Thermodynamics of Dihexanoylphosphatidylcholine Aggregation†

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ABSTRACT: Heats of dilution of aqueous solutions of dihexanoylphosphatidylcholine were determined by use of a flow microcalorimeter to monitor an exponential dilution gradient. Three different models of micelle formation were tested: monomer in equilibrium with micelles of fixed size, with micelles of varied size, or with small aggregates and micelles. The heat of dilution data for low solute concentration could be fit only by assuming the existence of premicellar aggregates. The critical micelle concentration determined calorimetrically is 0.016 ±0.002 M and is independent of the model. The enthalpy change for transfer of monomer into the micelle is 1.6 ± 0.2 kcal/mol; about one-third of this heat effect is produced in formation of the premicellar aggregation. Comparison of the calorimetric measurements with results obtained by using other methods indicates the complexity of the micellization process.

There have been numerous experimental and theoretical studies on micelle formation by simple amphiphiles. The extension of this work to more complex systems, such as the major membrane components, phospholipids, has been limited. Measurements of the critical micelle concentration and micelle size for a series of short-chain phosphatidylcholines have been reported by Tausk et al. (1974a–c), and some measurements on critical micelle concentrations of longer chain phospholipids have been carried out (Tanford, 1973). The preponderance of these studies have characterized the lamellar structures which spontaneously form in water. Most thermodynamic studies have been concerned with measurement of the gel to liquid-crystalline phase transition or predictions of the size of single-walled liposomes.

Measurements on short-chain phospholipids are important for two reasons: (1) Their relatively high critical micelle concentrations allow the micellization process to be studied by a wide variety of techniques; one expects that this knowledge of the behavior of short-chain compounds can be extended to develop an understanding of the interactions of biologically important phospholipids. (2) The short-chain phospholipids are frequently used as substrates for phospholipases (Wells, 1974), and thus the aggregation properties of these compounds need to be defined.

As part of an attempt to describe the thermodynamics of micelle formation by short-chain phosphatidylcholines and to develop a quantitative model for this process, we report in this paper measurements of the heat of dilution of micellar dihexanoylphosphatidylcholine. The results of these studies provide evidence for the existence of self-association below the critical micelle concentration (premicellar association). Correlation of these data with previous studies indicates that micelle formation by short-chain phosphatidylcholines is a multiequilibrium process.

Experimental Procedures

Materials. The preparation and purification of dihexanoylphosphatidylcholine (DiC_6)[1] and *sn*-glycero-3-phosphorylcholine (GPC) have been described (Wells, 1972; Yabusaki, 1975). All dilutions were made with deionized water. Ans and Dns-glycine were obtained from Eastman Organic Chemicals and Calbiochem, respectively.

Methods. The concentration of DiC_6 or GPC in stock solutions was determined by measuring the phosphorus content (Dittmer & Wells, 1969).

Heat of dilution data were generated continuously through measurement of a sample having an exponential gradient in DiC_6 concentration established, as described by Mountcastle et al. (1976), by use of an open vessel with an effective volume of about 6 mL. The gradient had a half-time of about 18 min. Solution was pumped continuously from the gradient vessel into an LKB flow microcalorimeter where it was diluted approximately 3:2 with water (see figure legends for dilution factors). Immediately after each experiment, the dilution system was calibrated by mixing solution from an exponential gradient in HCl with excess NaOH in the calorimeter. The HCl dilution data could be fit to a single exponential function with an error of less than 1% for all HCl concentrations.

Some dilution experiments were done without use of a gradient system, i.e., discontinuously, by mixing DiC_6 solutions of fixed concentration with water in an LKB flow microcalorimeter or in an LKB batch microcalorimeter.

All experiments were carried out a 25 °C. Calorimeters were calibrated by use of either the internal heater of the calorimeter or the heat of dilution of sucrose (Gucker et al., 1939). Flow rates were determined by weighing the amount of water pumped in 1 h. The calorimeter output was amplified by using a Keithley Model 150B instrument and was monitored by using a Sargent recorder.

For experiments using a DiC_6 concentration gradient, the calorimeter voltage signal was sampled with a Newport Model 2000A A-D converter and recorded every 5.31 s on paper tape. Approximately 1500 data points were collected during an experiment. The data were corrected for the response time of the calorimeter (Mountcastle et al., 1976) and then converted, using the initial DiC_6 concentration and the time constant for the exponential gradient determined from the HCl dilution experiment, to an array of DiC_6 concentrations and corresponding heats of dilution. For analysis of the data, approximately 100 eight-point averages were extracted from the full data set; these were selected to be as closely as possible evenly spaced in concentration.

Measurements of the cmc were made by using the change in fluorescence associated with dye binding, as previously described (Wells, 1974).

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[1] Abbreviations used: DiC_6, dihexanoylphosphatidylcholine; cmc, critical micelle concentration; Ans, 8-anilinonaphthalene-1-sulfonic acid; Dns (dansyl), 5-(dimethylamino)naphthalene-1-sulfonyl.

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Data Analysis. Three different models were tested for their fit to the heat of dilution data for DiC_6.

In the *phase-separation* model,[2] n monomers, M, associate to form a micelle, M_n (Tanford, 1973)

$$K_n = (M_n)/(M)^n \quad (1)$$

$$(M_t) = (M) + n(M_n) \quad (2)$$

where K_n is the association constant for the process

$$nM \rightleftharpoons M_n$$

(M_t) is the total concentration of amphiphile, (M) is the concentration of monomers, and (M_n) is the concentration of micelles.

The *premicellar-association* model is an extension of the phase-separation model; it allows for the existence of a small aggregate containing m monomers, M_m, and also of a micelle containing n of these small aggregates, M_{mn}

$$K_n = (M_{mn})/[K_m(M)^m]^n \quad (3)$$

$$(M_t) = (M) + m(M_m) + nm(M_{mn}) \quad (4)$$

where K_m is the association constant for the premicellization process

$$mM \rightleftharpoons M_m$$

and is defined as in eq 1, K_n is the association constant for the micellization process

$$nM_m \rightleftharpoons M_{mn}$$

(M_m) is the concentration of small aggregates, and (M_{mn}) is the concentration of micelles. For both the phase-separation and premicellar-association models, the number of monomers in a micelle was 36 (Tausk et al., 1974b).

The third model is that proposed by Tanford (1973), which allows micelles to contain any number of monomers, with the mole fraction of each species of size m being governed by the distribution equation

$$\ln X_m = -m\Delta G^\circ_m/RT + m \ln X_1 + \ln m \quad (5)$$

where X_1 is the mole fraction of monomer and ΔG°_m is the free energy of transfer of a monomer into a micelle of size m. ΔG°_m is given by

$$\Delta G^\circ_m = A + B(A_{H_m}) + C/A_{R_m} + D/(A_{R_m})^2 \quad (6)$$

where A represents the free energy of transfer of both hexanoyl side chains from water into the micelle, B represents the free energy of the hydrocarbon–water interface, A_{H_m} represents the area of the hydrocarbon–water interface in a micelle of size m, and C and D are constants which describe the polar head-group repulsion as a function of the area occupied by the polar head group, A_{R_m}, in a micelle of size m. For the calculations given here, it was assumed that the micelles are oblate ellipsoids. Allgyer & Wells (1978, 1979) give further details concerning these calculations for short-chain phosphatidylcholines.

The calorimetric heat of dilution, q_{obsd}, can be compared to the calculated value of the heat effect, q_{calcd}. For the phase-separation model

$$q_{calcd} = \Delta H[(M)_{final}f_{out} - (M)_{initial}f_{in}] \quad (7)$$

where (M) is the monomer concentration (mol/L) before or after dilution in the calorimeter, f is the flow rate (μL/s) into or out of the calorimeter, q_{calcd} is the heat flow (μcal/s), and ΔH is the enthalpy (cal/mol) of transferring monomer into a micelle. Equation 7 holds also for the Tanford model, for which it was usually assumed that ΔH is independent of micelle size. For the premicellar-aggregation model, eq 7 must be modified to allow different values of ΔH for insertion of monomer into small aggregate and into micelle. The three models were fit to the experimental data by using a Simplex-based search for the least-squares minimum (Nelder & Mead, 1965). For all three models, ΔH was treated as a variable parameter. Other variable parameters were the equilibrium constants in the phase-separation and premicellar-aggregation models and the constants A, B, C, and D of eq 6 for the Tanford model. The constants A_{H_m} and A_{R_m} were calculated according to Tanford (1973). For cases where the monomer concentration could not be given as an explicit function of the total concentration and the variable parameters, it was determined for the current choice of parameters by using an approximation routine. The conversion of mole fraction to molarity was done by using the value 400 mL/mol for the partial molar volume of DiC_6 (Tausk et al., 1974b).

The heat of dilution data are presented as the signal, q (μcal/s), divided by the flow rate, f (μL/s), to give the heat produced per unit volume of starting solution. The data from the flow calorimeter are thus presented in a form comparable to data from batch calorimetric experiments. For a discussion of the function q/f, see Johnson et al. (1979).

The critical micelle concentration (cmc) was defined as the concentration of total lipid at which 5% of the lipid is present in the form of micelles (Tanford, 1973).

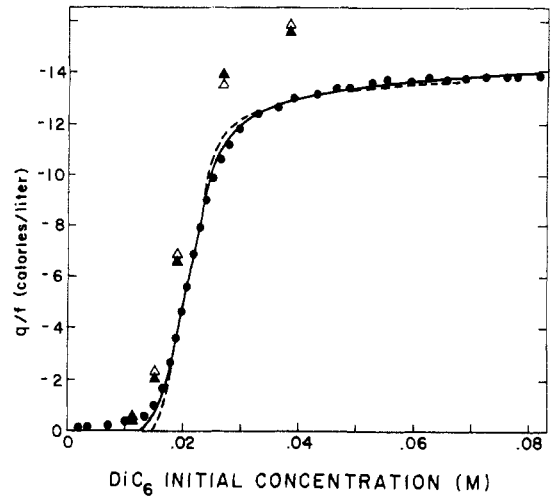

FIGURE 1: Heat of dilution of solutions of DiC_6 into H_2O at 25 °C. (●) Eight-point averages of dilution gradient data; initial $[DiC_6]$ = 0.0876 M; dilution factor = 0.63. (▲) Heats of dilution of DiC_6 solutions of fixed concentration measured by using a flow calorimeter; dilution factor = 0.59. (△) Heats of dilution of the same DiC_6 solutions measured by using a batch calorimeter; dilution factor = 0.59. The heavy curve represents the best least-squares fit of the Tanford model to the dilution gradient data; the dashed line represents the best fit of the phase-separation model, assuming n = 36; see Table I for parameter values.

Results and Discussion

Figure 1 shows data from a single exponential dilution experiment, in which the heat of dilution of DiC_6 was measured as a function of a continuously varied concentration. The dashed line is the best least-squares fit of the phase-separation model, assuming that a micelle contains 36 monomers. The parameter values are $K_a = 1.35 \times 10^{60}$ and $\Delta H = 1.37$ kcal/mol of monomer. This value of K_a corresponds to a cmc of 0.0167 M.

[2] The phase-separation model described here has been called the mass-action model (Wennerstrom & Lindman, 1979).

Table I: Thermodynamics of Association of Dihexanoylphosphatidylcholine into Micelles at 25 °C[a]

model	F	ΔH (kcal/mol of monomer)	cmc (mM)	species
phase separation	92	1.37	16.7[b]	1-mer-36-mer
Tanford	34	1.37	16.5[c]	distribution
premicellar association	7	0.55, 1.03	16.6,[d] 10–14.7,[e] 11.7–13.9[f]	1-mer-2-mer, 2-mer-36-mer

[a] F is a measure of the deviation of the fit from the gradient data given in Figure 1, and it is minimized during least-squares fitting procedures. ΔH is the best fit value for the enthalpy of transfer of monomer into the aggregate or micelle. The cmc values were calculated for the best fit equilibrium parameters, given in the footnotes. The error in ΔH, estimated from comparing values for different batches of DiC_6, is ±0.2 kcal/mol. For a given batch, the estimated fractional errors of the cmc and ΔH are less than 0.01. [b] $K_a = 1.35 \times 10^{60}$. [c] Parameters for eq 6: $A = 8079$, $B = 26.5$, $C = 96.3$, $D = 34\,807$. [d] $K_2 = 34.1$; $K_{36} = 2.33 \times 10^{40}$. [e] Literature values; see Allgyer & Wells (1979) for references. [f] Measured values for the preparation used in the calorimetric studies of Figure 1 and for the fitting described in this table.

The solid line in Figure 1 is the best least-squares fit of the Tanford model. This fit, which is slightly better than that of the phase-separation model, gives the same value of ΔH as found for the phase-separation model (see Table I for parameter values). The cmc, 0.0165 M, is approximately the same as for the phase-separation model. Expanding the Tanford model to allow for the existence of two types of micelles (Allgyer & Wells, 1978, 1979), differing in their enthalpy of transfer, does not improve the fit of the model to the data. Allowing for a dependence of ΔH on micelle size also does not significantly improve the fit.

Either model fits most of the data of Figure 1 well, except that neither predicts the heat of dilution seen at low DiC_6 concentration. An expansion of this region is shown in Figure 2. The heavy curve and dashed curve are calculated for the Tanford and phase-separation models, respectively, and are seen to deviate from the data for low concentration (<0.015 M DiC_6) beyond experimental uncertainty.[3]

In order to show that the heat effect at low concentration is not an artifact, several control experiments were done.

(1) The stock DiC_6 solution used to generate the gradient data given in Figure 1 was diluted 3:1 and 6:1, and each of these solutions was used in separate gradient experiments. The low concentration data for each of the three gradient experiments are plotted in Figure 2 to show that the effect is reproducible and not dependent on the concentration of the stock solution used to generate the gradient.

(2) A possible explanation of the heat of dilution seen at low concentration is that it represents some nonspecific electrostatic effect from the interaction of the zwitterionic head groups of the monomers, because the effect is observed for concentrations below the cmc. Due to the square-root dependence of the Debye–Hückel limiting law, such an effect could persist to a relatively low concentration. For examination of this possibility, a gradient experiment was carried out with glycerophosphorylcholine. No heat of dilution could be detected below about 0.02 M GPC. These data are presented in Figure 2.

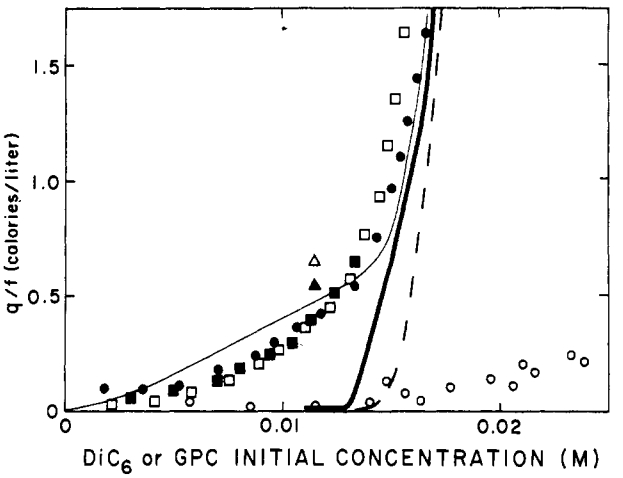

FIGURE 2: Heat of dilution of solutions of low concentration of DiC_6 or GPC into H_2O at 25 °C. The symbols ●, ▲, and △ are defined as in Figure 1. (□) Eight-point averages of data from dilution gradient with initial $[DiC_6] = 0.0292$ M. (■) Eight-point averages of data from dilution gradient with initial $[DiC_6] = 0.0146$ M. (○) Eight-point averages of data from dilution gradient of GPC of initial concentration of 0.144 M. Curves are as in Figure 1, except the thin line represents the best fit of the premicellar-association model to the data; see Table I for parameter values. The premicellar-association model does not deviate significantly from the data at the higher DiC_6 concentrations (not shown in this figure).

(3) In order to test for systematic error in the flow calorimetric results, batch calorimetric measurements of the heat of dilution were compared with flow measurements made without use of a gradient system. It is important to do this because flow measurements do not determine heat effects from slow processes having a characteristic time close to or greater than the residence time in the flow cell (50–100 s at the flow rates used in this work). The data given by the triangle symbols of Figures 1 and 2 show that the heats produced by diluting a set of DiC_6 solutions of fixed concentration in batch and flow calorimeters are in close agreement. This shows that there is no artifact associated with use of a flow system, i.e., that there are no undetected time-dependent heat effects.

The experiments on solutions of fixed DiC_6 concentration were done with a different preparation of DiC_6 than the gradient experiments, and the results differ in two respects: for the fixed concentration experiments, (1) the calculated cmc is slightly lower (0.014 vs. 0.0166 M), and (2) the heats of dilution at high concentration do not level off but increase slightly with increase in concentration. Although all samples were pure by accepted analytical criteria, the presence of minor contaminants cannot be excluded. Small amounts of salts of fatty acids might contribute significantly to the heat of dilution at high concentrations of DiC_6.

The small difference between the results obtained with different DiC_6 preparations does not affect the conclusion that dilute solutions of DiC_6 display a heat of dilution that cannot be explained by micelle formation or electrostatic interaction of the monomer head groups. The simplest explanation of this effect is the existence of small aggregates of DiC_6 at concentrations well below the cmc. The gradient data of Figures 1 and 2 were fit satisfactorily by a premicellar-association

[3] Other models (Allgyer & Wells, 1979; Wennerstrom & Lindman, 1979) that have been proposed allow, like Tanford's model, a distribution of micelle sizes. These models, also like Tanford's, do not predict a sufficient concentration of small aggregates to accommodate the thermal data obtained in this work. The very simple modification of the phase-separation model, in which small oligomers are explicitly included in separate equilibria, explain the data well. Clearly, a separate set of equilibria for formation of small oligomers could be added to the Tanford or another relatively sophisticated multiple equilibria model, and the thermal data could be fit well. We believe that it is inappropriate and unnecessary to do this in view of the good fit obtained with the simple model. Furthermore, the thermal parameter of interest, the enthalpy change for insertion of a monomer into a micelle, is the same for the phase-separation and Tanford models (Table I).

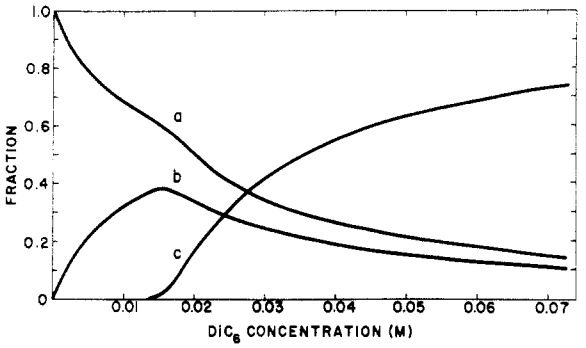

FIGURE 3: Weight fractions of monomer (a), dimer (b), and micelle (c) as a function of total DiC_6 concentration calculated by using the premicellar-association model and the parameters given in Table I.

model with aggregates of size $m = 2$–5, with the best for $m = 2$. Table I compares the best fit for the premicellar-association model with the best fits for the phase-separation and Tanford models. According to the premicellar-association model, about one-third of the enthalpy of transfer of monomer into micelle is associated with dimer formation. The cmc calculated for this model, assuming that the cmc refers to the large micelle, is 0.0166 M. The thin curve of Figure 2 shows that the fit of the dimer–36-mer model to the data is good, i.e., within the limits of experimental uncertainty.

The cmc for the DiC_6 preparation used in the gradient experiments was measured by using the fluorescence change associated with binding of Ans and Dns-glycine, which gave the values 0.0117 and 0.0139 M, respectively. These measurements are in accord with the values reported by Tausk et al. (1974a) and by others [see Allgyer & Wells (1979)]. The cmc determined for this sample from the calorimetric studies is 0.0165–0.0167 M and is independent of the model. Thus the calorimetric cmc is significantly greater than the cmc determined by other methods. As noted above, one batch of DiC_6 gave a cmc of 0.014 M, and in view of this, the cmc defined by calorimetry is 0.016 ± 0.002 M. Even with this cautionary uncertainty, the calorimetric cmc is higher than that measured by other methods.

The enthalpy of transfer of a DiC_6 molecule into the micelle is 1.6 kcal/mol, given by the sum of the enthalpy changes for the two steps of the premicellar-association model (Table I). The uncertainty in this value is estimated as 0.2 kcal/mol from the difference between batches of DiC_6 and the range of values obtained with the several models. The enthalpy of transfer is small and probably represents the balance of several effects of large but compensating enthalpy change. Thus it is difficult to develop an interpretation at a molecular level. There have been several previous calorimetric measurements of micelle formation with molecules other than DiC_6 (e.g., Jones et al., 1971; Kresheck & Hargraves, 1974; Paredes et al., 1976). These studies have found a similar small heat effect (0 ± 2 kcal/mol). Numerous analyses of the temperature dependence of the cmc (Kresheck, 1975) are in general agreement with this range of enthalpy values.

Perhaps the principal conclusion to be drawn from the calorimetric experiments is the existence of premicellar association for a phospholipid. Although premicellar association may be expected to be a general property of amphipathic molecules (Kresheck, 1975), these data represent the first demonstration of it for an analogue of a biologically relevant molecule. The thermodynamic description of solutions of short-chain phosphatidylcholines proposed by Allgyer & Wells (1979) must be extended to include premicellar association.

Figure 3 gives the concentrations of monomer, dimer, and micelle calculated by using the parameters of the premicellar-association model fit to the gradient data (Table I) as a function of the total concentration of DiC_6. The point to be emphasized is that at the cmc the dimer represents about 40% of the total solute and at the highest concentrations of DiC_6 studied the dimer represents more than 10% of the solute. Thus at both low and high DiC_6 concentrations, a small aggregate can make a significant contribution to the properties of the system.

The relationship between the processes observed by heat measurements and by other physical methods is not clear. The proposed transition in micellar form (Allgyer & Wells, 1979) is not observed calorimetrically. The calorimetric results are not in conflict with this suggestion, however, since the transition is understood not to be highly cooperative and the enthalpy change for the transition should be small. The relatively high value of the cmc determined by calorimetry has been noted. This may reflect a contribution of the premicellar aggregate to the properties observed by other methods. In any event, the process of micelle formation by short-chain phosphatidylcholines is complex and will require more complete physical data and more sophisticated theoretical descriptions to be adequately understood.

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