[Chem. Pharm. Bull.
32(4)1498—1502(1984)]

Fluorometric Analysis of the Micelle Formation Process of Surfactants in Aqueous Solution. II. Interaction of Cholate Molecules during Premicelle Formation

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(Received July 11, 1983)

The nature of the premicellar state of sodium cholate formed at the early stages of the micelle formation process has been analyzed using pyrene as a fluorescent probe. Formation of the premicellar aggregates of cholate is dependent on a diffusion-controlled process, and stabilization of the premicellar aggregates of cholate molecules appears to require both hydrophobic and electrostatic interactions. In addition, it has been demonstrated that there is a close relationship between the efficiency of the premicellar aggregate formation and the value of the critical micelle concentration of cholate. From these observations, it is suggested that the premicellar aggregates serve as nuclei in the micelle formation process.

Keywords——pyrene fluorescence; cholate; surfactant micelle; premicellar aggregate

It is widely accepted[1] that bile salt micelles play important roles in intestinal digestion, *i.e.* the solubilization and dispersion of dietary lipids. Therefore the process of formation and the structures of bile salt micelles must be of physiological importance.

In our previous papers,[2,3] we have demonstrated the usefulness of pyrene as a probe for analysis of the micelle formation process of all types of detergent as well as the formation of premicellar aggregates of anionic detergents including bile salts at the early stages of their micelle formation process. We have named this confined state of the early stages of micelle formation the "premicellar state."

Small *et al.*[4] and Oakenful and Fisher[5] have also demonstrated by means of conductimetry that cholate and deoxycholate molecules form small aggregates at concentrations far below the critical micelle concentrations (cmc) through hydrophobic interaction[4] and hydrogen bonding[5] among molecules. However, the significance of the formation of premicellar or small aggregates of bile salts in the micelle formation process is not clear at present.

In the present work, we have demonstrated by means of fluorometry using pyrene as a probe that the premicellar aggregates of cholate molecules are stabilized through both hydrophobic and electrostatic interactions, and that the nature of the premicellar state is closely related to the efficiency of complete micelle formation of the molecules.

Materials and Methods

Chemicals——Sodium cholate was purchased from Wako Pure Chemical Co. Pyrene obtained from Wako Pure Chemical Co. was recrystallized from ethanol before use and dissolved in ethanol at a concentration of 1 mM as a stock solution.

Fluorescence Measurements——Fluorescence measurements of pyrene were carried out using a Hitachi MPF-4 spectrofluorometer equipped with a rhodamine B quantum counter at 25 °C. The excimer formation efficiency of pyrene was expressed as the value of the excimer-to-monomer fluorescence ratio, I_E/I_M, which was calculated from the fluorescence intensities at 470 nm (for the excimer) and 392 nm (for the monomer) with excitation at 340 nm.

Determination of cmc——The cmc of cholate was determined from the intersection point of the two curves in the dose–response plot of the pyrene monomer fluorescence calculated by the non-linear least-squares method, as described in our previous paper.[3]

Results and Discussion

Concentration Effect of Sodium Cholate on the Fluorescence Properties of Pyrene

A typical concentration effect of sodium cholate on the fluorescence parameters of pyrene is illustrated in Fig. 1.

The monomer fluorescence at 392 nm of pyrene showed a biphasic response to increasing concentrations of cholate; there was a slight decrease at lower concentrations and a marked increase at higher ones. The curvature of the monomer fluorescence curve changed markedly at approximately 13 mM sodium cholate (confirmed by the non-linear least-squares method for best fitting), which is very close to its cmc value (13—15 mM) reported by other investigators.[6]

On the other hand, the excimer formation efficiency of pyrene expressed as the I_E/I_M ratio was markedly increased at cholate concentrations lower than the cmc. It is known[2,7] that excimer formation efficiency is dependent on the local concentration and also the orientation of the fluorophore. Therefore it is considered that the increase in I_E/I_M ratio of the probe with increasing bile salt concentration may be attributed to alteration in the organization of the surfactant molecules during the micelle formation process in a way that tends to increase the rate of collision and reorientation of the probe in favor of excimer formation. Similar phenomena were observed with other anionic detergents,[2,3] corresponding to the "pre-micellar state" designated in the previous paper.[2]

Effect of Ionic Strength and Divalent Cations

In order to analyze the relationship between the efficiencies of premicelle and micelle formations, we have explored the effect of KCl and divalent cations on premicellar aggregate formation of cholate, because the micelle formation of ionized surfactant is reported to be accelerated by addition of salts.[8]

Figure 2 shows the effect of ionic strength on the relation between excimer formation efficiency of pyrene and cholate concentration. With increasing KCl concentration in the medium, the maximal value of I_E/I_M ratio of the system decreased markedly and the concentration of sodium cholate at the maximum I_E/I_M ratio was lowered. We tentatively

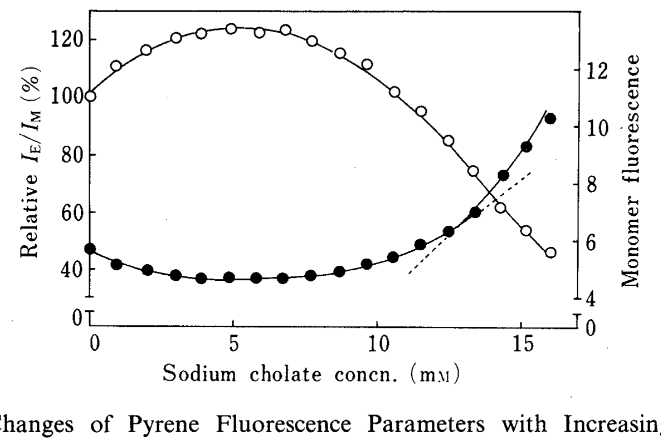

Fig. 1. Changes of Pyrene Fluorescence Parameters with Increasing Cholate Concentration

The concentration of sodium cholate was varied from 1 to 16 mM. The pyrene concentration was 0.5 μM. The I_E/I_M ratio (○) is expressed relative to that in the absence of sodium cholate. The monomer fluorescence intensity (●) is expressed in arbitrary units.

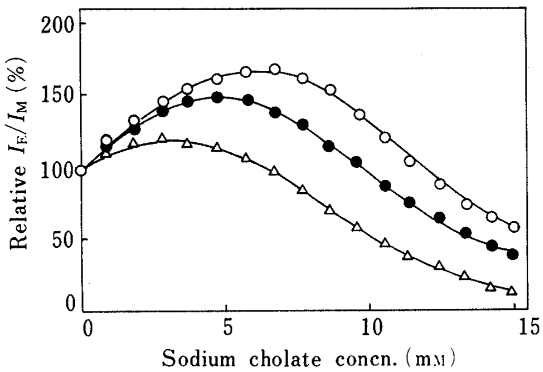

Fig. 2. Effect of KCl on the I_E/I_M Ratio of the Pyrene–Cholate System

Symbols: ○, no KCl; ●, 10 mM KCl; △, 30 mM KCl. Other conditions were the same as described in the legend to Fig. 1.

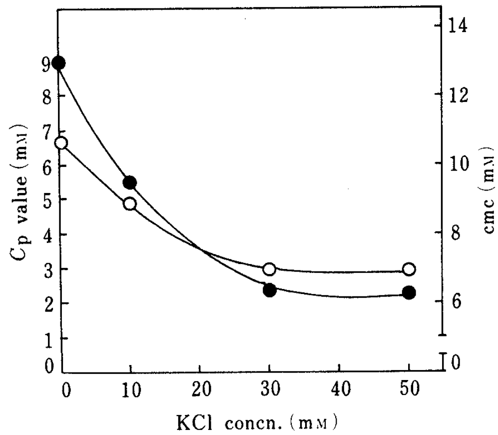

Fig. 3. Changes of the Cp Value and the cmc of Sodium Cholate with Increasing KCl Concentration

Symbols: ○, the Cp value; ●, the cmc value. KCl concentration was varied from 10 to 50 mM. Other conditions were the same as described in the legend to Fig. 1.

Table I. Effect of Divalent Cations on the Cp Value

Divalent cations	Cp (mM)
No addition	5.5—7.0
$CaCl_2$	3.8—4.6
$SrCl_2$	3.8—4.6
$MgCl_2$	4.0—5.5
$MnCl_2$	4.6—5.7

The added concentration of divalent cations was 10 mM. The term Cp is defined in the text. Other experimental conditions were the same as described in the legend to Fig. 1.

designate the surfactant concentration corresponding to the maximum I_E/I_M ratio as Cp, and this value was quantitatively determined from the maximum point in the dose–response curve of the pyrene excimer fluorescence, calculated by the non-linear least-squares method.

The relationship between the changes of the Cp and cmc values of sodium cholate at increasing KCl concentrations is shown in Fig. 3.

The profiles of the changes of these two parameters against increasing ionic strength were almost overlapping, suggesting a close relation between the formation of the premicellar state and the development of complete micelles.

The effect of divalent cations on the formation of premicellar aggregates of sodium cholate is summarized in Table I.

The presence of divalent cations in the system generally caused a considerable decrease of the Cp value or increased the efficiency of producing premicelles. In addition, it seems that cations having an ionic radius of 1.0—1.2 Å (Ca^{2+} and Sr^{2+}) were more effective in premicelle formation than those having an ionic radius of around 0.8 Å (Mg^{2+} and Mn^{2+}). It is clear that the efficiency of premicelle formation of sodium cholate is influenced by addition of divalent cations and by the ionic strength of the medium (Figs. 2 and 3), suggesting the importance of electrostatic interactions between the surfactant molecules for premicellar aggregate formation. Since these cations interact with negatively charged groups of the surfactant molecules, stimulation of premicelle formation by the addition of salts might result from a

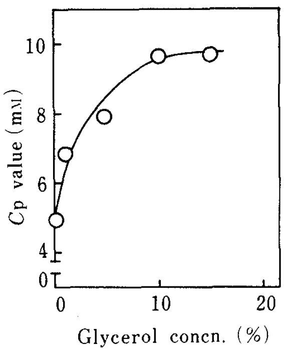

Fig. 4. Effect of Glycerol on the Cp Value of Sodium Cholate

Glycerol concentration was varied from 1 to 15%. Other conditions were the same as described in the legend to Fig. 1.

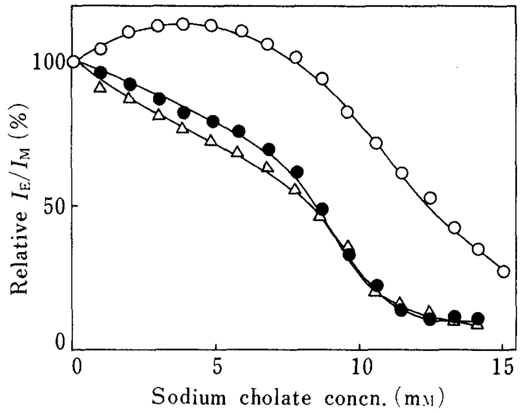

Fig. 5. Effect of Ethanol on the I_E/I_M Ratio of the Pyrene–Cholate System

Ethanol concentration: ○, no addition; ●, 0.1 M; △, 0.3 M. The concentration of sodium cholate was varied from 1 to 15 mM. Other conditions were the same as described in the legend to Fig. 1.

decreased Coulombic repulsion between the head groups of cholate molecules.

Effect of Viscosity

The effect of solvent viscosity on the efficiency of premicelle formation is shown in Fig. 4. The viscosity of the medium was varied by increasing the concentration of glycerol from 1 to 15%. The Cp value increased monotonically with increasing glycerol concentration at lower glycerol concentrations and reached an almost constant level above 10% glycerol. This suggests that the premicelle formation of cholate molecules is controlled by the viscosity around the surfactant molecules and that the efficiency of premicellar aggregate formation is reduced with increasing viscosity of the medium.

Effect of Ethanol on Excimer Formation Efficiency

Becher[9] and Flockhart[10] have demonstrated in their studies of the stability of detergent micelles in ethanol/water mixtures that the presence of ethanol in a detergent system causes disruption of hydrophobic interactions between the solute molecules. Thus, we explored the influence of addition of ethanol on the premicelle formation of sodium cholate.

As shown in Fig. 5, the addition of ethanol markedly reduced the excimer formation efficiency of pyrene over the concentration range of sodium cholate below 15 mM. This result indicates that ethanol interferes with the premicelle formation and that the association of cholate molecules in the premicellar state is somewhat stabilized through hydrophobic interactions at the nonpolar core. However, the premicelle formation efficiency of sodium cholate is also markedly influenced by addition of salts (Figs. 2 and 3 and Table I). From these results, therefore, it seems likely that the aggregates of cholate molecules in the premicellar state are stabilized through both hydrophobic and electrostatic interactions. Formation of hydrogen bonding between the molecules in the premicellar state may be a favorable factor for electrostatic interactions. Oakenful and Fisher[5] have suggested on the basis of conductimetric results that small aggregates of cholate and deoxycholate molecules are stabilized through hydrogen bonding between their hydroxy groups. In addition, Bennet *et al.*[11] have reported that di- and trihydroxy bile salts display self-association through multiple hydrogen bonding.

Our conclusions can be summarized as follows. (1) Formation of the premicellar state of bile salts is dependent on the viscosity around the molecules (*i.e.*, it is a diffusion-controlled process). (2) The structure of premicellar aggregates is stabilized through hydrophobic as well as electrostatic interactions among the molecules. (3) The efficiency of premicelle formation is closely related to that of micelle formation; premicelles probably function as nuclei or their equivalent in the micelle formation process of bile salt molecules.

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