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Studies on complexation between β-cyclodextrin and bile salts

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Summary

The thermodynamic parameters for the inclusion complexation of β-cyclodextrin and thirteen bile salts have been measured with flow microcalorimetry. The results show that most of the bile salts studied here form 1 : 1 complexes with the exception of deoxycholate and its conjugates which form 1 : 1 plus 1 : 2 complexes. The complexation affinities follow the order: deoxycholates > ursodeoxycholates ≈ chenodeoxycholates > cholates ≈ dehydrocholate. The inclusion modes were established with H-NMR for β-cyclodextrin and three bile salts. The thermodynamic data are consistent with the complex structures.

Introduction

The ligand binding interaction between β-cyclodextrin (βCD) and drugs is of interest in pharmaceutical research (Szejtli, 1988). As the complexes of βCD and like substances with drugs are considered for oral administration, the interactions of bile salts with βCD must be considered because of the possibility of exchange of the guest molecule with bile salts. The binding equilibrium constants of three bile salts to βCD have been estimated and used to explain the liberation mechanism of drug and βCD complexes (Miyajima et al., 1986). Drug absorption from the rat small intestinal lumen was also modified by βCD complexes (Nakanishi et al., 1989). The purpose of this investigation is to study more extensively the complexation between bile salts and βCD. Titration calorimetry (Lewis and Hansen, 1973; Briggner et al., 1986) and flow calorimetry (Hardee et al., 1978; Eftink and Harrison, 1981; Ueda et al., 1989) have been shown to be applicable to the determination of thermodynamic quantities for drug and cyclodextrin binding. The use of flow microcalorimetry for 1 : 1 stoichiometric binding reactions has been demonstrated (Tan and Lindenbaum, 1991). In this study, the flow microcalorimetry was utilized to investigate the complexation behavior between βCD and thirteen bile salts. Some of their H-NMR spectra were also determined to obtain information on the inclusion modes of βCD and bile salts.

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Materials and Methods

Materials

The βCD samples were purchased from Sigma and Fluka. The commercially available bile salts were either from Aldrich (98%): sodium cholate (C) and sodium deoxycholate (DC) or from Calbiochem: sodium chenodeoxycholate (CDC), sodium ursodeoxycholate (UDC), sodium glycocholate (GC), sodium glycodeoxycholate (GDC), sodium glycochenodeoxycholate (GCDC), sodium taurocholate (TC), sodium taurodeoxycholate (TDC). Sodium dehydrocholate (DHC) was from Fluka (97%). These compounds were used as received.

Sodium taurochenodeoxycholate (TCDC), sodium glycoursodeoxycholate (GUDC) and sodium tauroursodeoxycholate (TUDC) were prepared in our own laboratory using methods described in the literature (Lack et al., 1973). The TCDC purity was checked by TLC on silica gel (Kelly and Doisy, 1964). It yielded only a single spot on the plate. Standard addition experiments revealed that the maximum possible contamination by other conjugated and unconjugated bile salts was 1%.

Both the bile salts and βCD samples were analyzed for water content by thermogravimetry.

Deionized water was used throughout the study. All other chemicals were of analytical reagent grade.

Microcalorimetry

If the complexation is an n stepwise process of βCD binding to bile salt molecule A:

$$\beta CD + A \Leftrightarrow A \cdot \beta CD \qquad \Delta H_1, K_1$$
$$\beta CD + A \cdot \beta CD \Leftrightarrow A \cdot (\beta CD)_2 \qquad \Delta H_2, K_2$$
$$\cdots\cdots$$
$$\beta CD + A \cdot (\beta CD)_{n-1} \Leftrightarrow A \cdot (\beta CD)_n \qquad \Delta H_n, K_n$$

the overall reaction is:

$$n\beta CD + A \Leftrightarrow A \cdot (\beta CD)_n \qquad \Delta h_n, k_n$$

The experimental quantity which flow microcalorimetry measures is the rate of heat production P, and P may be expressed as:

$$P = R[A] \sum_{i=1}^{n} \Delta h_i k_i [\beta CD]^i \tag{1}$$

where R is the total flow rate of the two solutions, and [ ] stands for equilibrium concentration. The total concentrations of βCD and A, named as βCD_t and A_t, are

$$\beta CD_t = [\beta CD] + [A] \sum_{i=1}^{n} i k_i [\beta CD]^i \tag{2}$$

$$A_t = [A]\left(1 + \sum_{i=1}^{n} k_i [\beta CD]^i\right) \tag{3}$$

For single 1:1 binding, $n = 1$, the previous formulas have the simplest forms, and they may be correlated to each other. In this case, ΔH and K can be directly solved without requiring initial estimation and iterative calculation by use of the following equations (Tan and Lindenbaum, 1991):

$$\Delta H^4 (N\Sigma\alpha_i^2 - \Sigma^2\alpha_i) - \Delta H^3 (N\Sigma\alpha_i\beta_i - \Sigma\alpha_i\Sigma\beta_i) + \Delta H (N\Sigma\beta_i\gamma_i - \Sigma\beta_i\Sigma\gamma_i) - (N\Sigma\gamma_i^2 - \Sigma^2\gamma_i) = 0 \tag{4}$$

$$K = N/(\Delta H\Sigma\alpha_i - \Sigma\beta_i + \Sigma\gamma_i/\Delta H) \tag{5}$$

in which N is the number of data points, and α, β and γ are the combinations of the experimental quantities: $\alpha = A_t\beta CD_t R/P$; $\beta = A_t + \beta CD_t$; $\gamma = P/R$.

If $n = 2$, Eqns 1 and 2 may be written:

$$P = RA_t \frac{\Delta h_1 k_1 [\beta CD] + \Delta h_2 k_2 [\beta CD]^2}{1 + k_1 [\beta CD] + k_2 [\beta CD]^2} \tag{6}$$

$$\beta CD_t = [\beta CD] + A_t \frac{k_1 [\beta CD] + 2k_2 [\beta CD]^2}{1 + k_1 [\beta CD] + k_2 [\beta CD]^2} \tag{7}$$

Unfortunately, Eqns 6 and 7 cannot be unified into a single equation. For multiple equilibria, some methods have been developed to evaluate ΔH and K values from the calorimetric data (Izatt et al., 1968; Karlsson and Kullberg, 1976; Cerda et al., 1985). We have also derived a curve fitting program for this purpose. This program was run on a VAX computer. Values of Δh_1, Δh_2, k_1, k_2 and also the set of [βCD] corresponding to each βCD_t were initially estimated. By iteratively calling the subroutine UNLSF from IMSL (IMSL, 1987), the sum of squares of residuals, SSR,

$$SSR = \sum_{i=1}^{N} \left\{ (P_i - P_i^c)^2 + (\beta CD_{ti} - \beta CD_{ti}^c)^2 \right\}$$

converged to a minimum. P_i and βCD_{ti} are experimental data, P_i^c and βCD_{ti}^c are values calculated from Eqns 6 and 7, respectively.

Other formulas used in this work are: $\Delta G = -RT \ln K$; $\Delta S = (\Delta H - \Delta G)/T$; $\Delta H_1 = \Delta h_1$; $\Delta H_2 = \Delta h_2 - \Delta H_1$; $K_1 = k_1$; $K_2 = k_2/K_1$.

The operation of the flow microcalorimetry and dilution cell was the same as described elsewhere (Tan and Lindenbaum, 1991). The measurements were performed at 298.15 K. The densities of the buffer solutions were taken into account in calculating flow rates by volume.

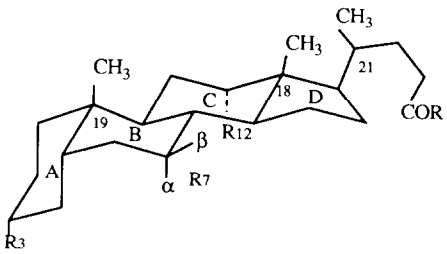
H-NMR method

The spectra were taken on a 300 MHz spectrometer using 5 mm sample tubes at room temperature (approx. 293 K). For each bile salt, five or six solutions with different molar ratios of βCD and the bile salt were prepared by dissolving a suitable amount of the bile salt in 5 mM βCD solution in D_2O solvent. The H-chemical shifts were referenced to the high peak of HOD which came from the solid samples and D_2O solvent. Repetitive measurements for βCD solutions showed a precision of 0.002 ppm. Referring to the literature, the six different protons on βCD (Demarco and Thakkar, 1970) and the protons connected to C_{18}, C_{19}, C_{21} and C_{12} of bile salt molecules (Barnes and Kirk, 1984) were easily distinguished.

Results and Discussion

A bile salt molecule has a rigid steroidal ring structure, as shown in Fig. 1. One side of the molecule has hydroxyl groups rendering this side of the molecule hydrophilic; the other side is hydrocarbon and hence hydrophobic in nature. Bile salts will therefore form micelles in aqueous solution at concentrations greater than the CMC (Sugihara et al., 1982; Vadnere and Lindenbaum, 1982). The enthalpy of micelle formation for DC was measured by calorimetry (Birdy, 1985). In order to study the heat of binding of bile salt to βCD, the heat of bile salt dissociation should be minimized. This was accomplished by varying the βCD concentration and keeping the bile salt concentration constant at a level of below 2.5 mM, lower than the CMCs of these bile salts (Hofmann and Small, 1967).

In addition to pH 7.4 Tris buffers (Tan and Lindenbaum, 1991), the reaction of C and βCD was measured in 0.02 M, 0.05 M and 0.10 M pH



Bile Salt	R_3	R_7	R_{12}	R
DHC	O=	O=	O=	ONa
C	OH	OH	OH	ONa
GC	OH	OH	OH	$NHCH_2CO_2Na$
TC	OH	OH	OH	$NHCH_2CH_2SO_3Na$
CDC	OH	α-OH	H	ONa
GCDC	OH	α-OH	H	$NHCH_2CO_2Na$
TCDC	OH	α-OH	H	$NHCH_2CH_2SO_3Na$
UDC	OH	β-OH	H	ONa
GUDC	OH	β-OH	H	$NHCH_2CO_2Na$
TUDC	OH	β-OH	H	$NHCH_2CH_2SO_3Na$
DC	OH	H	OH	ONa
GDC	OH	H	OH	$NHCH_2CO_2Na$
TDC	OH	H	OH	$NHCH_2CH_2SO_3Na$

Fig. 1. Structures and common names of bile salts used in this study.

7.2 phosphate buffer solutions and also in 0.02 M pH 9.0 Tris buffer solution. The results are almost the same and their average values are listed in Table 1. This suggests that the variation of the ionic species existing in the reaction system does not affect the complexation process. The pH change in this range also does not affect the bile salt stability.

The measurements for other bile salts were performed in 0.10 M pH 7.2 phosphate buffer solution except UDC which was measured at 0.02 M pH 9.0 Tris buffer solution because UDC was insoluble at pH 7.2.

The experimental data were first tested for a fit to the 1 : 1 model (Tan and Lindenbaum, 1991). The reactions of C, GC, TC, DHC, CDC, GCDC, TCDC, UDC, GUDC and TUDC yielded excellent fits to the 1 : 1 stoichiometric model. Fig. 2 represents some typical data and their fitting curves. For comparison of different bile salts, r, the ratio of βCD concentration to bile salt concentration, is used as the abscissa, and the ordinate is also expressed as the heat flux per mM bile salts. From the plateau heights of the three curves, it is apparent that the ΔH magnitudes are in the following order: UDC > CDC > C. The shapes of these three curves indicate that the K values of CDC and UDC are greater than that of C. Table 1 lists the thermodynamic parameters. Each reaction was repeated four times. The errors are expressed as standard deviation.

For reactions of DC, GDC and TDC, the heat

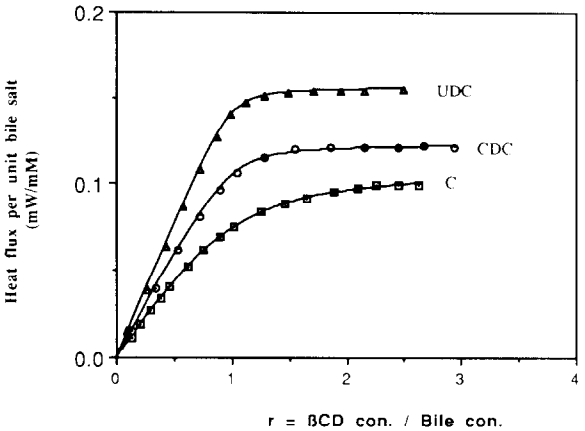


Fig. 2. Heat flux as a function of βCD concentration at 1.00 mM bile salt. The experimental bile salt concentrations are: C, 2.35 mM; CDC, 2.00 mM; UDC, 2.37 mM. The points are experimental data and the curves are derived from the 1 : 1 model.

fluxes were straight lines when $r < 1$. The heat fluxes continued to increase after $r > 1$. One example of the experimental data for DC is shown in Fig. 3. This type of curve did not fit the 1 : 1 model. It is assumed that each bile salt molecule has two binding sites for βCD. When $r < 1$, βCD binds to the first site (1 : 1 binding with large K). The ΔH for the first site binding may be obtained by using the data for $r < 1$ using Eqn 4. When $r > 1$, the extra βCD molecules may bind further to the second site on the bile salt molecule. After extracting the 1 : 1 binding part, the residual (the

TABLE 1

Thermodynamic parameters for the 1 : 1 β cyclodextrin–bile salt interactions

Bile salt	Abbreviation	$-\Delta H$	logK	$-\Delta G$	ΔS
Dehydrocholate	DHC	26.8 ± 0.3	3.38 ± 0.06	19.3	− 25.2
Cholate	C	26.0 ± 0.7	3.50 ± 0.05	20.0	− 20.1
Glycocholate	GC	26.7 ± 0.6	3.29 ± 0.06	18.7	− 26.8
Taurocholate	TC	22.9 ± 0.3	3.42 ± 0.04	19.3	25.2
Chenodeoxycholate	CDC	31.4 ± 0.7	4.36 ± 0.12	24.9	21.8
Glycochenodeoxycholate	GCDC	29.9 ± 0.2	4.50 ± 0.08	25.7	− 14.1
Taurochenodeoxycholate	TCDC	26.5 ± 0.2	5.04 ± 0.13	28.8	7.7
Ursodeoxycholate	UDC	39.1 ± 0.1	4.51 ± 0.06	25.7	− 44.9
Glycoursodeoxycholate	GUDC	38.4 ± 0.4	4.42 ± 0.10	25.2	− 44.3
Tauroursodeoxycholate	TUDC	34.2 ± 0.2	4.91 ± 0.17	28.0	− 20.8

K is in mol $^{-1}$ l; ΔH and ΔG are in kJ mol $^{-1}$; ΔS is in J mol $^{-1}$ K $^{-1}$.

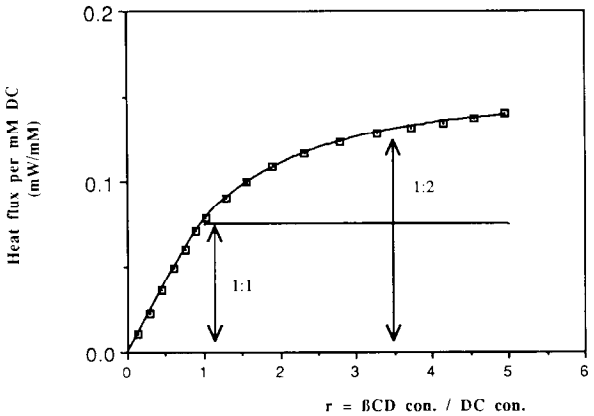


Fig. 3. Heat flux as a function of βCD concentration at 1.00 mM DC. The experimental DC concentration is 1.27 mM. The points are experimental data and the curve is derived from the 1 : 1 plus 1 : 2 model detailed in the text.

data over the horizontal line) can be again fitted to a 1 : 1 model and the ΔH_2 and K_2 are calculated. To verify the suggested mechanism, an experiment was carried out in which βCD was allowed to bind to the equimolar mixture of βCD and DC. The experimental data were fitted to the 1 : 1 model and the ΔH_2 and K_2 were nearly the same as those obtained above. For the overall 1 : 1 plus 1 : 2 reaction system, the ΔH_1, ΔH_2 and K_2 values estimated above and a guessed K_1 value were used as initial estimations for the curve fitting. The derived results are listed in Table 2. The fitting curve for the data in Fig. 3 is also shown.

The calorimetric experiments may suggest stoichiometries and inclusion modes of the βCD–bile salt complexes. In order to establish these structures, the proton NMR technique was employed to obtain the spectra of βCD with three typical bile salts: C, CDC and DC. From one spectrum, the chemical shifts of protons on the bile salt molecule as well as on βCD were obtainable. The induced shift, $\Delta\delta$, is defined as the difference in chemical shifts in the presence and absence of the other reactant. In this convention, a positive sign shows a downfield shift, and a negative sign shows an upfield shift. To express the induced shifts of βCD protons by bile salt molecules, the molar ratio of the bile salt to βCD is used as the abscissa. To express the induced shifts of bile salt protons by βCD, the molar ratio of βCD to the bile salt is used as the abscissa. The proton induced shifts determined in this fashion are shown in Fig. 4.

Even though there is no π electronic screening on the bile salt molecules, the major induced shifts of βCD and also bile salt protons are quite significant. It shows strong interactions between the host and guest molecules. The βCD proton shifts induced by the three bile salt molecules (graphs a, c and e in Fig. 4) verify that the bile salt molecules are included in the βCD cavity because its H_5, H_6 and H_3 shifts are greater than its H_1, H_2 and H_4 shifts. The fact that H_5 has a larger shift than H_3 and that H_6 has a relatively large shift indicates that βCD molecules bind toward bile salts from its primary hydroxyl side. The shapes of the curves confirm that the K value for C is less than those of CDC and DC.

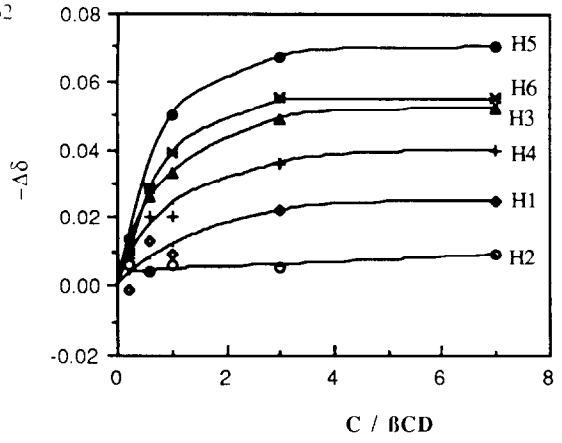
The complexation stoichiometries of CDC and DC systems may be inferred from the curve shapes. With large K values, the βCD proton shift curves induced by CDC reach their plateaus at the molar ratio of 1 for CDC/βCD; this implies that only the 1 : 1 complex exists throughout the molar ratio range, while the βCD proton curves induced by DC reach their plateaus at the molar ratio of 0.5 for DC/βCD and do not

TABLE 2

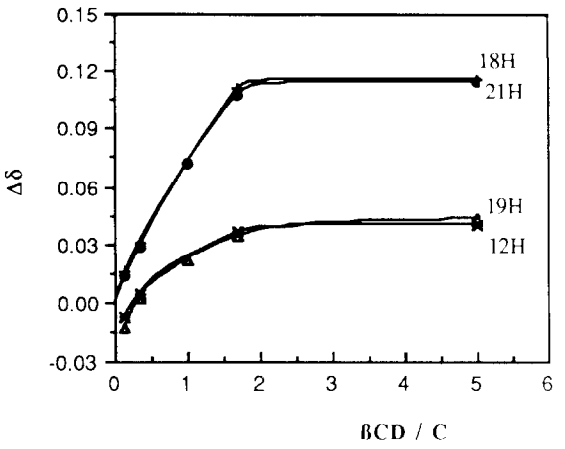
Thermodynamic parameters for the 1 : 1 plus 1 : 2 β-cyclodextrin–bile salt interactions

Bile salt	Abbreviation	$-\Delta H_1$	$-\Delta H_2$	$\log K_1$	$\log K_2$
Deoxycholate	DC	21.8 ± 1.0	19.1 ± 1.3	4.79 ± 0.05	2.86 ± 0.03
Glycodeoxycholate	GDC	22.6 ± 1.3	17.6 ± 1.3	4.27 ± 0.09	2.66 ± 0.15
Taurodeoxycholate	TDC	20.2 ± 0.6	17.6 ± 2.4	4.54 ± 0.13	2.73 ± 0.19

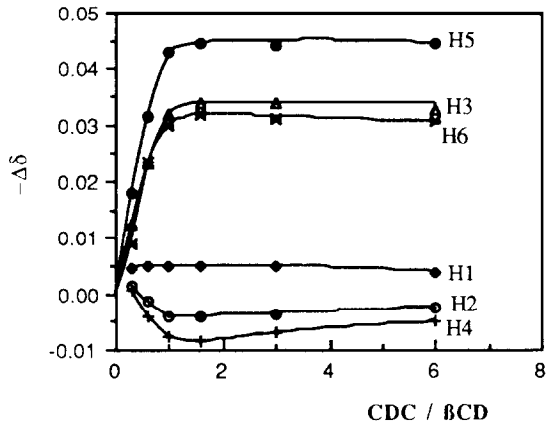
K_1 and K_2 are in mol^{-1} l; ΔH_1 and ΔH_2 are in kJ mol^{-1}.



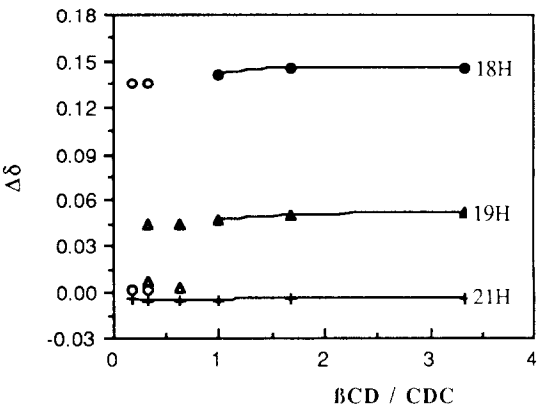
a. ßCD proton shifts induced by C



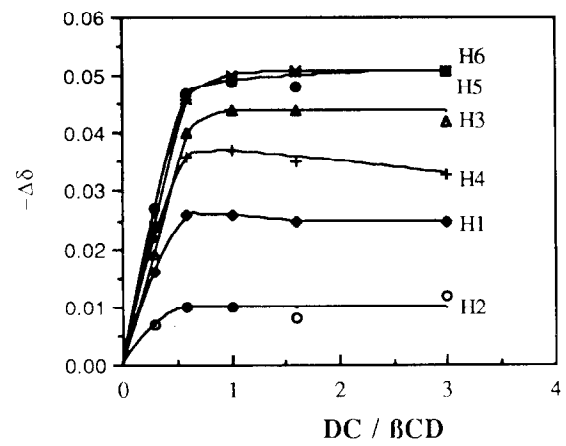
b. C proton shifts induced by ßCD



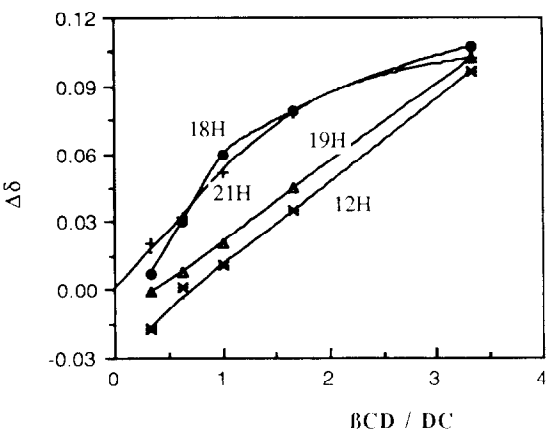
c. ßCD proton shifts induced by CDC



d. CDC proton shifts induced by ßCD



e. ßCD proton shifts induced by DC



f. DC proton shifts induced by ßCD

Fig. 4. Proton shifts induced by complexation of βCD and bile salt molecules. The abscissa is the molar ratio of the two molecules. The points are experimental data and the curves are the smooth connection of the points.

increase after molar ratio > 1. This suggests the existence of a 1:2 complex when the βCD concentration exceeds that of DC, and only a 1:1 complex when the βCD concentration is less than that of DC. The different order of curves in graph c compared to those on graphs a and e (Fig. 4) indicates that βCD is located on a different site on the bile salt steroid.

When looking at the bile salt proton shifts induced by the βCD molecule, it is easy to see the structural differences of the complexes due to different bile salt molecules. The common downfield phenomenon of guest molecular protons has been observed (Suzuki and Sasaki, 1979; Uekama et al., 1982). For C, it is suggested that βCD is located between the $18CH_3$ and $21CH_3$, with the primary hydroxyl side touching the D ring of the steroid, and it has the same effect on the two methyl protons. It is far away from 12H and $19CH_3$, and the effect on them is small. For DC, $18CH_3$ and $21CH_3$, 12H and $19CH_3$ have, respectively, nearly the same shift changes, which is similar to the case of C, when the molar ratio of βCD/DC is less than 1. It seems that the first binding site on DC steroid is at the same location as on C. When the molar ratio of βCD/DC is greater than one, the induced proton shifts continue to increase, and the increases of 12H and $19CH_3$ are more significant than those of $18CH_3$ and $21CH_3$. This demonstrates that there is a second binding site on the DC steroid and the second site is at the other side of the molecule, βCD extending to the 3OH group. For CDC, the $21CH_3$ has a small shift and the shift of its $18CH_3$ is greatest, and also the shift of $19CH_3$ is relatively large. It is reasonable to assume that the βCD molecule is located on the C ring of the CDC steroid, since there is no hindrance by the 12OH group.

The progressive shift of βCD protons with increasing concentration of the three bile salts (graphs a, c and e in Fig. 4) is due to the reversible association between the free and associated βCD species in solution. This association is fast on the NMR time scale and the guest molecules are rapidly spinning about the βCD's axis within its cavity (Demarco and Thakkar, 1970). The progressive shift behavior for C and DC with increasing βCD concentration (graphs b and f in Fig. 4) is probably also due to fast dynamic processes. However, in the spectra for CDC protons, the peaks for $18CH_3$ and $19CH_3$ did not shift progressively with increasing βCD concentration. When the molar ratio of βCD/CDC is less than 1, each peak splits into two peaks at the fixed positions, and the peak intensities are proportional to the molar ratios of βCD/CDC. For molar ratios > 1, the peak at the lower field disappears. This phenomenon is presumably due to the existence of distinguishable free CDC molecules and associated CDC species if the molar ratio of βCD/CDC is less than 1. If the molar ratio is greater than 1, all the CDC molecules are complexed. Based on the fact that the progressive shift for βCD protons and the splitting spectra for CDC protons occur in the same solution, it is concluded that the CDC molecule will be deformed after βCD molecule complexation; the exchange between complexed and uncomplexed CDC molecule is small.

The thermodynamic data are consistent with the proposed structures. For C, GC, TC and DHC, their 12OH or O= groups will hinder βCD binding beyond the D-ring, and their ΔH magnitude and K values are of the same scale and relatively smaller. For CDC and UDC and their conjugates, βCD may encroach further toward the bulk steroid and locates on the C-ring, making the interaction among the atoms of host and guest molecules much stronger, the ΔH magnitude and K values being larger than those of C, GC, TC and DHC. For DC, GDC and TDC, the first βCD binds to the same place on DC as on C, giving approximately the same value for ΔH. There is no 7OH group on the DC steroid; βCD may therefore extend toward the A-ring of the steroid after the first binding site has been saturated. The binding of two βCD molecules on the same bile salt molecule reduces the stability of the second binding site.

The complexation of βCD with steroids was the subject of a recent investigation (Liu et al., 1990) in which the 1:1 plus 1:2 complexation for βCD and four steroids was presented. They proposed the same complex structure as that suggested by the NMR data in this study. Our K

values are larger than others' (Miyajima et al., 1986). For cyclodextrin inclusion complexation, calorimetry may be more sensitive than fluorescence. This is also suggested by the ability of this technique to observe the 1:2 complexation for DC and its conjugates.

The side chains have no significant effect on the binding process, only the taurine side chain decreases the magnitude of ΔH by 4 kJ mol^{-1} for the 1:1 complexation systems. The fact that DHC shows the same results as C indicates that the position of the oxygen bearing group is much more important than whether the group is OH or O=. It is generally accepted that the binding affinity is not primarily dependent upon the chemical properties of the guest molecules, but that the most important factor is the relative size of the βCD cavity and the guest molecules. This would also be expected from the partition coefficients of the bile salts (Vadnere and Lindenbaum, 1982). The fact that K for C is smaller than those for the other three bile salts could imply a role of hydrophobic interaction in the complexation reaction. However, there is no linear correlation between the logK and $\log K_{app}$. It appears therefore that the spatial relationship between the guest and host molecules is most significant.

Several forces (Matsui and Mochida, 1979; Crowell et al., 1985) contribute to the inclusion complexation process: van der Waals forces, hydrogen bonds, hydrophobic interactions, changes in the environment of water molecules in both the βCD cavity and the hydrated guest molecules, and the possible deformation of the reactant molecules (e.g., the CDC molecular deformation as shown in the NMR data). The thermodynamic data represent the total contribution from all of the forces mentioned above. It would be highly speculative to attempt a more detailed explanation of the complexation mechanism. Comparing the results for the bile salts with the data for model substances or drugs binding to βCD from microcalorimetric measurements (cinnarizine (Ueda et al., 1989), flurbiprofen (Ueda and Perrin, 1986), adamantanecarboxylate (Crowell et al., 1985; Briggner et al., 1986), *p*-nitrophenolate (Eftink and Harrison, 1981)) and the drugs in Hardee's paper (Hardee et al., 1978), leads to one clear conclusion, namely that the bile salts bind more strongly to βCD than any of the other drug substances studied to date.

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