Proton magnetic resonance studies of the aggregation of taurine-conjugated bile salts[1]

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Abstract The concentration dependence of the 500 MHz ^{1}H-NMR spectra of taurocholate, taurochenodeoxycholate, taurodeoxycholate, and the monosulfate esters of taurochenodeoxycholate has been examined at 0.154 M NaCl in D_2O. The resonances of the C_{18}, C_{19}, and C_{21} methyl groups and the C_{23} methylene group are differentially broadened with respect to the C_{25} and C_{26} methylene and C_7 (or C_{12}) methine groups with increasing bile salt concentration for each of the bile salts studied. These data confirm hydrophobic association and indicate that the side chain contributes to the hydrophobic surface of the bile salt. The chemical shift difference of the anisochronous C_{23} methylene protons is different in monomer and aggregate form. The C_{25} methylene protons are isochronous in monomeric form but anisochronous in aggregate form. The concentration dependence of the observed chemical shifts has been analyzed to estimate the critical concentration associated with the onset of these changes. The conformer population about the C_{22}–C_{23} bond changes before the anisochronicity of the C_{25} methylene protons develops. This indicates that the C_{23} methylene group is affected by the intial stages of self-association, whereas specific motional constraints about the N–C_{25} bond in the taurine moiety are only induced in large primary micelles. The difference in the chemical shift of the C_{25} methylene protons depends on the structure of the bile salt. The relative magnitude of the shift differences is not altered by the presence of phosphatidylcholine. The data suggest that in primary micelles or mixed micelles the taurine moiety conforms to segregate the hydrophilic groups of the bile salt and effects greater van der Waals' contact between the hydrophobic surfaces.—**Stevens, R. D., A. A. Ribeiro, L. Lack, and P. G. Killenberg.** Proton magnetic resonance studies of the aggregation of taurine-conjugated bile salts. *J. Lipid Res.* 1992. **33:** 21–29.

Supplementary key words bile salts • ^{1}H-NMR micelle formation • taurocholate • taurodeoxycholate • taurochenodeoxycholate • tauro-chenodeoxycholate-3α-sulfate • taurochenodeoxycholate-7α-sulfate

Bile salts are polar amphiphilic substances that self-associate in water with increasing concentration (1). In contrast to typical ionic detergents that contain flexible hydrocarbon chains, bile salts are rigid non-planar molecules with hydrophilic and hydrophobic surfaces. This difference in molecular structure leads to an atypical pattern of aggregation involving stepwise association over broad concentration ranges (2).

Aggregation is thought to proceed by partition of the hydrophobic surfaces with one another (3). Evidence for this mode of interaction was provided by early ^{1}H-NMR studies (4, 5). The influence of bile salt structure on self-association suggests that the contiguous hydrophobic surface area is an important variable (6). More recent ^{1}H and ^{13}C NMR studies, however, have questioned whether the initial aggregation involves only intermolecular hydrophobic interactions and have proposed a role for hydrogen bonding (7, 8). The steroid side-chain structure also influences the concentration at which bile salts start to aggregate, suggesting that the side chain is involved in the hydrophobic interaction (6). While X-ray studies have demonstrated an interaction of the side chain with the hydrophobic surface of the steroid moiety in the crystal structure (9), no evidence from solution studies has been reported.

In this report, we present studies on the concentration dependence of the ^{1}H-NMR spectra of taurine-conjugated bile salts in the presence and absence of phosphatidylcholine at 500 MHz. Particular emphasis has been placed on the spectral parameters of the side chain and taurine moiety to examine their role in bile salt association. In addition to the more common bile salts taurocholate, taurochenodeoxycholate, and taurodeoxycholate, we have examined the monosulfate esters of taurochenodeoxycholate which

Abbreviations: TDC, taurodeoxycholate; TCDC, taurochenodeoxycholate; TC, taurocholate; TCDC-3-SO_4, TCDC-7-SO_4, 3α- and 7α-sulfate esters of taurochenodeoxycholate; CMC, critical micellar concentration; NMR, nuclear magnetic resonance; ppm, parts per million; 2D-COSY, two-dimensional correlated spectroscopy; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.

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contain a charged sulfate group on the hydrophilic surface of the bile salt. This structural modification should minimize secondary aggregate formation and permit evaluation of the NMR changes due to primary aggregation. The presence of a charged sulfate ester of the 3α- or 7α-hydroxyl should help clarify the role that intermolecular hydrogen bonding plays in primary bile salt aggregation.

MATERIALS AND METHODS

Sodium taurocholate (TC) was obtained from Calbiochem (San Diego, CA). Sodium taurochenodeoxycholate (TCDC), sodium taurodeoxycholate (TDC), and phosphatidylcholine (egg yolk lecithin-type V-E) were obtained from Sigma (St. Louis, MO). The sodium salts of [^{14}C]taurine-labeled 3α- and 7α-monosulfate esters of taurochenodeoxycholate (TCDC-3-SO_4 and TCDC-7-SO_4) were synthesized and purified using methods cited previously (10). TC, TDC, and TCDC were purified using Sep-Pak C_{18} cartridges, Waters (Milford, MA). Bile salts were lyophilized from aqueous solutions and desiccated under reduced pressure, 10^{-3} mm Hg, at 70°C for 5 days. Chromatographic purity (TLC and HPLC) was greater than 98%. Bile salt solutions were prepared in 99.9% D_2O, Cambridge Isotope Laboratories (Woburn, MA) and contained 0.154 M NaCl, except for TDC which contained 0.010 M Na_2HPO_4 and 0.134 M NaCl, pH 8.4, to minimize the formation of helical structures (11). The solutions were quantitated enzymatically (12) with prior solvolysis where necessary (13). Mixed micellar solutions were prepared by a method of co-precipitation (14).

^{1}H-NMR data were obtained at 290 ± 1 on 0.5 ml bile salt solutions in D_2O using 5-mm NMR tubes. The ^{1}H-NMR spectra were recorded on a General Electric GN-500 spectrometer operating at 500.12 MHz at the Duke NMR Center. The ^{1}H-NMR spectra were recorded using a spectral window of ± 1754 Hz with the transmitter frequency adjusted to focus on the 0–7 ppm region of the ^{1}H-NMR scale. These NMR spectra were digitized into 32768 computer points to yield a digital resolution of 0.21 Hz/point and data were processed with a line-broadening parameter of 0.01 Hz. Standard one-pulse ^{1}H-NMR experiments used an 8 - μsec pulse (70° flip angle), an acquisition period of 4.67 sec, and a delay of 2 sec. Chemical shifts were referenced to an external sample of 100 mM 2,2-dimethylsila-pentane-5-sulfonic acid (DSS), Aldrich (Milwaukee, WI), in a melting point capillary.

The line-widths of fully resolved resonances were estimated directly. For multiplets, a perpendicular from the peak of an outside line to the baseline was constructed. The line-width was estimated as twice the distance from the perpendicular to the outside edge of the line at half height. The line-widths of poorly resolved or highly complex multiplets were estimated by spectral curve analysis using General Electric Software, GEMCAP. The line-widths were corrected for artificial line broadening and magnetic field inhomogeneities which were ca. 0.5–0.7 Hz.

RESULTS

A perspective drawing and a 500 MHz ^{1}H-NMR spectrum of TCDC-3-SO_4 are shown for orientation purposes in **Fig. 1**. The proton resonances have been assigned on the basis of previously published data on sodium cholate (15). The methylene triplets of the taurine moiety at 3.5 and 3.0 ppm have been assigned to C_{25} and C_{26}, respectively. A persistent error in the assignment (16, 17) of the taurine methylene groups is reversed here on the basis of a published 2D COSY of TC in H_2O (18), published spectra of sulfonic acids (19), and the concentration dependence of the downfield methylene triplet (vide infra).

The line-widths of several resolved resonances representing the hydrophobic steroidal surface (C_{18} methyl), the side chain (C_{21} methyl and C_{23} methylene), the taurine moiety (C_{25} and C_{26} methylene), and the hydrophobic/hydrophilic interface (C_7 proton) are shown as a function of concentration for a hydrophilic bile salt (TCDC-3-SO_4) and a hydrophobic bile salt (TCDC) in **Fig. 2.**

The line-width behavior for the other hydrophilic bile salts, TCDC-7-SO_4 and TC, is similar to that of TCDC-3-SO_4. The behavior of the other hydrophobic bile salt, TDC, is similar to that of TCDC. In general, the C_{18}, C_{19}, and C_{21} methyl signals and the C_{23} methylene signals show the greatest line broadening and are broadened to the same extent for a particular bile salt. The C_{25} and C_{26} methylenes and the C_7 methine proton show the smallest line-broadening effects. The C_{12} methine proton of TDC, which represents the hydrophobic/hydrophilic interface for this bile salt, exhibits a concentration-dependent line broadening similar to that of C_7 of TCDC.

Small chemical shift changes are observed with increasing bile salt concentration. Relative to the external standard, the bulk of the resolved resonances outside the complex steroid envelope shift upfield, with the exception of a small downfield shift of the C_{21} methyl group. The C_{23} and C_{25} methylene proton resonances exhibit the most noticeable chemical shift changes. Barnes and Geckle (15) have previously reported an anisochronicity of the geminal protons of the C_{22} and C_{23} methylene groups of sodium cholate.

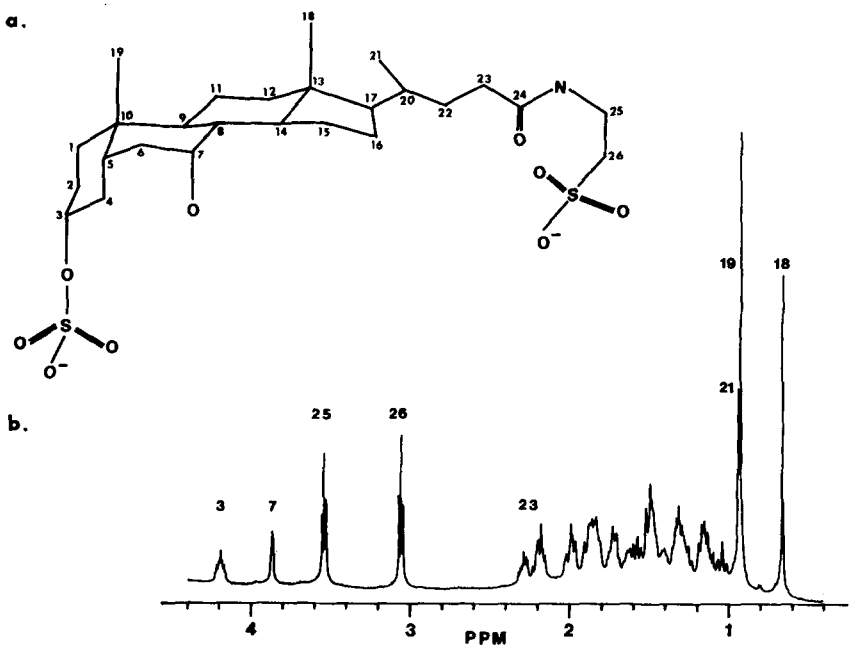

Fig. 1. a: A perspective drawing of taurochenodeoxycholate-3α-sulfate. b: A 500 MHz ^{1}H-NMR spectum of taurochenodeoxycholate-3α-sulfate at 5 mM.

This chemical shift non-equivalence arises from either attachment or proximity to the chiral C_{20} atom. In the present study, we note a pronounced upfield shift of the upfield C_{23} methylene signal with increasing bile salt concentration. The effect of concentration on the resonance of the C_{22} methylene protons is difficult to evaluate because of overlap with the steroid envelope signals. The C_{25} methylene group is a well-resolved triplet at low concentrations due to the vicinal spin-spin coupling with the C_{26} methylene group. However, as shown in **Fig. 3** for TCDC-3-SO_4, with increasing bile salt concentration the resonance becomes more complex and concentration-dependent. A previous NMR study of TC has shown that, in aggregate form, the C_{25} and C_{26} protons are linked by nuclear Overhauser effects due to motional constraints imposed on the taurine moiety (20). The present study shows that these motional constraints cause the two geminal protons to become chemical shift non-equivalent, i.e., anisochronous, and to reveal their geminal spin-spin coupling constant. Since the chemical shift difference is of the order of the coupling constant, the resonance exhibits second order effects (21). Thus, in aggregate form, the C_{25} protons have a typical AB pattern and are coupled to the C_{26} methylene protons with the same vicinal coupling constant, giving a multiplet structure of a quartet of triplets. This spectral change is observed for all the bile salts studied.

ANALYSIS

The concentration-dependent chemical shift changes of amphiphilic molecules may be used to estimate the critical micellar concentration, CMC (22, 23). When the rate of exchange between the monomeric and aggregated state is fast compared to the chemical shift difference of a resonance in the two states, $\Delta\delta$, (expressed in Hz), then the observed chemical shift relative to the monomer, $\Delta\delta_{obs}$, will be a weighted average and given by

$$\Delta\delta_{obs} = (C_m/C_t)\Delta\delta$$

where C_m and C_t are, respectively, the aggregate and the total amphiphile concentrations. As an initial approximation, a pseudophase model for aggregate formation is usually assumed, where $C_m = C_t - CMC$.

Therefore, we may write

$$\Delta\delta_{obs} = \Delta\delta - (CMC/C_t)\Delta\delta.$$

Thus, a plot of $\Delta\delta_{obs}$ against the reciprocal of the total amphiphile concentration theoretically will yield a straight line intersecting the abscissa at the reciprocal of the CMC and the ordinate at $\Delta\delta$.

In practice, some curvature of the points is expected, especially about the critical concentration, since aggregation phenomena rarely conform to a pseudophase model (24, 25). This is especially true in the case of bile salts (2, 26). Nevertheless, the ex-

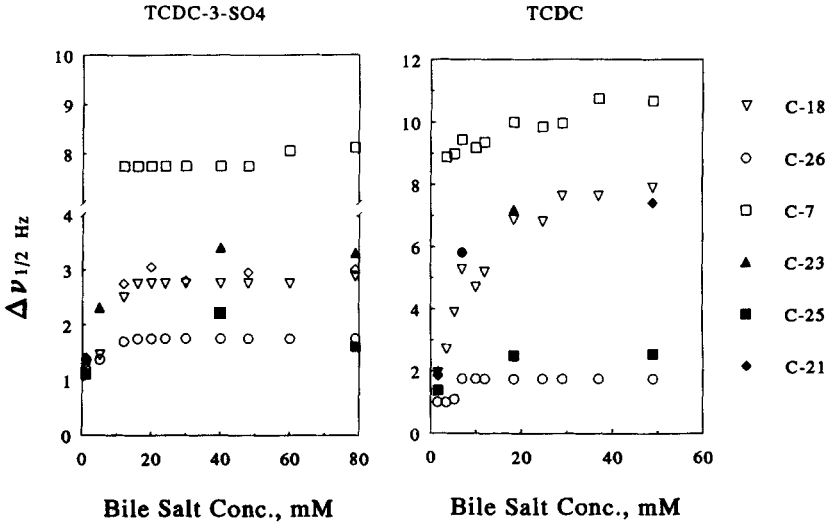

Fig. 2. The concentration dependence of the line-widths at half height, $\Delta\nu_{1/2}$, in Hz for selected resonances of taurochenodeoxycholate-3α-sulfate (TCDC-3-SO_4) and taurochenodeoxycholate (TCDC). The open symbols were determined by measurement and the solid symbols by curve analysis using GEMCAP of spectra with a digital resolution of 0.21 Hz/point.

trapolation of the linear portion of the curves should provide reasonable estimates of the critical concentrations associated with a given spectral change (22, 24, 25, 27).

This analytical approach is normally not possible for proton chemical shifts (28). The chemical shift changes are small and of the order of the magnetic susceptibility corrections that must be applied to the observed chemical shifts. However, in the present case, the concentration dependence of the C_{23} and C_{25} proton resonances may be analyzed using parameters that are independent of their intrinsic chemical shifts. The parameter used for the anisochronous C_{23} protons is the change in the difference of the chemical shift of the two protons and for the C_{25} methylene protons, the separation of the two central lines of the complex AB multiplet.

Reciprocal concentration plots of the parameter for the C_{23} protons of TC, TCDC, TDC, and TCDC-3-SO_4 are shown in **Fig. 4**. TCDC-7-SO_4 was similar to TCDC-3-SO_4 and is not shown. No strictly linear portions of the curves were found for TC, TCDC, and TDC. However, it is clear from the reciprocal concentration plots that, for example, TC exhibits a marked upswing of the curve between 200 and 100 M^{-1} (5–10 mM). The upper limit of the critical concentration range for these bile salts was estimated by linear regression analysis over a limited concentration range, as indicated in Fig. 4. The critical concentrations and shift differences are given in **Table 1.**

Reciprocal concentration plots for the separation of the two central lines of the C_{25} proton multiplet are given in **Fig. 5, A and B.** The results of the linear

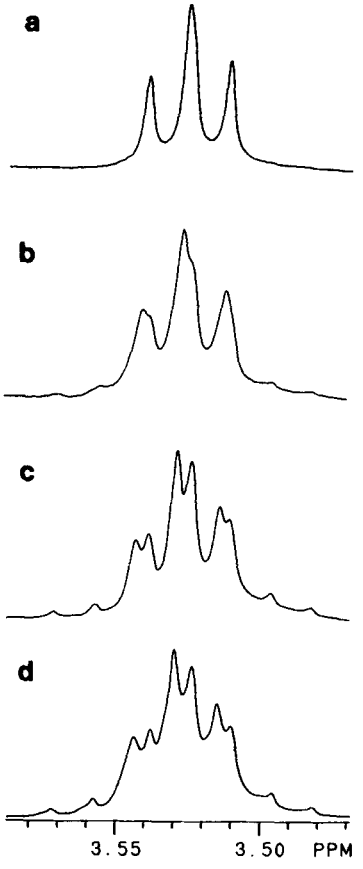

Fig. 3. The concentration dependence of the C_{25} methylene protons of taurochenodeoxycholate-3α-sulfate at (a) 11 mM, (b) 18 mM, (c) 27 mM, and (d) 47 mM. It can be shown (21) that the separation of the two central lines of the AB quartet equals [2C-J], where $C = ((J^2+D^2)^{1/2})/2$. J and D represent the geminal spin-spin coupling constant and chemical shift difference of the anisochronous C_{25} protons.

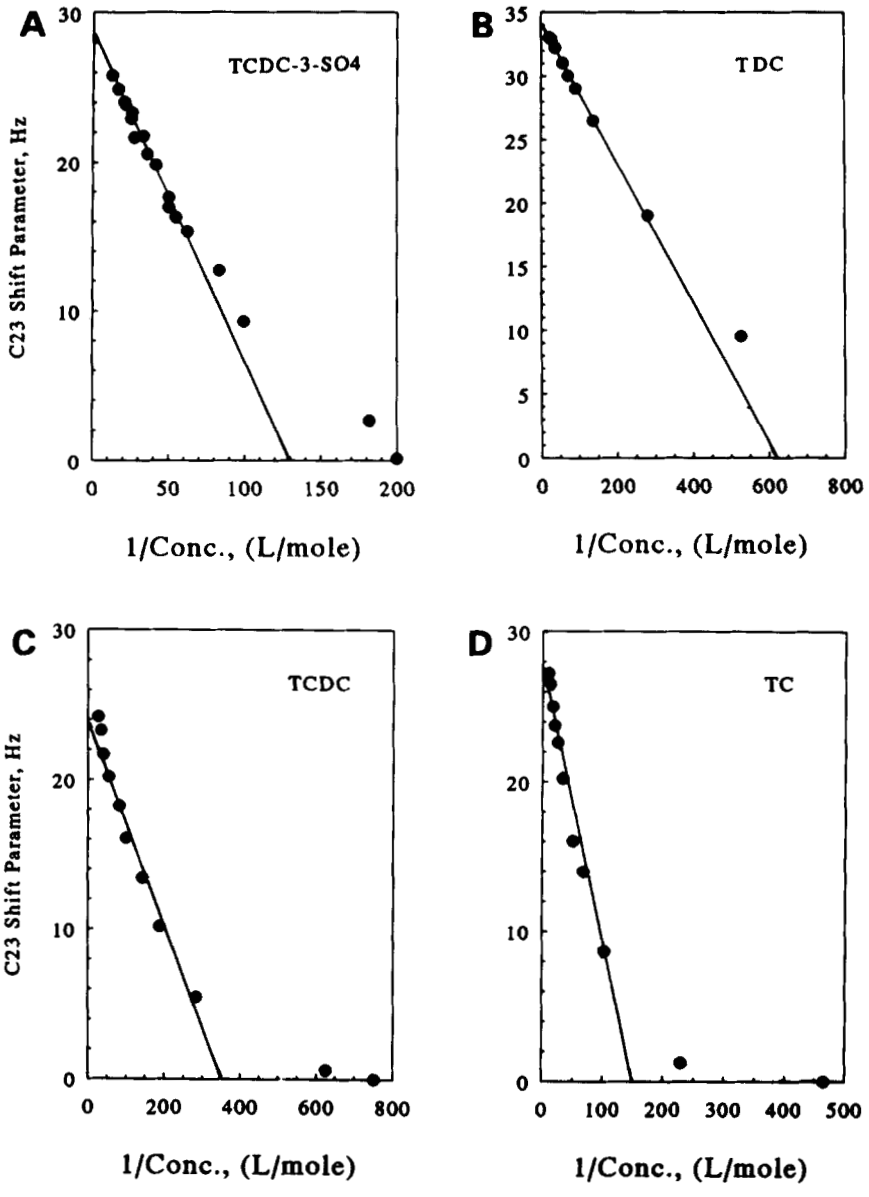

Fig. 4. Reciprocal concentration plots of the C_{23} methylene shift parameters of the taurine-conjugated bile salts. The shift parameters, expressed in Hz, were measured relative to taurocholate at 2.2 mM, taurochenodeoxycholate at 0.4 mM, taurodeoxycholate at 0.4 mM, and taurochenodeoxycholate-3α-sulfate at 1 mM. Taurochenodeoxycholate-7α-sulfate is not shown but is similar to taurochenodeoxycholate-3-sulfate.

regression analysis for the critical concentration associated with the onset of the second order effect are given in Table 1. Included in the table are the calculated value of the chemical shift difference of the geminal protons in aggregate form and the measured geminal spin-spin coupling constant. The line separations for TCDC, TCDC-3-SO_4, and TCDC-7-SO_4 are in the 2–5 Hz range. With a digital resolution of 0.2 Hz/point, reasonable linear plots are obtained. The analysis is more difficult for TC since the maximum value line separation is only about 2 Hz. The analysis is not possible for TDC because the magnitude of the chemical shift difference is much less than the geminal coupling constant and the intrinsic line-width of the resonance. Nevertheless, at the higher concentrations, the outer lines of the complex multiplet are discernible, which permits estimation of the coupling constant and chemical shift difference.

We have briefly examined the spectral appearance of the C_{25} methylene protons in mixed micellar solutions of these bile salts and phosphatidylcholine at a molar ratio of 3:1. The complex second order structure is preserved. The spectral parameters are included in Table 1. While the association of bile salts

TABLE 1. Critical concentrations and ^{1}H-NMR parameters for C_{23} and C_{25} methylene protons

Bile Salt	C_{23}		C_{25} (Micellar)			C_{25} (Mixed Micellar)	
	Conc.[a]	Shift[b]	Conc.[c]	Shift[d]	J[e]	Shift[d]	J[e]
	mM	Hz	mM	Hz	Hz	Hz	Hz
TDC	1.7	33		3.1	1	4.0	14
TCDC	2.8	24	2.9	9.2	14	15.1	13.6
TC	6.4	30	22	6.7	14.1	9.6	13.9
TCDC-3-SO_4	7.6	28	14.3	13.1	14.0	15.7	13.4
TCDC-7-SO_4	9.3	29	16.4	12.5	14.0		

[a]Upper limit of the critical concentration associated with the change in chemical shift difference of the anisochronous C_{23} methylene protons.
[b]Increase in chemical shift difference in aggregate form associated with the upper limit of the critical concentration.
[c]Critical concentration associated with the anisochronicity C_{25} methylene protons.
[d]Chemical shift difference of the anisochronous protons in aggregate form.
[e]Geminal spin-spin coupling constant.

with phosphatidylcholine leads to an increase in the chemical shift difference for each bile salt studied, the relative magnitude remains invariant.

DISCUSSION

The present study has used ^{1}H-NMR to study the effects of aggregation on molecular mobility of specific sites of taurine-conjugated bile salts with particular emphasis on the steroid side change and taurine moiety. Spectral line-widths and chemical shift changes are used to monitor overall mobility and specific motional constraints, respectively.

The greater line broadening of the steroid methyl groups relative to the more hydrophilic sites at C_7 and C_{26} is consistent with the original findings of Small, Penkett, and Chapman (4). The introduction of a charged sulfate group on the hydrophilic surface of the molecule does not influence the pattern of differential line broadening. This argues against a substantive role for hydrogen bonding in the formation of primary bile salt aggregates as claimed by others (7, 8, 29). The steroid methyl resonances of the more hydrophobic bile salts, TCDC and TDC, broaden to a greater extent than those of the more hydrophilic bile salts, TC, TCDC-3-SO_4, and TCDC-7-SO_4. This decreased mobility is most likely due to the tighter packing in aggregates of dihydroxy bile salts, consistent with microviscosity (30) and molal volume (31) measurements. After a rapid increase in the linewidth, the steroidal methyl signals of the dihydroxy bile salts continue to broaden with increasing concentration, in contrast to those of the more hydrophilic bile salts. This is probably due to secondary aggregation; quasi-elastic light-scattering measurements indicate a modest growth of aggregates of these dihydroxy bile salts with increasing concentration at 0.15 M NaCl (32, 33).

The signals of the C_{21} methyl and C_{23} methylene in the side chain are broadened to the same extent as the steroid methyl resonances, suggesting that they are also involved in the hydrophobic interaction. The taurine methylene groups broaden to the least extent and remain relatively narrow for each of the bile salts studied. This suggests that they are not involved in the hydrophobic interaction. These observations provide support for conclusions from studies of the effect of bile salt structure on the CMC that the side chain but not the taurine moiety contributes to the hydrophobic surface which is removed from the aqueous environment upon aggregate formation (6).

The chemical shift changes of the C_{23} and C_{25} methylene protons indicate that the conformer populations about the C_{22} – C_{23} bond in the side chain and the N – C_{25} bond in the taurine moiety are modified when the bile salts aggregate. The analysis of the concentration dependence of these changes shows that C_{23} protons are affected before the anisochronicity of the C_{25} methylene protons develops. This suggests that conformational changes in the side chain are associated with the initial stages of aggregation, e.g., dimer formation, whereas motional constraints about the N – C_{25} bond in the taurine moiety only occur with significant growth of the aggregate, i.e., the "mature" primary micelle. The difference between the two critical concentrations reflects the range over which the early stages of aggregation are taking place. The narrower range observed for TCDC compared to TC is in agreement with current concepts of this aspect of bile salt association (34).

The difference in chemical shift of the two C_{25} methylene protons, i.e., the conformer populations about the N – C_{25} bond, depends on the position of the hydrophilic substituents of the steroid nucleus of the bile salt (see Table 1). It is decreased by the presence of an hydroxyl group in the 12α-position

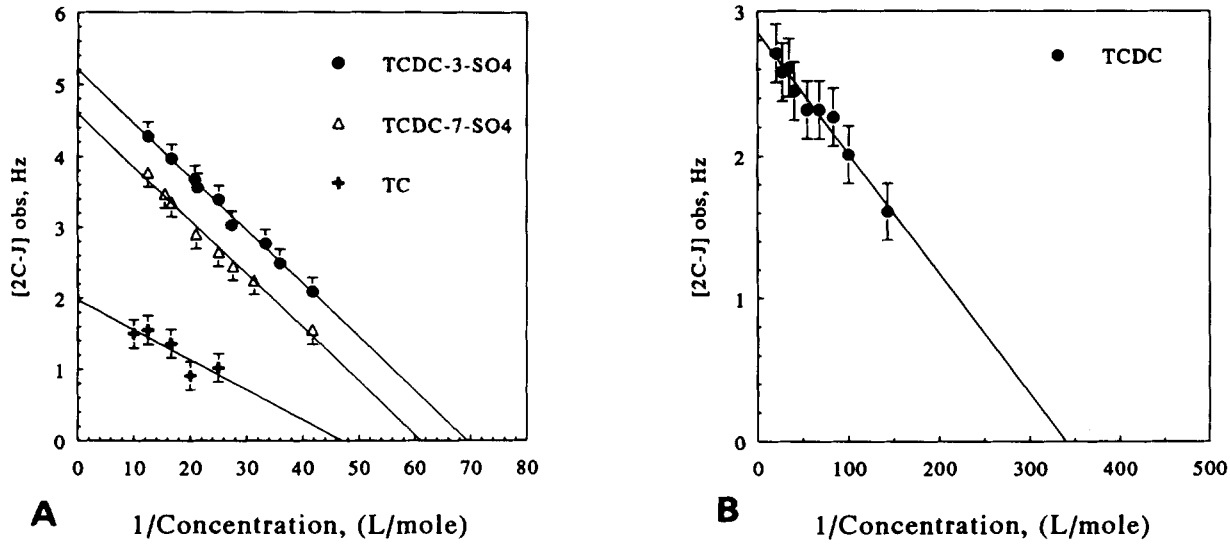

Fig. 5. Reciprocal concentration plots of the separation of the central two lines [2C–J] of the second order multiplet of the C_{25} methylene proton resonances of (A) taurocholate (+), taurochenodeoxycholate-3α-sulfate (●), and taurochenodeoxycholate-7α-sulfate (Δ) and (B) taurochenodeoxycholate. The error bars represent a digital resolution of 0.21 Hz/point.

and is increased by an hydroxyl in the 7α-position. Sulfation at 3α or 7α further amplifies the chemical shift difference. The magnitude and relative size of the shift difference are essentially preserved in mixed micellar systems of these bile salts with phosphatidylcholine. This suggests that the molecular interactions that lead to specific motional constraints about the N – C_{25} bond and anisochronicity of the C_{25} protons are similar whether the bile salts self-associate or form mixed micelles with phosphatidylcholine. These molecular interactions must, therefore, be essentially intramolecular rather than intermolecular.

The chemical shift changes of the C_{23} and C_{25} protons may be interpreted from the point of view of a previous study of a metal ion-bile salt complex which proposes that the conjugated side chain exists in one predominant conformation in which the segregation of polar groups is enhanced to facilitate hydrophobic interactions (35). The delayed onset of the C_{25} anisochronicity and its variation with bile salt structure may also be rationalized in this context. For example, the different conformer populations of the taurine in TDC and TCDC may reflect the segregation or alignment of the sulfonate and peptide carbonyl with the 3α- and 12α- or 3α- and 7α-hydroxyl groups, respectively, in an attempt to minimize their contiguous hydrophilic surface and allow greater van der Waals' contact between the hydrophobic surfaces when they become incorporated in the larger aggregates.

It has been generally assumed that bile salt micelles have a globular structure(1, 34). An alternative arrangement, based on the crystal structure, of a staggered bilayer in which the side chain interacts with the hydrophobic surface of the steroid has been proposed (9). While the present study does not specifically address intermolecular interactions, it is difficult to account for the dependence of the anisochronicity of the C_{25} methylene protons on micellar growth and bile salt structure in such an arrangement.

In summary, the present ^{1}H-NMR study confirms that bile salts hydrophobically associate and demonstrates that aggregation results in conformational changes of the taurine-conjugated side chain. The concentration dependence of the accompanying observed chemical shifts has been used to estimate the critical concentrations associated with these conformational changes.

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