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ORIGINAL CONTRIBUTION

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Solubilization and precipitation of cholesterol in aqueous solution of bile salts and their mixtures

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Abstract Solubilization of cholesterol by mixed micelles of sodium chenodeoxycholate with sodium ursodeoxycholate was investigated in carbonate-tetraborate buffer (Kolthoff) solution at pH 10 and 37 °C. It was found that the mixing of the two bile salts gives a negatively synergetic effect on solubilization of cholesterol. The solubilizing power of bile salts for cholesterol was remarkably influenced with the change in mole fraction of sodium ursodeoxycholate (X_{UDC}).

The behavior of bile salt solutions saturated with cholesterol was examined by measuring the surface tension. Two break points were observed in the curves of surface tension vs. concentration. The break points seem to correspond to a CMC in the absence of solubilized cholesterol and another CMC in the presence of solubilized cholesterol inside bile salt micelle.

Key words Solubilization – surface tension – bile salt – cholesterol – mixed micelle

Introduction

While the physicochemical properties of bile acids and their salts have already been extensively reviewed by a number of researchers [1–4], few fundamental studies using bile salt mixtures have been reported yet. It is noteworthy that the functional molecular aggregates found in nature are composed of two or more kinds of surface-active species. Hence, the study on cholesterol dissolution by bile salt mixtures is important and meaningful for comprehending some functions in biological systems [5].

It is generally said that the formation of cholesterol gallstones proceeds by two steps [6]. In the first step, when the amount of cholesterol exceeds the capacity of bile to solubilize cholesterol, the solubilized cholesterol probably begins to partially precipitate in the form of microcrystals. In the second step, the microcrystals of cholesterol unite and grow to form a macroscopic gallstone. Namely, it is thought that the critical step in the formation of cholesterol gallstone is initiated by nucleation of excess cholesterol. The behavior of the equilibrium cholesterol solubilization into bile salt solutions may be correlated to cholesterol crystal nucleation [7–9]. Here, it would be necessary to clarify the conditions that cause the precipitation of cholesterol.

The most important difference between sodium chenodeoxycholate (NaCDC) and sodium ursodeoxycholate (NaUDC) lies in the orientation of hydroxyl group at the position 7. That is, NaCDC has 7 α OH while NaUDC has 7 β OH. This difference, apparently small, has been known to show a great influence on colloidal properties such as lipids or cholesterol solubilizations [10–12].

In the present study, focusing our attention on the stability of mixed micelles related to the structure difference, we have investigated the solubilization of cholesterol and the surface tension of the mixed micellar solutions with cholesterol.

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Experimental

Materials

Bile salts were purchased from Calbiochem (San Diego, California, USA) and the purity was found to be generally about 96% by thin layer chromatography. Cholesterol monohydrate (ChM) was crystallized from cholesterol (Wako Pure Chem. Ind., Japan) dissolved in 95% EtOH at 60 °C [10]. All solvents used were purified by two or three distillations. All inorganic salts were of analytical grade and used as received from Nacalai Tesque, Kyoto, Japan.

Method

In the measurement of cholesterol solubilization, sealed test tubes containing a fixed amount of cholesterol monohydrate in carbonate-tetraborate buffer (Kolthoff) at pH 10.0 with addition of respective bile salts or their various mixtures were shaken in a thermostated water bath at 37 °C for 24 h. Air in the test tubes was completely replaced with N_2 gas to avoid oxidation of cholesterol monohydrate during the process. After the incubation, each solution was centrifuged (3000 rpm, 10 min) and filtered using 0.01 μm membrane filter (detergent free). The concentration of solubilized cholesterol monohydrate was measured by the enzymatic assay (Determiner C555, Kyowa Medex Co., Ltd. Japan) using a JASCO UVIDEC-320 Spectrophotometer.

For the surface tension measurement, the bile salt solutions with solubilized cholesterol were prepared at different concentrations by diluting the stock solution saturated with cholesterol, that is, at equilibrium of solubilization. From the mixed solutions in certain concentration range, precipitation of cholesterol took place when in the dilution process. When the precipitation became visible, the precipitate was removed by filtration with 0.05 μm membrane filter. Surface tensions of the solution were measured by a Wilhelmy-Type Surface Tensiometer (CBVP-A1, Kyowa Interface Science Co., Ltd. Japan) at 30 °C. The equilibrium value was checked by repeated measurements in 10-min intervals.

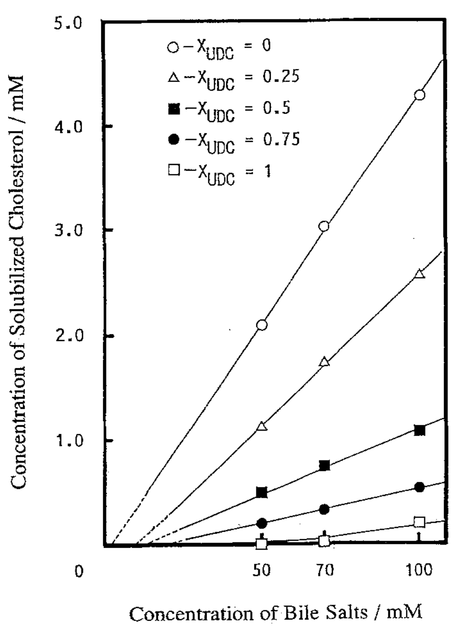
Results and discussion

Solubilization

The solubilization of ChM was observed as a function of mole fraction of NaUDC, $X_{UDC} = 1$, 0.75, 0.5, 0.25, 0, in the bile salt mixture at three total concentrations of NaCDC-NaUDC mixture, i.e., 50 mM, 70 mM, and 100 mM. Figure 1 shows the amount of solubilized ChM against the total bile salt concentration at 37 °C as a function of mole fraction, X_{UDC}, in the mixture of NaCDC and NaUDC. Extending over the wide concentration range, the solubilized amount of ChM increases linearly with increase of bile salt concentration. The linearity may suggest, as will be discussed below, that the size and shape of bile salt micelles solubilizing ChM are almost constant, while the gradual change in slope for X_{UDC} is likely to correspond to a change in the micellar structure with increase in concentration as will be described later. Even in the case of $X_{UDC} = 1$, it is inferred that the solubilization behavior of NaUDC may become similar to the other mixed systems if the total concentration is increased.

As for the structural change of solubilizing micelles, Abe et al. have reported that even if a micelle swelled, accompanied by solubilization of a small amount of nonpolar solubilizates (e.g. sodium dodecyl sulfate, hexadecyl polyoxyethylene ethers/*n*-octane, 1-octanol), at a certain limited concentration range the size and shape of the solubilizing micelle no longer change in the range higher than the limit [13]; this is in addition to the reports showing several examples that micellization accompanies little change in size and shape [14, 15]. Above all, Murata et al. have studied the ChM solubilization by sodium deoxycholate (NaDC) to examine, by means of light scattering measurements, if the size (accordingly the aggregation number) of ChM- solubilizing NaDC micelles

Fig. 1 Plots of solubilized cholesterol amount against the bile salts concentration for NaCDC-NaUDC mixed system at 37 °C



changes with both concentrations of NaDC and ChM, and found that at higher concentration range NaDC forms symmetric (spherical) micelles whose aggregation number is kept around 45 irrespective of the concentration change [16]. In the study of ChM solubilization by bile salts as a function of temperature, pH and pNa, Sugihara et al. also reported that a good linearity was obtained for the NaDC-ChM mixed system in the correlation of the amount of solubilized ChM with the bile salt concentration, while no linear relation was seen for the sodium cholate (NaC)-ChM mixed system; the latter was attributed to the stepwise association with increasing NaC concentration [17], as pointed out by Chang and Cardinal [18]. The facts mentioned above may at least be the case for NaCDC-ChM mixed system.

In Fig. 2, the results in Fig. 1 were replotted against X_{UDC}. It exhibits that the solubilization of ChM rapidly decreases with increasing the ratio of NaUDC in the bile salt mixture, and that all the curves negatively deviate from a linearity, i.e., the additivity rule. This negative deviation implies that the mixing of NaUDC with NaCDC causes disadvantage of ChM solubilization. For instance, one can recognize that pure 50 mM NaCDC solution at $X_{UDC} = 0$ solubilizes 2.1 mM ChM, while 100 mM bile salt solution at $X_{UDC} = 0.5$, in which 50 mM NaCDC and 50 mM NaUDC are contained, solubilizes ChM only to the extent of 1.3 mM. As is seen in the example, the addition of NaUDC to NaCDC solution leads to reduction of the function of ChM solubilization. This suggests that the mixed micelle formation of NaCDC with NaUDC does not seem to be ideal mixing [19], especially when ChM participates in the mixed micelle formation. As the reason, a steric hindrance resulting from participation of ChM is considered to reduce the stability of mixed micelle in thermodynamical as well as structural aspects and, accordingly, the solubilizing power decreases. Salvioli et al. showed that the mixing of glycochenodeoxycholate and glycoursodeoxycholate with phosphatidylcholine gave the same negative effect on solubilization of ChM as in our study [20].

In order to analyze the results in Fig. 1 quantitatively, we use the previously defined solubilizing power Sp, i.e., the slope of the curve in Fig. 1, as follows [17];

$$Sp = dW/d(C - C_0) = dW/dC ,$$

where W is the molar concentration of solubilized ChM, C the total concentration of bile salts, and C_0 the critical concentration of bile salts to solubilize ChM, which was determined by extrapolation from the linear relation as shown in Fig. 1. Figure 3 shows a dependence of Sp^{-1} on X_{UDC}. The reciprocal solubilizing power (Sp^{-1}) means the number of bile salt molecules per molecule of solubilized ChM. Sp^{-1} values for NaCDC and NaUDC can be determined to be 22 and 250 respectively, from both sides in

Fig. 2 Change of concentration of solubilized cholesterol with mole fraction, X_{UDC}, in the mixture of NaCDC and NaUDC

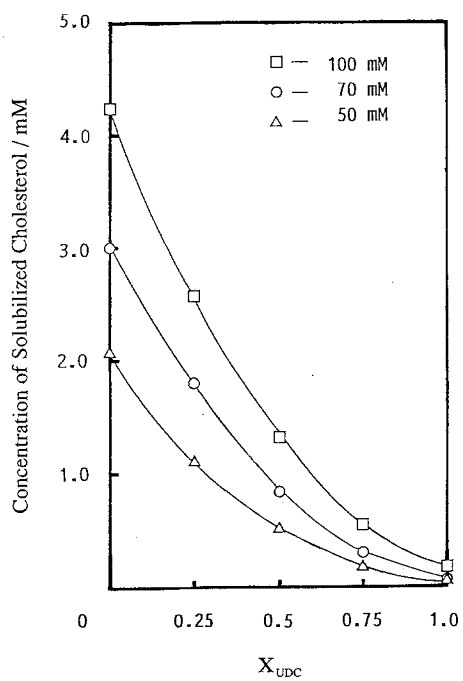


Fig. 3 Change of the reciprocal of solubilizing power, S^{-1}, with mole fraction, X_{UDC}. Closed circles are estimated by interpolation in Fig. 2

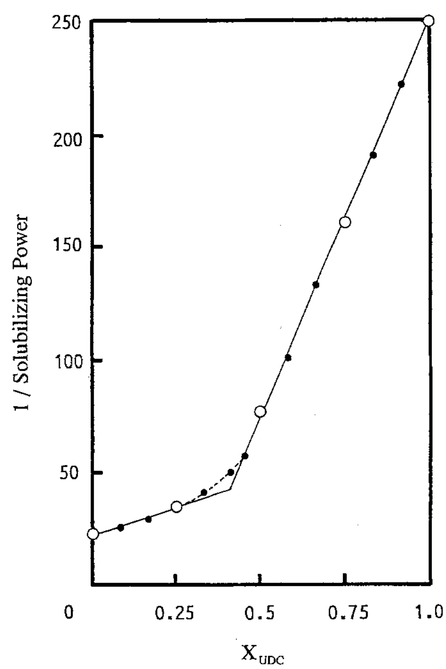


Fig. 3, i.e., at $X_{UDC} = 0$ and 1. The solubilizing power of NaCDC to ChM turned out to be approximately 11 times more than that of NaUDC . The solubilization of ChM [10, 12, 17, 21] and acid form of each bile salt [21] has been examined in aqueous bile salt solutions, and the results are summarized in Table 1. In Table 1, the solubilizing powers of NaCDC and NaUDC were compared in terms of the molecular number of each bile salt required to solubilize a ChM molecule. The result informs that Sp^{-1} values in three papers for NaCDC are generally superior to those of NaUDC including the acid forms of bile salts.

As seen in Fig. 3, the Sp^{-1} of bile salts to ChM given as a function of mole fraction of NaUDC changes abruptly when X_{UDC} is approximately 0.4. This suggests that the state of NaCDC-rich micelle is somewhat different from that of NaUDC-rich micelle. Accordingly, the micelles formed may also be different between NaCDC and NaUDC in terms of interior structure of micelles, aggregation number, and the mode of interaction of ChM with bile salts.

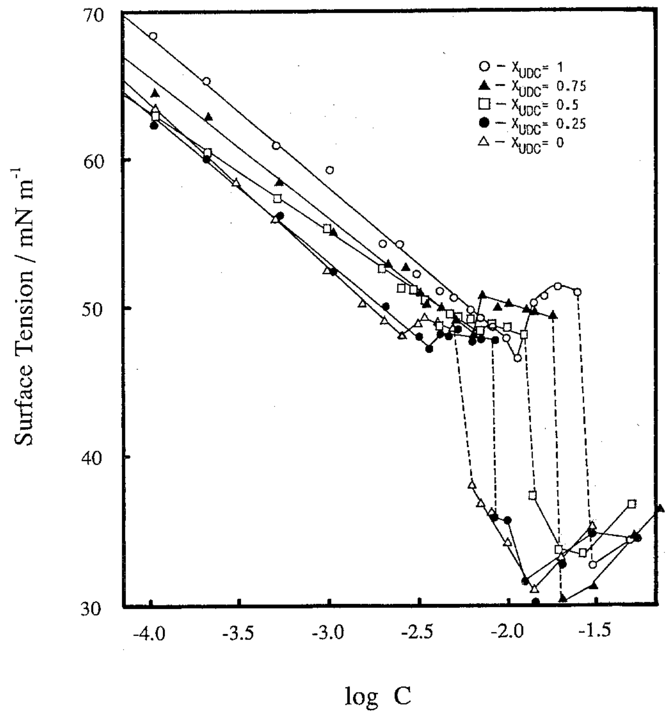


Fig. 4 Plots of surface tension against the logarithm of the bile salts concentration for NaCDC-NaUDC mixed system at 30 °C

Surface tension

The surface tension behavior was examined for the mixed micellar solutions which were prepared by diluting a stock solution equilibrated with ChM. The results are given as a function of the logarithmic concentration of bile salt for different NaCDC and NaUDC mixtures in Fig. 4. The bile salt/ChM concentration ratio of stock solutions ranges from 100 mM/0.2 mM at $X_{UDC} = 1$ to 100 mM/4.3 mM at $X_{UDC} = 0$. The surface tension of the bile salt-ChM solution decreases gradually to the minimum point with increasing bile salt concentration, then remains almost constant over a certain concentration range and at a concentration suddenly shows a sharp fall (indicated by a dotted line) and then a minimum. That is, all the surface tension curves of bile salt solution in the presence of solubilized ChM have two break points at lower and higher concentrations as shown in Fig. 4. Of these results, the typical one is given in Fig. 5 and the break points are designated as CMC-L and CMC-H respectively for the sake of convenience. Moreover, the regions partitioned by the break points are denoted by A, B, and C as shown in Fig. 5. The broken lines in Fig. 5 indicate the boundary of the precipitation region of ChM crystals on the dilution process. In the region A at the concentration range lower than CMC-L, the surface tension decreases gradually down to point CMC-L. Judging from the surface tension value, in this region, bile salt species is only considered to be adsorbed at the air/water interface without forming micelles. The minimum points of CMC-L agree fairly well with the critical micelle concentration (CMC) values of bile salts in the absence of ChM obtained by the drop volume method [19]. The respective values at different mole fractions of NaUDC, X_{UDC} are given in Table 2. On the other hand, the mixed micelles containing slightly solubilized ChM may exist in the region B between the break points of CMC-L and CMC-H. In the region B, as the precipitation of ChM crystals was observed on the dilution process, surface tension of the solution of which the precipitate was removed by filtration was measured. The growth of bile salts micelles had been measured over several temper-

Table 1 Solubilization data of NaCDC and NaUDC

	ChM			each one's own acid form;
	Igimi[(a)]	Montet[(b)]	Nagadome[(c)]	HA[(d)]
$(S_{CDC})^{-1}$	16	24	22	8
$(S_{UDC})^{-1}$	303	384	250	75
$(S_{CDC})/(S_{UDC})$	18	16	11	9

[(a)] Igimi et al. (1981) and Carey et al. (1981) in 100 mM BS at 37 °C, pH 10.0.
[(b)] Montet et al. (1987) in 30 mM BS at 37 °C, pH 9.5.
[(c)] Nagadome et al. in 100 mM BS at 37 °C, pH 10.0; the present study.
[(d)] Montet et al. (1987) in 20 mM BS at 25 °C; recalculated from their $[A^-]/[HA]$ data.

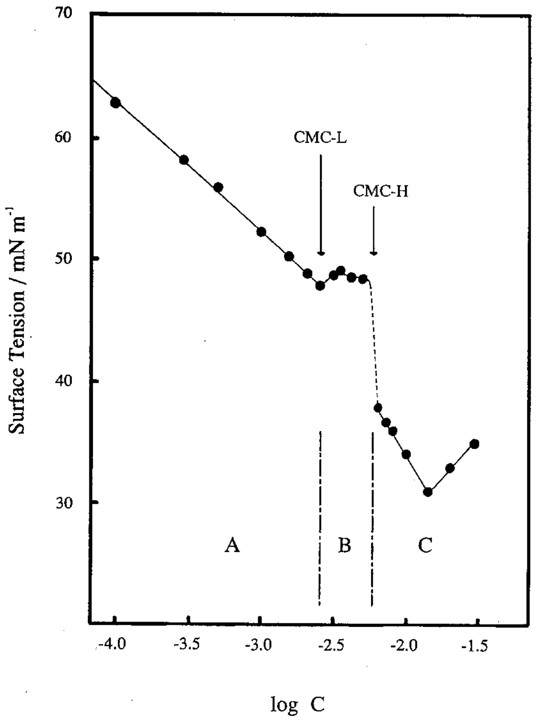


Fig. 5 A typical change of surface tension with the logarithmic concentration obtained for the system of pure NaCDC with cholesterol at 30 °C

Table 2 The data of CMC-L, CMC-H and CMC (determined by drop volume method) for NaCDC-NaUDC mixed system

X_{UDC}	CMC-L[(a)]	CMC[(b)]	CMC-H[(c)]
0	2.6	2.9	3.6
0.25	3.3	3.6	8.3
0.5	4.2	5.0	14
0.75	6.2	7.0	20
1	11.2	10.4	30

[(a)] Critical concentration of poorly cholesterol solubilizing micelle formation.
[(b)] CMC determined by drop volume measurement[19].
[(c)] Critical concentration of more stably cholesterol solubilizing micelle formation.

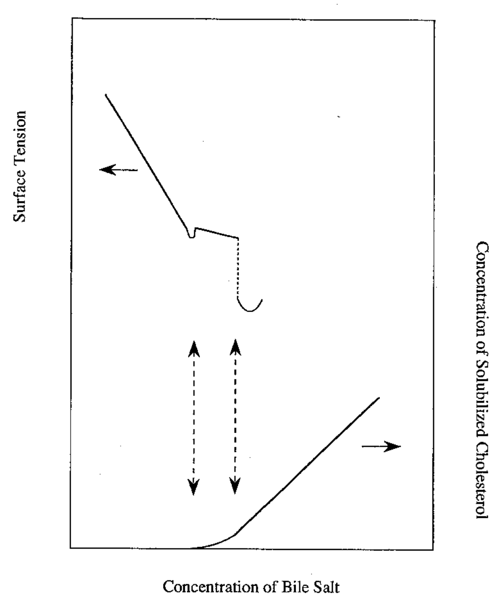
atures and salt concentrations [1, 22]. The plateau suggests that there exist the micelles supersaturated with ChM in this region, because precipitation of ChM crystals was observed in the mixed micellar solution of bile salt on the dilution process. These are conveniently called unstable mixed micelles.

In the region C, the mixed micelles equilibrated thermodynamically with ChM are formed at the concentration of CMC-H. These are conveniently called stable mixed micelles. Comparing with the results obtained in Fig. 1, the value of CMC-H is found to correspond nearly with the critical concentration of bile salt solubilizing ChM [17]. At the higher concentration above CMC-H, the precipitation of ChM crystals was not observed by the naked eye during the dilution process, and the surface tension still changed and showed a minimum, however, the value ranged between 30 and 40 $\mathrm{mN\,m^{-1}}$.

Again considering that the respective solutions at different concentrations were prepared by diluting a stock micellar solution saturated with ChM, the dilution process may accompany breaking partially mixed micelles and, thus, ChM excluded from micelles may escape, to some extent, toward the air/water interface. This causes surface tension depression, because the surface activity of ChM is much greater than that of bile salts. The surface tension value of the stock mixed micellar solution is given by the point at the highest concentration for each curve in Fig. 4. At the minimum in the region C, the air/water interface is considered to be covered mostly with ChM, so the surface tension values around the minimum are not of real thermodynamic equilibrium of ChM solubilization.

The relationship between solubilization and surface tension is schematically shown in Fig. 6. The region between the two break points in the scheme of surface tension corresponds just to that between the critical starting point of solubilization and the point extrapolated along the linear relationship in the scheme of solubilization. In this region, therefore, the micelles solubilizing ChM would

Fig. 6 Schematic diagram of the correspondency of the relations surface tension vs. bile salt concentration and solubilized cholesterol concentration vs. bile salt concentration



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be unstable and seem to be growing to a certain size at this range. Thus, the unstable mixed micelles of bile salts may take part in the formation of cholesterol gallstones, and may play an important role in the crystal nucleation of cholesterols in the gallbladder.

As a concluding remark, the NaCDC-NaUDC mixed micelles proved to reduce the solubilizing power due to the structure hindrance with each other and it was found that there exists the concentration range of unstable mixed micelle in solubilization of ChM by bile salts. In addition, the results gave a clue to understanding the biological activities of bile salts and gallstone formation in gallbladder.

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