Specific Interactions between Sodium Deoxycholate and Its Water-Insoluble Analogues. Mechanisms for Premicelle and Micelle Formation of Sodium Deoxycholate

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Interactions between sodium deoxycholate (NaDC, host) and its water-insoluble analogues (guests) have been studied in water (pH 10) to clarify the mechanisms for the premicelle and micelle formation of NaDC. Turbidity measurements have been used as a convenient method to study the interactions between the guest and host molecules. At least two α-hydroxy groups attached to the C-3 and C-12 positions of a steroid nucleus are required for the guest steroids to interact with NaDC below the critical micelle concentration. This strongly suggests the formation of a hydrogen-bonded dimer of NaDC as a premicelle whose formation is assisted by the hydrophobic environment provided by the α-plane of the steroid. The guest steroids having a hydroxy group at the C-3 position and a polar head group at the C-24 position, which can participate in hydrogen bonding, are solubilized by the NaDC micelles. The results support the mechanism for the NaDC micelle formation involving hydrogen bonding between the C-3 hydroxy group of a premicelle and the C-24 carboxylate anion of another premicelle.

Introduction

Bile salts are biologically important detergents whose micellar behavior is considerably different from that of ordinary detergents having a long alkyl chain and a hydrophilic head group.[1-4] The bile salts consist of a steroid nucleus, up to three α-hydroxy groups (C-3, C-7, and/or C-12 positions), and a hydrophilic head group at the C-24 position. Although many detailed studies have been carried out, there is still a controversy on the mechanism for micelle formation of bile salts in pure water. On the basis of the ^{1}H NMR data of sodium cholate (NaC) and sodium deoxycholate (NaDC), Small and co-workers have presented a "back-to-back model" that involves a spontaneous association of the bile salt molecules through a hydrophobic interaction between β-planes of the steroid to form small micelles and further association of the small micelles through hydrogen bonding at higher bile salt concentrations.[5,6] Although the back-to-back model may be understandable because it is essentially the same as the model for ordinary micelles, the very complex NMR patterns of these bile salts and the small changes in the chemical shifts as a function of the bile salt concentration cause the difficulty in evaluating this model. Oakenfull and Fisher measured the conductances of the aqueous NaC and NaDC solutions and assumed that a premicelle, probably a dimer, is formed through hydrogen bonding and the hydrogen-bonded dimer is a basic building block of the micelles.[7,8] Such model can be called a "face-to-face model". Although the face-to-face model has been criticized,[9] this model can explain the reason the hydroxy groups of NaC and NaDC are directed toward the concave surfaces of these steroids whereas the hydroxy group of cholesterol, a precursor of the bile salts, is attached at the opposite direction. In analogy with the case of the back-to-back model, however, there is little experimental evidence for this model. Another model for the deoxycholate micelle has been presented by Giglio and co-workers, who demonstrated a "helix model" on the basis of the results of X-ray, SAXS, EXAFS, ESR, and NMR analyses of the bile salt crystals and/or the highly concentrated bile salt solutions.[10-12] Since the structure of the micelle usually depends on various conditions such as detergent concentration, temperature, salt added into the bulk solution, etc., it is difficult to evaluate whether the helix model can be applied for the bile salt solution near the critical micelle concentration (cmc) or not.

The present study deals with the mechanisms for the premicelle and micelle formation of NaDC. We chose NaDC as an amphiphilic bile salt because NaDC is a common bile salt used in the works where the above three models are presented. Since it was very difficult to evaluate the formation of the premicelle at the NaDC concentrations below the cmc by means of the usual spectroscopic methods, we employed a novel method. Namely, the interactions between the NaDC molecule (host) and its water-insoluble analogues (guests) were investigated by measuring the turbidities of the solutions. Although this method is very simple, the interaction between the host and guest can be detected very sensitively. The structures of the water-insoluble analogues used in this study are shown in Figure 1. All guest steroids (**1-16**) are insoluble in water, and their aqueous dispersions are turbid. It can be expected that an aqueous turbid dispersion becomes transparent if the guest steroid interacts with water-soluble NaDC. On the basis of the results for the host-guest interactions, we deduced the mechanisms for the formation of premicelle and micelle of NaDC. The results strongly suggest the formation of a hydrogen-bonded dimer as a premicelle as well as the formation of a micelle formed by hydrogen bonding between the C-24 carboxylate anion of a premicelle and the C-3 hydroxy group of another premicelle.

Experimental Section

Materials. Reagent grade of deoxycholic acid (DCA, 3α,12α-dihydroxy-5β-cholan-24-oic acid) was purchased from Nacalai Tesque (Kyoto), and its purity was checked by measuring the melting point. Equimolar aqueous NaOH was added to an aqueous 0.05 M DCA solution, and then water was removed under reduced pressure. The residue was washed with acetonitrile and recrystallized from aqueous acetone. Sodium deoxycholate (NaDC, sodium 3α,12α-dihydroxy-5β-cholan-24-oate) thus obtained was dried under vacuum at 120 °C for 4 h. The C-24 methyl esters (**1**, **2**, **4**, **6-8**) were prepared from the corresponding bile acids according to the procedures described in the literature.[13] The crude esters were purified by silica gel column chromatog-

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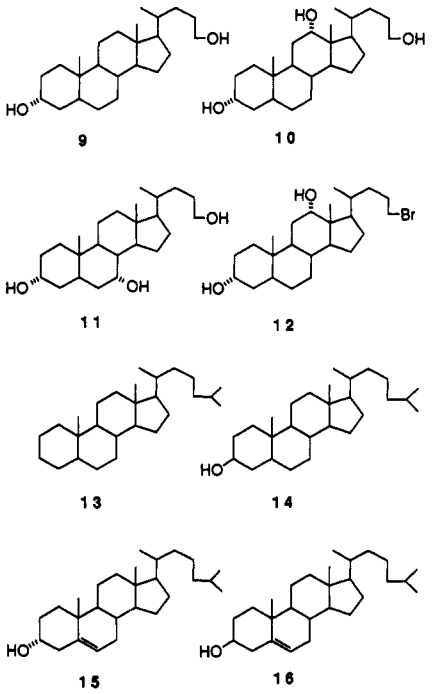

Figure 1. Structures of water-insoluble steroids used in this study as the guest molecules.

TABLE I: Yields and Melting Points of Guest Steroids

compd	yield, %	mp, °C
methyl 5β-cholan-24-oate (**1**)	35	79–81
methyl 3α-hydroxy-5β-cholan-24-oate (**2**)	89	116–118
methyl 3β-hydroxy-5β-cholan-24-oate (**3**)	3.5	74–76
methyl 3α,12α-dihydroxy-5β-cholan-24-oate (**4**)	79	99–100
methyl 3β,12α-dihydroxy-5β-cholan-24-oate (**5**)	5	124–125
methyl 3α,7α-dihydroxy-5β-cholan-24-oate (**6**)	55	58–60
methyl 3α,7β-dihydroxy-5β-cholan-24-oate (**7**)	65	59–61
methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate (**8**)	76	83–85
3α-hydroxy-5β-cholan-24-ol (**9**)	75	128–130
3α,12α-dihydroxy-5β-cholan-24-ol (**10**)	57	93–94
3α,7α-dihydroxy-5β-cholan-24-ol (**11**)	47	103–104
24-bromo-3α,12α-dihydroxy-5β-cholane (**12**)	7	68–70
cholest-5-en-3α-ol (**15**)	17	137–139

raphy eluting with chloroform. The epimerization of the hydroxy group at C-3 position of **4** to get **5** was carried out according to the procedures described in the literature.[14] The same procedures were applied for the preparation of **3**. The 5β-cholan-24-ol derivatives (**9–11**) were obtained by reducing the corresponding methyl esters (**2**, **4**, **6**) with $LiAlH_4$ in absolute THF. 24-Bromo-3α,12α-dihydroxy-5β-cholane (**12**) was prepared by the reaction of **10** with Br_2–triphenylphosphine in absolute acetonitrile, and the crude **12** was purified by silica gel column chromatography eluting with chloroform. The epimerization of cholesterol **16** to give epicholesterol **15** was performed according to the method presented previously.[15] The yields and melting points of the materials prepared in this study are listed in Table I. Other materials were purchased (Nacalai Tesque) and used without further purification.

Determination of the Cmc. Surface tensions of the aqueous NaDC solutions in water at pH 10 (NaOH) were measured at 23 ± 2 °C to determine the cmc by using a Kyowa Kaimenkagaku Digi-O-Matic ESB-IV (a Whilhelmy method). The determined cmc of NaDC was 5×10^{-3} M, which is in good agreement with the reported value ($(4–5) \times 10^{-3}$ M).[16–19] The I_1/I_3 ratio of the pyrene monomer fluorescence[20–22] also indicated the cmc of NaDC to be 5×10^{-3} M.

TABLE II: Conditions of Turbidity Measurements

compd	final concn of guest, M	final content of MeOH, % (v/v)	wavelength, nm
1	1.9×10^{-4}	1.5	235
2	7.5×10^{-5}	0.4	229
3	1.8×10^{-4}	0.7	245
4	1.5×10^{-4}	0.6	400
5	1.3×10^{-4}	0.5	310
6	1.5×10^{-4}	0.4	300
7	1.5×10^{-4}	0.4	250
8	6.0×10^{-4}	0.8	260
9	6.0×10^{-4}	2.0	250
10	1.5×10^{-4}	2.0	291
11	1.5×10^{-4}	2.0	310
12	1.0×10^{-4}	1.0	250
13	6.0×10^{-5}	2.4	400
14	2.0×10^{-4}	2.0	250
15	2.0×10^{-4}	2.0	250
16	2.0×10^{-4}	2.0	250

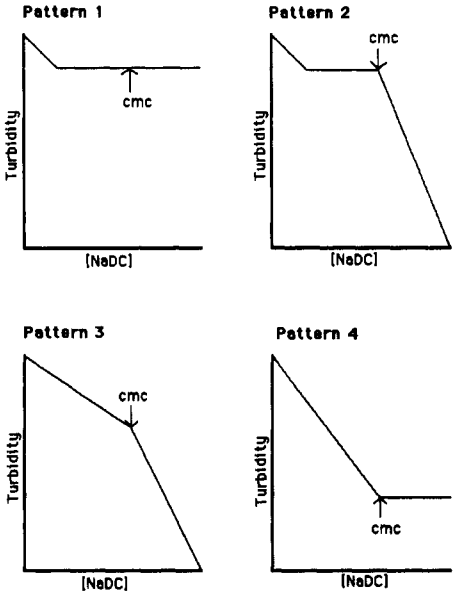

Figure 2. Patterns of the turbidity–NaDC concentration profiles.

Turbidity Measurement. The turbidities of the mixtures of the guest steroids and NaDC in water at pH 10 were measured at 23 ± 2 °C as a function of NaDC concentration. An appropriate volume of the methanol solution of guest steroid was added to the aqueous NaDC, and the solution was sonicated by a bath-type sonicator (a Branson B-12, 50 W) for 2 min. The apparent concentrations of the guest steroids, the final contents of methanol, and the wavelengths for the turbidity measurements, which were chosen to show appropriate optical densities, are shown in Table II. The turbidities were followed by a Shimadzu MPS-5000 spectrophotometer 30 min after the samples were prepared. We confirmed that the pH value adjusted by NaOH did not change during measurements.

Results

Patterns of Turbidity Changes. The turbidities of the aqueous dispersions of the guest steroids were measured in water at pH 10 (NaOH) as a function of the NaDC concentration. The turbidity changes could be classified into four patterns, which are shown in Figure 2. In the case of pattern 1, the turbidities decrease to some extent in the NaDC solutions up to [NaDC] = $(1–3) \times 10^{-3}$ M and become constant above these NaDC

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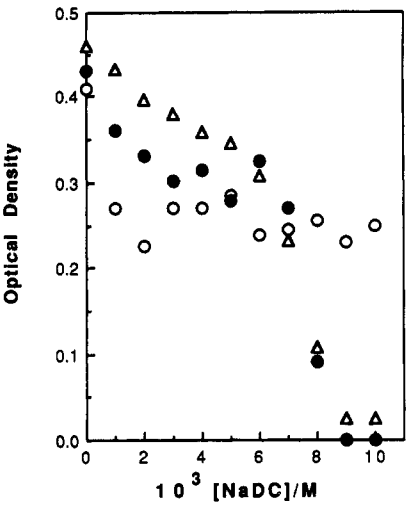

Figure 3. Turbidity changes of the aqueous dispersions of **1**, **2**, and **4** as a function of [NaDC]: ○, **1**; ●, **2**; △, **4**.

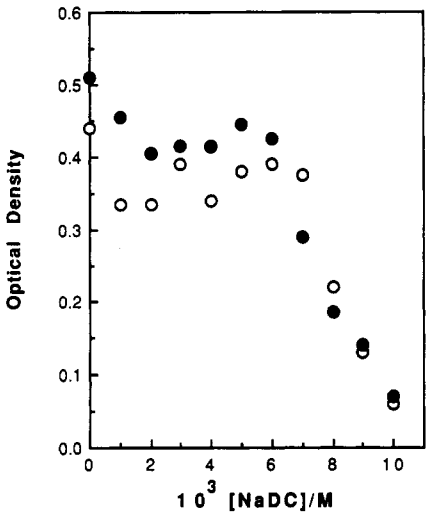

Figure 4. Turbidity changes of the aqueous dispersions of **3** and **5** as a function of [NaDC]: ○, **3**; ●, **5**.

concentrations. Pattern 2 is similar to pattern 1 until the cmc, but the solutions become transparent at the concentrations above the cmc. In the case of pattern 3, the turbidity continuously decreases with increasing the NaDC concentration both below and above the cmc. The case where the turbidity decreases upon addition of NaDC up to the cmc and becomes constant in the NaDC solution above the cmc belongs to pattern 4.

Interactions between NaDC and Methyl Cholanoates. All methyl cholanoates (**1-8**) used in this study were insoluble in water. Figures 3–5 show the turbidity–NaDC concentration profiles for the methyl cholanoates, and their patterns classified by using Figure 2 are summarized in Table III.

The guest steroid **1**, which does not have any hydroxy group in its steroid nucleus, reveals pattern 1. The fact that the turbidity does not change in the NaDC solution above the cmc clearly indicates that **1** cannot be solubilized by the NaDC micelle. The guest steroids having a hydroxy group at the C-3 position such as **2** and **3**, however, are solubilized by the NaDC micelle (pattern 2). The profiles for the guest steroids **4** and **8**, which have the α-hydroxy groups at the C-3 and C-12 positions in common, show pattern 3. In the case of **8**, the decrease in the turbidity is very remarkable in the NaDC solution below the cmc. We regard the turbidity–NaDC concentration profile for **8** as a modified pattern of pattern 3. It is clear that both **4** and **8** are solubilized by the NaDC micelle. The steroid **5** is an epimer of **4**, and, interestingly, its pattern (pattern 2) is different from that for **4** (pattern 3). Although both **4** and **5** can penetrate into the NaDC micelle, the behavior in the NaDC solutions below the cmc is different between

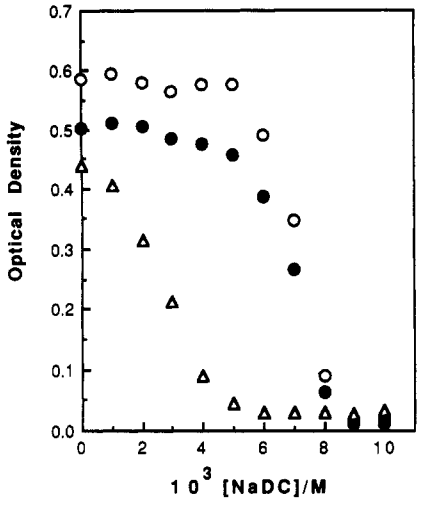

Figure 5. Turbidity changes of the aqueous dispersions of **6–8** as a function of [NaDC]: ○, **6**; ●, **7**; △, **8**.

TABLE III: Patterns of Turbidity–NaDC Concentration Profiles for Various Guest Steroids

compd	C-3	C-7	C-12	C-24	pattern
1	H	H	H	$COOCH_3$	1
2	α-OH	H	H	$COOCH_3$	2
3	β-OH	H	H	$COOCH_3$	2
4	α-OH	H	α-OH	$COOCH_3$	3
5	β-OH	H	α-OH	$COOCH_3$	2
6	α-OH	α-OH	H	$COOCH_3$	2
7	α-OH	β-OH	H	$COOCH_3$	2
8	α-OH	α-OH	α-OH	$COOCH_3$	3
9	α-OH	H	H	OH	2
10	α-OH	H	α-OH	OH	3
11	α-OH	α-OH	H	OH	2
12	α-OH	H	α-OH	Br	4
13[a]	H	H	H	2-propyl	1
14[a]	β-OH	H	H	2-propyl	1
15[b]	α-OH	H	H	2-propyl	1
16[b]	β-OH	H	H	2-propyl	1

[a] Cholestane derivative. [b] Cholesterol derivative.

these epimers. The guest **6** and its epimer **7** having two hydroxy groups at the C-3 and C-7 positions exhibit pattern 2 but do not show the initial decrease in the turbidity at NaDC concentrations much lower than the cmc.

On the basis of these findings, it can be concluded that the hydroxy group at the C-3 position of the methyl cholanoates is necessary for the solubilization of these water-insoluble steroids by the NaDC micelle.

Consider now the turbidity changes in the NaDC solutions below the cmc. In the cases of patterns 1 and 2, the turbidities decrease to some extent at NaDC concentrations much lower than the cmc. The steroids **1–3** and **5** exhibit these profiles. The degree of the decrease in the turbidity at low NaDC concentrations seems to depend upon the hydrophobicity of the guest molecules. ΔOD is defined as

$$\Delta OD = (OD_0 - OD)/OD_0 \quad (1)$$

where OD_0 is the optical density of the dispersion in the absence of NaDC, and OD represents the average optical density of the dispersion in the region where the turbidity becomes constant at a NaDC concentration range below the cmc ($(2–5) \times 10^{-3}$ M). ΔOD for **1–3** and **5** are 0.36, 0.28, 0.17, and 0.17, respectively. Assuming that the β-hydroxy group in the steroid nucleus determines the increase in the hydrophilicity of the β-plane, the hydrophobicity of the β-planes of the guest steroids seems to increase in the order **5**, **3**, **2**, and **1**. ΔOD increases in this order, suggesting that the initial decrease in the turbidity is ascribed to a complexation between NaDC and the guest steroid through a hydrophobic interaction. There are the regions where the turbidities are almost constant in patterns 1 and 2. In this region,

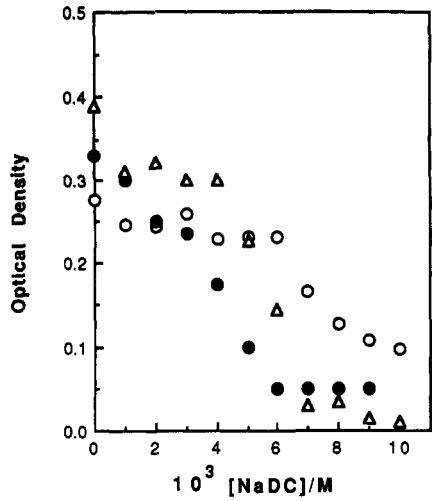

Figure 6. Turbidity changes of the aqueous dispersions of **9–11** as a function of [NaDC]: ○, **9**; ●, **10**; △, **11**.

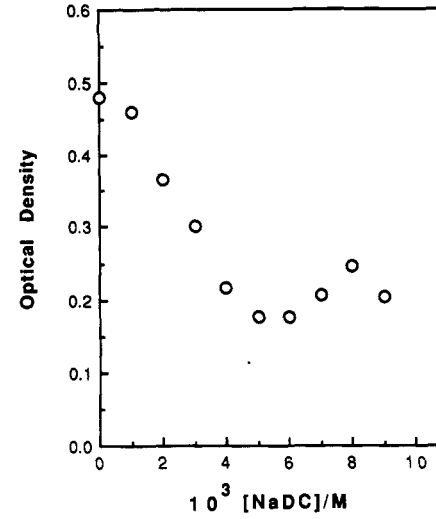

Figure 7. Turbidity change of the aqueous dispersion of **12** as a function of [NaDC].

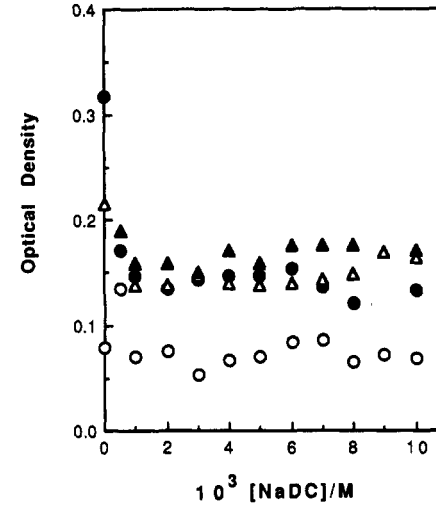

Figure 8. Turbidity changes of the aqueous dispersions of **13–16** as a function of [NaDC]: ○, **13**; ●, **14**; △, **15**; ▲, **16**.

further interaction between NaDC and the guest may not occur. The guests **4** and **8**, which have two α-hydroxy groups at the C-3 and C-12 positions in common, show a continuous decrease in turbidity at the NaDC concentrations both below and above the cmc. The results cannot be explained in terms of the hydrophobic interaction as the only binding force for molecular association between NaDC and guest steroid. If only hydrophobic interaction was the binding force, the decrease in the turbidities of the steroids **1–3** would be greater than that for **4** and **8**. The experimental results do not fit this expectation. For example, a large decrease in the turbidity was observed in the case of **8**, which has three hydroxy groups. We have to consider an interaction in addition to the hydrophobic interaction to explain the molecular association between NaDC and **4** or **8**. The most plausible interaction is simultaneous hydrogen bonding between the α-hydroxy groups at the C-3 position of NaDC and at the C-12 position of **4** or **8** and the α-hydroxy groups at the C-12 position of NaDC and the C-3 position of **4** or **8**.

Interactions between NaDC and Cholanols. The turbidity–NaDC concentration profiles for the cholanols **9–11** are shown in Figure 6, and their patterns are listed in Table III. The guests **9** and **11** have an α-hydroxy group at the C-3 position but not at the C-12 position. In analogy with their corresponding methyl cholanoates, these guest steroids exhibit pattern 2. On the other hand, **10**, which is a derivative of deoxycholic acid, strongly interacts with NaDC in solutions below the cmc. The results are essentially the same as those obtained for the methyl cholanoates, and therefore it is confirmed that the α-hydroxy groups at the C-3 and C-12 positions of the guest are important for interaction with NaDC below the cmc.

Interaction between NaDC and 12. The profile obtained for **12** (Figure 7) is very suggestive. The guest **12** exhibits pattern 4. It seems that **12** interacts with NaDC below the cmc but is not solubilized by the NaDC micelle. This result can be explained reasonably: the α-hydroxy groups at the C-3 and C-12 positions of **12** interact with the α-hydroxy groups at the C-12 and C-3 positions of NaDC, respectively, through hydrogen bonding to form a face-to-face association complex in the NaDC solution below the cmc, but above the cmc **12** cannot penetrate into the NaDC micelle because of the absence of a polar head group at the C-24 position that can participate in hydrogen bonding.

From the results obtained for the methyl cholanoates and the cholanols, it becomes clear that the hydroxy group at the C-3 position of the guest steroid is necessary for solubilization of the guest steroids by the NaDC micelle. The methyl cholanoates and the cholanols have the carboxylate ester and hydroxy groups, respectively, at the C-24 position, but **12** does not have a polar head group that can contribute to the formation of a hydrogen bond. It seems, therefore, that the polar head group such as –COOR or –OH, which can participate in hydrogen bonding, at the C-24 position as well as the hydroxy group at the C-3 position of the guest steroid is necessary for interaction between the guest and the NaDC micelle. It is reasonable to consider that the solubilization of the guest steroid by the NaDC micelle requires simultaneous hydrogen bonding between the hydroxy group at the C-3 position of the guest and the carboxylate anion of the host and the polar head group at the C-24 position of the guest and the hydroxy group at the C-3 position of the host.

Interactions between NaDC and Cholestane Derivatives. Cholestane (**13**), 3β-hydroxycholestane (cholestanol, **14**), cholest-5-en-3α-ol (epichoresterol, **15**), and cholest-5-en-3β-ol (cholesterol, **16**) reveal pattern 1 as shown in Figure 8. A series of these guest steroids does not have an α-hydroxy group at the C-12 position and a polar head group at the C-24 position. On the basis of the assumption described above, these steroids would not be expected to form the hydrogen-bonded complex of the guest and the host in the NaDC solution below the cmc as well as the guest-embedding NaDC micelle in the NaDC solution above the cmc. The results obtained for **13–16** are consistent with this expectation.

Discussion

During conversion of cholesterol into bile acids in the liver, an epimerization of the β-hydroxy group at the C-3 position of cholesterol as well as α-hydroxylation of the steroid nucleus occurs to give cholic acid and deoxycholic acid. Except for ursodeoxycholic acid, all hydroxy groups introduced to the natural bile salts are directed to a concave surface of the steroid nucleus. The

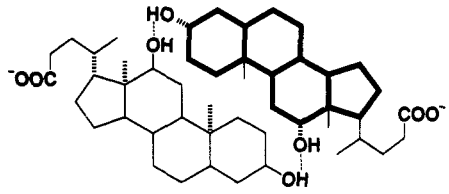

Figure 9. Plausible structure of the hydrogen-bonded dimer of NaDC.

hydroxy groups, therefore, provide a hydrophobic β-plane and a relatively more hydrophilic α-plane of bile salt. It is evident that the hydroxy groups play an important role in solubilization of the bile salts in an aqueous medium. For example, sodium salts of cholanic acid and lithocholic acid are sparingly soluble in water, whereas NaDC are well soluble. The back-to-back model[5,6] for the micelle formation of NaDC is understandable on the basis of the nature of the bile salts described above. Namely, in this model, a small micelle is assumed to be formed by facing the hydrophobic β-planes of the NaDC molecules through a hydrophobic interaction. Although this model is currently accepted,[9,20,22-25] our results obtained cannot be explained by the back-to-back model. If the hydrophobic interaction was the main binding force for primary association of the NaDC molecules, the more hydrophobic guest steroids would interact with NaDC more efficiently. Contrary to this expectation, the hydrophobic guest steroids such as **1** and **13–16** do not interact strongly with NaDC both below and above the cmc. Our turbidity measurements clearly indicate that both α-hydroxy groups at the C-3 and C-12 positions of the guest steroid are necessary for interaction with the NaDC molecule, which also has two α-hydroxy groups at the C-3 and C-12 positions, in the NaDC solution below the cmc (see the results for **4**, **8**, **10**, and **12**). The most reasonable explanation for this specific interaction is the formation of the hydrogen-bonded complexes of the guests and NaDC. This explanation presented for the host–guest interaction can be expanded to the discussion of the self-association of the NaDC molecules below the cmc. Neglecting an electrostatic repulsion between the carboxylate anions, it is quite reasonable to consider the formation of the hydrogen-bonded dimer of NaDC as a premicelle whose plausible structure is illustrated in Figure 9. This model is exactly the same as that demonstrated by Oakenfull and Fisher.[7,8] The electrostatic repulsion between the carboxylate anions is minimized in this model. A space-filling molecular model suggests that the face-to-face dimer is sterically reasonable. Of course, hydrogen bonding is a very weak interaction in aqueous media. In the case of the NaDC association, however, the hydrogen bonds are formed at two points, and their formation may be assisted by a hydrophobic environment provided by the α-plane of the steroid. The situation is very similar to that of proteins whose conformation are determined by hydrogen bonds in microscopically hydrophobic environments provided by polypeptides. Enantioselective complexations between bilirubin and oligosaccharides[26] and nucleosides[27] in water are in the same category. The hydrogen-bonded dimer of NaDC has also been suggested by Tanaka and coworkers.[17,28,29]

The present results on the solubilization of the guest steroids into the aqueous NaDC above the cmc can be utilized to evaluate the mechanism of the NaDC micellization. The hydroxy group at the C-3 position and the polar head group at the C-24 position, which can form a hydrogen bond with a hydroxy group, are essential for solubilization of the guest steroids by the NaDC micelles. On the basis of this finding, we can deduce a mechanism for the micelle formation of NaDC that involves hydrogen bonding between the hydroxy group at the C-3 position of a premicelle and the carboxylate anion at the C-24 position of another premicelle. A similar mechanism has already been suggested,[5,7,30] and the formation of a hydrogen bond between the α-hydroxy group at the C-3 position of a NaDC molecule and the carboxylate anion of another NaDC molecule has been detected by an X-ray analysis.[10]

Judging from the models presented in this study, the salts of deoxycholic acid and cholic acid are expected to form the hydrogen-bonded dimer below the cmc, but the salts of lithocholic acid and ursodeoxycholic acid do not. The higher cmc of sodium ursodeoxy cholate ($>1 \times 10^{-2}$ M) may be ascribed to the absence of the hydrogen-bonded dimer of this bile salt. Further study is now in progress. Meanwhile, the solubilization phenomena of the water-insoluble guest steroids employed in this study into the NaDC solution containing NaCl are quite different from those in the absence of NaCl. Effects of inorganic salts on micellar structures will be our next subject.

Acknowledgment. This work was supported by Grant-in-Aid for Science Research on Priority Areas, "Multiplex Organic Systems" (01649006), from the Ministry of Education, Science and Culture, Japan. We acknowledge the experimental assistance by Yuuko Wakabayashi and Yutaka Imanishi.

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