Bile acids and bile alcohols in a child with hepatic 3β-hydroxy-Δ^5-C$_{27}$-steroid dehydrogenase deficiency: effects of chenodeoxycholic acid treatment[1]

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Abstract Duodenal bile, urine, plasma, and feces from a child with hepatic 3β-hydroxy-Δ^5-C$_{27}$-steroid dehydrogenase deficiency were analyzed by fast atom bombardment mass spectrometry and gas chromatography–mass spectrometry to investigate the formation and excretion of abnormal bile acids and bile alcohols. The biliary bile salts consisted of glycocholic acid (25%) and of sulfated and glycine conjugated di- and trihydroxycholenoic acids (55%), two C$_{27}$ bile acids, and eleven sulfated bile alcohols (mainly tetrols, 20%), all having 3β,7α-dihydroxy-Δ^5 or 3β,7α,12α-trihydroxy-Δ^5 ring structures. In plasma, sulfated cholenoic acids constituted 65% and unconjugated 3β,7α-dihydroxy-5-cholestenoic acid 25% of the total level, 71 μg/ml. The urinary excretion of the former was 30.4 mg/day and that of unsaturated bile alcohol sulfates, mainly pentols, 7 mg/day. The predominant bile acid in feces was an unconjugated epimer of 3β,7α,12α-trihydroxy-5-cholenoic acid, and small amounts of cholic acid were present. The minimum total excretion was 11.3 mg/day. Treatment with chenodeoxycholic acid resulted in marked clinical improvement and normalized liver function tests. Further studies are needed to define the mechanism of action. Plasma bile acids decreased to 1.6 μg/ml and urinary excretion to 3.4 mg/day. Chenodeoxycholic and ursodeoxycholic acids became predominant in all samples. The fecal excretion of unsaturated cholenoic acid sulfates increased to 40 mg/day compared to 89 mg/day of saturated bile acids. The results provide further support for a defective hepatic 3β-hydroxy-Δ^5-C$_{27}$-steroid dehydrogenase deficiency, and indicate that the 3β-hydroxy-Δ^5 bile acids are formed via 7α-hydroxycholesterol. The formation of glycocholic acid may be due to an incomplete enzyme defect or to transformation of the 3β-hydroxy-Δ^5 structure by bacterial and hepatic enzymes during an enterohepatic circulation. —**Ichimiya, H., B. Egestad, H. Nazer, E. S. Baginski, P. T. Clayton, and J. Sjövall.** Bile acids and bile alcohols in a child with hepatic 3β-hydroxy-Δ^5-C$_{27}$-steroid dehydrogenase deficiency: effects of chenodeoxycholic acid treatment. *J. Lipid Res.* 1991. **32:** 829–841.

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The biosynthesis of bile acids is an important metabolic pathway for the elimination of cholesterol. The major synthetic pathway is composed of several steps and more than ten enzymes are involved (1). Cerebrotendinous xanthomatosis (CTX) and the disorders of peroxisomal β-oxidation represent inborn errors of bile acid biosynthesis in which side chain oxidation is affected (1–3). Recently, two novel types of inborn errors involving the transformation of the 3β-hydroxy-Δ^5 structure to the 3α-hydroxy-5β(H) configuration have been proposed (4, 5). One of these was found in a Saudi Arabian infant with cholestatic jaundice who excreted large amounts of 3β,7α-dihydroxy- and 3β,7α,12α-trihydroxy-5-cholenoic acids in urine. Since 5β-cholanoic acids, e.g., cholic, chenodeoxycholic and deoxycholic acids were not detected in urine or plasma, a defect of hepatic 3β-hydroxy-Δ^5-C$_{27}$-steroid dehydrogenase was proposed (4). Treatment with chenodeoxycholic acid was suggested as a means of suppressing the production of possibly hepatotoxic 3β-hydroxy-Δ^5 bile acids and normalizing the bile acid pool. However, the patient was lost to follow-up and this suggestion could not be

Abbreviations: FABMS, fast atom bombardment mass spectrometry; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; 7-ketolithocholic acid, 3α-hydroxy-7-oxo-5β-cholanoic acid; RI, retention index.

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tested until a few years ago. To evaluate the effects of the treatment and to get further information about the pathways of bile acid biosynthesis in this patient, samples of bile, urine, plasma, and feces were analyzed qualitatively and quantitatively by fast atom bombardment mass spectrometry (FABMS), gas-liquid chromatography (GLC) and gas-liquid chromatography-mass spectrometry (GLC-MS). The analytical results are described in this paper; the clinical data documenting the successful treatment have been reported separately (6).

MATERIALS AND METHODS

Patient

The family history and the initial presentation of this patient (MU2) were described in ref. 4. He was lost to follow-up but then presented at the King Faisal Specialist Hospital and Research Centre, Riyadh, at the age of 2 years 9 months. He was physically underdeveloped with persistent jaundice, hepatic dysfunction, and vitamin D malabsorption. Supportive therapy with several vitamins (A, K, E, and large doses of D) was then started but liver function did not improve. A detailed case history is given in ref. 6. Samples of serum, bile, and urine for analyses of bile acids and bile alcohols were collected at the age of 4 years 1 month, and samples of feces and urine were obtained at 4 years 4 months immediately before starting the treatment with chenodeoxycholic acid (Chendol, Weddel, U.K.). The initial dose was 125 mg twice daily (18 mg/kg) which was reduced to 125 mg daily after 2 months. Samples of serum and/or urine were taken 3, 4, 6, 7, and 13 months after the beginning of the treatment. Samples of bile and feces were collected after 13 months of the treatment that has now continued for nearly 3 years.

Sample collection and extraction of bile acids and alcohols

Urine was collected in 24-h periods, each fresh sample being immediately frozen and stored at −20°C. An aliquot (25 ml) of the mixed sample was passed through a Sep-pak C_{18} cartridge (Waters Associates, Milford, MA) prewashed with chloroform-methanol, methanol, and distilled water. The cartridge was then washed with 5 ml of distilled water and sent by mail to Stockholm. After a further wash with 5 ml water, the bile acids and alcohols were eluted with 8 ml of methanol and made to 10 ml with distilled water. Plasma (2 ml) was diluted with 8 ml of 0.1 M aqueous sodium hydroxide and heated at 60°C for 15 min (7). The bile acids and alcohols were then extracted as described for urine. Unstimulated bile was obtained by duodenal intubation and 2-ml portions were diluted and extracted as described for plasma. Feces were collected for 3 consecutive days, each sample being immediately frozen at −20°C. After thorough homogenization, an aliquot (about 20 g) was sent to Stockholm. To a weighed sample (60 mg wet weight feces), 1.8 ml of distilled water, 8 ml of ethanol, and ^{14}C-labeled lithocholic acid (to monitor recoveries) were added. The 80% ethanolic solution was heated at 60°C for 15 min and then kept in the ultrasonic bath for 15 min. After centrifugation, the supernatant was collected. Ten ml of 80% ethanol was added to the residue and treated in the same way. Finally, 10 ml of chloroform-methanol 1:1 (v/v) was added and treated in the same way. This procedure quantitatively extracts common bile acids and sterols (M. Axelson, B. Mörk, and J. Sjövall, unpublished results). The pooled extracts were evaporated to 1-2 ml aqueous solution and 6 ml of methanol and 2-3 ml of distilled water were added to give an about 60% methanolic solution. This was passed through a column of 200 mg Preparative C_{18} (Waters) and the collected effluent was evaporated to give an about 10% methanolic solution. This was again passed through the Preparative C_{18} column. After a wash with 5 ml each of distilled water and 10% methanol, the sterols, bile alcohols, and bile acids were eluted with 10 ml of methanol-chloroform 95:5 (v/v) (8).

Analysis by fast atom bombardment mass spectrometry (FABMS)

Aliquots of the 80% methanolic extracts (10 μl), corresponding to 25 μl urine, plasma, or 2 μl bile, were applied under a gentle stream of N_2 to the FAB target coated with glycerol matrix. Negative ion FABMS was performed on a VG 7070E double focusing instrument equipped with a FAB source, an Ion Tech atom gun operating with xenon at 8 keV, and a VG 11-250 data processing system (VG Analytical, Manchester, U.K.) (9). The accelerating voltage was 6 kV and resolution was about 1000. Spectra with an m/z range 800 to 80 were recorded at a scan rate of 10 s per decade.

Analysis by gas-liquid chromatography and gas-liquid chromatography-mass spectrometry

The principle of the sample preparation was a combination of group separation by anion-exchange chromatography (triethylaminohydroxypropyl Sephadex LH-20, TEAP-20) and treatment under mild conditions to avoid production of artefacts. Using authentic methyl 3β,7α-dihydroxy-5-cholenoate (a kind gift from Dr. K. Uchida, Shionogi Research laboratories, Osaka, Japan), the stability of the labile 3β,7α-dihydroxy-Δ^5 structure was examined under various conditions.

After addition of about 15×10^3 cpm of ^{14}C-labeled lithocholic acid (NEN, Dreieich, FRG, sp act 1.5 GBq/mmol), aliquots of the extracts were adjusted to 95% methanolic solutions that were passed through a column of TEAP-LH-20 (60 × 4 mm) (10). The effluent and ad-

ditional washings with 5 ml each of 95% methanol and methanol–chloroform 1:1 (v/v) were combined to give a fraction comprising neutral compounds. Then, unconjugated, glycine-, taurine-conjugated, and sulfated bile acid fractions were eluted stepwise with 5.5 ml of 0.15 M acetic acid in 95% methanol, 7 ml of 0.30 M acetic acid/ammonia in 95% methanol (pH 5.0), 7 ml of 0.30 M acetic acid/ammonia in 95% methanol (pH 6.6), and 10 ml of 0.3 M ammonium carbonate in 80% methanol, respectively (10, 11). Glycine- and taurine-conjugated bile acids were usually collected together as amidated bile acids by eluting with 7 ml of 0.3 M acetic acid/ammonia in 95% methanol (pH 6.6). The unconjugated bile acid fraction was taken to dryness in vacuo and the residue was methylated with freshly prepared diazomethane. Triacontane (C_{30}) and hexatriacontane (C_{36}) were added as internal standards prior to trimethylsilylation (10). An aliquot (1/20) was used to determine the recovery of radioactivity. The 3β,7α-dihydroxy-Δ^5 structure was not affected by the evaporation and trimethylsilylation.

After addition of 10 ml distilled water, the amidated bile acid fraction was concentrated to about 10 ml and extracted with a bed of 100 mg of Sepralyte (Analytichem International Inc., Harbor City, CA). About 15 × 10^3 cpm of ^{14}C-labeled taurocholic acid (Amersham International plc., U.K., sp act 2 GBq/mmol) was added to check the efficiency of the hydrolysis. Since the 3β,7α-dihydroxy-Δ^5 structure was destroyed during alkaline hydrolysis, hydrolysis with cholylglycine hydrolase (12) was carried out. Deconjugated bile acids were extracted with Sepralyte, rechromatographed on TEAP-LH-20, and derivatized.

Sulfated bile acids were solvolyzed as described by Hirano et al. (13) with some modifications. After extraction with 100 mg of Sepralyte and addition of about 15 × 10^3 cpm of ^{3}H-labeled 3β-hydroxy-5-androsten-17-one sulfate (NEN, sp act 400 GBq/mmol) to monitor recovery, the sulfated bile acids were incubated with 1 ml of tetrahydrofuran (freshly distilled)–methanol–trifluoroacetic acid 900:100:1 (v/v/v) for 15 min or 120 min. Evaporation of the reaction mixture resulted in destruction of the 3β,7α-dihydroxy-Δ^5 structure; therefore, the reaction mixture was neutralized with 0.1 M sodium hydrogen carbonate and diluted with 10 ml of distilled water. The bile acids were then extracted with 100 mg Sepralyte and separated into neutral, unconjugated, and amidated bile acid fractions on a column of TEAP-LH-20. The neutral fraction was collected for determination of the efficiency of solvolysis. Amidated bile acids were further hydrolysed with cholylglycine hydrolase.

For quantitative analysis of bile alcohols, the extracts were first solvolyzed and then separated into bile alcohols, nonamidated, and amidated bile acids on TEAP-LH-20. Another aliquot of the urine extract was separated into unconjugated, glucuronidated, monosulfated, and disulfated bile alcohol fractions on Lipidex-DEAP (Packard Instrument Co., Downers, Grove, IL) and the glucuronide fraction was treated with *Helix pomatia* intestinal juice (14). Sepralyte was used to extract bile acids and bile alcohols from aqueous solutions and TEAP-LH-20 for final clean-up unless otherwise stated. Neutral compounds were derivatized to trimethylsilyl (TMS) ethers.

GLC was performed on a Carlo Erba HRGC 5300 gas chromatograph (Farmitalia Carlo Erba, Milano, Italy) equipped with an on-column injection system and a Spectra-Physics SP 4270 integrator (Spectra-Physics, Darmstadt, FRG). Two fused silica capillary columns (25 m × 0.32 mm) were used, coated either with a 0.25-μm layer of cross-linked methyl silicone (Quadrex Corp., New Haven, CT) or with 0.2-μm layer of UC 1625 (silicone gum containing 25% 4-phenoxyphenyl, 2% vinyl and 73% methyl groups, KSV Chemicals, Helsinki, Finland). The temperature of the oven was 80°C during the injection, then programmed to 270 or 280°C at a rate of 25°C/min and then kept at the final temperature. Helium was used as carrier gas at 50–100 kPa. Retention indices (RI, Kovats) were calculated from the retention times of triacontane and hexatriacontane added to the sample. Quantitation of individual compounds was based on the comparison of the peak area with that of hexatriacontane assuming a response factor of 1.0.

GLC–MS was carried out using a VG 7070E instrument equipped with an all-glass falling-needle injection system and a Dani 3800 gas chromatograph. The same column as that used in the GLC analyses was directly connected and ended in the ion source. Helium was used as carrier gas. The operating conditions were as follows: oven temperature 265°C, temperatures of connecting line and ion source 250°C, the ionization voltage 70 eV, the trap current 200 μA, the accelerating voltage 6kV, the resolution about 1000. The spectra were taken by repetitive magnetic scanning (2 s per decade) over the *m/z* range 850–50.

RESULTS

Analysis of bile before treatment

Analysis by FABMS. The negative ion spectrum of a bile extract is shown in **Fig. 1** (left). As in the spectra of urine and plasma extracts (Fig. 1, left), peaks corresponding to the quasimolecular ions, $[M-1]^-$, of sulfated dihydroxycholenoic (*m/z* 469) and trihydroxycholenoic (*m/z* 485) acids and their respective glycine conjugates (*m/z* 526 and 542) were observed. The latter two peaks were the major ones in the spectrum of bile. The peak at *m/z* 464 indicates the presence of glycine-conjugated trihydroxycholanoic acid(s). The peaks at *m/z* 497, 513, and 529 correspond to quasimolecular ions of sulfated cholestenetriol(s), cholestenetetrol(s), and cholestenepentol(s), respectively, and *m/z* 577 and 593 may represent disulfates of cholestene-

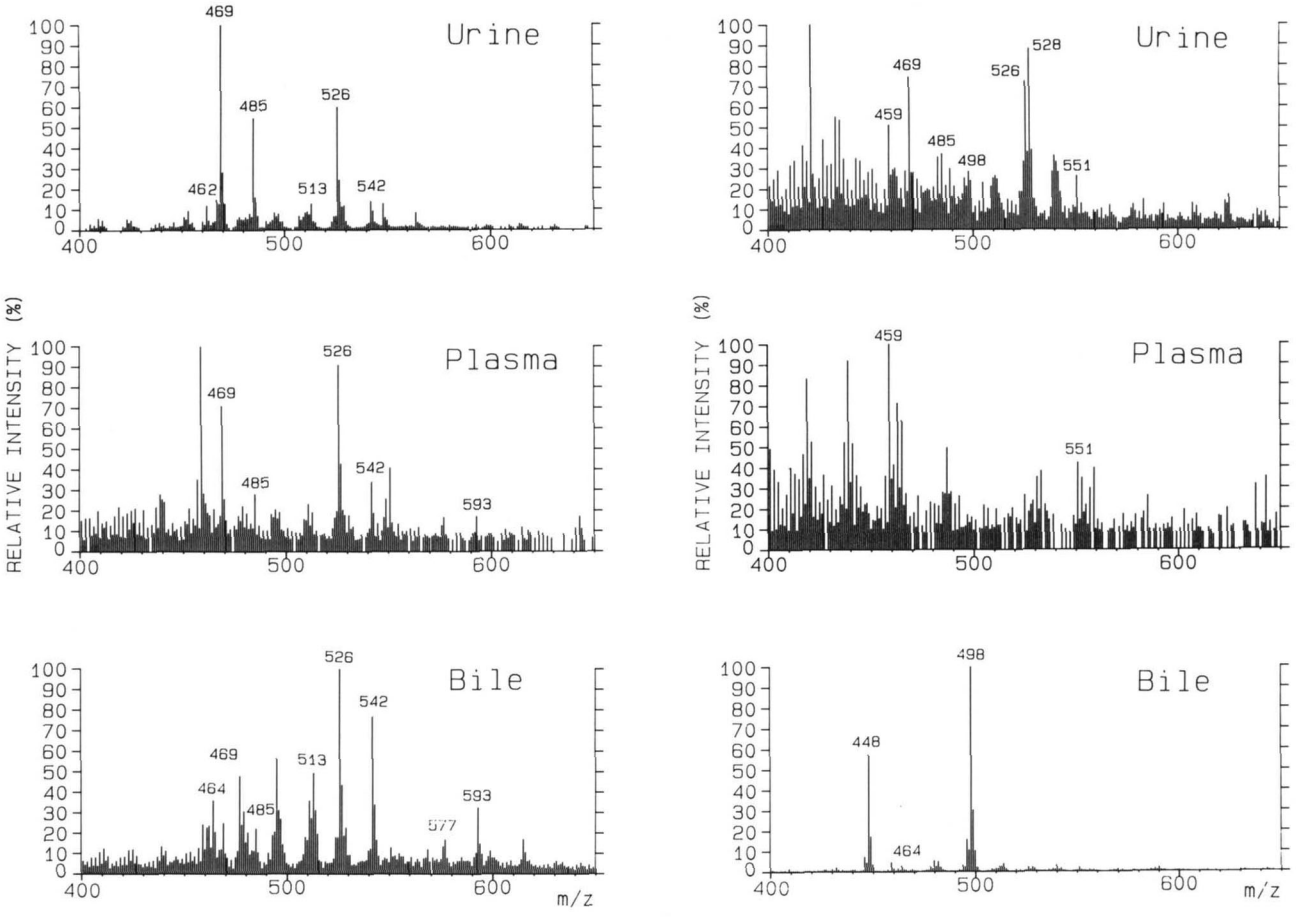

Fig. 1. The high mass regions of the negative ion FAB mass spectra of extracts of urine, plasma, and bile collected before (left) and during (right) treatment with chenodeoxycholic acid.

triol(s) and -tetrol(s), respectively. The peaks at *m/z* 495 and 511 are compatible with the presence of sulfated cholestenoic acids with one and two hydroxyl groups, respectively. Alternatively, these ions as well as *m/z* 477 and 479 could be due to conjugated steroid hormone metabolites. Peaks corresponding to quasimolecular ions of glucuronidated bile acids were not observed. The spectra of samples taken during treatment (Fig. 1, right) are discussed below.

FABMS analysis of the three fraction from TEAP-LH-20 (**Fig. 2**) provided further information. The glycine-conjugated bile acid fraction gave a major peak at *m/z* 464. A minor peak at *m/z* 448 corresponds to the quasimolecular ion of glycine-conjugated dihydroxycholanoic acid(s). Peaks at *m/z* 446 and 462 indicate glycine-conjugated di- and trihydroxycholenoic acids, respectively. Ions of unsaturated bile alcohol sulfates (*m/z* 497, 513, and 529) were abundant in the spectrum of the taurine-conjugated bile acid fraction which is expected to contain sulfated neutral steroids. The bile acid sulfate fraction gave the expected peaks at *m/z* 469, 485, 526, and 542. The presence of *m/z* 593 indicates that this represents a disulfated cholesteneterol rather than a glucuronidated cholestenetriol which would be eluted in the glycine or taurine conjugate fractions. The peaks at *m/z* 495 and 511 are probably due to cholestenoic acid sulfates while *m/z* 495, 497, and 511 in the spectrum of the taurine-conjugate fraction are likely to represent conjugated steroid hormone metabolites.

Analysis by GLC and GLC–MS. In order to simplify the fractionation of bile alcohol and bile acid conjugates, the bile extract was subjected to solvolysis prior to ion-exchange chromatography. The gas chromatographic analyses of compounds in the three fractions from TEAP-LH-20 are shown in **Fig. 3**. The compounds were characterized by their mass spectra and chromatographic behavior (retention index), and tentative identifications were made on biological grounds. In addition to 3β,7α-dihydroxy- and 3β,7α,12α-trihydroxy-5-cholenoic acids, nine bile alcohols and two C_{27} bile acids listed in **Table 1** were identified. With the exception of compounds 6 and 7, the common mass spectrometric finding was the presence of an intense peak at *m/z* (M–90) indicating the ease with which the allylic trimethylsiloxy group is lost (4, 15).

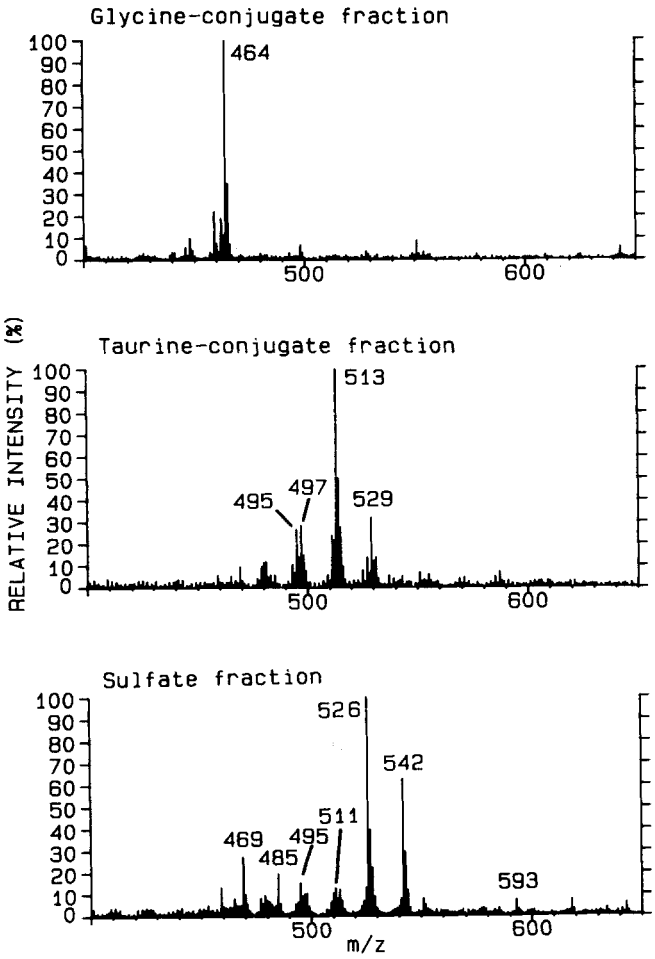

Fig. 2. The high mass regions of the negative ion FAB mass spectra of fractions obtained by chromatography on TEAP-LH-20 of an extract of bile collected before treatment with chenodeoxycholic acid.

The mass spectra of the TMS ether derivatives of compounds 1, 2, and 4 (**Fig. 4**) showed an intense peak at *m/z* 632 (M-90). Additional loss of trimethylsilanol yielded ions at *m/z* 542 and 452. The ions at *m/z* 341 and 251 resulting from the loss of side chain and successive loss of two and three trimethylsilanols indicated that these compounds were cholestenetetrols having three trimethylsiloxy groups in the steroid skeleton and one in the side chain. Minor peaks at *m/z* 129 and 209, but no significant peak at *m/z* 196 suggested a 3β-hydroxy-Δ^5 structure (4). In addition to these common fragment ions, diagnostic ions arising by side chain cleavages (16) were observed. The spectrum of the derivative of compound 1 gave a side chain fragment ion at *m/z* 145 composed of carbons 24 to 27, and a series of fragment ions at *m/z* 499, 409, and 319 derived from the scission of the bond between C-24 and C-25 (loss of mass 43) and successive loss of two to four molecules of trimethylsilanol. Based on these results the structure of compound 1 is suggested to be 5-cholestene-3β,7α,12α,24-tetrol. The derivative of compound 2 gave an intense side chain fragment ion at *m/z* 131 composed of carbons 25–27 and typical of the 25-trimethylsiloxy structure (16). The spectrum of compound 4 showed no side chain ions except for a minor ion at *m/z* 103 indicative of a trimethylsiloxy group at C-26 (16). These results, the molecular ions at *m/z* 722, and the retention time relationships suggest that the structures of compounds 2 and 4 are 5-cholestene-3β,7α,12α,25-tetrol and 5-cholestene-3β,7α,12α,26-tetrol, respectively.

The derivative of compound 3 gave a molecular ion at *m/z* 634, an intense base peak at *m/z* 544 (M-90), and a smaller peak at *m/z* 454. The peaks at *m/z* 253, 233, 208,

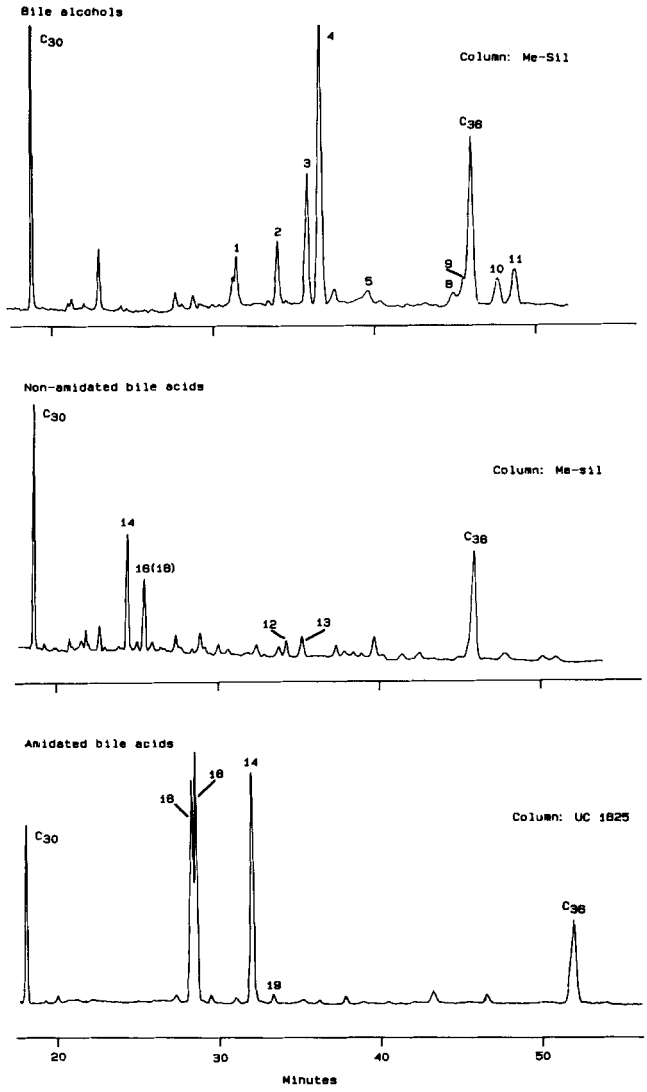

Fig. 3. Capillary GLC profiles of bile alcohols (TMS ether derivatives on a methyl silicone column) and nonamidated and amidated bile acids (methyl ester TMS ether derivatives on methyl silicone and UC 1625 columns, respectively) in bile collected before treatment with chenodeoxycholic acid. The bile extract was solvolyzed; the three fractions were isolated by chromatography on TEAP-LH-20; and the amino acid moieties were removed by enzyme hydrolysis. Numbers refer to the compounds listed in Table 1.

TABLE 1. Bile alcohols and bile acids in samples collected before treatment with chenodeoxycholic acod

No.	Compound[a]	Retention Index[b] Me-Sil	UC 1625
1.	5-Cholestene-3β,7α,12α,24-tetrol	3344	3205
2.	5-Cholestene-3β,7α,12α,25-tetrol	3396	3252
3.	5-Cholestene-3β,7α,26-triol	3432	3398
4.	5-Cholestene-3β,7α,12α,26-tetrol	3447	3322
5.	27-Nor-5-cholestene-3β,7α,12α,24,25-pentol	3499	3293
6.	Cholestenepentol	3548	3335
7.	Cholestenepentol	3566	3344
8.	5-Cholestene-3β,7α,12α,24,25-pentol	3582	3355
9.	5-Cholestene-3β,7α,12α,24,25-pentol	3593	3366
10.	5-Cholestene-3β,7α,25,26-tetrol	3630	3494
11.	5-Cholestene-3β,7α,12α,25,26-pentol	3644	3416
12.	3β,7α-Dihydroxy-5-cholestenoic acid	3411	3555
13.	3β,7α,12α-Trihydroxy-5-cholestenoic acid	3429	3468
14.	3β,7α-Dihydroxy-5-cholenoic acid	3181	3334
15.	3β-Hydroxy-5-cholenoic acid	3192	3475
16.	3β,7α,12α-Trihydroxy-5-cholenoic acid	3208	3260
17.	3,7,12-Trihydroxy-5-cholenoic acid[c]	3232	3289
18.	Cholic acid	3210	3271
19.	Chenodeoxycholic acid	3198	3364

[a]The identifications of bile alcohols are tentative (see text).
[b]Retention index of TMS ether or methyl ester TMS ether derivatives; Me-Sil, cross-linked methyl silicone column; UC 1625, silicone gum containing 25% 4-phenoxypropyl, 2% vinyl, and 73% methyl groups.
[c]Found only in feces.

and 129 that are observed in the mass spectrum of the methyl ester TMS ether of 3β,7α-dihydroxy-5-cholenoic acid (4) suggested a 3β,7α-bis-trimethylsiloxy-Δ^5 structure. A fragment ion at *m/z* 103 was also observed. The retention index of the derivative of compound 3 was 27 units lower than that of the TMS ether of 5-cholestene-3β, 26-diol and 315 units higher than that of the TMS ether of 5-cholestene-3β,7α-diol (**Table 2**). This can be compared with the shift of retention times upon introduction of TMS ether groups in reference compounds. Thus, the addition of a 7α-trimethylsiloxy group to the TMS ethers of cholesterol and methyl 3β-hydroxy-5-cholestenoate decreases the retention indices (ΔRI) by 15 and 25 units, respectively, on a methyl silicone column (10). The addition of a 26-trimethylsiloxy group to cholesterol TMS ether increases the RI by 325 units. On the basis of these results, compound 3 was tentatively identified as 5-cholestene-3β,7α,26-triol. This was later confirmed following synthesis of the authentic compound (J. Shoda, M. Axelson, and J. Sjövall, unpublished results).

The derivative of compound 5 gave a peak at *m/z* 706 (M-90) and a side chain fragment ion at *m/z* 117 composed of carbons 25–26 and a series of fragment ions at *m/z* 589, 499, 409, and 319 assumed to be derived from

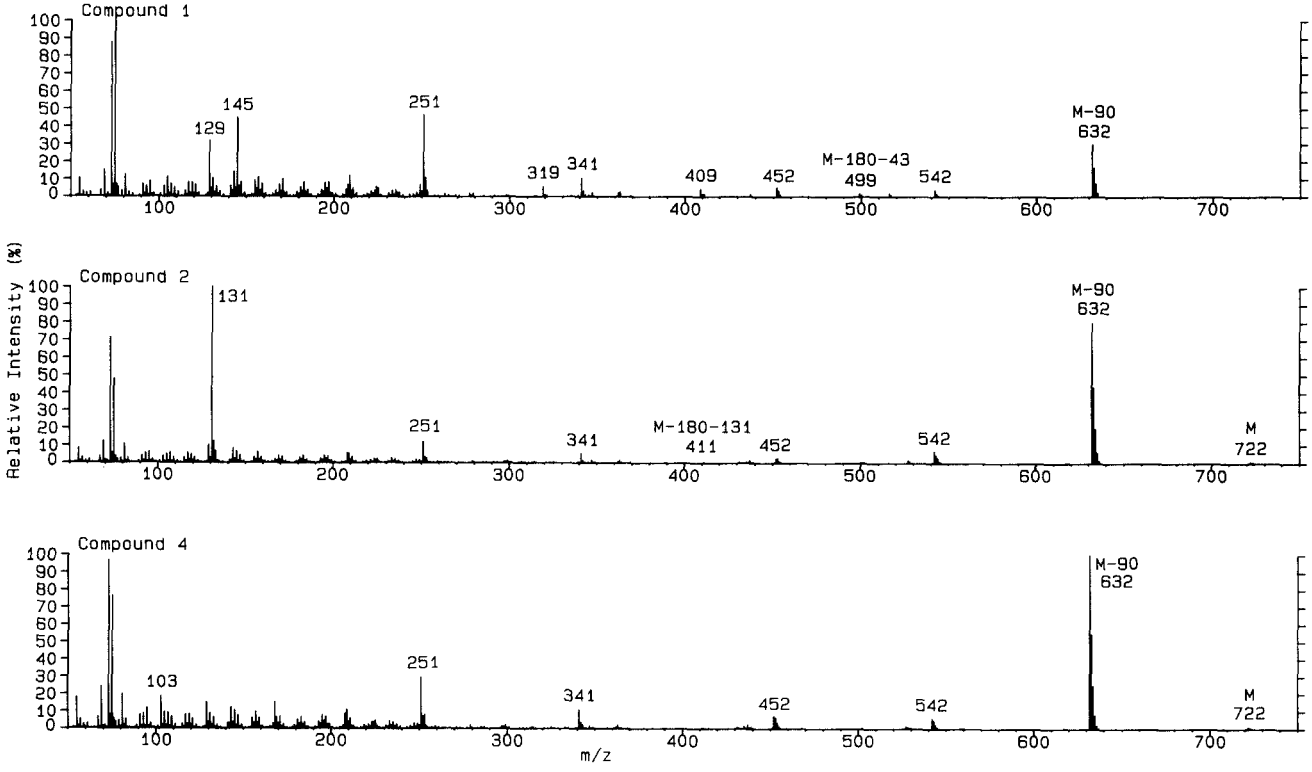

Fig. 4. Mass spectra of the TMS ether derivatives of compounds 1, 2, and 4 (Table 1) obtained in the GLC/MS analysis of bile alcohols in bile collected before treatment with chenodeoxycholic acid.

TABLE 2. Retention indices of reference compounds

No.	Compound[a]	Retention Index[a] Me-Sil	UC 1625
	5-Cholestene-3β-ol	3132	3254
	5-Cholestene-3β,7α-diol	3117	3124
	5-Cholestene-3β,7β-diol	3298	3272
	(23R)-5-Cholestene-3β,23-diol	3320	3361
	(23S)-5-Cholestene-3β,23-diol	3323	3362
	5-Cholestene-3β,25-diol	3399	3472
	5-Cholestene-3β,26-diol	3459	3557
	5β-Cholestane-3β-ol	3059	3137
	5β-Cholestane-3α,7α,12α-triol	3138	3073
	5β-Cholestane-3α,7α,12α,26-tetrol	3441	3324
	3α,7α-Dihydroxy-5β-cholestanoic acid	3427	3575
	3α,7α,12α-Trihydroxy-5β-cholestanoic acid	3431	3465
	3β-Hydroxy-5-cholenoic acid	3192	3475
	3β,7α-Dihydroxy-5-cholenoic acid	3182	3334
	3β,7α-Dihydroxy-4-cholenoic acid	3188	3359
	3β,7α,12α-Trihydroxy-4-cholenoic acid	3199	3284

[a]Retention index of TMS ether or methyl ester TMS ether derivatives; Me-Sil, cross-linked methyl silicone column; UC 1625, silicone gum containing 25% 4-phenoxypropyl, 2% vinyl, and 73% methyl groups.

the loss of a side chain fragment of mass 117 (cleavage of the bond between C-24 and C-25) and successive loss of one to four molecules of trimethylsilanol. A 3β,7α,12α-tris-trimethylsiloxy-Δ^5 structure was assumed by the presence of the minor ions at *m/z* 129, 209, 251, and 341. These findings and the analogies to the spectrum of the saturated 3α,5β(H) isomer (17) suggest that compound 5 is 27-nor-5-cholestene-3β,7α,12α,24,25-pentol.

The mass spectra of the derivatives of compounds 8 and 9 were very similar and typical of bile alcohols with a 24,25-bis-trimethylsiloxy structure (17). A very intense peak at *m/z* 131 was considered to be a side chain fragment ion formed by scission between C-24 and C-25, and a series of feeble fragment ions at *m/z* 720, 630, 540 to represent losses of trimethylsilanols from the molecular ion. Based on these results the structure 5-cholestene-3β,7α,12α,24,25-pentol is assumed.

The retention index of the derivative of compound 10 was 14 units lower than that of the derivative of compound 11. The mass spectrum of compound 10 showed a molecular ion at *m/z* 722, an intense peak at *m/z* 632 (M–90), and a series of fragment ions at *m/z* 619, 529, 439, and 349 resulting from loss of a side chain fragment of mass 103 and successive losses of one to three trimethylsilanols. Peaks were present at *m/z* 129, 253, and 343. A peak at *m/z* 219 was thought to be a side chain fragment ion composed of carbons 25–27 (16). These results suggest that compound 10 is 5-cholestene-3β,7α,25,26-tetrol.

Two series of fragment ions were observed in the spectrum of the derivative of compound 11. One series at *m/z* 720 (an intense peak), 630, and 540 was derived from successive loss of one to three molecules of trimethylsilanol. The other at *m/z* 707, 617, 527, 437, and 347 was thought to result from loss of a side chain fragment of mass 103 and successive loss of zero to four molecules of trimethyl silanol. It also gave a side chain fragment ion at *m/z* 219 and modest peaks at *m/z* 251 and 341 indicating that three trimethylsiloxy groups were lost from the ring system. Thus, compound 11 was tentatively identified as 5-cholestene-3β,7α,12α,25,26-pentol.

The retention index and mass spectrum of the derivative of compound 12 were identical to those of the methyl ester TMS ether of 3β,7α-dihydroxy-5-cholestenoic acid (10). The mass spectrum of the derivative of compound 13 showed a molecular ion at *m/z* 678, an intense peak at *m/z* 588 (M–90), and peaks due to further loss of trimethylsilanol. Peaks at *m/z* 129, 209, 251, and 341 suggested a 3β,7α,12α-tris-trimethylsiloxy-Δ^5 structure. Based on these findings it is assumed that compound 13 is 3β,7α,12α-trihydroxy-5-cholestenoic acid.

The retention indices and mass spectrum of the derivatives of compounds 14 and 16 were identical to those of the methyl ester TMS ethers of 3β,7α-dihydroxy-5-cholenoic and 3β,7α,12α-trihydroxy-5-cholenoic acids, respectively.

The derivative of compound 18 was eluted together with that of compound 16 on a methyl silicone column, but separated on a UC 1625 column (Fig. 3). The mass spectrum and RIs on both columns were identical to those of the methyl ester TMS ether of cholic acid. The RIs on both columns and the mass spectrum of the derivative of compound 19 were identical to those of methyl ester TMS ether of chenodeoxycholic acid. The RIs of the TMS ethers of all isomers of methyl 3,7,12-trihydroxy- and methyl 3,7-dihydroxy-5β-cholanoates (synthesized by Prof. F. C. Chang and obtained from the Bader Library of Rare Chemicals at the Aldrich Chemical Co. Inc., Milwaukee, WI) were determined on both columns (11) and the data for the bile acids in bile only fitted those for the 3α,7α- and 3α,7α,12α-isomers, respectively.

The total concentration of bile acids was 324 μg/ml duodenal bile (**Fig. 5**). The major constituents, 3β,7α-dihydroxy-5-cholenoic and 3β,7α,12α-trihydroxy-5-cholenoic acids, were present mainly as sulfated glycine conjugates. Contrary to expectation, a considerable fraction consisted of cholic acid, about 30% of the total bile acids, present mainly as a nonsulfated glycine conjugate. Chenodeoxycholic acid constituted less than 2% of the total. Cholestenoic acids, detected in the nonamidated bile acid fraction (after solvolysis), constituted 1.5% of the total bile acids.

The total concentration of sulfated bile alcohols was 66.5 μg/ml, corresponding to 21% of the bile acid concentration. Cholestenetetrols were predominant (about 60%) and 5-cholestene-3β,7α,12α,26-tetrol and 5-cholestene-3β,7α,26-triol constituted 45% and 19%, respectively of the total bile alcohols. No bile alcohols were detected in the neutral fraction. Saturated bile alcohols and the C_{27}

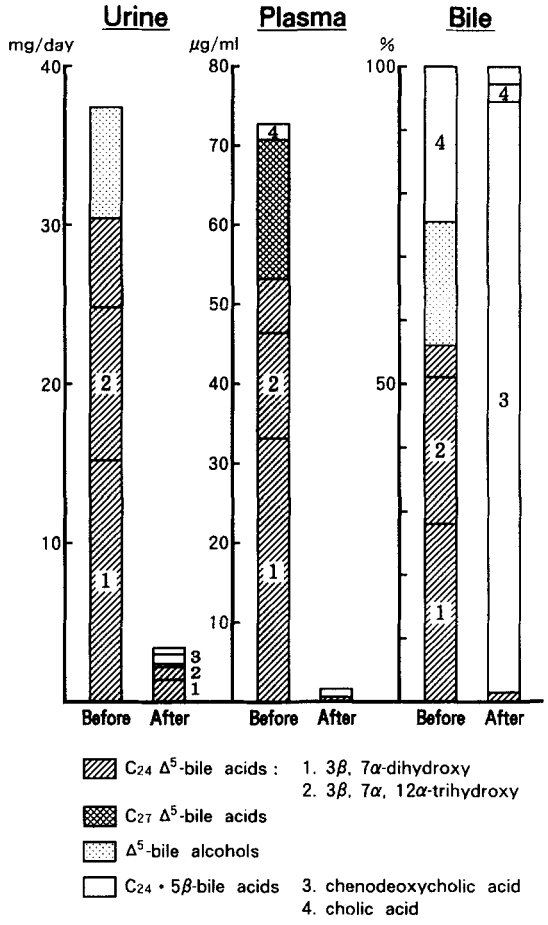

Fig. 5. Concentrations and composition of bile acids and bile alcohols determined by GLC analyses of urine, plasma and bile collected before and during (after) treatment with chenodeoxycholic acid.

bile acids present in normal human bile (18–20) were not detected.

Analysis of urine, plasma, and feces before treatment

Urine. Two samples of urine collected 3 months before and immediately prior to treatment with chenodeoxycholic acid were analyzed. The results were very similar and data are reported for the sample collected 3 months before treatment. The 3β,7α-dihydroxy-5-cholenoic acid was predominant, excreted mainly as a sulfated glycine conjugate. In contrast, about 60% of 3β,7α,12α-trihydroxy-5-cholenoic acid was excreted as a nonsulfated glycine conjugate. Trace amounts of cholic acid and 3β-hydroxy-5-cholenoic acid (compound 15) were detected, but chenodeoxycholic and cholestenoic acids were not found. Considerable amounts of bile alcohol sulfates were excreted in urine. 5-Cholestene-3β,7α,12α,26-tetrol was the major compound (26% of the total bile alcohols), whereas only traces of 5-cholestene-3β,7α,26-triol were detected. The relative proportion of cholestenepentols was higher (47% of the total) than in bile. Two additional cholestenepentols (compounds 6 and 7) were detected in urine. The mass spectra of their derivatives were similar and resembled those of the derivatives of compounds 8 and 9. Thus, the side chain fragment ion at *m/z* 131 was very intense and there was a series of ions at *m/z* 710, 620, and 530. However, the ion at *m/z* 710 (M–90) had a higher relative intensity and the compounds were eluted earlier than the derivatives of compounds 6 and 7. These compounds may be isomers of 5-cholestene-3β,7α,12α,23,25-pentol. Only trace amounts of bile alcohols were detected in the glucuronide fraction. The excretion rates of bile acids and bile alcohols in urine were 30.4 and 7.0 mg/day, respectively.

Plasma. The total concentration of bile acids in plasma was 71.2 μg/ml, the majority (65%) being present as sulfates. However, unconjugated 3β,7α-dihydroxy-5-cholestenoic acid was abundant (25% of the total). GLC–MS analysis suggested the presence of cholic acid in trace amounts. Bile alcohols were not analyzed by GLC–MS.

Feces. In feces, more than 95% of the bile acids were unconjugated. The predominant compound 17 constituted 64% of the total. The derivative gave a spectrum almost identical to that of the derivative of 3β,7α,12α-trihydroxy-5-cholenoic acid. However, the RI was slightly higher on both columns (Table 1). If one assumes, on the basis of the almost identical spectra, that compound 17 and 3β,7α,12α-trihydroxy-5-cholenoic acid are epimers, the RI relationships are compatible with a 3α,7α-dihydroxy-Δ^5 structure (cf. retention times of the TMS ethers of 7α-hydroxycholesterol and its 3α epimer (15)). The RI of 7β-hydroxycholesterol TMS ether is 91 units higher than that of the 7α-isomer, which is a larger difference than that observed for the two trihydroxycholenoic acids in feces. Smaller amounts of 3β,7α,12α-trihydroxy-5-cholenoic acid, cholic acid, and a dihydroxycholanoic acid (possibly deoxycholic acid) were detected, but 3β,7α-dihydroxy-5-cholenoic acid was not found. Compounds present in amounts too small for identification gave mass spectra indicative of unsaturated ketonic bile acids.

Bile alcohols were not found in the neutral fraction, neither before nor after solvolysis. The major compounds in this fraction were cholesterol (22.7 mg/day), sitosterol (11.3 mg), campesterol (2.7 mg), and stigmasterol (2.3 mg). The daily loss of bile acids in feces was calculated to be at least 11.3 mg. This is a minimum figure since nothing is known about the possible bacterial metabolism of steroids with 3,7-dihydroxy-Δ^5 structures to compounds not detected by the analytical method. The failure to find any of the biliary bile alcohols in feces may indicate such a loss. The excretion of cholic acid in feces was very small, about 0.5 mg/day.

Effects of chenodeoxycholic acid treatment

Clinical. The results of liver function tests before and after treatment are shown in **Fig. 6**. The patient had been stunted before treatment and showed obvious signs of rickets. Dark urine, itching, and abnormal liver function tests all indicated the presence of persistent cholestatis. His rickets improved to some extent biochemically, radiologically, and clinically after the supportive therapy with several vitamins. However, the liver function deteriorated as evaluated by ASAT and ALAT. The treatment with chenodeoxycholic acid was started at the age of 4 years 4 months. Two months later, the liver function tests were markedly improved. Total bilirubin and transaminase values decreased to normal levels. Clinically, he felt better with less itching and the urine returned to normal color. Based on these clinical data, the dose of chenodeoxycholic acid was reduced to 125 mg daily. He has been well and free from adverse effects until the present (7 years old).

Bile acids. A comparison of the FAB mass spectra given by samples collected before (Fig. 1, left) and during (Fig. 1, right) treatment showed that chenodeoxycholic acid induced striking changes. During treatment, the bile extract gave very intense peaks corresponding to quasimolecular ions of taurine-conjugated (*m/z* 498) and glycine conjugated (*m/z* 448) dihydroxycholanoic acid(s). Very small peaks were seen at *m/z* 526 and 542 indicative of 3β-hydroxy-Δ^5 bile acids, and at *m/z* 464 and 514 indicating conjugated trihydroxycholanoic acid(s). Although peaks corresponding to quasimolecular ions of 3β-hydroxy-Δ^5 bile acids were observed in analyses of urine during treatment, their intensities relative to that of the glycerol matrix (*m/z* 459 and 551) were much lower than before treatment. The peak corresponding to sulfated glyco-dihydroxycholanoic acid(s) (*m/z* 528) was predominant. The spectra of crude plasma extracts showed no peaks of bile acids during treatment.

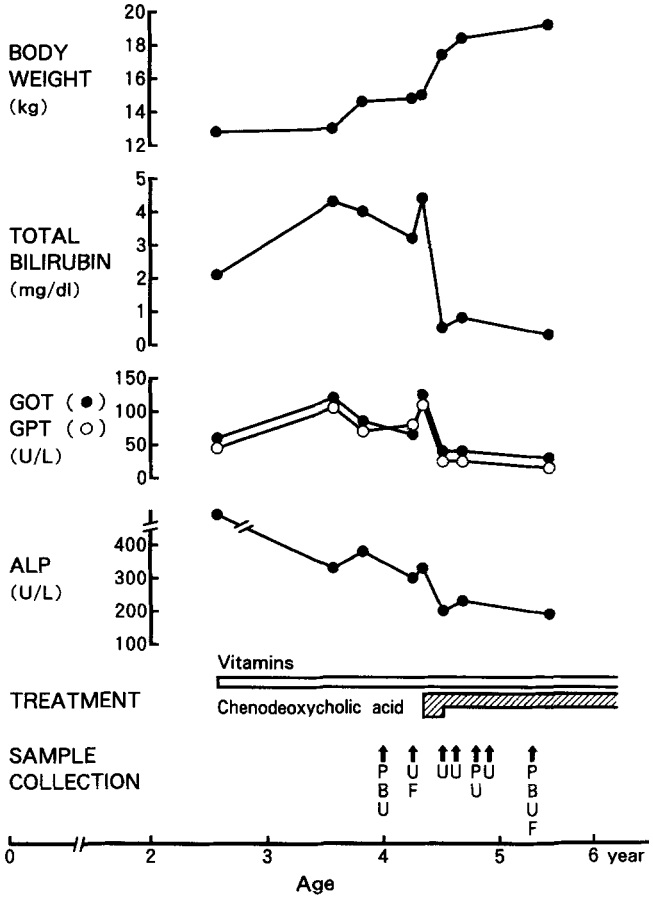

Fig. 6. Clinical and laboratory data and times of sampling of urine (U), plasma (P), bile (B), and feces (F) before and during treatment with chenodeoxycholic acid.

These findings were confirmed quantitatively by GLC and GLC–MS analyses (Fig. 5). The daily urinary excretion of bile acids was 3.4 mg/day, i.e., 9 times lower than before treatment. Chenodeoxycholic and ursodeoxycholic acids constituted 31% of the total. Bile alcohols were below the detection limit.

The total bile acid concentration in plasma was 1.55 μg/ml, i.e., normal, and 5β-cholanoic acids (chenodeoxycholic, ursodeoxycholic, and cholic acids) predominated. Thus, the levels of 3β-hydroxy-Δ^5 bile acids were several orders of magnitude lower than before treatment.

The excretion of bile acids in feces also showed striking changes (**Table 3**). Chenodeoxycholic and ursodeoxycholic acids predominated mainly in unconjugated form, constituting about 53% and 9%, respectively, of the total bile acids. Small amounts of 7-ketolithocholic acid (4%) and an isomer of chenodeoxycholic acid (2%) were also present. Contrary to expectation, the excretion of 3β-hydroxy-Δ^5 bile acids was higher than before treatment (40.0 vs. 11.3 mg/day). In contrast to the case before treatment, the unsaturated bile acids were excreted almost exclusively as sulfates.

Bile alcohols were below the detection limit in feces. The neutral fraction contained cholesterol and plant sterols, but, in contrast to the pretreatment sample, the sample collected after about 1 year of treatment contained 5β-reduced bacterial metabolites. The excretion of cholesterol and 5β-cholestane-3β-ol was about 126 and 72 mg/day, respectively, and that of total plant sterols about 109 mg/day.

DISCUSSION

The major pathway of bile acid biosynthesis from cholesterol is thought to begin with 7α-hydroxylation, a rate-limiting step, and continue with conversion of the 3β-hydroxy-Δ^5 to a 3α-hydroxy-5β(H) configuration via a 3-oxo-Δ^4 intermediate with or without 12α-hydroxylation

TABLE 3. Excretion of bile acids in feces before and during treatment with chenodeoxycholic acid (125 mg/day, orally)

Bile Acids	Before Treatment				During Treatment			
	U	GT	S	Total	U	GT	S	Total
Saturated bile acids[a]								
5βB-2α,7α-diol	nd	nd	nd	nd	63.9	2.6	0.9	67.8
5βB-3α,7β-diol	nd	nd	nd	nd	10.7	0.3	nd	11.0
5βB-3α-ol-7-one	nd	nd	nd	nd	4.4	0.3	nd	4.7
B-3,7-diol	nd	nd	nd	nd	2.1	0.1	0.5	2.7
5βB-3α-ol	nd	nd	nd	nd	0.2	0.1	0.2	0.5
5βB-3α,7α,12α-triol	0.3	0.1	0.1	0.5	2.1	0.4	nd	2.5
Others	0.2	nd	nd	0.2	nd	nd	nd	nd
Total saturated	0.5	0.1	0.1	0.7	83.4	3.8	1.6	88.8
Unsaturated bile acids[a]								
B^5-3B-ol	nd	nd	nd	nd	nd	nd	3.5	3.5
B^5-3β,7α-diol	nd	nd	nd	nd	nd	0.2	17.0	17.2
B^5-3β,7α,12α-triol	0.6	0.1	0.2	0.9	0.8	0.2	10.5	11.5
B^5-3,7,12-triol	7.3	0.1	nd	7.4	nd	nd	nd	nd
Others	2.2	nd	nd	2.2	nd	nd	7.8	7.8
Total unsaturated	10.1	0.2	0.2	10.5	0.8	0.4	38.8	40.0
Total bile acids	10.6	0.3	0.3	11.2	84.2	4.2	40.4	128.8

U, GT, and S refer to unconjugated, glycine-taurine-conjugated, and sulfated bile acids, respectively; nd, not detected.

[a] B, cholanic acid; Greek letters denote configuration of hydroxyl groups; superscript indicates position of double bond.

(1). Alternative pathways have been demonstrated in which side-chain shortening occurs before the nuclear modifications are completed, giving rise to 3β-hydroxy-Δ^5-C_{24} bile acid intermediates (21–24). The finding in our patient of 3β-hydroxy-Δ^5 bile acids with absence of cholic and chenodeoxycholic acids led to the hypothesis that 3β-hydroxy-Δ^5 steroid dehydrogenase was deficient in this child (4). The hypothesis predicts that 3β,7α-dihydroxy-5-cholenoic acid is produced from 7α-hydroxycholesterol via 5-cholestene-3β,7α,26-triol and 3β,7α-dihydroxy-5-cholestenoic acid (Yamasaki pathway (24)). Since intermediates with a shortened side chain do not serve as substrates for 12α-hydroxylase (1) the formation of 3β,7α,12α-trihydroxy-5-cholenoic acid is assumed to proceed mainly via 5-cholestene-3β,7α,12α,26-tetrol and 3β,7α,12α-trihydroxy-5-cholestenoic acid. Depending on the activity of the microsomal pathway, 5-cholestene-3β,7α,12α,25-tetrol and 5-cholestene-3β,7α,12α,24,25-pentol are alternative intermediates. All of these postulated intermediates have been found in the present study, lending strong support to the hypothesis that the patient has a deficiency of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase. Our alternative mechanism for the defective bile acid synthesis was that sulfation of the 3β-hydroxy group protected it from oxidation. The presence of the metabolites identified could then only be explained by hydroxylation and side-chain oxidation of sulfated intermediates, which seems rather unlikely especially in view of the high proportion of nonsulfated trihydroxy-cholenoic acid in urine. Direct evidence that the patient has a deficiency of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase was recently obtained by measurements of enzyme activities in fibroblast cultures using 7α-hydroxycholesterol as substrate (25). In contrast to cells from healthy subjects, cells from MU2 showed no enzyme activity.

The relatively high excretion of bile alcohols, both in bile and urine, suggests a rate limitation in the side-chain oxidation. This could be due either to a lower affinity of the enzymes catalyzing 26-hydroxylation and β-oxidation for the 3β-hydroxy-Δ^5 substrates or to a rapid sulfation of these steroids making them unavailable for further metabolism. Bile acid biosynthesis is compartmentalized: 7α-hydroxylation, 3β-hydroxy-Δ^5 oxidation, and 12α-hydroxylation take place in the endoplasmic reticulum, while 26-hydroxylation is mitochondrial and β-oxidation at least partly peroxisomal (1). Sulfotransferases, active on 3β-hydroxy-Δ^5-C_{19} and -C_{21}-steroids, are cytosolic (26), so that sulfation of intermediates transported between the above organelles is a likely event. The composition of the sulfated bile alcohols in bile is quite compatible with such a process. Rapid sulfation could also explain why the bile alcohols do not appear in the urine as glucuronides, as is

the case for the saturated bile alcohols in urine in healthy infants and patients with liver disease (14, 27). The presence of 27-nor-5-cholestene-3β,7α,12α,24,25-pentol as a major component in the urine suggests that the removal of the terminal carbon atom occurred to the same extent as in healthy infants (14, 17).

Cholic acid constituted about 30% of the bile acids in bile. The daily loss of cholic acid was very much smaller than that of 3β-hydroxy-Δ^5 bile acids, suggesting that only small amounts of cholic acid were formed and effectively retained in the enterohepatic circulation. This may have played an important role for the maintenance of bile secretion and for the survival of the child. The mechanism of formation of the cholic acid is not known. The lack of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase activity in fibroblasts does not exclude the possibility of an incomplete enzyme defect. This could be analogous to the formation of saturated steroid hormone metabolites in infants with the incomplete form of 3β-hydroxysteroid dehydrogenase deficiency affecting steroid hormone biosynthesis (28). The liver contains at least two different 3β-hydroxy-Δ^5 steroid dehydrogenases (29) with different specificities. While the enzyme involved in the biosynthesis of steroid hormones is different from that used in bile acid biosynthesis (29, 30), its activity towards most of the bile alcohols formed by MU2 is not known. The presence in MU2 of the enzyme required for steroid hormone production can be inferred from the absence of an endocrinological disorder (4).

Alternatively, cholic acid might be formed by transformation of 3β,7α,12α-trihydroxy-5-cholenoic acid during an enterohepatic circulation. Potential intermediates were not detected in feces although traces of unidentified unsaturated ketonic bile acids were present. Furthermore, the analogous conversion of cholesterol to 5β-cholestan-3β-ol was not observed in the sample analyzed. Regardless of the mechanism of formation, it is notable that chenodeoxycholic acid was only a trace component in bile, whereas the dihydroxycholenoic acid was more abundant than the trihydroxycholenoic acid in all samples analyzed.

A comparison of results obtained in the analysis of urine samples collected at the ages of 3 months (4) and about 4 years reveals an increased proportion of the dihydroxycholenoic acid at 4 years. This may be analogous to the normal increase of the chenodeoxycholic acid/cholic acid ratio with age (31). Alternatively, it may reflect progressive liver damage (32). Cholic acid was not detected in plasma or urine at 3 months but was present at 4 years. While this may reflect methodological improvements, it cannot be excluded that enzymes capable of converting 3β,7α,12α-trihydroxycholenoic acid into cholic acid have developed in the intestinal microflora or in host tissues. A decreasing rate of sulfation making more unsaturated bile acid available for oxidation is another possibility, which is compatible with the finding of an increased proportion of the nonsulfated glycine conjugate of the trihydroxycholenoic acid in urine at 4 years of age.

While 3β-hydroxy-5-cholenoic acid produces cholestasis (33) this effect has not been observed with 3β,7α-dihydroxy-5-cholenoic acid (34). However, in the presence of 3β-hydroxysteroid dehydrogenase, the latter acid is converted to chenodeoxycholic acid (34). This enzyme is lacking in MU2 where one or both of the Δ^5 bile acids or their sulfates may induce cholestasis or fail to produce bile acid-dependent bile flow. The treatment with chenodeoxycholic acid was attempted in order to suppress cholesterol 7α-hydroxylase and hence the formation of toxic 3β-hydroxy-Δ^5 bile acids, to stimulate the bile secretion and hence the elimination of toxic substances, and to improve the absorption of fats and fat soluble vitamins. This treatment markedly improved the clinical status and normalized the liver function tests (6). The levels of bile acids in plasma were drastically reduced, as was the bile acid excretion in urine. The unsaturated bile acids in bile were replaced by chenodeoxycholic acid and the bile alcohols disappeared. The beneficial effects of oral chenodeoxycholic acid were recently confirmed in a newly diagnosed child with 3β-hydroxy-Δ^5-C_{27}-steroid deficiency (35). Our results are compatible both with a reduced formation and an increased biliary elimination of the abnormal endogenous bile acids. The data do not permit a definitive conclusion regarding the extent of inhibition of the endogenous synthesis. The absence of detectable amounts of sulfated bile alcohols in feces during the treatment might indicate an inhibition of the 7α-hydroxylation of cholesterol. However, the daily fecal excretion of 3β-hydroxy-Δ^5 bile acids during treatment was about 40 mg or 2.3 mg/kg body weight. This is in the range of the normal bile acid production in healthy children of this age (36). The urinary excretion of 3β-hydroxy-Δ^5 bile acids and bile alcohols before treatment was also about 40 mg/day while the fecal excretion was only about 11 mg/day. The latter value is a minimum figure since the major bile acid was an unconjugated isomer of 3β,7α,12α-trihydroxy-5-cholenoic acid, indicating that the biliary bile acids had undergone bacterial modifications. Thus, it is possible that unknown metabolites have escaped detection in the analytical procedure.

During treatment with chenodeoxycholic acid, the unsaturated bile acids in feces were sulfated and had the same structure as in bile. This indicates absence of bacterial sulfatase(s) present before treatment. This is likely to promote the elimination of the endogenous bile acids since the strongly acidic sulfates are poorly reabsorbed both by the active and passive reabsorption processes (37). The change of intestinal microflora, possibly induced by the treatment with chenodeoxycholic acid, is also evidence from the formation of 5β-reduced metabol-

ites of cholesterol and plant sterols which were absent from feces before treatment.

In summary, the results show that treatment with chenodeoxycholic acid is very effective in improving the clinical condition and the liver function. The mechanism may be twofold: inhibition of cholesterol 7α-hydroxylase and increase of the bile acid-dependent secretion of bile with elimination of the endogenous, possibly hepatotoxic, Δ^5 bile acids. A decrease of the bacterial sulfatase activity may also be important in this child. The relative importance of inhibition of 7α-hydroxylase and stimulation of bile secretion could possibly be evaluated by treatment with ursodeoxycholic acid which does not inhibit the 7α-hydroxylase. Fast atom bombardment mass spectrometry of plasma and urine samples is a simple and effective method to monitor and evaluate the effects of such treatment.

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