of the model and cite their points of failure.

Qualitative Description of the Model

The driving force for micelle formation is the favorable bulk free energy change associated with removal of the hydrocarbon chain from the aqueous environment. Formation of the hydrocarbon core of the micelle results in creation of a hydrocarbon–water interface. The properties of this interface are such as to introduce two opposing surface free energy terms which modulate the bulk free energy term. On one hand there is an attempt to bring the polar head groups in the surface as close together as possible in order to reduce the area of hydrocarbon–water contact. On the other hand there are repulsive interactions between the polar head groups which act to increase the separation between the head groups. At some optimal surface area per amphiphile there will be a balance between these two opposing forces (Tanford, 1972).

The problem of developing a theoretical model basically involves estimation of the optimal area and determination of a micelle size and shape which will give that area (Tanford, 1972). For entropic reasons the micelle with smallest aggregation number which permits the optimal area will be favored. The structure with the smallest aggregation number which can provide the optimal area is a sphere with a radius equal to the length of the alkyl chain. In the case of a single-chain amphiphile, a sphere does not in fact allow the area to be optimal, and other shapes such as disks or torroids are invoked (with the constraint that the dimension in one direction cannot exceed the length of the alkyl chain). In this manner quite satisfactory agreement between theoretical and experimental values for the cmc, aggregation number, and concentration dependence of the aggregation number can be achieved. In general, the observed aggregation number is less than twice the value predicted for a spherical micelle, and the aggregation numbers are rather narrowly distributed about the optimal value.

Table I: Comparison of One- and Two-Chain Amphiphiles

compd	V_{hc}[a]	l_c[b]	$\bar{N}$ of sphere[c]	obsd $\bar{N}$
DiC_6	323.8	7.825	6	34–38
DiC_7	377.6	9.090	8	50–200[d]
DiC_8	431.4	10.355	11	600–2500[d]
C_{11} alkyl betaine	323.3	15.415	47	58[e]
C_{13} alkyl betaine	377.1	17.945	64	87[e]
C_{15} alkyl betaine	430.9	20.475	83	130[e]

[a] Calculated from eq 3. [b] l_c = length of the extended alkyl chain = $1.5 + 1.265n_c$. [c] $\bar{N}$ of sphere = $(4/3)\pi l_c^3/V_{hc}$. [d] Strongly concentration dependent. [e] Swarbrick & Daruwala (1970).

The major problems involved in developing a theoretical model for micelle formation by short-chain phosphatidylcholines are (1) the aggregate size is considerably larger than predicted for a spherical micelle and (2) the size distribution is heterogeneous and this heterogeneity is strongly dependent on alkyl chain length. This is clearly shown in Table I in which are compared observed aggregation numbers and those calculated for a sphere for phosphatidylcholines and alkyl betaines of comparable hydrocarbon volume. In addition to the fact that the size of the micelles formed by the short-chain phosphatidylcholines deviates strongly from those predicted by a spherical model, it may be noted that the length of the alkyl chain is too small to form a stable bilayer (Israelachvili et al., 1976).

In addition to the geometric problems, a theoretical model must also account for the fact that both ^1H and ^{13}C NMR data (Hershberg et al., 1976; Schmidt et al., 1977) indicate the presence of two types of micelles at concentrations of amphiphile above the cmc and that an adequate explanation of the anomalous kinetics for hydrolysis of dihexanoylphosphatidylcholine also requires the presence of two types of micelles.

The model finally adopted in this study proposes the existence of two types of micelles in solutions of short-chain phosphatidylcholines. The first micelle (micelle I) is proposed to be an oblate ellipsoid or discoid as is described by the approach of Tanford (1973, 1977). The second micelle (micelle II) is proposed to arise from micelle I through a surface phase transition. The result of this transition is to produce a micelle with spherical ends connected by a long cylinder of, alternatively, a long ribbonlike structure. The properties of this micellar form have been described by Israelachvili et al. (1976). The most important attribute of this micellar form is that it gives rise to polydispersity of aggregation numbers. It was further assumed that the transition

from micelle I to micelle II occurs at a critical micelle size. The properties of the model are such that micelle I is the predominant species at amphiphile concentration from the cmc to approximately 4 times the cmc, whereas micelle II is the predominant species at concentrations greater than 4 times the cmc.

Mathematical Description of the Model

The thermodynamics of micelle formation have been discussed in detail by Tanford (1972, 1973, 1974a,b, 1977), Israelachvili et al. (1976), Ruckenstein & Nagarjan (1975), and Nagarajan & Ruckenstein (1977). The fundamental equation for micelle formation is given by

$$X_m = mX_1^m \exp(-m\Delta G^\circ_m/RT) \quad (1)$$

which relates the mole fraction (X_m) of a micelle of aggregation number m to the unitary free energy of transfer (ΔG°_m) of a single amphiphile from aqueous solution to a micelle of size m and the mole fraction of monomer (X_1). Once ΔG°_m is defined as a function of m, eq 1 can be used as a distribution function to determine the amount of amphiphile incorporated into micelles of various sizes. The problem therefore is to develop expressions for ΔG°_m.

Micelle I

The full statement of the free energy of transfer in Tanford's treatment is

$$\Delta G^\circ_m = A + B(n_c - 1) + C(A_{Hm} - D) + RT(E/A_{Rm} + F/A_{Rm}^2) \quad (2)$$

The first two terms on the right side of this equation are size independent and represent the free energy of transfer of the hydrocarbon chain from aqueous solution to the interior of the micelle. The last two terms are size dependent and represent the interfacial energy of the hydrocarbon–water interface and polar head group repulsion, respectively. The constant A constitutes the methyl groups contribution to the free energy. An estimate of the value of this constant was taken as 85% of twice the value Tanford utilized (−2000 cal/mol). This is consistent with the idea that two alkyl chains on the same molecule in the monomeric state tend to partially associate with each other and thus reduce the free energy gained by transfer to a hydrocarbon environment (Tanford, 1973). The value of the constant B which represents the methylene groups contribution ($n_c - 1$ is the number of methylene carbons in a single alkyl chain) was established from the slope of a plot of $RT \ln X_1$ at the cmc's vs. n_c for various diacylphosphatidylcholines. The derived value of −1216 cal/mol is approximately 85% of twice the value which Tanford (1974a) used for single-chain amphiphiles. We have specifically included all CH_2 groups since it is likely that the glycerol backbone which interfaces with the aqueous environment would be analogous to the first CH_2 group of a single-chain amphiphile which, as Tanford points out, should not be included in the calculations.

The constant C represents the interfacial tension of the hydrocarbon–water interface, A_{Hm} represents the area per molecule at the interface, and D represents the portion of the interface covered by the polar head group. There is disagreement between Tanford (1974a) and Israelachvili et al. (1976) as to how this term should be treated. In the former case the area is calculated at the distance of closest approach of a water molecule to the van der Waals boundary whereas in the latter case the area is calculated at the van der Waals boundary. The value of C depends on which approach is used. We have considered C an adjustable parameter and, following Tanford, used as initial estimates of C 25 ± 5 (cal/mol)/Å^2. We established the value of D as 41 Å^2, the limiting area of lecithin molecule at the oil–water interface (Taylor et al., 1973). The last term of eq 2 represents the polar head group contribution to the free energy where A_{Rm} is the area per polar head group. An initial estimate of the values of E and F was calculated from the fit of this term to the low-pressure region of the π–A curves of diacylphosphatidylcholines at the oil–water interface (Llerenas & Minigins, 1976).

In order to apply eq 2, it is necessary to calculate A_{Hm} and A_{Rm} as a function of micelle size, which of course depends on what shape is assumed for the micelles. This particular problem is undoubtedly the most contentious issue in developing a theory of micelle formation and has been discussed in detail by Israelachvili et al. (1976). A primary determinant of the shape of the aggregate is the fact that one dimension of the hydrocarbon core cannot exceed the length of the extended alkyl chain. This dimension is expected to be close to the optimal length based on configurational energy alone and was calculated according to Tanford. A second factor to be taken into consideration is that the volume of the hydrocarbon core depends only on aggregation number and is given by

$$V_{hc} = 2m(27.4 + 26.9n_c) \quad (3)$$

As mentioned above, in the case of two-chain amphiphiles such as phosphatidylcholines, the ratio of the volume of the hydrocarbon core to the length of the alkyl chain is large and establishes a limit on the size of a spherical aggregate which is much smaller than the observed aggregation numbers. Aggregates above the spherical limit were assumed to be of a regular shape, and calculations were made for both oblate and prolate ellipsoids of revolution. As pointed out by Israelachvili et al. (1976), these shapes, while giving a correct estimate of the *average* area per molecule, can only be considered approximate due to the radius of curvature of the edges. In spite of this limitation, the ellipsoids were used in these calculations since other shapes are more difficult to treat mathematically and there is not sufficient data on the shape of short-chain lecithin micelles to suggest logical alternatives. By use of the length of an extended alkyl chain for the semiminor axis of the ellipsoid and the equation for the volume of the ellipsoid, the semimajor axis of the ellipsoid can be calculated as a function of aggregation number. These calculated axes refer to the smooth hydrocarbon surface and were increased by a value δr_{hc} to account for the distance of closest approach of a water molecule (1.5 Å) and the undulating character of the surface (1.5–2.5 Å). For our calculations the value of δr_{hc} was taken as 3.0 Å. By use of the enlarged semiaxes, the area per molecule at the level of the hydrocarbon–water surface, A_{Hm}, can be calculated.

In a similar manner, the semiaxes calculated for the smooth hydrocarbon surface were increased by a factor δr_{hg} to allow estimation of the area per molecule at the level of the polar head group, A_{Rm}. To obtain an estimate of δr_{hg}, we constructed molecular models of the four sterically acceptable conformations for diheptanoylphosphatidic acid which have a parallel arrangement of the acyl chains as determined by intramolecular energy minimization calculations (Vanderkooi, 1973). The choline residue was added, assuming a gauche conformation (Sundaralingam & Jensen, 1965), and the distance from the α-methylene carbon of the acyl chain to the midpoint of the phosphate–quaternary nitrogen dipole was measured. The average value was 5.0 Å and was taken as δr_{hg}.

With A_{Hm} and A_{Rm} calculated as described above, eq 2 was used to calculate ΔG°_m as a function of m, and eq 1 was then

used to obtain X_m as a function of m. All values of X_m for which $\ln X_m > -50$ were summed to give $\sum X_m$, and the number ($\bar{m}_n$) and weight ($\bar{m}_w$) average aggregation numbers were calculated by the usual procedures.

The calculated values of $\bar{m}_n$, $\bar{m}_w$, and cmc (defined as the value of $X_1 + \sum X_m$ where $\sum X_m$ is 5% X_1) were compared to experimental data. The adjustable parameters C, E, and F of eq 1 were changed and the calculations iterated until a best fit of the calculated values and experimental data was obtained.

Micelle II

The existence of a second micellar species was incorporated into the model by assuming that a "phase transition" occurs when the aggregate reaches a critical size (m_c). A phase transition in this context simply represents a reduction in $\Delta G°_m$. There are no data which specify the source of this reduction: it may result from an intermolecular chain–chain interaction or a change in the polar head group interaction (either reduction of head-group repulsion or establishment of head-group attraction). Precedence for such a phase transition is clearly found in monolayer studies at both the oil–water (Taylor et al., 1973; Llerenas & Mingins, 1976) and air–water interfaces (Cohen et al., 1976). For aggregates above size m_c, we assume that growth occurs by lengthening without change in the cross-sectional area normal to the long axis. The growth of the micelle can be envisaged as occurring in two stages; first the hemispherical or hemiellipsoidal ends are formed and then at some critical size, m_c, further growth occurs by addition to one axis of the micelle. There is some evidence to support this type of geometry: the light-scattering data of Tausk et al. (1974c) suggest that the DiC_8 micelle may resemble a random coil.

Israelachvili et al. (1976) have developed the necessary thermodynamic treatment for this geometry by replacing $\Delta G°_m/RT$ in eq 1 with $\Delta G°_\infty/RT - \alpha/m$ to give

$$X_m = m[X_1 \exp(\Delta G°_\infty/RT - \alpha/m)]^m \qquad (4)$$

where α is a constant characteristic of the amphiphile and $\Delta G°_\infty$ is the free energy of transfer of an amphiphile to a micelle as m approaches infinity. The mole fraction of aggregate, $\sum X_m$, can be calculated from

$$\sum X_m = \frac{m_c Y^{m_c} e^{-\alpha}}{1 - Y}\left[1 + \frac{Y}{m_c(1 - Y)}\right] \qquad (5)$$

and the average aggregation number, $\bar{m}$, can be calculated from

$$\bar{m} = m_c + \frac{Y}{1 - Y}\left[1 + \frac{1}{m_c(1 - Y) + Y}\right] \qquad (6)$$

(Israelachvili et al., 1976), where Y is $X_1 \exp(\Delta G°_\infty/RT)$. Use of eq 5 and 6 allows calculation of the aggregate concentration and $\bar{m}$ for given values of m_c, α, and Y without requiring numercial integration.

The dependence of $\ln X_m$ on m as described by eq 4 is quite broad. The range of Y values that pertain to a particular amphiphile is determined by the value of $\Delta G°_\infty$ and the monomer concentration (X_1) range which is physically achievable. For example, the approximate Y values at the cmc are 0.88 for DiC_6, 0.90 for DiC_7, and 0.98 for DiC_8.

In calculating the contributions of micelles I and II at a given monomer concentration, we used eq 1 with $\Delta G°_m$ established by eq 2 for aggregation numbers up to m_c and the summation of these mole fractions ($\sum_{m=2}^{m_c} X_m$) to define the contribution of micelle I. Above aggregate size m_c, eq 5 was used to calculate the concentration ($\sum_{m=m_c}^{\infty} X_m$) of the second type of aggregate (micelle II) in equilibrium with the given concentration of monomer. The values of α, m_c, and $\Delta G°_\infty$ were then adjusted to achieve a best fit to experimental data over the range of total substrate concentration employed.

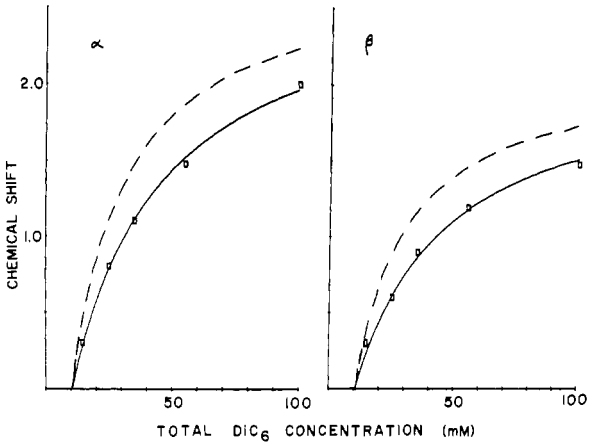

FIGURE 1: ^{13}C NMR chemical shifts as a function of the concentration of dihexanoylphosphatidylcholine (DiC_6). The data of Schmidt et al. (1977) for ^{13}C-enriched DiC_6 at either the α- or β-carbonyl have been replotted with the chemical shift measured with respect to that of the pure monomer (open squares). The dashed lines represent the predicted dependence of the chemical shift on concentration by using eq 8 with cmc = 10.5 mM and $\Delta\alpha_M = 2.78$ or $\Delta\beta_M = 2.17$. The solid lines represent the predicted dependence of the chemical shift on concentration by using eq 7 with cmc = 10.5 mM, K = 36.7 mM, and $\Delta\alpha_M = 2.78$ or $\Delta\beta_M = 2.17$.

Analysis of NMR Data

^1H nuclear magnetic resonance studies of aggregation of DiC_6 and DiC_7 in aqueous solution (Hershberg et al., 1976) suggest the existence of two micellar species at high concentrations. NMR spectra for solutions of DiC_6 and DiC_7 at concentrations up to 4 times the respective cmc's indicate a rapid exchange of amphiphile between the monomeric and micellar states since the α-methylene protons of the carboxylic acid side chains demonstrate a single averaged multiplet spectrum made up of two partially overlapping triplets. At approximately 4 times the cmc, the appearance of an additional splitting of the α-methylene signals indicates the existence of two magnetically distinct environments in the aggregated state. The magnitude of the splitting indicates that the individual molecules are in slow exchange between the two environments.

Using DiC_6 specifically ^{13}C enriched at either the α- or β-carbonyl, Schmidt et al. (1977) have measured the ^{13}C nuclear magnetic resonance chemical shifts as a function of concentration in aqueous solutions. These data are replotted in Figure 1 in which the shift is measured with respect to the pure monomer resonance. The shifts of both the α- and β-carbonyl resonances ($\Delta\alpha$ and $\Delta\beta$, respectively) describe hyperbolic curves. This is the expected behavior for the chemical shift of nuclei in fast exchange between two environments, e.g., monomer and micelle (Hershberg et al., 1976).

Since the chemical shift data appear to be hyperbolic, it should be possible to fit them with an equation of the form

$$\Delta\alpha = \frac{S}{K + S}\Delta\alpha_M \qquad (7)$$

where $S = S_{total}$ – cmc represents the concentration of micellar lipid, K is an arbitrary constant, $\Delta\alpha$ is the observed shift, and $\Delta\alpha_M$ the shift for pure aggregate (a similar equation applies to $\Delta\beta$).

With cmc = 10.5 mM and $\Delta\alpha_M = 2.78$ or $\Delta\beta_M = 2.17$, the solid lines in Figure 1 are calculated with K = 36.7 mM. The root mean square error (RMSE) for the fit to the α-carbonyl

data was 0.037, and the correlation coefficient for the fit of the linear transformation of eq 7 was 0.9959. For the β-carbonyl data the RMSE was 0.059 and the correlation coefficient was 0.9552. The RMSE was increased approximately 10% when K was changed by ±0.5 mM or cmc was changed by ±0.2 mM. The only meaning we ascribe to K is that it can be used to define the total concentration of DiC_6 at which $\Delta\alpha = (1/2)\Delta\alpha_M$, i.e., 47.3 mM. It is significant that this concentration is approximately 4 times the cmc and corresponds to the concentration at which Hershberg et al. (1976) noted the appearance of additional ^1H NMR splittings of the α-methylene signals. If DiC_6 were present in only two environments (monomer and a single aggregate form), eq 7 would become

$$\Delta\alpha = \frac{S}{\text{cmc} + S}\Delta\alpha_M \tag{8}$$

where $S/(\text{cmc} + S)$ is the mole fraction of aggregate. Then $\Delta\alpha$ should equal $(1/2)\Delta\alpha_M$ when S = cmc, i.e., at a total concentration of approximately 21 mM. This clearly does not agree with the experimental data.

We interpret these results to indicate that above the cmc DiC_8 can exist in two micellar forms, micelle I (MI) and micelle II (MII). ^1H NMR detects the presence of both micelles; however, ^{13}C NMR detects primarily MII; i.e., the ^{13}C shift of MII is large compared to the ^{13}C shift of MI. As a first approximation, the entire ^{13}C shift may be ascribed to MII; and the fraction of micellar lipid present as MII can be calculated from

$$\frac{(\text{MII})}{S} = \frac{\Delta\alpha}{\Delta\alpha_M} = \frac{S}{K + S} \tag{9}$$

or the concentration of MII can be calculated from

$$(\text{MII}) = \frac{S^2}{K + S} \tag{10}$$

and the concentration of MI can be calculated from

$$(\text{MI}) = S - (\text{MII}) \tag{11}$$

It should be noted that the ratio of the concentration of MI to MII is given by

$$(\text{MI})/(\text{MII}) = K/S \tag{12}$$

which indicates that a simple equilibrium between MI and MII cannot adequately describe the situation.

Figure 2 shows the calculated concentrations of micelles I and II as a function of the total DiC_6 concentration by using eq 10 and 11 and assuming that cmc = 10.5 mM and K = 36.7 mM. A formal limitation to this analysis is that it assumes that the monomer concentration remains constant at 10.5 mM, i.e., it is a phase-separation model. This assumption is not entirely correct; however, the inaccuracy generated by its use is not greater than that reflected by the data themselves.

Analysis of Kinetic Data

The simplest approach to the analysis of the kinetics of hydrolysis of micellar substrates is to assume that the solution contains two substrates for the enzyme, monomer and micelle, each characterized by its own K_m and V_{max}. The appropriate equation is (Wells, 1974a)

$$v_t = \frac{V_{max}^{mon}[(\text{mon})/K_m^{mon}] + V_{max}^{agg}[(\text{agg})/K_m^{agg}]}{1 + [(\text{mon})/K_m^{mon}] + [(\text{agg})/K_m^{agg}]} \tag{13}$$

where v_t is the total reaction velocity, V_{max}^{mon} and K_m^{mon} are the kinetic constants for the monomer substrate, and V_{max}^{agg} and K_m^{agg} are the kinetic constants for the micellar substrate.

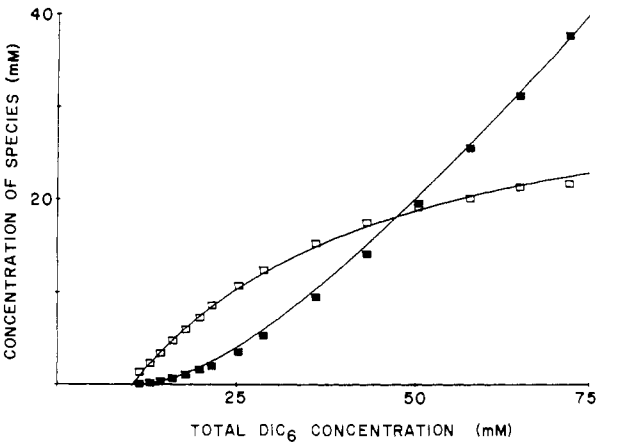

FIGURE 2: Concentration of micelle I (I, open squares) and micelle II (II, closed squares) as a function of total DiC_6 concentration. The lines were calculated according to eq 10 and 11 by using cmc = 10.5 mM and K = 36.7 mM. The concentration of micelle I depicted by the open squares was calculated by using eq 1 and 2. The concentration of micelle II depicted by the closed squares was calculated by using eq 5.

V_{max}^{mon} and K_m^{mon} were obtained by fitting the kinetic data below 10 mM to a simple one-substrate kinetic equation by the method of Eisenthal & Cornish-Bowden (1974). In the present case at 25 °C, pH 8.0, and 1 mM $CaCl_2$, the results were K_m^{mon} = 4.2 mM and V_{max}^{mon} = 25 (μequiv/min)/mg. Initial estimates for V_{max}^{agg} were obtained by using the data at high substrate concentration (>25 mM). Using a phase-separation model in which (mon) = cmc when the substrate concentration is greater than the cmc and (agg) = S_t − cmc and varying K_m^{agg} and V_{max}^{agg}, we obtained the data shown in Table II. Although a reasonable fit to the monomer region and the high concentration region (>35 mM) can be achieved, the fit between 10 and 35 mM was poor. (This defines the "anomalous" region.) The fit could not be improved without allowing both K_m^{agg} and V_{max}^{agg} to depend in an arbitrary manner on substrate concentration.

We next analyze the kinetic data in terms of the two-micelle model suggested from the NMR studies. In this case eq 13 is expanded to

$$v_t = \frac{V_{max}^{mon}\frac{(\text{mon})}{K_m^{mon}} + V_{max}^{MI}\frac{(\text{MI})}{K_m^{MI}} + V_{max}^{MII}\frac{(\text{MII})}{K_m^{MII}}}{1 + \frac{(\text{mon})}{K_m^{mon}} + \frac{(\text{MI})}{K_m^{MI}} + \frac{(\text{MII})}{K_m^{MII}}} \tag{14}$$

Using the constants for the monomer described above and assuming a phase-separation model, we varied K_m and V_{max} for MI and MII in order to achieve the best fit (Figure 4). The constants used to achieve this fit were K_m^{mon} = 4.2 mM, V_{max}^{mon} = 25 (μequiv/min)/mg; K_m^{MI} = 3.25 mM, V_{max}^{MI} = 25 (μequiv/min)/mg; K_m^{MII} = 3.30 mM, V_{max}^{MII} = 13 950 (μequiv/min)/mg. The root mean square error for the 15 data points above cmc was 98.1. The calculated and experimental values are given in Table I in order to better judge the fit. A 5% increase in the error was observed with the following changes: V_{max}^{MII} ± 100 (μequiv/min)/mg; K_m^{MI} ± 0.25 mM; K_m^{MII} ± 0.25 mM. Allowing V_{max}^{MI} to vary from 0–150 (μequiv/min)/mg did not affect the fit. The important conclusion to be drawn from these calculations is that the bulk of the rate acceleration observed above the cmc can be ascribed to the same micellar species which is responsible for the ^{13}C chemical shift, namely, MII. The sigmoidal nature of the velocity vs. substrate concentration curve results from both

Table II: Observed and Calculated Velocities for the Hydrolysis of Dihexanoylphosphatidylcholine by *C. adamanteus* Phospholipase A_2

substrate (mM)	obsd velocity[a]	calcd velocity[a] single-micelle model[b]	^{13}C NMR analysis[c]	theoretical model[d]
1.4	6.1	6.3	6.3	6.3
2.9	10.3	10.2	10.2	10.2
4.3	12.5	12.7	12.7	12.7
5.8	14.5	14.5	14.5	14.5
7.2	15.7	15.8	15.8	15.8
8.6	16.9	16.8	16.8	16.8
10.0	22.0	17.6	17.6	17.6
11.5	117	236	47	88
13.0	180	552	159	229
14.4	355	835	353	398
16.1	595	1165	620	662
17.9	900	1498	929	968
19.8	1425	1834	1272	1308
21.5	1520	2120	1584	1486
25.2	2015	2705	2239	2182
28.7	2810	3213	2831	2824
35.9	4055	4140	3918	3946
43.0	4800	4927	4822	4823
50.2	5600	5621	5617	5610
57.4	6400	6229	6262	6336
64.5	6850	6760	6863	6810
71.7	7215	7240	7315	7332

[a] Micromoles per minute per milligram at pH 8.0, 1 mM Ca^{2+}, and 25.0 °C. [b] Using eq 13; $K_m^{mon} = 4.2$ mM, $V_{max}^{mon} = 25$ (μmol/min)/mg; $K_m^{agg} = 20$ mM, $V_{max}^{agg} = 15\,500$ (μmol/min)/mg. [c] Using eq 14: $K_m^{mon} = 4.2$ mM, $V_{max}^{mon} = 25$ (μmol/min)/mg; $K_m^{MI} = 3.25$ mM, $V_{max}^{MI} = 25$ (μmol/min)/mg; $K_m^{MII} = 3.30$ mM, $V_{max}^{MII} = 13\,950$ (μmol/min)/mg. [d] Using eq 14: $K_m^{mon} = 4.2$ mM, $V_{max}^{mon} = 25$ (μmol/min)/mg; $K_m^{MI} = 3.0$ mM, $V_{max}^{MI} = 25$ (μmol/min)/mg; $K_m^{MII} = 2.25$ mM, $V_{max}^{MII} = 12\,050$ (μmol/min)/mg.

the large V_{max} of the MII substrate and the enzyme binding to MI. In fact, the fit in the 10–35 mM range depends critically on the value of K_m^{MI}.

Evaluation of the Theoretical Model

The initial model was based on an adaptation of Tanford's approach (eq 1–3). For DiC_6 aggregates the experimental values chosen for fitting were $\bar{m}_w$ of 34–37 for an aggregate concentration of 0–25 mM (Tausk et al., 1974b) and a cmc of 10.5 mM, the value calculated from the ^{13}C NMR data (Schmidt et al., 1977). The cmc for DiC_6 has been variously reported from 7.5 to 14.6 mM (Roholt & Schamowitz, 1961; Pieterson, 1973; Wells, 1974a,b; Tausk et al., 1974b). Since the data generated from the theoretical model are to be compared to equivalent data from ^{13}C NMR, we choose this value for the cmc. Excellent agreement with these values of the cmc and $\bar{m}_w$ was obtained by using the following values for the parameters of eq 2: $A = 3478$; $B = 1216$; $C = 28.5$; $D = 94.6$; $F = 32\,143$. A clear preference for oblate ellipsoids was demonstrated at all m values. The distribution of aggregate sizes was narrow and did not change dramatically with increasing concentration above the cmc.

Having achieved agreement between the observed DiC_6 values of cmc and $\bar{m}_w$ and the predicted values of the theoretical model, we examined the calculated concentrations and specific physical parameters of the aggregate (A_{Hm}, A_{Rm}, $\bar{m}_n$, and $\bar{m}_w$) to determine if a correlation could be established between the calculated change in a given parameter and the change in velocity demonstrated with phospholipase A_2 between 10 and 35 mM total substrate concentration. Neither the aggregate concentration nor the physical parameters of the aggregate exhibited a change which could be related to the DiC_6–PLA_2 kinetic results, and calculation of the velocity of the reaction as a function of total substrate concentration using eq 13 gave results essentially indistinguishable from the phase-separation model when the same values for the kinetic constants were used.

In addition to the fact that the above model could not explain the DiC_6–PLA_2 kinetic data, additional observations indicated that this model was too simple to account for the thermodynamics of short-chain lecithin aggregation.

First, when calculations for DiC_7 aggregation were attempted, using the same values for the adjustable parameters of eq 2 as were used for DiC_6 above, the calculated cmc agreed with the experimental cmc, but the dependence of $\bar{m}_w/\bar{m}_n$ on aggregate concentration did not agree with the experimental data (Tausk et al., 1974b). The calculated values of $\bar{m}_w/\bar{m}_n$ were very close to 1.02 over the entire concentration range, whereas the experimental values range from 1.49 to 2.12. Alteration of the adjustable parameters did not produce the observed heterogeneous distribution. Tanford (1974a) generated $\bar{m}_w/\bar{m}_n$ values less than 1.10 and 1.26 for oblate and prolate ellipsoids, respectively. Nagarajan & Ruckenstein (1977) generated values less than 1.03 with their statistical thermodynamic treatment.

In addition, none of the physical parameters of the aggregates could be used to explain the existence of the third molecular environment detected by ^{13}C and ^1H NMR studies.

The second form of the model incorporated a second micellar species (micelle II) which arose from the first (micelle I) through a phase transition. It was assumed that the transition resulted from a change in the polar head group interaction which in effect reduced the head-group repulsion. For purposes of calculation, this was incorporated into the model by assuming that the values of E and F in eq 2 changed (becoming E' and F') at some $m = m_c$, which had the effect of reducing $\Delta G°_m$ at all values of m equal to and greater than m_c. This essentially produces a bimodal micellar distribution; i.e., two discrete micelle populations exist above the cmc. The details of this approach have been published (Allgyer & Wells, 1978).

Assuming that micelle II was responsible for the high velocities observed above the cmc, it was possible to fit the PLA_2–DiC_6 data adequately. The bimodality produced in the aggregate distributions of this model was consistent with the ^1H NMR data of Hershberg et al. (1976) and the ^{13}C NMR data of Schmidt et al. (1977) and provided the heterogeneity required by the DiC_7 physical data. However, this model was judged unsatisfactory because it could not adequately predict the properties of DiC_8 micelles.

Tausk et al. (1974c) have characterized the association of DiC_8 at room temperature in 0.2 M LiI (since DiC_8 is not soluble in water at room temperature). They reported that the weight-average micellar weight increases strongly with the concentration ($\bar{m}_w$ changes from 500 to 5000 over a concentration range of cmc to 20 mM). Also, this strong dependence of aggregation number on concentration implies large polydispersity of aggregate size at a given concentration. Attempts to reproduce the observed DiC_8 $\bar{m}_w$ values by adjusting m_c, E', and F' were totally unsuccessful.

In the final form of the model the second micellar species still arises from micelle I by a phase transition, but its behavior is defined by a thermodynamic formulation attributable to Israelachvili et al. (1976) rather than that of Tanford which applies to micelle I.

In applying this model to DiC_6, we used the criteria that it should be able to fit the PLA_2 kinetic data and that the

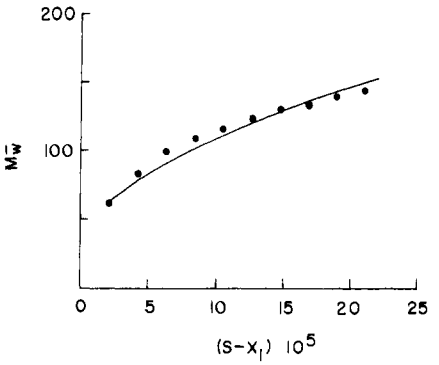

FIGURE 3: Weight-average aggregation number ($\bar{m}_w$) of diheptanoyllecithin (DiC_7) as a function of aggregate mole fraction ($S - X_1$). The closed circles are the experimental values of Tausk et al. (1974b), and the line represents the calculated values by using eq 14.

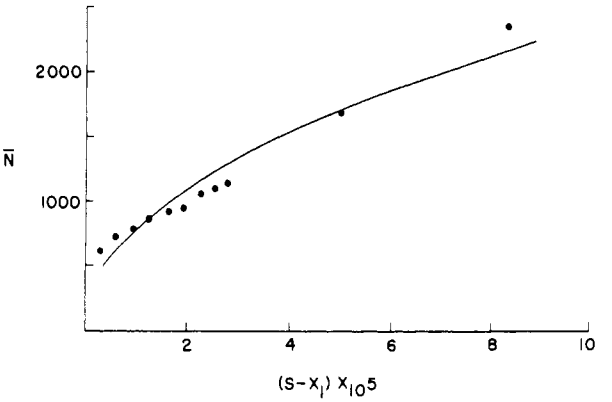

FIGURE 4: Average aggregation number ($\bar{N}$) of dioctanoyllecithin as a function of aggregate mole fraction ($S - X_1$). The solid circles are the experimental values of Tausk et al. (1974c), and the line represents the calculated values by using eq 6 and assuming $\bar{m}_w \approx \bar{N}$.

calculated concentration of micelle II should agree with the calculations made from the ^{13}C NMR data. The best fit was achieved with values of $\Delta G°_\infty/RT = 8.49$, $\alpha = 15.32$, and $m_c = 53$. Using eq 14 and the kinetic constants $K_m^{mon} = 4.2$ mM, $V_{max}^{mon} = 25$ (μequiv/min)/mg, $K_m^{MI} = 3.0$ mM, $V_{max}^{MI} = 25$ (μequiv/min)/mg, $K_m^{MII} = 2.25$ mM, and $V_{max}^{MII} = 12050$ (μequiv/min)/mg, we obtained the values shown in Table II. The RMSE was 79.3, which indicates a somewhat better fit than that achieved with the ^{13}C NMR treatment. The calculated concentrations of micelle I and II by using the theoretical model are compared to the corresponding calculations from ^{13}C data in Figure 2. The RMSE for the concentrations used in fitting the kinetic data were 0.35 for MI and 0.59 for MII.

An attempt was made to fit the DiC_7 aggregate weight data of Tausk et al. (1974b) by using

$$\bar{m}_w \simeq \frac{\bar{m}_w \sum_{m=2}^{m_c} X_m + \bar{m} \sum_{m=m_c}^{\infty} X_m}{\sum_{m=2}^{\infty} X_m} \qquad (15)$$

to approximate the average aggregate weight. Good agreement between the calculated values (solid line in Figure 3) and the experimental values (solid circles) was achieved with $\alpha = 16.50$, $\Delta G°/RT = 10.44$, and $m_c = 60$.

The DiC_8 aggregate weight data of Tausk et al. (1974c) were fit by assuming that micelle I makes a negligible contribution to the aggregate population (micelle II becomes the predominant species at approximately 4 times the cmc of 0.018 mM) and that $\bar{m}_w$ is approximated by $\bar{m}$. Good agreement between the calculated values (solid line in Figure 4) and the experimental values (solid circles) was achieved with $\alpha = 23.40$, $\Delta G°_\infty/RT = 12.31$, and $m_c = 100$.

The predictions of the final form of the model are reasonably consistent with all these sets of data, although there is disagreement in some details. In particular, the cmc for DiC_6 is lower than that reported by Tausk et al. (1974a), and the molecular weight predictions, while indicating the correct trend, do deviate significantly from the experimental data. However, considering the general lack of agreement between experimental data from different investigators and the rather nonideal behavior of lecithin solutions at high concentrations, which is not explicitly considered in the model, we find that the overall agreement between calculated and experimental data is quite satisfactory.

Discussion

In order to give some perspective to this work it is worth pointing out some of the difficulties encountered in developing the model. All previous theoretical studies on soluble micellar systems, as opposed to bilayers of long-chain phospholipids, had been carried out on single-chain amphiphiles with quite satisfactory results (Tanford, 1973; Israelachvili et al., 1976). In these cases aggregation numbers do not increase strongly with length of the alkyl chain and micelle shapes do not deviate strongly from spherical. In contrast, the aggregation numbers for diacylphosphatidylcholines increase strongly with chain lengths and the shapes deviate considerably from spherical. Because of the uncertain nature of the shapes of the lecithin micelles, the values of the constants used to develop the model must also be considered as only first approximations. The important result of this study is that equations were developed which predict satisfactorily the properties of the lecithin micelles. We make no claim that these represent a unique set of equations and constants; however, the results do make testable predictions which could be used to further refine the approach.

The most important conclusions to be drawn from this study are that two types of micelles exist in solutions of short-chain lecithins above the cmc and that, in the case of *Crotalus adamanteus* PLA_2 hydrolysis of DiC_6, the rate enhancement observed above the cmc is totally attributable to the appearance of the second type of micelle. Since the model was developed by combining two prototype micelles, i.e., DiC_6 near the cmc represents micelle I and DiC_8 represents micelle II, it is interesting to note that the conclusion that DiC_6 micelle II is the substrate for phospholipase A_2 is in accord with the general observation that DiC_8 is the best substrate for this enzyme (de Haas et al., 1971; Wells, 1974a). Furthermore, V_{max} for hydrolysis of DiC_6 micelle II [(12–14) $\times 10^3$ (μequiv/min)/mg] compares favorably to values for DiC_8 under a variety of conditions [(5–15) $\times 10^3$ (μequiv/min)/mg] (Misiorowski & Wells, 1973; Wells, 1974a). That the DiC_6 micelle II and DiC_8 have approximately the same kinetic constants for hydrolysis by phospholipase A_2 suggests the optimal interfacial structure is approximately independent of chain length. In fact, a comparison of the apparent first-order rate constants (V_{max}/K_m) for hydrolysis of DiC_6 micelle II [(2–3) $\times 10^6$ s^{-1} M^{-1}], DiC_8 micelles [(1–9) $\times 10^6$ s^{-1} M^{-1}], DiC_8 monolayers [(2–4) $\times 10^7$ s^{-1} M^{-1} (Shen et al., 1975; Cohen et al., 1976)], and DiC_8 in ether–water (4×10^6 s^{-1} M^{-1}) suggests that the optimal structure may be obtained regardless of the manner in which the interface is created. These observations and the fact the micelle I is a poor substrate for the enzyme lend credence to the suggestion that the conformation of the substrate molecules in the interface is the

important factor in the interfacial activation of phospholipase A_2 (Wells, 1974a).

Since the mechanism of interfacial activation of lypolytic enzymes in general and phospholipases A_2 specifically [e.g., Verger et al. (1973), Wells (1974a), Deems et al. (1975), Roberts et al. (1977), Slotboom et al. (1977), Rietsch et al. (1977), and Wells (1978)] has been a subject of much interest, it would be useful to know the differences in the physical characteristics of lecithin molecules which are elements of a micelle I aggregate (poor substrate) or a micelle II aggregate (excellent substrate). Schmidt et al. (1977) concluded that the ^{13}C shifts observed upon micelle formation can be attributed to a decrease in the hydration of the carbonyls. Since we conclude that the ^{13}C shift of micelle II is much larger than that of micelle I, it appears reasonable to suggest that the difference in kinetic characteristics of micelle I and micelle II is related to dehydration of the carbonyl groups, the site of phospholipase A_2 action. The hypothesis that the extent of hydration of the ester carbonyls might affect the activity of pancreatic lipase has been developed by Brockerhoff (1968) and has received some support from the data of Entressangles & Desnuelle (1974). In the case of phospholipase A_2, reports from this laboratory on the activity of the enzyme in organic solvents (Misiorowski & Wells, 1973, 1974; Poon & Wells, 1974; Wells, 1974b) have also established the importance of the extent of substrate hydration in modulating enzyme activity. A thorough understanding of the nature of the transition from micelle I to micelle II may better define the kinetically critical aspects of the conformation of substrate molecules.

The model we present successfully portrays the physical and kinetic characteristics of the short-chain lecithins as they are presently known, and its development has extended our understanding of both the nature and behavior of aggregates of these phospholipids. There is, however, need for considerable refinement of both the experimental data and the theoretical treatment. More extensive physical data, in particular, detailed ^{13}C NMR studies, would be most helpful. No attempt has been made to address the phenomenon of premicellar aggregation, since essentially no pertinent physical data for this region exist. The current model reflects our ignorance of the behavior of two-chain, short-chain amphiphiles whose polar head group interactions may not be simply repulsive. It consists of two essentially geometric treatments joined by an ill-defined "phase transition". The geometric models, while mathematically convenient, should not be construed to be explicitly accurate. It is our impression that adequate definition of the phase transition and a further understanding of the intermolecular forces within lecithin aggregates should permit eventual development of a single generalized theoretical treatment which would accurately describe the physical properties of all lecithins regardless of the length of the acyl chain.

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