Solubilization of Cholesterol in Two Binary Mixed Micelles of Bile Salt and Nonionic Surfactant

Hiroyuki Asano[a], **Hiroki Sasamoto**[a] and **Minoru Ueno**[a,b,*]
[a]Department of Applied Chemistry, Faculty of Science, Science University of Tokyo, 1-3 Kagurazaka, Shinjuku-ku, Tokyo 162, Japan and [b]Institute of Colloid and Interface Chemistry, 1-3 Kagurazaka, Shinjuku-ku, Tokyo 162, Japan

The saturated amounts of solubilized cholesterol (C_{ch}) in mixed micelles of sodium cholate (NaC) and octaoxyethylene glycol mono *n*-decyl ether ($C_{10}E_8$) and of sodium deoxycholate (NaDC) and $C_{10}E_8$ were measured by enzyme assay at 25, 29, 33 and 37°C. The C_{ch} values in both systems increase with the total surfactant concentration. Because the mixed micelles for both systems tend to form $C_{10}E_8$-rich micelles near the critical micellar concentration (CMC) of the mixed system, the curves of cholesterol solubility approached the C_{ch} curve for $C_{10}E_8$ alone near the CMC. The tendency of C_{ch} to decrease in both systems with increasing mole fractions of bile salts resembled that of the mean aggregation number of micelles. Thermodynamic analyses of cholesterol solubilization showed that the free energy of solubilization, if considered as the transfer of cholesterol from solid state to micellar environment, increased with increasing mole fraction of bile salt. The enthalpy of cholesterol solubilization ($\Delta H_{S \to M}$) decreased with the mole fraction of bile salts and showed break points around the mole fraction of 0.75 for the NaC-$C_{10}E_8$ system and at 0.60 for the NaDC-$C_{10}E_8$ system, respectively. These phenomena resemble earlier hydrophobicity data for mixed micelles by fluorescence measurements. Furthermore, C_{ch} values for the NaDC-$C_{10}E_8$ system were larger than those for the NaC-$C_{10}E_8$ system because of the structural differences at the 7α hydroxyl group between NaC and NaDC. This fact was confirmed by thermodynamic calculations.

KEY WORDS: Cholesterol, octaoxyethylene glycol mono *n*-decyl ether, sodium cholate, sodium deoxycholate, solubilization.

Bile salts are important biosurfactants, and bile salts' micelles differ from micelles found in solutions of classical surfactants because of their unusual chemical structure, *i.e.*, their molecules have a hydrophobic surface, which is the convex side of the steroid nucleus, and a hydrophilic surface, which is the polyhydroxylated concave side of this nucleus (1–7). Accordingly, micelles of bile salts are rigidly formed by hydrophobic interaction and hydrogen bonding between the bile salts' molecules. On the other hand, nonionic surfactant molecules have an alkyl chain as the hydrophobic moiety and a polyoxyethylene chain as the hydrophilic moiety, and they form loosely packed micelles in solution.

In a previous paper (8), the unusual properties of bile salts [sodium cholate (NaC) and sodium deoxycholate (NaDC)] have been researched by an addition of a nonionic surfactant [octaoxyethylene glycol mono *n*-decyl ether ($C_{10}E_8$)] to their solutions. The micellar properties of aqueous binary solutions consisting of these bile salts and $C_{10}E_8$ are as follows: Each mixed system is a nonideal mixture in solution, and the mole fraction of bile salt in the mixed micelles near the critical micellar concentration (CMC) is less than that of the total solution. The hydrophobicity of the interior of the mixed micelles increases gradually up to a mole fraction of 0.75 for the NaC-$C_{10}E_8$ system and 0.57 for the NaDC-$C_{10}E_8$ system. Above these values, the hydrophobicity increases steeply with an increase of bile salt. From these facts it can be assumed that the mixed micelles change from $C_{10}E_8$-rich micelles to bile salt-rich micelles, in particular for the NaDC-$C_{10}E_8$ system because of the stronger hydrophobicity of NaDC. Furthermore, the aggregation number decreases dramatically with an increasing mole fraction of bile salt in the low-mole fraction range because of strong cohesion and the large volume of bile salt molecules.

Accordingly, the solubilization of cholesterol in these mixed solutions can be expected to be influenced by these mixed-micelle properties. In this work, cholesterol solubility in the mixed micelles of two combination systems (NaC-$C_{10}E_8$ and NaDC-$C_{10}E_8$) at various temperatures will be discussed on the basis of the earlier results for these mixed micelles and the data from the enzyme assay (9,10). Then, the relationship between the properties of these mixed micelles and cholesterol solubility in these systems will be found out from all results.

EXPERIMENTAL PROCEDURES

Materials. NaC (Mikuni Chemicals, Tokyo, Japan) and NaDC (Sigma Chemical, St. Louis, MO) were purified several times by recrystallization from a mixture of ethanol and methanol or from ethanol alone, and then Soxhlet extraction was performed with acetone or *n*-hexane for 72 h. $C_{10}E_8$ (Nikko Chemicals, Tokyo, Japan) was purified by gel chromatography (Wakogel C-200; Wako Chemicals, Osaka, Japan) with acetone and *n*-hexane mixed equivalently; the eluate then gave a single spot by thin-layer chromatography. These surfactants were confirmed to be highly pure by the fact that there was no minimum in the surface tension *vs.* the concentration curves around each of the CMCs.

Cholesterol (Kokusan Chemical, Tokyo, Japan) was recrystallized three times from hot ethanol and evacuated to dryness in a dessicator. The reagent for enzyme assay of cholesterol, Sanassay F-CHO, was obtained from Sankoh Junyaku (Tokyo, Japan).

To prevent hydrolysis of the bile salts, aqueous solutions of surfactants were prepared with tris/HCl buffer solution adjusted at pH 9.00 ± 0.05 with ionic strength of 0.026.

Methods. Before all measurements, aqueous solutions for NaC, NaDC and $C_{10}E_8$ were prepared separately, as well as binary mixtures of each bile salt and $C_{10}E_8$ with mole fractions of 0.25, 0.50, 0.75 and 0.9, respectively.

The assay of solubilized cholesterol in micellar solutions was performed as follows: Cholesterol (5 mg) in 10 mL of micellar solution and fixed volumes of the micellar solution were added to glass-stoppered bottles, and the air in these bottles was replaced by nitrogen gas. The sample bottles were then treated with an ultrasonic generator for

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*To whom correspondence should be addressed.

a fixed time and immersed in a constant-temperature bath adjusted at 25 ± 0.05°C, 29 ± 0.05°C, 33 ± 0.05°C or 37 ± 0.05°C from two weeks to a month to attain equilibrium of the solutions. After filtration through Millipore filters (pore size: 0.22 μm) (Milford, MA), the saturated amounts of the solubilized cholesterol in the micellar solutions were measured by using the color-development enzyme for cholesterol (9,10). The absorbance of this coloration was measured at 555 nm with a Shimadzu spectrophotometer UV-120-02 (Kyoto, Japan).

RESULTS AND DISCUSSION

Dependency of cholesterol solubilization on total surfactant concentration at 25 and 37°C. Figure 1 shows the saturated amounts of solubilized cholesterol, C_{ch}, in the aqueous binary solution of NaC and $C_{10}E_8$ at 25 and 37°C, respectively, as a function of total surfactant concentration. Similarly, C_{ch} for the NaDC-$C_{10}E_8$ system at 25 and 37°C is shown in Figure 2a and 2b, respectively.

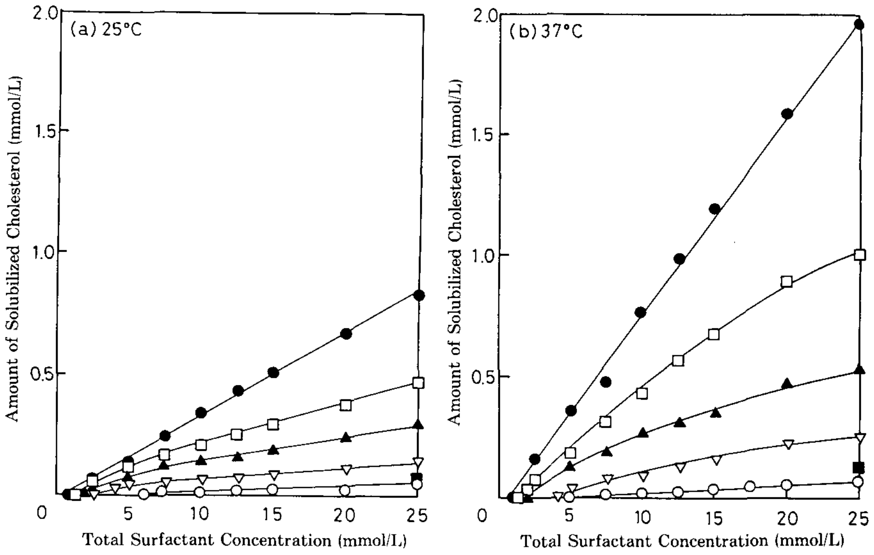

FIG. 1. Saturated amount of solubilized cholesterol at 25 (a) and 37°C (b) for the sodium cholate (NaC)–octaoxyethylene glycol mono *n*-decyl ether system *vs.* total surfactant concentration at various mole fractions: Mole fraction of NaC = 0.00 (●), 0.25 (□), 0.50 (▲), 0.75 (▽), 0.90 (■), 1.00 (○).

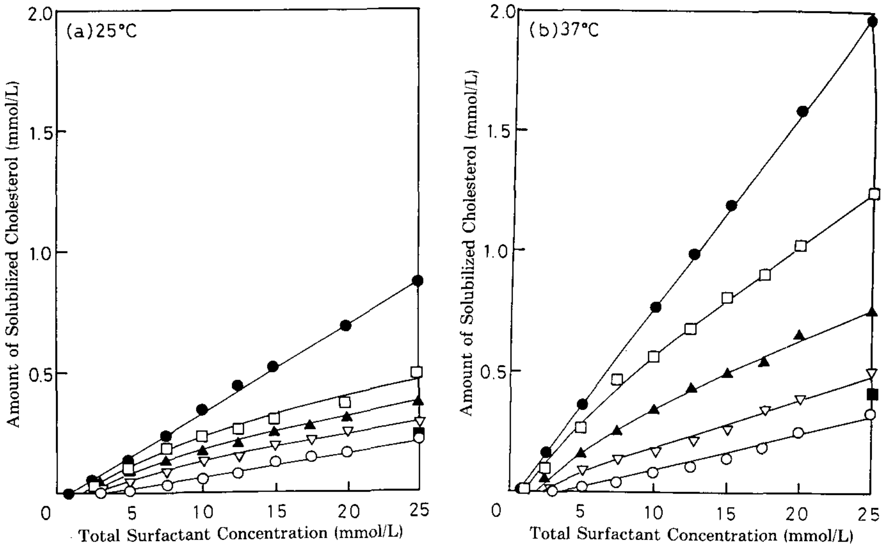

FIG. 2. Saturated amount of solubilized cholesterol at 25 (a) and 37°C (b) for the sodium deoxycholate (NaDC)–octaoxyethylene glycol mono *n*-decyl ether system *vs.* total surfactant concentration at various mole fractions: Mole fraction of NaDC = 0.00 (●), 0.25 (□), 0.50 (▲), 0.75 (▽), 0.90 (■), 1.00 (○).

The C_{ch} values for $C_{10}E_8$ alone at 25 and 37°C increased linearly with the increase of total $C_{10}E_8$ concentration, and the slope of C_{ch} values at 37°C was far larger than that at 25°C. In general, as the polyoxyethylene part of $C_{10}E_8$ is dehydrated by the rise in temperature, the hydrophobicity of $C_{10}E_8$ micelles becomes greater, and the mean aggregation number of $C_{10}E_8$ micelles increases with the rise in temperature (11). Accordingly, this suggests that the C_{ch} value in $C_{10}E_8$ solution is considerably influenced by a rise in temperature.

On the other hand, the C_{ch} values for NaC and NaDC alone at 25 and 37°C also increased linearly with increasing total NaC or NaDC concentration, respectively. However, the slope for each system was far lower than that for $C_{10}E_8$, and the difference of the C_{ch} values between 25 and 37°C at each total concentration of NaC or NaDC alone was less than that of $C_{10}E_8$. These are reasons why bile salt molecules tend to form rigid micelles as the result of strong hydrophobic interactions and hydrogen bonding (1,7). Furthermore, the C_{ch} values for NaDC alone at each temperature were larger than those for NaC alone. In our previous paper (8), we reported that NaDC molecules without the 7 α hydroxyl group would form more hydrophobic micelles in solution than NaC molecules with its 7 α hydroxyl group. This was based on the pyrene fluorescence data. Accordingly, the large C_{ch} value for NaDC alone may be due to the high hydrophobicity of NaDC micelles.

For the mixed NaC-$C_{10}E_8$ and NaDC-$C_{10}E_8$ systems, the C_{ch} values at 25 and 37°C also increased with total surfactant concentration. And C_{ch} values at 37°C for each mole fraction of bile salt in both systems were larger than those of 25°C because of the dehydration of $C_{10}E_8$ molecules in the mixed micelles. The values of C_{ch} for the NaDC-$C_{10}E_8$ system were larger than for NaC-$C_{10}E_8$ system. The reason is that the intramicelles for the NaDC-$C_{10}E_8$ system are more hydrophobic than those for the NaC-$C_{10}E_8$ system, and that the mean aggregation numbers of the mixed micelles in the NaDC-$C_{10}E_8$ system are larger than those in the NaC-$C_{10}E_8$ system (8). Furthermore, it is interesting that, for both mixed systems, these curves approached a straight line for $C_{10}E_8$ alone near the CMC and became logarithmic at low total surfactant concentrations. In our earlier papers (8,12,13), we have indicated that mixed micelles consisting of bile salts and $C_{10}E_8$ form $C_{10}E_8$-rich micelles near the CMC, which was based on theoretical calculations of the micellar compositions from the excess thermodynamic quantities of Motomura *et al.* (14). Accordingly, this phenomenon can be attributed to the $C_{10}E_8$-rich micelles at low total surfactant concentration.

Dependency of cholesterol solubilization on mole fraction of bile salts. To examine the dependency of cholesterol solubilization on the mole fraction of bile salts, the C_{ch} values at 25, 29, 33 and 37°C were measured in micellar solutions that were fixed at 25 mmol/L of total surfactant concentration. At this concentration, all the surfactants are perfectly in micellar forms as shown by CMC data for each system at both 25 and 37°C as obtained by surface tension measurements (Table 1) (11).

The C_{ch} values at 25, 29, 33 and 37°C in these micellar solutions, fixed at 25 mmol/L total surfactant concentration, as a function of the mole fraction of bile salt are shown in Figure 3a for the NaC-$C_{10}E_8$ system and in Figure 3b for the NaDC-$C_{10}E_8$ system, respectively. The values are listed in Tables 2 and 3.

TABLE 1

Critical Micelle Concentration (CMC) of Each System at 25 and 37°C

Mole fraction of bile salt in the solution (X_{bile})	CMC (mmol/L)[a]			
	NaC-C_{10} system		NaDC-$C_{10}E_8$ system	
	25°C	37°C	25°C	37°C
0.00	1.00	0.80	1.00	0.80
0.25	1.17	0.99	1.11	0.93
0.50	1.53	1.33	1.25	1.15
0.75	2.28	2.07	1.63	1.60
0.90	4.28	3.83	2.42	2.06
1.00	6.93	6.83	3.16	2.77

[a]NaC, sodium cholate; NaDC, sodium deoxycholate.

The C_{ch} values for NaC and NaDC alone at 25, 29, 33 and 37°C were considerably smaller than those for $C_{10}E_8$ alone. The reason is that the solubilization of cholesterol into bile salts' micelles is made difficult by the rigid micelles and low aggregation number of bile salts. However, the C_{ch} values for NaDC alone at each temperature were larger than those for NaC alone because of the high hydrophobicity of NaDC molecules.

For the mixed systems, the C_{ch} values at each temperature decreased abruptly with increasing mole fraction of bile salts, and these curves resembled the curves found in earlier research for the variation of mean aggregation number (15,16) of the mixed micelles for bile salt–$C_{10}E_8$ systems as a function of the mole fraction (8,11–13). These facts indicate that C_{ch} depends considerably on the mean aggregation number of the micelles. Furthermore, in both systems, the more the mole fraction of bile salts decreased, the larger the C_{ch} values became with a rise in temperature. This may be due to the increase of the hydrophobicity of $C_{10}E_8$ caused by the dehydration in the polyoxyethylene moiety.

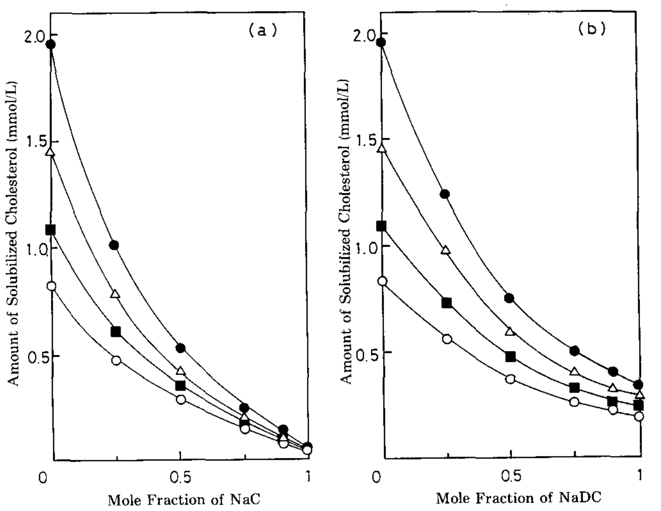

FIG. 3. Saturated amount of solubilized cholesterol at 25 (○), 29 (■), 33 (△) and 37°C (●) for (a) NaC-$C_{10}E_8$ system and (b) (NaDC)–sodium deoxycholate-octaoxyethylene glycol mono *n*-decyl ether system *vs.* mole fraction of sodium cholate (NaC) or NaDC. (The total surfactant concentration of each system is fixed at 25 mmol/L.)

TABLE 2

Saturated Amount of Cholesterol and the Values of Various Thermodynamic Parameters of the NaC-$C_{10}E_8$ System at Total Surfactant Concentration of 25 mmol/L[a]

Mole fraction of NaC in the solution (X_{NaC})	Saturated amount of cholesterol [C_{ch} (mmol/L)]				Free energy of solubilization [$\Delta G_{S \to M}$(kJ/mol)]				Enthalpy of solubilization [$\Delta H_{S \to M}$ (kJ/mol)]
	25°C	29°C	33°C	37°C	25°C	29°C	33°C	37°C	
0.00	0.831	1.093	1.458	1.965	8.547	7.996	7.402	6.776	52.55
0.25	0.481	0.607	0.791	1.020	9.872	9.433	8.899	8.379	47.22
0.50	0.280	0.348	0.424	0.534	11.20	10.81	10.45	10.00	40.51
0.75	0.140	0.172	0.195	0.242	12.91	12.57	12.42	12.02	33.86
0.90	0.071	0.082	0.092	0.098	14.60	14.41	14.34	14.35	24.46
1.00	0.044	0.050	0.056	0.061	15.78	15.66	15.59	15.55	19.93

[a]Abbreviations: NaC-$C_{10}E_8$, sodium cholate-octaoxyethylene glycol mono *n*-decyl ether; C_{ch}, solubilized cholesterol.

TABLE 3

Saturated Amount of Cholesterol and the Values of Various Thermodynamic Parameters of the Sodium Deoxycholate (NaDC-$C_{10}E_8$) System at Total Surfactant Concentration of 25 mmol/L[a]

Mole fraction of NaDC in the solution (X_{NaDC})	Saturated amount of cholesterol [C_{ch} (mmol/L)]				Free energy of solubilization [$\Delta G_{S \to M}$(kJ/mol)]				Enthalpy of solubilization [$\Delta H_{S \to M}$ (kJ/mol)]
	25°C	29°C	33°C	37°C	25°C	29°C	33°C	37°C	
0.00	0.831	1.093	1.458	1.965	8.547	7.996	7.402	6.776	52.55
0.25	0.560	0.731	0.978	1.240	9.500	8.976	8.375	7.895	49.52
0.50	0.379	0.487	0.605	0.793	10.46	9.974	9.564	9.009	45.88
0.75	0.264	0.327	0.409	0.504	11.34	10.96	10.55	10.15	41.06
0.90	0.226	0.268	0.333	0.407	11.72	11.45	11.06	10.70	37.58
1.00	0.198	0.244	0.299	0.341	12.06	11.69	11.33	11.15	35.12

[a]NaC-$C_{10}E_8$, sodium cholate-octaoxyethylene glycol mono *n*-decyl ether; C_{ch}, solubilized cholesterol.

Thermodynamic analysis of cholesterol solubilization. The standard free energy ($\Delta G_{S \to M}$) and the enthalpy ($\Delta H_{S \to M}$) of solubilization, considering transfer of cholesterol from the solid state to the micellar environment, were calculated from the C_{ch} values in each micellar solution at 25 mmol/L of total surfactant concentration to discuss thermodynamically the cholesterol solubility in the micelles of the NaC-$C_{10}E_8$ and NaDC-$C_{10}E_8$ systems (17–20).

$\Delta G_{S \to M}$ values for each mole fraction of bile salts and each temperature are given by Equation 1,

$$\Delta G_{S \to M} = -RT \ln X_{ch} \qquad [1]$$

where R is the molar gas constant, T is the absolute temperature and X_{ch} is the mole fraction of cholesterol in the solutions.

Figure 4a shows plots of $\Delta G_{S \to M}$ values for the NaC-$C_{10}E_8$ system at 25, 29, 33 and 37°C *vs.* the mole fractions of bile salt. Similar curves for the NaDC-$C_{10}E_8$ system are shown in Figure 4b. The $\Delta G_{S \to M}$ values are also listed in Tables 2 and 3, respectively. Both figures show that $\Delta G_{S \to M}$ values for $C_{10}E_8$ alone were lower than those of bile salt alone. $\Delta G_{S \to M}$ for each mixed system increased with an increase in the mole fraction of bile salt, and in the range of low mole fractions of bile salt, the curves of the $\Delta G_{S \to M}$ values deviated downward with the rise in temperature. These facts suggest that $C_{10}E_8$ micelles solubilize the cholesterol easier than micelles of bile salts, and the ability of $C_{10}E_8$ solubilizing cholesterol depends appreciably on the temperature. Furthermore, the $\Delta G_{S \to M}$ values of NaDC alone at each temperature showed lower values than those of NaC alone. This fact confirms that the micelles of NaDC molecules without the 7 α hydroxyl group solubilize the cholesterol more easily than those of NaC.

However, the $\Delta G_{S \to M}$ values for each system in Figure 4 show positive values, and this indicates that the solubilization of cholesterol from the solid state to the micellar environment is not a spontaneous phenomenon. The reason

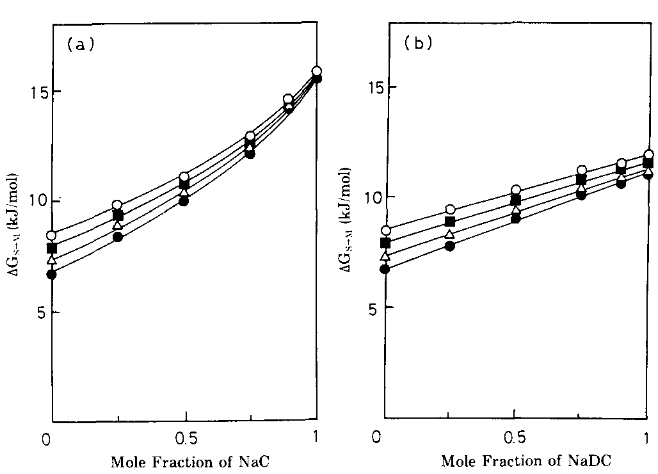

FIG. 4. Free energy of solubilization for transfer of cholesterol from the solid state to a micellar environment at 25 (○), 29 (■), 33 (△) and 37°C (●) for (a) sodium cholate (NaC)–octaoxyethylene glycol mono *n*-decyl ether system and (b) NaDC-$C_{10}E_8$ system *vs.* mole fraction of NaC or NaDC. (The total surfactant concentration of each system is fixed at 25 mmol/L.) See Figure 3 for abbreviations.

is that cholesterol is almost insoluble in water, and the standard free energy of cholesterol's dissolution from the solid state to the water phase, $\Delta G_{S\rightarrow W}$, is an enormous value (30.5 kJ/mol at 25°C) (17). Accordingly, it is necessary to consider the saturated level of solubilized cholesterol from the solid state to water phase (in the absence of a surfactant) and to calculate the standard free energy for the solubilization of cholesterol from water phase to the micellar environment ($\Delta G_{W\rightarrow M}$). Consequently, the $\Delta G_{W\rightarrow M}$ values at 25°C were calculated as shown in Table 4. From these results, the transfer of cholesterol from the water phase to the micellar environment of both systems is considered a natural process thermodynamically.

$\Delta H_{S\rightarrow M}$ values were calculated as follows in Equation 2:

$$\Delta H_{S\rightarrow M} = -R[d\ln X_{ch}/d(1/T)] \qquad [2]$$

Because the saturated amount of dissolved cholesterol in water is negligibly small, the $\Delta H_{S\rightarrow M}$ values can be calculated without the consideration of the cholesterol's transfer from the solid state to the water phase. On the basis of this assumption and the CMC data of each solution, Arrhenius' plots for X_{ch} and T values of each system were drawn. Figure 5 shows plots of the natural logarithm of X_{ch} for the NaC–$C_{10}E_8$ system (Fig. 5a) and the NaDC–$C_{10}E_8$ system in Figure 5b as a function of the reciprocal of T. As each plot of natural logarithms of X_{ch} *vs.* 1/T gave a straight line, the $\Delta H_{S\rightarrow M}$ value of each system was calculated by using Equation 2.

Plots of $\Delta H_{S\rightarrow M}$ for both single and mixed micellar systems of NaC–$C_{10}E_8$ and NaDC–$C_{10}E_8$ *vs.* the mole fraction of bile salt are shown in Figure 6. Because all $\Delta H_{S\rightarrow M}$ values for both systems are positive, cholesterol solubilization in solutions of NaC–$C_{10}E_8$ and NaDC–$C_{10}E_8$ is endothermic.

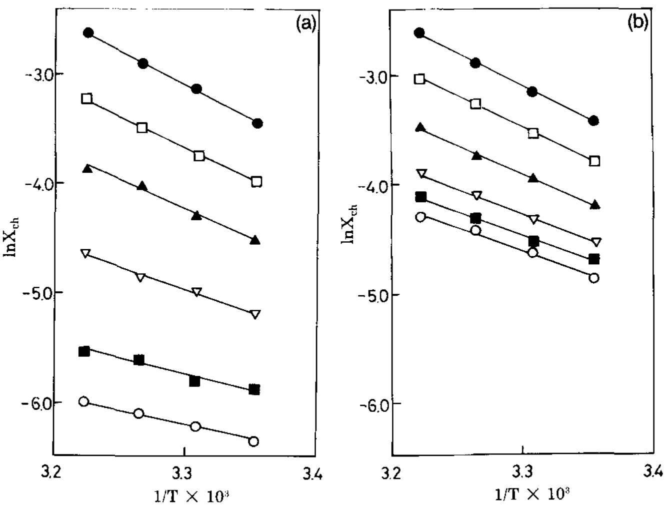

FIG. 5. Natural logarithm of X_{ch} for (a) NaC–$C_{10}E_8$ system and (b) NaDC–$C_{10}E_8$ system *vs.* 1/T. Mole fraction of bile salts = 0.00 (●), 0.25 (□), 0.50 (▲), 0.75 (▽), 0.90 (■), 1.00 (○). See Figure 4 for abbreviations.

In each mixed system, $\Delta H_{S\rightarrow M}$ decreased with an increase in the mole fraction of bile salt and showed break points around mole fraction 0.75 for the NaC–$C_{10}E_8$ system and 0.60 for NaDC–$C_{10}E_8$, respectively. Above these mole fractions, the $\Delta H_{S\rightarrow M}$ values decreased more steeply. This variation of $\Delta H_{S\rightarrow M}$ values for each system is similar to that of the I_1/I_3 values, which correspond to the ratio of the first and third peaks in the monomeric pyrene fluorescence emission spectrum in our previous work (8). This I_1/I_3 value is know to be an excellent index of the polarity for the intramicelles in the probe microenvironment (21–23). From this I_1/I_3 measurement, we have presumed the micellar shape of binary mixtures of bile salts and $C_{10}E_8$ to change from $C_{10}E_8$-rich micelles to bile salt-rich micelles at the break points. Accordingly, this resemblance suggests that cholesterol solubilization in solutions of NaC–$C_{10}E_8$ is influenced by the polarity of the mixed micelles and the change of the micellar shape from $C_{10}E_8$-rich micelles to bile salt-rich micelles.

$\Delta H_{S\rightarrow M}$ values for the NaDC–$C_{10}E_8$ system are higher than those for the NaC–$C_{10}E_8$ system. Theoretical analyses for the mixed micelles (8) show that, as NaDC molecules are easier to mix with $C_{10}E_8$ molecules, the

TABLE 4

The Free Energy of Solubilization of Cholesterol for Transfer from the Water Phase to a Micellar Environment ($\Delta G_{W\rightarrow M}$, at 25°C)[a]

Mole fraction of bile satls in the solution (X_{bile})	Free energy of solubilization ($\Delta G_{W\rightarrow M}$ (kJ/mol))	
	NaC–$C_{10}E_8$ system	NaDC–$C_{10}E_8$ system
0.00	−21.97	−21.97
0.25	−20.65	−21.02
0.50	−19.32	−20.06
0.75	−17.61	−19.18
0.90	−15.92	−18.80
1.00	−14.74	−18.46

[a]See Tables 2 and 3 for abbreviations.

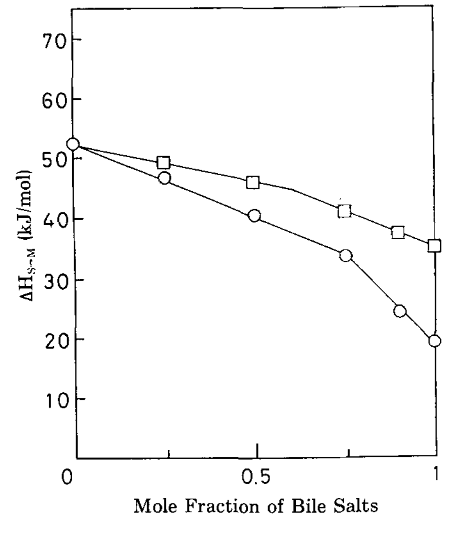

FIG. 6. The enthalpy of solubilization, assuming transfer of cholesterol from the solid state to a micellar environment for NaC–$C_{10}E_8$ system (○) and NaDC–$C_{10}E_8$ system (□) *vs.* mole fraction of bile salts. (The total surfactant concentration of each system is fixed at 25 mmol/L.) See Figure 4 for abbreviations.

mole fraction of $C_{10}E_8$ in a mixed micelle for the NaDC-$C_{10}E_8$ system is higher than that for the NaC-$C_{10}E_8$ system. Accordingly, cholesterol solubility into the mixed micelles of the NaDC-$C_{10}E_8$ system tends to depend more on temperature, and the cholesterol molecules are more easily solubilized into NaDC-$C_{10}E_8$ micelles.

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