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Transport behavior of four bile salt micelles and cholesterol solubilized by their micelles across porous membrane

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Abstract The transport behavior of bile salts (BSs) solubilizing cholesterol (Ch) or none across an artificial membrane was investigated for sodium salts of deoxycholic acid (NaDC), chenodeoxycholic acid (NaCDC), ursodeoxycholic acid (NaUDC) and cholic acid (NaC) in tetraborate–carbonate buffer solution at pH 10.0 and 37 °C. The study demonstrated that the surfactant properties such as critical micellization concentration (CMC) and micellar size or diffusion coefficient were determinable from the flux or permeability measurements. The comparison among the respective pure systems of BSs led to a conclusion that the micellar size was in the order of NaDC > NaCDC > NaUDC > NaC and determined CMC values were in agreement with those in literature.

The magnitude of solubilizing power (capacity) of BS for Ch was found to decrease in the order of NaDC > NaCDC > NaC > NaUDC; this order is in accordance with that of the empirical hydrophobicity index. The hydrodynamic radii for the singly dispersed species and the micellar species of the respective BSs and of Ch-solubilizing micelles were estimated from the permeability data; the radii of the Ch-solubilizing micelles are approximately 12–15 Å and interestingly, smaller than those of the respective BS alone micelles ranging from 14 to 22 Å.

Key words Membrane transport – solubilization – bile salt micelles – cholesterol – permeability coefficient

Introduction

Much knowledge on the important role of bile salts (BSs) in biological activities and interesting colloid of properties has so far been accumulated [1–8]. Nevertheless, the detailed function of the BSs has not been elucidated yet, partly because the mechanisms in biological and physicochemical action are too much complex to understand and partly because it is really hard to prepare *in vivo* mimic situation for the *in vitro* experimental work.

In the preceding work, we investigated the lipid-transport mediated by micelles of sodium salt of deoxycholic acid (NaDC) as well as the formation of NaDC micelle or its mixed micelle with monooleoylglycerol (MO) as a simple model system using artificial membranes [9]. The studies on transport behavior across the membrane gave us some valuable knowledge on the transport mechanism of dietary lipids in living bodies in addition to critical micellization concentration (CMC) and micellar sizes of the micelles.

In order to extend the understanding regarding BS–lipids interrelation to other kinds of BS homologues and an other type of lipid, a comparative study was carried out in the present paper; four different BSs and cholesterol (Ch) were chosen to investigate the transport behavior

across the artificial membrane, paying attention to the interaction between BSs and Ch. Because the digestive absorption has been known to take place predominantly in the vicinity of the microvilli, the porous membrane with 0.1 μm pore size which corresponds to the maximum space of microvillus was employed as an artificial membrane in the same manner to the preceding study [10, 11]. Regarding these BSs, in particular, there lies an important difference in the orientation of hydroxyl group at the position 7 on steroid skeleton of sodium chenodeoxycholate acid (NaCDC) and sodium ursodeoxycholate (NaUDC). The difference between 7α OH of NaCDC and 7β OH of NaUDC seems to be small but it has been known to give a great influence in solubilization of Ch which is a lipid [12–14] and in adsorption onto different solids in water [15–17].

In this study, the results obtained through the transport behavior observation and the solubilization measurement will be discussed in relation to CMCs, micellar sizes, and diffusion coefficients of the respective BS micelles or BS–Ch mixed micelles.

Experimental

Materials

Four bile salts (BSs) were used in this study: sodium salts of deoxycholic acid (NaDC), chenodeoxycholic acid (NaCDC), ursodeoxycholic acid (NaUDC) and cholic acid (NaC). All of these salts were obtained from Calbiochem. Co. CA, USA. Cholesterol (Ch) was used as received from Sigma Co., USA. *p*-Aminobenzoic acid (PABA) and other inorganic salts were reagent grades and used as received from Wako Pure Chem. Ind. Thrice-distilled water was used for all the solutions. In most cases a porous nitrocellulose membranes (purchased from Sartorius Co.) were employed and their pore size was 0.1 μm with 100 μm thickness.

Diffusion cell

The diffusion apparatus and its handling were the same as that reported previously [9].

Preparation of mixed solutions

Known amounts of Ch and BSs were dissolved in tetraborate-carbonate buffer solution (0.05 M $Na_2B_4O_7$ + 0.05 M Na_2CO_3) at pH 10.0 in test tubes with a glass stopper and the mixed solutions were shaken and incubated in a thermostated water bath at 37 °C for 24 h. During the preparation, air on the mixed solution was completely replaced with dry N_2 gas to avoid oxidation of Ch. The buffer solutions involving only BSs were prepared with shaking at 37 °C for 2 h at least.

Measurement

In all experiments, the starting sample solution and the buffer solution with or without addition of a known amount of BS were placed in the left (Chamber L) and right (Chamber R) hand sides of the membrane, respectively. The aliquot with every time interval was withdrawn from the sampling solution in the Chamber R using a microsyringe (Hamilton Co.) and the increases of BSs and Ch concentrations with time were determined by means of a spectrophotometer (JASCO UVIDEC-320). Prior to the measurement, a diffusion measurement across the membrane was carried out using PABA as a standard diffusate [11]. In the case of PABA, the concentration change with time across the porous membrane was analyzed by taking 10 μl of the sampling solution for each measurement. The concentration of Ch was determined by applying the enzymatic assay (Determiner TC555, Kyowa Medex Co., Ltd., Japan) to each 50 μl of the sampling solution [18, 19]. For each 10 μl of the sampling solution taken out, concentration change of BS with time was measured according to the enzymatic assay (ENZABILE-2, Daiichi Pure Chem. Co., Ltd., Japan) [20].

Results and discussion

Bile salt systems

The diffusivity of BS across the porous membrane was at first investigated for the respective BS only systems dissolved in buffer solution at 37 °C. Here, it is noted that the buffer solution at pH 10.0 and ionic strength 0.15 (the same as physiological saline solution, 0.15 M NaCl) was used to avoid precipitation due to hydrolysis of BSs. Unstable transport behavior was often observed in the early stage, and it took a certain time to get a linear relation between the concentration and time. In the concentration changes of BSs in Chamber R against the lapsed time except for the initial stage, a satisfactory linearity was confirmed to hold throughout the BS concentration range. This suggests that the transport of the system is at a steady state. The flux of BS across the membrane, $J_{(BS)}$, can be obtained by dividing the slope by the effective membrane area [9, 21], and the results obtained are plotted as a function of BS concentration as shown in Fig. 1. It should be stressed that there

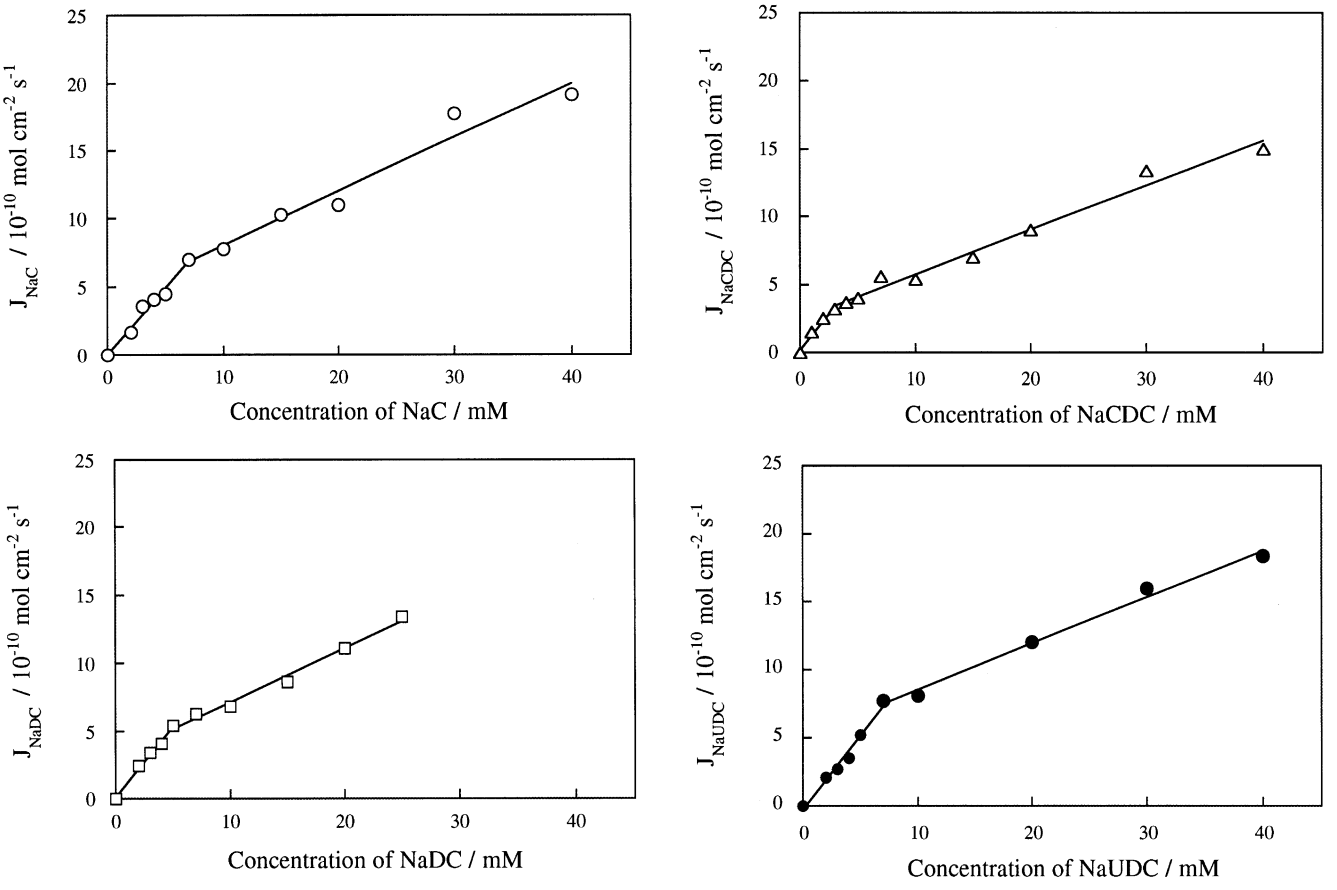

Fig. 1 Flux (J) changes with concentration for the respective BSs in buffer solution at 37 °C: NaC (○), NaDC (□), NaCDC (△) and NaUDC (●)

appears a sharp kink point at a certain BS concentration for all BSs investigated. The break means that the change of transport process was caused by micelle formation of BSs. The concentration giving the break point was found to correspond to the literature value of CMC [21], and these values were ca. 8 mM for NaC, ca. 5 mM for NaDC, ca. 3 mM for NaCDC and ca. 8 mM for NaUDC.

Generally, the flux, J_M of a solute M across the membrane is described simply as a function of solute concentration, C_M,

$$J_M = P_M \Delta C_M , \quad (1)$$

where P_M is a proportionality constant called the permeability coefficient of M, and ΔC_M, the concentration difference of M between Chambers L and R. As seen in Fig. 1, two straight lines intersect each other for every BS solution system. The concentration giving the intersection may be regarded as CMC, as described above. Thus, the slopes below and above CMC correspond to the permeability coefficients peculiar to the monomer and micellar states, respectively. In other words, the transport process across the porous membrane is subject to the molecular (or micellar) size of BSs.

According to Eq. (1), the permeability for each process can be obtained from the slope in Fig. 1. In general, P is a function of the diffusion coefficient, tortuosity and porosity factors, and membrane thickness as follows: $P = fD/h$, where D is the diffusion coefficient, f the frictional coefficient of the membrane and h the membrane thickness. Moreover, $f = \varepsilon/\tau$, the f value reflects porosity factors (τ) and tortuosity (ε) of the membrane [23]. If factors other than the diffusivity are considered as constant characteristics of the membrane employed, the permeability coefficient would be attributed only to the diffusion coefficient of solute through pores in the membrane. On the other hand, since a PABA molecule is sufficiently smaller than pore sizes used in this study, the diffusion coefficient of PABA in aqueous solution can be used as a reference value even in the porous membrane. Thus, the following relation may be applied to estimate the diffusion coefficients at

below and above CMC.

$$\frac{P_{\mathrm{PABA}}}{D_{\mathrm{PABA}}} = \frac{P_{\mathrm{BS}}}{D_{\mathrm{BS}}} . \quad (2)$$

Further, this expression was considered applicable even at different temperatures by assuming the temperature dependence negligible. Here, the literature value of D_{PABA} ($D_{\mathrm{PABA}} = 8.4 \times 10^{-6}\ \mathrm{cm^2\,s^{-1}}$ at 25 °C [11, 24]) was quoted so that the unknown diffusion coefficients of BSs at monomeric and micellar states can be estimated. From the preliminary experiment, P_{PABA} value was determined to be $1.4 \times 10^{-4}\ \mathrm{cm\,s^{-1}}$ at 25 °C. According to the above treatment, P_{BS} and D_{BS} values were obtained and tabulated in Table 1, indicating clearly that monomeric BS molecules can migrate through the membrane faster than the aggregates.

It is of interest to estimate the monomeric and micellar sizes of BS through the porous membrane using the Stokes–Einstein relation, $D = kT/6\pi\eta r$, where k is the Boltzman constant, T the Kelvin temperature, η the viscosity of the continuous phase, and r the hydrodynamic radius. Strictly speaking, the viscosity η should be of buffer solution used, but, instead, the value of water ($\eta = 0.695$ cP at 37 °C) was employed. The radii estimated are also included in Table 1. The micellar sizes are in the order NaDC > NaCDC > NaUDC > NaC. It should be noticed that this order is in agreement with the data determined by photon correlation spectroscopy measurement in 0.15 M NaCl at pH 10 [25] or the data determined by means of light scattering measurement using taurine conjugated bile salts [26]. The hydrodynamic radius value for each BS roughly corresponds to the literature value [25].

The aggregation number for each BS was tentatively calculated from the expressions derived by Mazer et al. as follows [26]:

$$n = \begin{cases} n_0\,(r/15)^3 & \text{for } r < 15\ \text{Å}\,, \\ n_0\,(a_{\mathrm{m}}/15) & \text{for } r \geq 15\ \text{Å}\,, \end{cases} \quad (3)$$

where n_0 is the aggregation number corresponding to spherical micelle with a hydrodynamic radius, r of 15 Å and a_{m} is the semimajor axis of a prolate ellipsoid with a semiminor axis (b_{m}) of 15 Å. In this treatment, n_0 was taken as 10. The volume of a sphere is assumed to be equal to that of a prolate ellipsoid, and a_{m} is determined from the following expression, $\frac{4}{3}\pi r^3 = \frac{4}{3}\pi a_{\mathrm{m}} b_{\mathrm{m}}^2$ (prolate ellipsoid) [27]. Using Eq. (3) with $n_0 = 10$, it was found that the n (mean aggregation number) values obtained in this study are 8 for NaC micelles, 31 for NaDC micelles, 12 for NaCDC micelles and 12 for NaUDC micelles. The result that only NaDC has a larger aggregation number satisfies the early observations by several authors [4].

Bile salts – cholesterol mixed systems

In this section, focusing our attention on mixed micelle formation depending on the micellar structure of each BS species, the experimental results of the solubilization of Ch as well as transport behavior of Ch-solubilizing micelles are described.

At first, Fig. 2 shows the plots of the amount of solubilized Ch against the four BS concentrations at 37 °C. Extending over the wide concentration range, the solubilized amount of Ch increases linearly with increase of BS concentration. The linearity may suggest that the size and shape of BS micelles solubilizing Ch are almost constant irrespective of the BS concentration change. In order to analyze the results in Fig. 2 quantitatively, we use solubilizing power (capacity) Sp, i.e., the slope ($\mathrm{d}C_{(\mathrm{Ch})}/\mathrm{d}C_{(\mathrm{BS})}$) of the curve in Fig. 2 [4, 28]. The reciprocal of solubilizing power (Sp^{-1}) means the number of BS molecules per molecule of solubilized Ch, and the results were summarized together with two other hydrophobic indices (HIs) in Table 2. The first HI has been defined by Miyajima et al. on the basis of the geometrical evaluation of hydrophobic surface area [29] (called the geometrical HI [15–17]), and the other HI has been introduced by Heuman on the basis of the logarithms of BS capacity factors determined using reverse-phase high performance liquid chromatography (HPLC) [30] (called the empirical HI [15–17]). Both HIs indicate that the larger the value, the stronger the hydrophobicity. In the table, the

Table 1 Hydrodynamic data of bile salts in monomeric and micellar states

	P (10^{-5} cm s^{-1})		D (10^{-6} cm^2 s^{-1})		r (Å)	
	Monomer	Micelle	Monomer	Micelle	Monomer	Micelle
NaC	9.8	4.0	5.9	2.4	5.5	14
NaDC	10	2.5	6.0	1.5	5.4	22
NaCDC	11	3.3	6.6	2.0	5.0	16
NaUDC	11	3.4	6.6	2.1	5.0	16

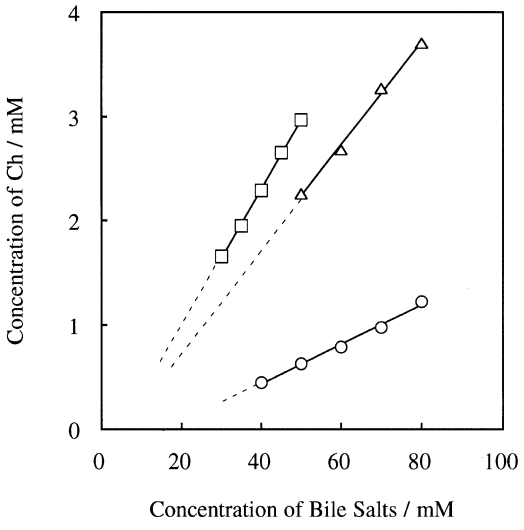

Fig. 2 Plots of solubilized cholesterol amount against the BS concentration at 37 °C: NaC (○), NaDC (□) and NaCDC (△)

Table 2 Comparison of solubilization data with hydrophobic indices

	Sp^{-1} (solubilizing power)	Miyajima's HI	Heuman's HI
NaC	52	6.91	+ 0.13
NaDC	15	7.25	+ 0.72
NaCDC	20	7.27	+ 0.59
NaUDC	250*	5.48	− 0.31

* A reference value [28].

magnitude of solubilizing power to Ch decreases in the following order, NaDC > NaCDC > NaC > NaUDC. It should be noted that this order is the same as the empirical HI. A comparison between NaDC (3α, 12α) and NaCDC (3α, 7α), which are dihydroxy BS in terms of Sp^{-1} and each HI, indicates that other than the location of hydroxyl group they differ little. On the other hand, a comparison of NaUDC (3α, 7β) with NaC (3α, 7α, 12α) which is a trihydroxy BS indicates that a β-oriented hydroxyl group results in more effective hydrophilicity than two hydroxyl groups of 7α and 12α.

Secondly, the flux of Ch, $J_{(Ch)}$ in Ch–BS mixed micellar solutions are plotted against solubilized Ch concentration as shown in Fig. 3. According to nonequilibrium thermodynamics, the phenomenological equations for the mixed miceller solution can be subjected to two kinds of driving forces, so that $J_{(Ch)}$ can be described by the following expression:

$$J_{(Ch)} = P_{11}\,\Delta C_{(Ch)} + P_{12}\,\Delta C_{(BS)}\,, \tag{4}$$

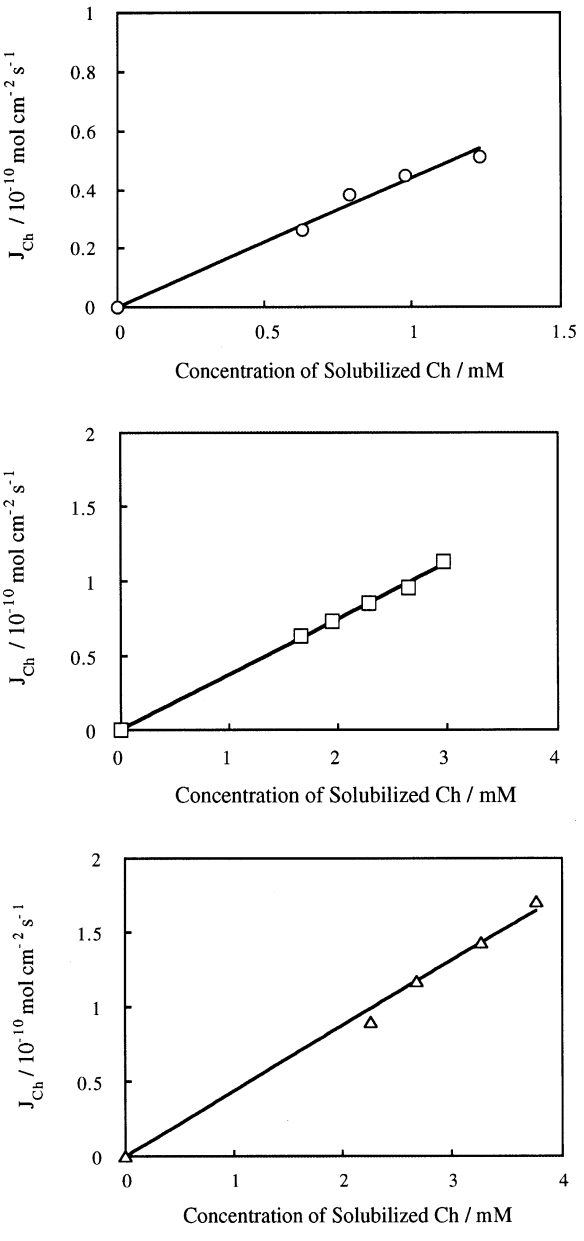

Fig. 3 The plots of fluxes (J_{Ch}) of cholesterol against concentration of solubilized cholesterol for three BS/Ch mixed systems: NaC (○), NaDC (□) and NaCDC (△)

where P_{11} is the major proportionality constant and P_{12} is the minor proportionality constant for $J_{(Ch)}$. Provided that the mixed solution consists of the BS only micelle and the mixed micelle with Ch, $\Delta C_{(Ch)}$ and $\Delta C_{(BS)}$ are the concentration differences due to an aggregate species of the mixed micelles with Ch and that of the BS micelles without Ch, respectively. Here, P_{11} is defined as the proportionality coefficient between the flux and the corresponding Ch concentration difference, and P_{12} represents the physical

quantities concerning the interaction between the BS alone micelle and the mixed micelle with Ch in the mixed solution. In Fig. 3, interestingly, $J_{(Ch)}$ linearly increases with increase in Ch concentration for each BS–Ch mixed system, whereas no linear relationship was found between $J_{(Ch)}$ and BS concentration. This suggests that the value of P_{12} may be negligibly small compared with that of P_{11} and that the BS concentration hardly contributes to the Ch transport. The curves of $J_{(Ch)}$ in Fig. 3 tell us that $J_{(Ch)}$ corresponds just to the flux of Ch–BS mixed micelles themselves, and the linearity of $J_{(Ch)}$ against Ch concentration change suggests that $P_{(Ch)}$ is a constant independent of Ch concentration change. Using the slopes from the flux vs. Ch concentration curves, the permeability coefficient of Ch-solubilizing mixed micelles was calculated and listed in Table 3.

Similar to the above section, the hydrodynamic radius of the aggregate was estimated to be approximately 12–15 Å. Interestingly, these values are small compared with those of pure BS micelles without Ch, implying that the solubilization of Ch by BS leads to a reduction of micellar size. In the preceding paper [9], we have already reported some results similar to the present paper, i.e., the hydrodynamic radius of monooleoylglycerol (MO)–NaDC mixed micelles (in 0.15 M NaCl saline solution at 37 °C through artificial membranes) have been found to be small compared with those of pure NaDC micelles. MO molecules solubilized may probably enhance the interaction between MO and NaDC molecules by a better contact with the respective hydrophobic groups in a mixed micelle [9]. However, Ch having a rigid steroid skeleton exhibited a larger extent of micellar size reduction compared to MO with a flexible hydrocarbon chain.

As for the interaction between BS and Ch molecules, Armstrong and Carey [31] presumed that Ch may be solubilized in BS micellar solutions by not only hydrophobic but also hydrophilic association with the external (hydrophilic) surface of BS micelles rather than the hydrophobic core of the micelle's interior. In addition, Mazer et al. reported that the dimerization of primary micelles may result in micelle–solvent contact of the micellar surface when the primary micelles are bound [26]. We conclude that the micelle–solvent interaction (a hydrogen-bond interaction between H_2O and OH groups) would be reduced temporarily when Ch molecules were bound by the external surface of BS micelles, leading to a steric hindrance to the aggregation of some primary micelles, then the micellar size of BS–Ch mixed micelles is a little reduced.

Table 3 Hydorodynamic data of the respective bile salt micelles solubilized cholesterol

	P (10^{-5} cm s^{-1})	D (10^{-6} cm^2 s^{-1})	r (Å)
NaC-Ch	4.4	2.7	12
NaDC-Ch	3.7	2.2	15
NaCDC-Ch	4.4	2.7	12
NaUDC-Ch	—	—	—

Although the mixed micelle formation or lipid solubilization by micelles, in general, accompanies a free energetical stabilization (a decrease of free energy due to mixing) and thus a decrease in CMC, when BS's CMCs are determined by a Ch solubilizing method, the resultant CMC values are slightly reduced compared with CMC values determined from surface tension methods [4, 28]. This may suggest that Ch solubilization by BS micelles is not due to mixing of solubilizate–solubilizer but due to adsorption of solubilizate onto solubilizer micelle's surface. If so, the extent of energetical stabilization in the adsorption type of solubilization is likely to be smaller than the mixing type solubilization.

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