High resolution nuclear magnetic resonance spectroscopy of bile salts: individual proton assignments for sodium cholate in aqueous solution at 400 MHz

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Abstract The 400 MHz ^{1}H-nuclear magnetic resonance spectrum of sodium cholate in dilute aqueous solution has been successfully resolved using a combination of decoupling, partial relaxation, and decoupled partial relaxation techniques. The individual carbon resonances in the ^{13}C-NMR spectrum of sodium cholate have also have assigned. Assignments of individual methylene protons were made by consideration of the molecular structure of sodium cholate and the expected couplings and ^{1}H-nuclear Overhauser enhancement experiments. Verification of the assignments of the methine protons was made by application of single frequency ^{1}H-decoupled ^{13}C-NMR. Variation of pH* from 6.0 to 11.0 did not alter the individual chemical shifts except for those between 2.12 δ and 2.30 δ, originating from the protons on the C_{23} position adjacent to the ionizable carboxyl group. The chemical shifts of the proton resonances were independent of concentration below 5 mM. Above 10 mM (micellar region), the proton chemical shifts were altered slightly and some band broadening occurred. These data are consistent with the formation of small micellar aggregates (up to N = 4) of cholate molecules.—**Barnes, S., and J. M. Geckle**. High resolution nuclear magnetic resonance spectroscopy of bile salts: individual proton assignments for sodium cholate in aqueous solution at 400 MHz. *J. Lipid Res.* 1982. **23:** 161–170.

Supplementary key words micelles • partially relaxed decoupled proton NMR spectra • nuclear Overhauser enhancement analysis • single frequency ^{1}H-decoupled ^{13}C-NMR

Bile salts are the body's detergents and are synthesized from cholesterol in the liver. They are secreted into the duodenum via the bile. In the bile and the small intestine they aggregate to form micelles in conjunction with phospholipid or fatty acid. These mixed micelles enable the solubilization of substances such as cholesterol which have very low aqueous solubility. The physical chemistry of this process has been investigated by a variety of methods, including freezing point depression (1), light scattering (2, 3) and, to a limited extent, nuclear magnetic resonance (NMR) spectroscopy (4–9). The latter technique has been applied for the most part for determining the structure of synthetic bile acids, e.g., ref 10–14. Small, Penkett, and Chapman (4) and Martis, Hall, and Thakkar (5) were able to demonstrate the difference in line broadening between protons on the non-polar and polar faces of the bile salt molecule as micelle formation was stimulated. However, these studies were limited by the inadequate resolution of individual proton peaks at the field strength used (2 Tesla or lower). Whereas the methyl groups at C_{18}, C_{19}, and C_{21} were easily identified (0.5–1.0 δ), as were the protons epimeric to the hydroxyl groups (3–4 δ), the remainder of the protons (in sodium cholate, a further 24) formed a broad hump of unresolved resonances between 1.0–2.3 δ. The availability of high field strength (8 Tesla) superconducting magnets raised the possibility that at least partial resolution of the resonances between 1.0–2.3 δ could be achieved. This was confirmed by studies performed on the permethyl ether methyl ester derivatives of bile salts (15).

In the present investigation the ^{1}H-NMR spectrum of an aqueous solution of sodium cholate (3α, 7α, 12α-trihydroxy-5β-cholan-24-oate) (see Fig. 1 for molecular structure) has been examined at a frequency of 400 MHz. Using a combination of decoupling, partial relaxation, and decoupling combined with partial relaxation techniques and single frequency proton decoupled ^{13}C-NMR, the proton assignments have been determined for the first time.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TSP, sodium trimethylsilyl-1,1,2,2-tetradeutero-propionate; δ, parts per million downfield from TSP; NOE, nuclear Overhauser enhancement; pH*, the glass electrode reading of pH in deuterium oxide without corrections for isotope effects; FT-NMR, Fourier transform nuclear magnetic resonance; cholic acid, the trivial name for 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; 23-norcholic acid, the trivial name for 3α,7α,12α-trihydroxy-5β-cholan-23-oic acid.

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MATERIALS AND METHODS

Materials

Cholic acid was obtained from Sigma Chemical Co. (St. Louis, MO) and 23-norcholic acid from Research Plus Labs (Denville, NJ). Deuterium oxide, deuterium chloride, and sodium deuteroxide were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Methods

Preparation of bile salt solutions. Cholic acid was purified by repeated crystallization from ethanol–benzene. It was placed in vacuo over phosphorus pentoxide for several days to remove moisture and solvents. A sample of 8.2 mg was weighed out and D_2O and NaOD were added to a final volume of 5.0 ml and pH* of 7.0, thus producing a 4 mM solution. Other concentrations were prepared in a similar manner. More acidic solutions were prepared by careful titration with 0.1 M DCl. Titration of the 4 mM solution until precipitation just began produced a pH* of 6.0.

Purification of 23-norcholic acid was accomplished by preparative TLC on silica gel G 20 × 20 cm glass plates (Fisher Chemical Co., Norcross, GA) using the solvent system hexane–ethyl acetate–acetic acid–propan-2-ol 2:1:1:0.2 (by vol). It was eluted from the silica with methanol and recrystallized from aqueous methanol, uncorrected melting point 203°C. This material gave single peaks on capillary GLC (12 m × 0.25 mm ID coated with Poly S-179) as the tris trimethylsilyl ether methyl ester and as the trimethyl ether methyl ester derivatives (15). After extensive drying in vacuo, 4.6 mg was weighed out and made up in 3 ml of D_2O and NaOD to give a 4 mM solution, pH* 7.0.

NMR measurements. Proton and ^{13}C-NMR spectra were measured on a Bruker WH-400 superconducting spectrometer equipped with a temperature controller, Aspect 2,000 computer and disk storage.

Fourier transform ^{1}H-NMR spectra of sodium cholate (pH* 7.0) a D_2O were obtained in 5-mm tubes at 297 ± 2°K employing TSP as the internal chemical shift reference (0.00 δ). Spectral conditions were as follows; sweep width 4,000 Hz; quadrature phase detection; 32 K points; pulse flip angle 66° (9 μsec); 16 to 32 scans; acquisition time 4.096 seconds; additional delay 0 to 5 seconds; pulse repetition rate 4.096 to 9.096 seconds. Resolution enhanced spectra (16) were obtained from 256 scans.

Homonuclear proton decoupling experiments were carried out using the standard hardware available on the Bruker WH-400 spectrometer; the decoupler was gated off during data acquisition. Fourier difference spectra (17) were obtained using blocks of four or eight scans with the decoupler on-resonance and off-resonance, respectively (8 cycles). Decoupler power ranged from 9 to 18 db below 0.2 watts (0.025 to 0.003 watts). Since the decoupling power was gated off approximately 80% of the time, the average power level at the sample was about 20% of the above.

Nuclear Overhauser effect (NOE) analysis was performed using a similar procedure to the Fourier difference decoupling experiment described above. The important difference was that the decoupler was turned off during data acquisition. As before, off-resonance data were also recorded. Because of low signal-to-noise, 256–1024 scans were necessary for demonstration of NOE effects.

Partially relaxed ^{1}H-NMR spectra were obtained using a 180°-τ-90° sequence. For a τ value of 300 msec, the methine proton resonances were nulled, leaving a methylene spectrum. Similarly for a τ value of 155 msec, the methylene proton resonance were nulled giving a methine spectrum (inverted). Combination of partial relaxation and ^{1}H-decoupling techniques enabled the production of a difference spectrum of either the methine or methylene protons, greatly simplifying interpretation.

Carbon-13 FT-NMR spectra (100.62 MHz) of sodium cholate (4 to 40 mM in D_2O) were obtained in 10-mm tubes using dioxane (δ = 67.4) as the internal chemical shift reference. Normal parameters for broad band ^{1}H-decoupled spectra were as follows: sweep width 8,000 to 10,000 Hz; data table size 16 K; acquisition time ca. 1 sec; pulse flip angle 90° (26 μsec); pulse repetition rate (acquisition time plus additional delay) 3 to 7 seconds; 128 to 8,192 scans accumulated. The proton decoupling power during acquisition was ca. 3 watts and was lowered to 0.5 watts during the additional delay to prevent heating of the sample. Its center frequency was 2.00 δ in the proton spectrum. Carbon linewidths ($\Delta\Upsilon_{1/2}$) under these conditions were less than 1 Hz for quaternary carbons and less than 5 Hz for protonated carbons. To minimize truncation effects and improve signal to noise, the FID was multiplied by an exponential function before transformation. This resulted in an additional line broadening of approximately 1 Hz for all resonances.

Single frequency ^{1}H-decoupled ^{13}C-spectra of sodium cholate (20 mM) were obtained with unmodulatd continuous wave, low power (0.06 to 0.2 watts) ^{1}H-decoupling (18), and at a series of ^{1}H-decoupling frequencies ranging from 0.5 δ to 3.0 δ. This technique correlated the methine ^{1}H with ^{13}C-chemical shift assignments, since a decoupled ^{13}C line resulted only when the decoupler frequency was on-resonance with the proton directly bonded to that carbon. Partially decoupled ^{13}C-resonances were observed in all other cases (J-reduced spectra). For methylene carbons, the signal pattern was complex, except when both protons had the same chemical shift.

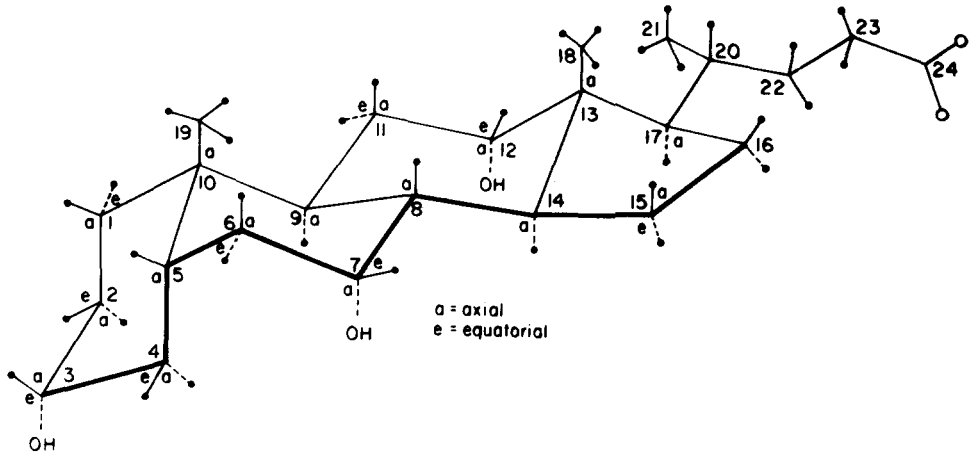

Fig. 1. Molecular structure of sodium cholate. This is a schematic diagram; while effort has been made to create a perspective effect, artistic license has been used for clarity. The orientation of each proton with respect to the plane of the molecule is given by the dotted (below the plane) and solid (in or above the plane) lines. In the text this orientation is denoted by the suffixes α and β, respectively. Orientation with respect to each ring is denoted by the terms equatorial (in the plane) and axial (out of the plane). No axial-equatorial assignments were made for the protons on carbon atom 16 since this is equivocal (see Ref. 19).

RESULTS

The molecular structure of sodium cholate is shown in schematic form (**Fig. 1**). The orientation of the side chain has been drawn for convenience. The ^{1}H-NMR spectrum between 1.0 and 2.3 δ of sodium cholate (4 mM, pH* 7.0) at 400 MHz is shown in **Fig. 2A.** A substantial improvement in resolution has occured compared to previously published data (4). The resolution could be further enhanced by use of a double exponential function (16) (Fig. 2B).

Some simplification of the spectrum was obtained by the use of partial relaxation techniques (see Methods). The resonances from the methine protons could be nulled, leaving the resonances from methylene protons at carbon atoms 1, 2, 4, 6, 11, 15, and 16 (**Fig. 3**); alternatively, the resonances from the methylene protons could be nulled resulting in a spectrum containing the methine proton resonances (inverted) at $C_{5\beta}$, $C_{8\beta}$, $C_{9\alpha}$, $C_{17\alpha}$, and C_{20}. Coincidentally, the resonances from the methylene protons on C_{22} and C_{23} had relaxation times similar to the ring methine protons because of faster isotropic tumbling in the side chain (Fig. 3).

Decoupling experiments

The major strategy for identification of individual proton resonances was the use of decoupling at proton resonances already assigned; comparison was then made with off-resonance spectra. The expected proton cou-

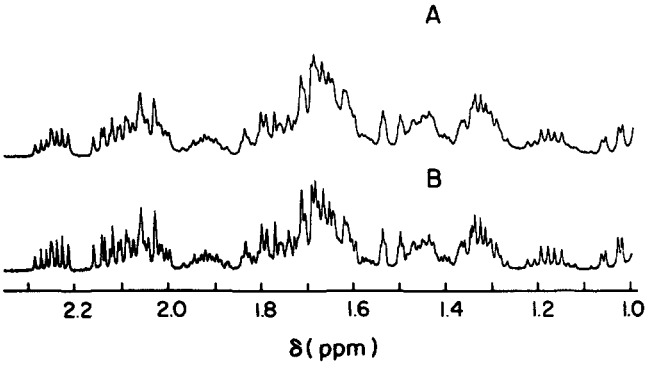

Fig. 2. ^{1}H-NMR spectra of sodium cholate at 400 MHz. The ^{1}H spectra of an aqueous 4 mM sodium cholate solution was obtained at 400 MHz (A). Increased resolution was realized in (B) by multiplying data by a double exponential function.

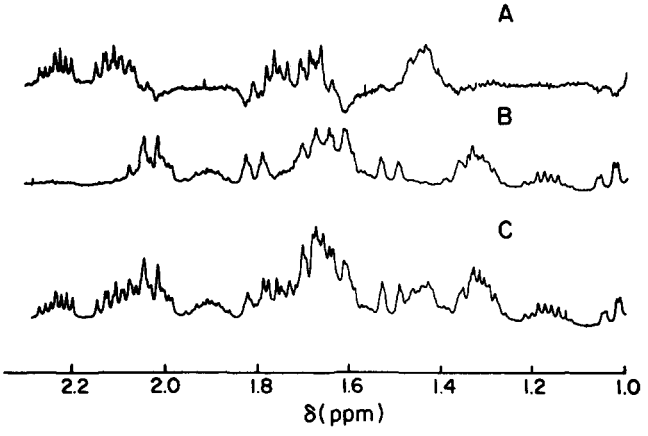

Fig. 3. Methine and methylene ^{1}H-NMR spectra of aqueous 4 mM sodium cholate. Methine spectrum (A) was obtained using a 180°-τ-90° sequence with a τ value of 155 msec and methylene spectrum (B) with a τ value of 300 msec. Only the chemical shift range 1.0–2.3 δ is depicted. The normal spectrum is also shown (C).

plings are shown in **Table 1.** This approach was refined by carrying out partial relaxation techniques in conjunction with decoupling (see Methods). In this way changes could be seen in the methylene and methine spectra independently (see **Fig. 4**). The observed resonance patterns in the decoupled difference spectra obtained by decoupling individual protons are summarized in **Table 2.**

In the first series of experiments, four resonances were decoupled, at $C_{3\beta}$ (3.51 δ), $C_{7\beta}$ (3.91 δ), $C_{12\beta}$ (4.09 δ), and C_{21}-CH_3 (0.98 δ). These resonances were not overlapped by any other proton resonances and were readily identified by inspection of their resonance patterns, as noted by other investigators (4, 10). The simplest one was $C_{12\beta}$ since it could only affect the two methylene protons at C_{11}. The decoupled difference spectrum showed changes centered at 1.60 δ (Fig. 4A). Decoupling of $C_{7\beta}$ affected the methylene protons at C_6 (centered at 1.52 and 2.01 δ) and the methine proton, $C_{8\beta}$ (centered at 1.67 δ). This is shown in Fig. 4B and C. Decoupling of $C_{3\beta}$ affected two pairs of methylene protons, at C_2 and C_4 (centered at 1.36, 1.60, and 2.05 δ) (Fig. 4D). These could not be distinguished at this stage. Decoupling of C_{21}-CH_3 only affected the methine proton at C_{20} (centered at 1.45 δ) (Fig. 4E). This proton was coupled with $C_{17\alpha}$, C_{21}-CH_3 and C_{22}-CH_2 and was a very broad envelope of resonances.

In the next series of studies, the newly assigned proton resonances were used for further decoupling experiments. Decoupling at 1.60 δ (C_{11}-CH_2) caused a change centered at 2.08 δ in the methine spectrum. This could only be due to $C_{9\alpha}$, although overlap with other proton resonances at the decoupling frequency occurred. To verify the assignment, decoupling was carried out at 2.08 δ; the methine spectrum showed the required change centered at 1.67 δ ($C_{8\beta}$). Decoupling at 1.45 δ (C_{20}-CH) produced a single change in the methine spectrum centered at 1.64 δ which was assigned to $C_{17\alpha}$.

Of the peak clusters seen in the methylene spectrum, the one centered at 1.92 δ was not altered by the previous experiments. The possibilities were the methylene protons on C_1, C_{15}, C_{16}, and C_{22}. Decoupling at 1.92 δ produced changes centered at 1.64 δ in the methine spectrum which had been assigned to $C_{17}\alpha$. This proton could only

TABLE 1. Theoretical interactions of decoupling at specific protons

Decoupled Proton	Methine Spectrum	Methylene Spectrum
1($\alpha\beta$)[a]		1($\alpha\beta$),2(α,β)
2($\alpha\beta$)	3β	1(α,β),2($\alpha\beta$)
3β		2(α,β),4(α,β)
4($\alpha\beta$)	3β,5β	4($\alpha\beta$)
5β		4(α,β),6(α,β)
6($\alpha\beta$)	5β,7β	6($\alpha\beta$)
7β	8β	6(α,β)
8β	7β,9α,14α	
9α	8β	11(α,β)
11($\alpha\beta$)	9α,12β	11($\alpha\beta$)
12β		11(α,β)
14α	8β	15(α,β)
15($\alpha\beta$)	14α	15($\alpha\beta$),16(α,β)
16($\alpha\beta$)	17α	15(α,β),16($\alpha\beta$)
17α	20	16(α,β)
18-CH_3		
19-CH_3		
20	17α,21-CH_3[b],22-CH_2[b]	
21-CH_3	20,22-CH_2[b]	
22-CH_2	20,23-CH_2[b]	
23-CH_2	22-CH_2[b]	

[a] ($\alpha\beta$) signifies either/or; (α,β) signifies both protons.
[b] These protons appear in the methine spectrum under the conditions used.

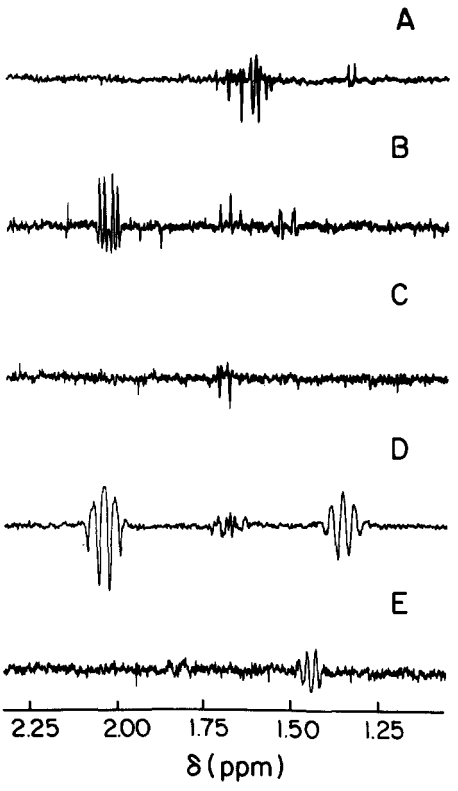

Fig. 4. ^{1}H-NMR decoupled difference spectra of aqueous 4 mM sodium cholate. Each of these spectra is the difference between on- and off-resonance decoupled ^{1}H-spectra. In A, on-resonance decoupling was at $C_{12\beta}$ (4.08 δ) using non-relaxed spectra. The peak patterns are due to the two overlapping C_{11} protons ($C_{11\alpha}$, 1.64 δ and $C_{11\beta}$, 1.60 δ). In B and C, decoupling was at $C_{7\beta}$ (3.91 δ). In B, the non-relaxed spectrum is shown containing the two C_6 protons ($C_{6\alpha}$, 2.01 δ and $C_{6\beta}$, 1.52) and the $C_{8\beta}$ proton (1.68 δ). In C, the methine spectra is shown containing only the $C_{8\beta}$ methine proton. In D, decoupling was at $C_{3\beta}$ (3.51 δ) using non-relaxed spectra. The peak patterns showing the large decoupling were $C_{2\alpha}$ (1.36 δ) and $C_{4\alpha}$ (2.04 δ). The smaller changes were due to $C_{2\beta}$ (1.60 δ) and $C_{4\beta}$ (1.60 δ). In E, decoupling was at C_{21}-CH_3 (0.98 δ) using methine spectra, producing a single pattern due to the C_{20} proton.

TABLE 2. Summary of decoupling experiments

Provisionally Assigned Decoupled Proton	Chemical Shift (δ)	Center of Pattern in Difference Spectrum		
		Full	Methylene	Methine
$C_{12\beta}$	4.09	1.60		
$C_{7\beta}$	3.91		1.52, 2.01	1.67
$C_{3\beta}$	3.51	1.36, 1.60, 2.05		
C_{21}-CH_3	0.98			1.45
$C_{11\alpha\beta}$	1.60			2.08
$C_{9\alpha}$	2.08			1.67
C_{20}	1.45			1.64
C_{16}	1.92		1.17, 1.32	1.64
C_{15} or C_{16}	1.17		1.32, 1.92	1.80
C_{15} or C_{16}	1.32		1.06, 1.17, 1.81, 1.92	1.64
C_1	1.01		1.36, 1.60, 1.81	
C_4 and C_6	2.04			1.48
C_{23}	2.25	1.32, 1.74, 2.12		

be coupled with protons at C_{16} and C_{20}. Since the resonances of C_{20}-CH (1.45 δ) were far from the decoupling frequency, the resonances centered at 1.92 δ were assigned to one of the methylene protons at C_{16}. Decoupling at 1.92 δ (C_{16}) produced changes in the methylene spectrum centered at 1.17 and 1.32 δ. These changes should have been due to the other C_{16} proton and to the C_{15} methylene protons.

Decoupling at 1.17 δ produced a marked change centered at 1.80 δ in the methine spectrum. If the group of resonances centered at 1.17 δ represented the other C_{16} proton, then a change would have been expected at 1.64 δ—none was observed. Therefore, the resonances centered at 1.17 δ must have been due to one of the C_{15} protons, and furthermore, the changes centered at 1.80 δ must have been due to the $C_{14\alpha}$ proton. In the methylene spectrum, changes occurred centered at 1.92 and 1.32 δ, i.e., those at 1.92 δ were from one of the C_{16} protons (as before) and those at 1.32 δ were either from C_{15} or C_{16} or both. Decoupling at 1.32 δ confirmed that one of the C_{16} protons was present, since the $C_{17\alpha}$ proton resonance pattern (at 1.64 δ) was altered in the methine spectrum. In the methylene spectrum, changes were seen centered at 1.06, 1.17, 1.81, and 1.92 δ. Those at 1.17 δ and 1.91 δ were identified, as noted before, as one each of the C_{15} and C_{16} protons, respectively.

The portion of the spectrum between 2.1 and 2.3 δ has been previously suggested as being due to C_{23}-CH_2 (4). These peak patterns were the only part of the ^{1}H-NMR spectrum that was sensitive to pH* (see below, Effect of pH*) and on this basis they were assigned to C_{23}-CH_2. When the C_{23}-CH_2 resonance pattern was decoupled at 2.25 δ, marked changes occurred centered at 2.12 δ, i.e., the C_{23}-CH_2 protons were anisochronous, and also at 1.74 δ and 1.32 δ (**Fig. 5**). The latter two resonances were assigned to the C_{22} methylene protons; again the two protons being anisochronous. Further confirmation of the identity of the resonances centered at 2.12 δ and 2.25 δ was obtained by examination of the ^{1}H-NMR spectrum of 23-norcholate. Although the resonance patterns were almost identical, the proton chemical shifts for this bile salt were slightly downfield compared to cholate (**Fig. 6**). This was most marked for the protons on the carbon atom adjacent to the carboxyl group. For 23-norcholate this is C_{22} as opposed to C_{23} for cholate. The resonance pattern was also simplified, as coupling for this methylene group occurs only with the methine proton at C_{20}.

The only remaining resonance pattern in the methylene spectrum apparently centered at 1.06 δ was assigned to one of the C_1 protons. When this proton was decoupled, changes were observed in the methylene spectrum centered at 1.36, 1.60, and 1.81 δ. Since those centered at 1.36 and 1.60 δ were observed when decoupling $C_{3\beta}$, they must be due to the C_2 protons and that at 1.81 δ must be the other C_1 proton. Hence the C_4 protons were

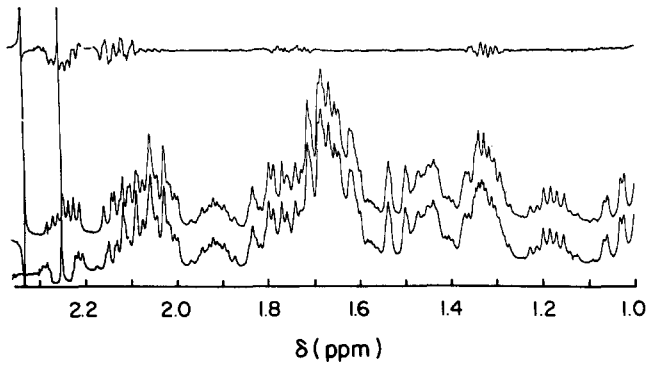

Fig. 5. Identification of the C_{22} methylene proton resonances. The bottom spectrum is that decoupled at C_{22} (2.25 δ) and the center one is the off-resonance spectrum. The uppermost spectrum is the difference of two lower spectra. The peak patterns are seen for the other C_{23}-proton (2.12 δ) and the two C_{22} protons (1.32 δ and 1.74 δ).

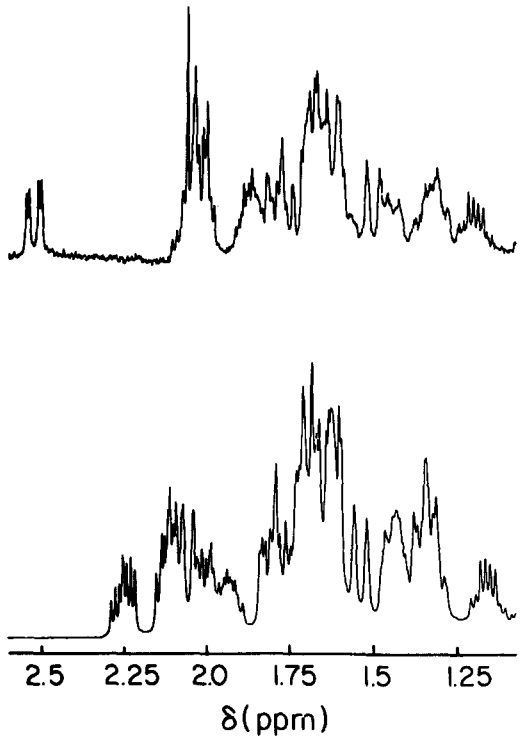

Fig. 6. ^{1}H-NMR spectrum of 4 mM aqueous sodium 23-norcholate. Each trace is part of the spectrum between 1.0–2.5 δ. The upper spectra is that from 23-norcholate, the lower one from cholate. The prominent difference is the absence of the C_{23} protons (at 2.12 δ and 2.25 δ) and the large downfield shift of the C_{22} protons (2.50 δ).

the other changes seen when decoupling $C_{3\beta}$, i.e., those centered at 1.60 and 2.04 δ.

The location of the resonances due to the $C_{5\beta}$ proton was made by decoupling at 2.04 δ (C_4 and C_6 protons), the methine spectrum showing a marked change centered at 1.48 δ.

NOE Experiments

Further confirmation of the assignments for some of the methylene and methine protons were obtained in NOE experiments, since NOE enhancements are dependent on the sixth power of the distance between the saturated protons and the affected proton. Thus NOE enhancements can discriminate between two methylene protons which are not equidistant from the saturated protons assuming, of course, that an NOE enhancement can be measured.

The two methyl groups (C_{18} and C_{19}) attached to the quaternary carbon atoms C_{13} and C_{10}, respectively, were saturated and the NOE differences spectra were examined. From these two experiments, further assignments of individuals methylene proton resonances were made, those of C_6, C_{11}, and C_{15}. Saturation of C_{19} methyl protons (at 0.92 δ) produced NOEs centered at 1.01, 1.43, 1.60, 1.64, 1.81, and 2.01 δ. Those at 1.01 and 1.81 δ were from the two C_1 protons. The NOE enhancements were approximately equal (2%). This is consistent with the molecular structure, the two protons being symmetrically oriented about the plane of the C_1-C_{10}-C_{19} bonds and being equidistant from the C_{19} methyl group. Those at 1.60 and 1.64 δ were identified as $C_{11\beta}$ and $C_{8\beta}$, respectively. Both these protons also showed NOEs when the C_{18} methyl group (0.72 δ) was saturated. The NOE at 2.01 δ is due to $C_{6\beta}$; thus the other C_6 resonance centered at 1.52 δ is from $C_{6\alpha}$.

Saturation of C_{18} methyl protons caused NOEs at 1.42 δ (C_{20}), 1.69 ($C_{15\alpha,\beta}$) and 4.08 δ ($C_{12\beta}$), in addition to effects on $C_{8\beta}$ and $C_{11\beta}$. The NOE at 1.69 δ was assigned to the $C_{15\beta}$ proton because of structural considerations; thus $C_{15\alpha}$ was at 1.17 δ. The magnitude of the NOE was about 2% in those cases where integration was not affected by overlap and was always positive.

Identification of individual methylene protons

Methylene protons were distinguished by NOE experiments (C_6, C_{11}, and C_{15}—see above) and by consideration of their resonance peak patterns (for C_1, C_2, and C_4). From the molecular structure and the bond angles between the adjacent protons (19), $C_{1\beta}$ should have two large 14 Hz couplings, one geminal coupling to $C_{1\alpha}$ and a 180° vicinal coupling to $C_{2\alpha}$, and one small 2–3 Hz vicinal coupling a $C_{2\beta}$. The pattern centered apparently at 1.06 δ was consistent with this, except that one part of the expected triplet of doublets was absent. However, the adjacent C_{21}-CH_3 doublet overlapped. By using a 180°-τ-90° sequence where τ was 255 msec, the C_{21}-CH_3 peaks were nulled and the missing C_1 doublet was observed (**Fig. 7**). Thus C_1 was a pattern centered at 1.01 δ. The other resonance pattern for $C_{1\alpha}$ was at 1.81 δ with one large negative 14 Hz geminal coupling to $C_{1\beta}$ and two small 2–3 Hz vicinal couplings to $C_{2\alpha}$ and $C_{2\beta}$. Of the two C_2 protons (at 1.36 and 1.60 δ), the one at 1.36 δ was assigned to $C_{2\alpha}$ because of its large 14 Hz 180° vicinal coupling to $C_{3\beta}$. The resonance pattern for the C_4 proton at 2.04 δ contained two large 14 Hz couplings and one small 3 Hz coupling. This could only occur for the $C_{4\alpha}$ proton, showing geminal coupling with $C_{4\beta}$ and 180° vicinal coupling with $C_{3\beta}$. Other methylene protons, at C_6 and C_{15}, were identified by NOE experiments.

Proton assignments by partial ^{1}H-decoupled ^{13}C-NMR

Assignments for the $C_{5\beta}$, $C_{8\beta}$, $C_{9\alpha}$, $C_{14\alpha}$, $C_{17\alpha}$, and C_{20} methine protons were confirmed by partial ^{1}H-decoupled ^{13}C-NMR. The ^{13}C-chemical shift assignments were

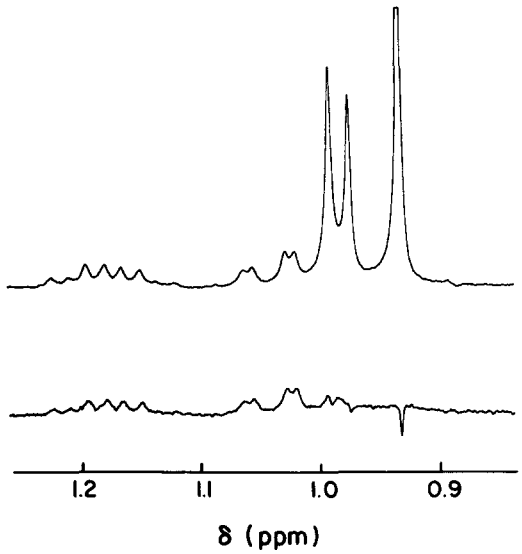

Fig. 7. Recovery of hidden $C_{1\beta}$ doublet using partial relaxation ^{1}H-NMR. The upper trace is the ^{1}H-spectrum between 0.9–1.2 δ. The lower trace was obtained by using a 180°-τ-90° sequence with a value of 255 msec to null the C_{21}-CH_3 doublet.

derived from previously published data (4). Carbon atoms with methine protons were distinguished from those with methylene protons since, as the methine proton was decoupled, the signal for the carbon atom to which it was attached changed from a multiplet to a singlet. Because of the band pass of the decoupler (25 Hz), the $C_{5\beta}$ (1.48 δ) and C_{20} (1.45 δ) protons could not be distinguished by this technique. However, their identification was previously established by decoupling of the C_{21}-methyl group as shown in Fig. 4E. In addition, the chemical shifts of the resonances of the methylene protons on carbon atoms 1,2,4,6,11,15,16,22, and 23 were confirmed. The assignments of the proton resonances in the ^{1}H-spectrum of sodium cholate are thus shown in **Table 3.** The ^{13}C-chemical shifts for sodium cholate in aqueous solution are given in **Table 4.**

Effect of pH*

Careful titration of a 4 mM sodium cholate solution to pH* 6.0 where precipitation of cholic acid was just beginning to occur, caused small changes (0.01 δ upfield) in the spectrum only at C_{23}-CH_2 (2.12 δ and 2.25 δ). After more DCl was added, substantial precipitation of cholic acid occurred and the pH* rose to 6.37. At this point the downfield shift of C_{23}-CH_2 was eliminated and the spectrum was identical to that at pH* 7.0. Peak broadening did not occur at pH* 6.0.

Effect of concentration

No differences could be detected between the ^{1}H-spectra of sodium cholate at 1 mM and 5 mM. Above 5 mM there were some small changes in chemical shifts (0.02–0.04 δ) that were concentration dependent. Peak broadening was observed above 5 mM, being approximately twofold for the C_{18} and C_{19} methyl groups at 50 mM.

TABLE 3. Assignments of individual proton resonances[a]

Proton	Center of Resonance Pattern 4 mM	20 mM
1(αβ) CH_2-	1.01(β), 1.81(α)	1.02(β), 1.81(α)
2(αβ) -CH_2-	1.36(α), 1.60(β)	1.36(α), 1.60(β)
3β HO -CH	3.51	3.51
4(αβ) -CH_2-	1.60(β), 2.04(α)	1.60(β) 2.05(α)
5β -CH	1.48	1.46
6(αβ) -CH_2	1.52(α), 2.01(β)	1.54(α), 2.01(β)
7β HO -CH	3.91	3.99
8β -CH	1.68	1.64
9α -CH	2.07	2.09
11(αβ) -CH_2-	1.60(β), 1.64(α)	1.60(β), 1.62(α)
12β HO -CH	4.08	4.08
14α -CH	1.80	1.78
15(αβ) -CH_2-	1.17(α), 1.69(β)	1.15(α), 1.70(β)
16(αβ) -CH_2-	1.32[b], 1.92[b]	1.33[b], 1.94[b]
17α -CH	1.66	1.70
18 -CH_3	0.73	0.73
19 -CH_3	0.93	0.93
20 -CH	1.45	1.43
21 -CH_3	0.98	0.98
22 -CH_2-	1.32, 1.74	1.34, 1.74
23 -CH_2-	2.12, 2.25	2.12, 2.25

[a] Given as ppm relative to TSP (δ = 0.00).
[b] The resonances remain unassigned.

TABLE 4. ^{13}C-Chemical shifts for sodium cholate in aqueous solution (20 mM)

Carbon Atom	Type	Chemical Shift[a]
1	CH_2	30.1
2	CH_2	33.1
3	HO-CH-	69.4
4	CH_2	39.2
5	CH	41.8
6	CH_2	34.5
7	HO-CH-	72.5
8	CH	39.8
9	CH	27.2
10	C	35.1
11	CH_2	28.3
12	HO-CH-	74.2
13	C	46.9
14	CH	42.4
15	CH_2	23.6
16	CH_2	28.0
17	CH	47.6
18	-CH_3	12.7
19	-CH_3	17.5
20	CH	36.1
21	-CH_3	22.7
22	CH_2	35.4[b]
23	CH_2	35.4[b]
24	COOH	185.5[c]

[a] In parts per million, relative to dioxane (67.4 ppm).
[b] The resonance for C_{23} is pH*-dependent and moves downfield with increasing pH*.
[c] Value obtained at pH* 8.0.

Identification of individual proton resonances at 20 mM was carried out as described in detail at 4 mM (Table 3).

DISCUSSION

The use of 400 MHz ^{1}H-NMR has given sufficient resolution of the ^{1}H-NMR spectrum of aqueous sodium cholate to permit assignments for each of the protons in this type of molecule for the first time. In addition, 100 MHz ^{13}C-NMR has been used to obtain ^{13}C-spectra of this bile salt at concentrations below its critical micellar concentration.

The high field NMR has assisted this investigation in several ways, first, by increasing resolution and second, by improving signal-to-noise enabling lower concentrations to be studied. In addition, since measurements could be made below the critical micellar concentration, the bile salts were in the monomeric rather than polymeric form. The signals are inherently better resolved.

Proton assignments were made using a series of decoupling and NOE difference experiments. For bile salt molecules the general method for ^{1}H-assignment involves the functional groups, e.g., hydroxyl, carboxyl, and the C_{21} methyl group, in a pivotal approach using decoupled difference spectroscopy, often combined with partial relaxation measurements. Secondly, mere separation of methine and methylene ^{1}H-resonances by partial relaxation measurements was not sufficient for ^{1}H-assignments in the bile salt molecules. By combining the techniques of partial relaxation, to isolate the methine and methylene ^{1}H-resonances, and ^{1}H-decoupling difference spectra, it was possible to observe couplings to methine and methylene protons individually. This method, as far as we know, has not been used before; however, future studies on molecules of this degree of, or more, complexity will benefit from its application. For sodium cholate, decoupling of the protons ($C_{3\beta}$, $C_{7\beta}$, and $C_{12\beta}$) epimeric to the hydroxy groups and decoupling of the methyl doublet from C_{21} methyl group identified ten proton resonance patterns, although this included four pairs of methylene protons. Using these new assignments, the remainder of the protons were identified using the same basic technique. Only the C_1 protons were assigned in an arbitrary way, i.e., by elimination.

The decoupled difference experiments provided less clear information on which proton from each pair of methylene protons corresponded to the two observed chemical shifts. This was mostly overcome by NOE experiments and inspection of the recently published crystal structure of sodium cholate monohydrate (19), assuming that the rigidity of the steroid nucleus makes such data applicable.

Although the axial-equatorial difference in chemical shift for methylene protons in six-membered rings is usually 0.4 δ (20), no consistent differences were observed for the methylene protons in sodium cholate. This, in part, was due to the *cis*-fused A ring. Assignment of $C_{11\beta}$ (1.60 δ) and $C_{11\alpha}$ (1.62 δ) came from consideration of the coupling patterns and from the observation that NOE experiments on C_{18} and C_{19} methyl groups caused an effect only at 1.60 δ, not at 1.62 δ. $C_{11\beta}$ is 1,3 diaxial with respect to the C_{19}-methyl group, and approximately equidistant to both methyl groups (2.0 Å). The C_1 methylene protons had similar NOE enhancements in experiments on the C_{19} methyl group, being symmetrically sited about the C_1-C_{10}-C_{19} plane. Consideration of coupling with the C_2 methylene protons permitted assignment of $C_{1\beta}$ (1.01 δ) and $C_{1\alpha}$ (1.81 δ). The axial $C_{1\alpha}$ was shielded by $C_{11\alpha}$ (internuclear distance 2.0 Å) which could account for its large downfield shift relative to $C_{1\beta}$. The C_2 protons were identified on the basis of coupling to $C_{3\beta}$; only $C_{2\alpha}$, the axial proton, should have been strongly coupled since the bond angle was 180°. The greater chemical shift for $C_{2\alpha}$ than predicted was probably due to shielding of $C_{2\alpha}$ by $C_{9\alpha}$ (internuclear distance 1.8 Å). Strong coupling of $C_{4\alpha}$ with $C_{3\beta}$ was also used to resolve the C_4 protons. In this case, $C_{4\alpha}$ is shielded by the hydroxyl group ($C_{4\alpha}$-O_7 internuclear distance is 1.5 Å), so much so that the equatorial-axial difference in chemical shift is reversed.

For the C_6 protons, assignment of $C_{6\beta}$ was made on the basis that it had an NOE enhancement (at 2.01 δ) when the C_{19} methyl group was saturated. Although $C_{6\beta}$ is an axial proton, considerable shielding occurs from the methyl group (C_{19}) (internuclear distance 1.5 Å), causing a downfield shift. In a similar manner the $C_{15\beta}$ proton was identified by the NOE effect when saturating the C_{18} methyl group (1.9 Å). From the crystal structure data on sodium cholate (19), $C_{15\beta}$ is axial; however, the shielding from the C_{18} methyl group causes a downfield shift. No NOE could be detected for $C_{16\beta}$ from saturating the C_{18} methyl group. This is reasonable since the internuclear distance from the crystal structure is 2.5 Å; since the NOE falls off with the sixth power of distance, the NOE would be reduced four-fold compared to that on $C_{15\beta}$, taking the effect below the signal-to-noise threshold (0.5%). The two sets of resonance patterns for the C_{16} protons could not be distinguished either by NOE or proton decoupling experiments.

The NOE experiments also confirmed the orientation of the C_{20} proton. Saturation of the C_{19} methyl group caused an NOE effect of 2% on C_{20}. This result suggests that the internuclear distance between the C_{18} methyl group and the C_{20} proton is about 2–2.2 Å. This can only occur if the proton is orientated axially to the B, C, and D rings, i.e., with the C_{21} methyl group equatorial

to these rings. Saturation of the C_{21} methyl group doublet confirmed this since a strong (2%) NOE was observed for $C_{12\beta}$.

Both the methylene groups in the side chain had two peak patterns. The pair ascribed to C_{22}-CH_2 (at 1.32 and 1.74 δ) were anticipated. Despite rapid rotation of the methylene group it cannot average out its orientation with respect to the asymmetric carbon atom at C_{20}. The two proton resonances for C_{23}-CH_2, showing a small difference in chemical shift (2.12 and 2.25 δ), were not expected. This phenomenon has not been described before and may be a consequence of using higher frequency radiation. Identification of these resonances for the C_{23}-CH_2 protons was made by their sensitivity to changes in pH* in the range 6.0–6.3. At this pH*, 20–25% of the cholate anions become protonated. This caused small, but significant alterations (0.01 δ) to the observed chemical shifts. The chemical shifts for these protons from pure cholic acid, i.e., in the absence of the anion, may be somewhat larger. However, limited aqueous solubility of cholic acid prevented such a measurement.

Concentration effects in the range 1–50 mM for proton chemical shifts were small (mostly 0.02–0.04 δ) and there was a twofold increase in linewidth of the C_{18} and C_{19} methyl groups as noted previously (4, 5). Since the NOEs were always positive it is possible to place an upper bound on the size of the aggregates in solution. For a positive NOE, the square of the product of the correlation time (τ_c) and the resonance frequency (measured in radians, i.e., 2.5×10^9/sec) must be less than 1.25 (21), i.e., τ_c is somewhat less than 4×10^{-10} sec. This value for τ_c is observed for molecules of molecular weight of 1000–1500, i.e., not larger than a tetramer. This result is consistent with the observations of Small (3), using analytical ultracentrifugation. Indeed, it is possible that the linewidth changes, previously reported (4) and confirmed in this study, are due to increased microviscosity rather than to significant aggregation.

While this work was in progress, Hall and Saunders (22) published a paper on the proton assignments of 1-dehydrotestosterone, employing a strategy similar to that used in the present work. However, proton assignment for the sodium cholate presented a far more difficult task since there is no side chain in 1-dehydrotestosterone, the A-ring protons are few and comparatively easily observed, there is no 5-proton at the A-B ring junction, and the D-ring protons are influenced by the 17β hydroxyl group (22).

This study opens up the way to carry out studies of the physical chemistry and molecular structure of bile salts in micelles in aqueous solution. Since ^{1}H-NMR and ^{13}C-NMR can yield information on interaction, motion, and location of individual portions, i.e., of the bile salt molecule in micelles, it is complementary to other techniques, quasielastic laser light scattering (23) and low angle x-ray diffraction (24), currently being used to investigate the assembly of lipids in biliary bile salt micelles.

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