Ketonic bile acids in urine of infants during the neonatal period

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Abstract Ketonic bile acids have been found to be quantitatively important in urine of healthy infants during the neonatal period. In order to determine their structures, the bile acids in urine from 11 healthy infants were analyzed by gas-liquid chromatography-mass spectrometry (GLC-MS) and three samples with particularly high levels of ketonic bile acids were selected for detailed studies by ion exchange chromatography, fast atom bombardment mass spectrometry, microchemical reactions, and GLC-MS. The major ketonic bile acid was identified as 7α,12α-dihydroxy-3-oxo-5β-chol-1-enoic acid, not previously described as a naturally occurring bile acid. The positional isomer 7α,12α-dihydroxy-3-oxo-4-cholenoic acid, recently described as a major urinary bile acid in infants with severe liver diseases, was also excreted by most infants. Three acids related to cholic acid were identified: 7α,12α-dihydroxy-3-oxo-, 3α,12α-dihydroxy-7-oxo-, and 3α,7α-dihydroxy-12-oxo-5β-cholanoic acids. Five bile acids having one oxo and three hydroxy groups were also present. Based on mass spectra and biological considerations two of these were tentatively given the structures 1β,7α,12α-trihydroxy-3-oxo- and 1β,3α,12α-trihydroxy-7-oxo-5β-cholanoic acids. Some of the others had a hydroxy group at C-4 or C-2. The levels of ketonic bile acids were higher on the third than on the first day of life, and lower after 1 month. The formation and excretion expecially of 3-oxo bile acids is proposed to result from changes of the redox state in the liver in connection with birth. —**Wahlén, E., B. Egestad, B. Strandvik, and J. Sjövall.** Ketonic bile acids in urine of infants during the neonatal period. *J. Lipid Res.* 1989. **30:** 1847–1857.

Supplementary key words 7α,12α-dihydroxy-3-oxo-5β-chol-1-enoic acid • 7α,12α-dihydroxy-3-oxo-4-cholenoic acid • human • fast atom bombardment mass spectrometry • gas-liquid chromatography-mass spectrometry

Ketonic bile acids are usually considered to arise by bacterial oxidation of primary and secondary bile acids (1–4). Thus, they are important constituents in human feces (5), but only small amounts are normally found in bile (3, 6, 7), blood (8) and urine (9), partly due to the ability of the liver to reduce oxo groups during the enterohepatic circulation (10–13). In the course of a study of the excretion of bile acids in urine of newborn infants (14), mass spectrometric evidence was obtained for the presence of a number of ketonic bile acids. Their rate of excretion seemed to vary with age. The purpose of this study was to identify these compounds.

MATERIALS

Four healthy male premature (32–34 gestational weeks) and seven healthy male fullterm (39–40 gestational weeks) infants were studied during the first month of life. Clinical data of the infants are given in **Table 1**. All deliveries and pregnancies were normal except for the mother of two premature twins who had a slight cholestasis of pregnancy. No infant had hypoxia. Cases 1–5 and 8–11 were breastfed during the study period, cases 6 and 7 were formula-fed after the third day of life. Except for physiological jaundice, requiring brief phototherapy for some premature infants, no medical problem was noted during the neonatal period. Urine was collected in plastic bags, starting immediately after birth (day 1) and continuing for 24-h periods at days 3–4, 6–8 (prematures), and 30–35. The samples were immediately frozen at – 20°C until analyzed. Informed consent was given by the parents and the study was approved by the Ethical Committee of the Karolinska Institutet.

METHODS

For identification purposes, three urine samples with recognized high levels of ketonic bile acids (cases 6, 7, and 10 at 3–4 days of age) were analyzed in a gentle way to avoid production of artifacts. Extractions and separations were monitored by fast atom bombardment mass spectrometry (FABMS) and final analysis was made by capillary column gas-liquid chromatography (GLC) and capillary gas-liquid chromatography-mass spectrometry (GLC-MS).

Abbreviations: GLC, gas-liquid chromatography; MS, mass spectrometry; FABMS, fast atom bombardment mass spectrometry; TMS, trimethylsilyl. The terms diolone and triolone are used for bile acids containing one oxo group and two and three hydroxyl groups, respectively.

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TABLE 1. Clinical data and approximate urinary excretion of ketonic bile acids in infants

Case	Gestational Age	Birth Weight	Birth Length	Bile Acid Excretion			
				Day 1	Days 3-4	Days 6-8	Day 30
	wk	*g*	*cm*	*μmol/24 h*			
1	40	3140	48	0.12			n.d.[a]
2	40	3960	51	0.03	0.61		n.d.
3	39	3345	51	0.06	0.11		n.d.
4	39	4150	54	0.19	0.52		n.d.
5	39	3460	50	1.25	6.50		1.23
6	39	4430	54	0.14	1.23		n.d.
7	39	2960	50		2.06		0.02
8	34	2730	48			0.35	n.d.
9	34(twin)	1925	43	0.15	0.27	n.d.	0.08
10	34(twin)	2340	45	0.22	0.74	0.07	n.d.
11	32	1850	43	0.23	0.30	0.19	0.23

[a]Not detected.

Chemicals

Solvents were of analytical grade (Merck, Darmstadt, FRG) and redistilled in an all-glass apparatus. Water was deionized before redistillation. All reagents were of analytical grade or better. Glassware was cleaned in an ultrasonic bath. Reference compounds were those used in previous studies (9, 15).

Extraction and fractionation of urinary bile acids

Twenty-five ml of the 24-h urinary sample was passsed through a 20 × 4 mm bed of Sepralyte (octadecylsilane-bonded silica, Analytichem International, Inc., Harbor City, CA). After a wash with 5 ml of water and 5 ml of 0.01 M HCl, the bile acids were eluted with 10 ml of methanol. Bile acids in the methanol eluate were then fractionated on a lipophilic ion exchanger, Lipidex-DEAP (Packard Instrument Co., Downers Grove, IL) (9). The unconjugated and glycine- and taurine-conjugated bile acid fractions were further analyzed. After enzyme hydrolysis of the two latter fractions with cholylglycine hydrolase (16), bile acids were rechromatographed on Lipidex-DEAP. Sepralyte was used to extract bile acids from aqueous solutions unless otherwise stated.

Microchemical reactions

Methyl esters were prepared with fresh diazomethane which was added in diethyl ether to samples dissolved in 1 ml methanol-diethyl ether 1:9 (v/v), and left at 4°C for 15 min. Trimethylsilyl (TMS) ethers were prepared by heating at 60°C for 30 min in pyridine-hexamethyldisilazane-trimethylchlorosilane 3:2:1 (by vol). Samples were taken to dryness under a stream of nitrogen and immediately dissolved in hexane. Oximes were prepared by addition of 5 mg of hydroxylammonium chloride and 50 μl of pyridine to the dried sample and heating at 60°C for 30 min (17). The pyridine was removed under a stream of nitrogen, 1 ml of water was added, and the products were extracted with 3 × 1 ml of ethyl acetate. The combined extracts were washed with 0.5 ml of water. Oximes of conjugated bile acids were extracted with Sepralyte. Oxo groups were reduced with 1 mg of sodium borohydride added to the bile acid samples dissolved in 1 ml of ethanol. The reaction was terminated with 0.01 M hydrochloric acid after 60 min and bile acids were extracted with 3 × 1 ml of ethyl acetate. The combined extracts were washed with 0.5 ml of water. Double bonds were hydrogenated by treating the bile acid methyl ester for 30 min with hydrogen/platinum(IV) oxide in 1 ml of acetic acid. The reaction was terminated by addition of 5 ml of water and the product was extracted with Sepralyte.

Fast atom bombardment mass spectrometry (FABMS)

FABMS was performed on a VG 7070E double focusing mass spectrometer equipped with a fast atom bombardment ion source, an Ion Tech atom gun, and a DS 2350 data system (VG Analytical, Manchester, UK). The sample, in 4-10 μl 70% aqueous methanol (corresponding to 0.2-0.5 ml of urine), was applied under a gentle stream of nitrogen to the FAB target coated with the glycerol matrix. Xenon (8 keV) was used as bombarding atoms. Spectra of negative ions were recorded between *m/z* 750 and 100 at a scan rate of 20 s × decade^{-1}. The accelerating voltage was 5 kV and the resolution about 1000.

Gas-liquid chromatography (GLC) and gas-liquid chromatography-mass spectrometry (GLC-MS)

GLC was performed on a Carlo Erba HRGC 4160 gas chromatograph (Farmitalia Carlo Erba, Milano, Italy)

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connected to an SP 4270 integrator (Spectra-Physics, Darmstadt, FRG). The column was a 25 m × 0.32 mm i.d. fused silica capillary coated with cross-linked methyl silicone (0.25 μm film thickness; Quadrex Corp., New Haven, CT). Helium was used as a carrier gas at 50–100 kPa. Samples were injected on-column in 0.5–1.2 μl hexane at 60°C and the temperature was then taken directly to 200°C and after 5 min to 290°C at 30°C × min^{-1}. Retention indices were calculated from the retention times of even-numbered C_{28}–C_{38} n-alkanes. GLC-MS was carried out on the VG 7070E mass spectrometer with the EI/CI ion source and a Dani 3800 gas chromatograph. The same type of capillary column was used as in the GLC analysis. It was kept at 280°C, directly connected and ending in the ion source. All all-glass falling needle injection system was used. The ionization energy was 22.5 eV and the trap current was 200 μA. Spectra were recorded by repetitive magnetic scanning between *m/z* 750 and 20 at a scan rate of 2 s × $decade^{-1}$. The accelerating voltage was 6 kV and the resolution was about 1000. Retention times were calculated relative to that of the TMS ether of methyl cholate. methyl cholate.

Measurement of the rate of excretion

Semiquantitative estimates of the excretion of ketonic bile acids were obtained using the method described previously (14). Amounts of bile acids were calculated from peak areas obtained in GLC analyses on a packed column with Hi-Eff 8 BP as stationary phase. No correction was made for possible differences in response between ketonic and nonketonic bile acids. The identities of the peaks and their correspondence with the peaks obtained on the methyl silicone capillary column were established by GLC-MS.

Statistics

Spearman's rank correlation coefficient was estimated to detect a possible association between two variables.

RESULTS

FABMS analyses

FABMS of the three urine extracts studied in detail (cases 6, 7 and 10 at 3–4 days of age) produced negative ion spectra indicating the presence of saturated and unsaturated ketonic bile acids (**Fig. 1**). The peaks correspond to quasimolecular ions, $[M-1]^-$, of taurine conjugated bile acids with three (*m/z* 514) and four (*m/z* 530) hydroxy groups, and corresponding compounds with one (*m/z* 512 and 528) and two (*m/z* 510 and 526) double bonds. The latter compounds were assumed to contain an oxo group, a carbon–carbon double bond, or both. FABMS alone will not discriminate between these possibilities since the elemental composition is the same. Corresponding ions of glycine conjugates are found at *m/z* 464, 480, 462, 478, 460, and 476 and of unconjugated bile acids at *m/z* 407, 423, 405, 421, 403, and 419. The latter peaks were hardly recognizable before fractionation into conjugate groups due to their low response under FAB conditions. The peak at *m/z* 544 in the sample from case 7 corresponds to the quasimolecular ion of a sulfated and glycine-conjugated trihydroxy bile acid. The peaks at *m/z* 383, 399, 411, 427, 429, and 443 present in the spectra of all samples correspond to quasimolecular ions of sulfated androstene and pregnene derivatives (18). The FAB spectra showed large differences between the samples, but in general there was a predominance of taurine-conjugated bile acids. The absence or presence of only small amounts of sulfated bile acids is notable and confirms the results of previous GLC

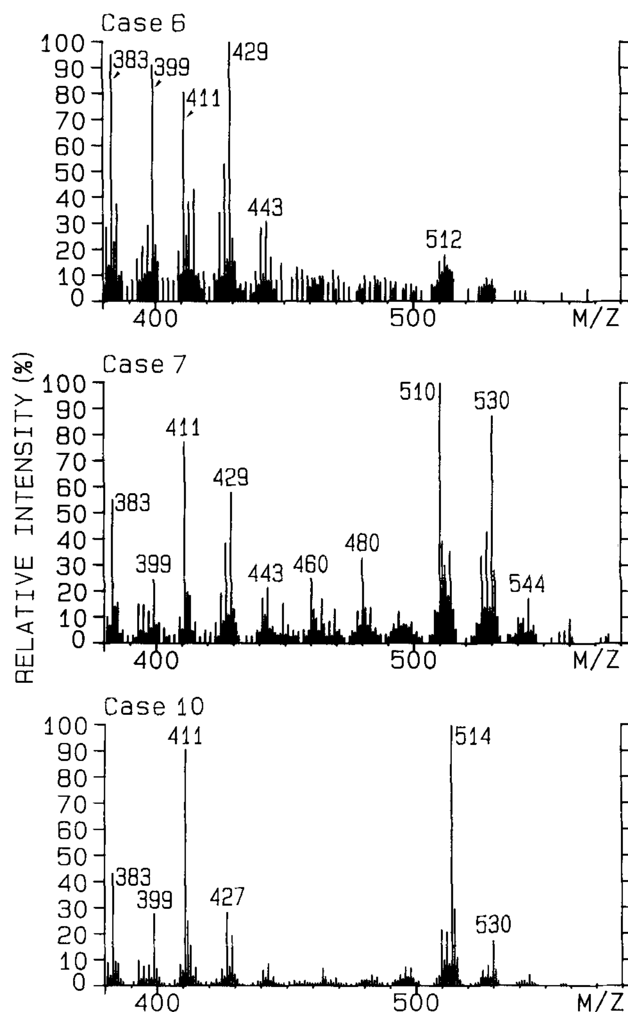

Fig. 1. The high mass regions of the negative ion FAB mass spectra of extracts of urine collected from three infants during their third day of life. The samples, corresponding to 400 μl urine, were applied in 8 μl 70% aqueous methanol under a stream of nitrogen to the FAB target already covered with the glycerol matrix.

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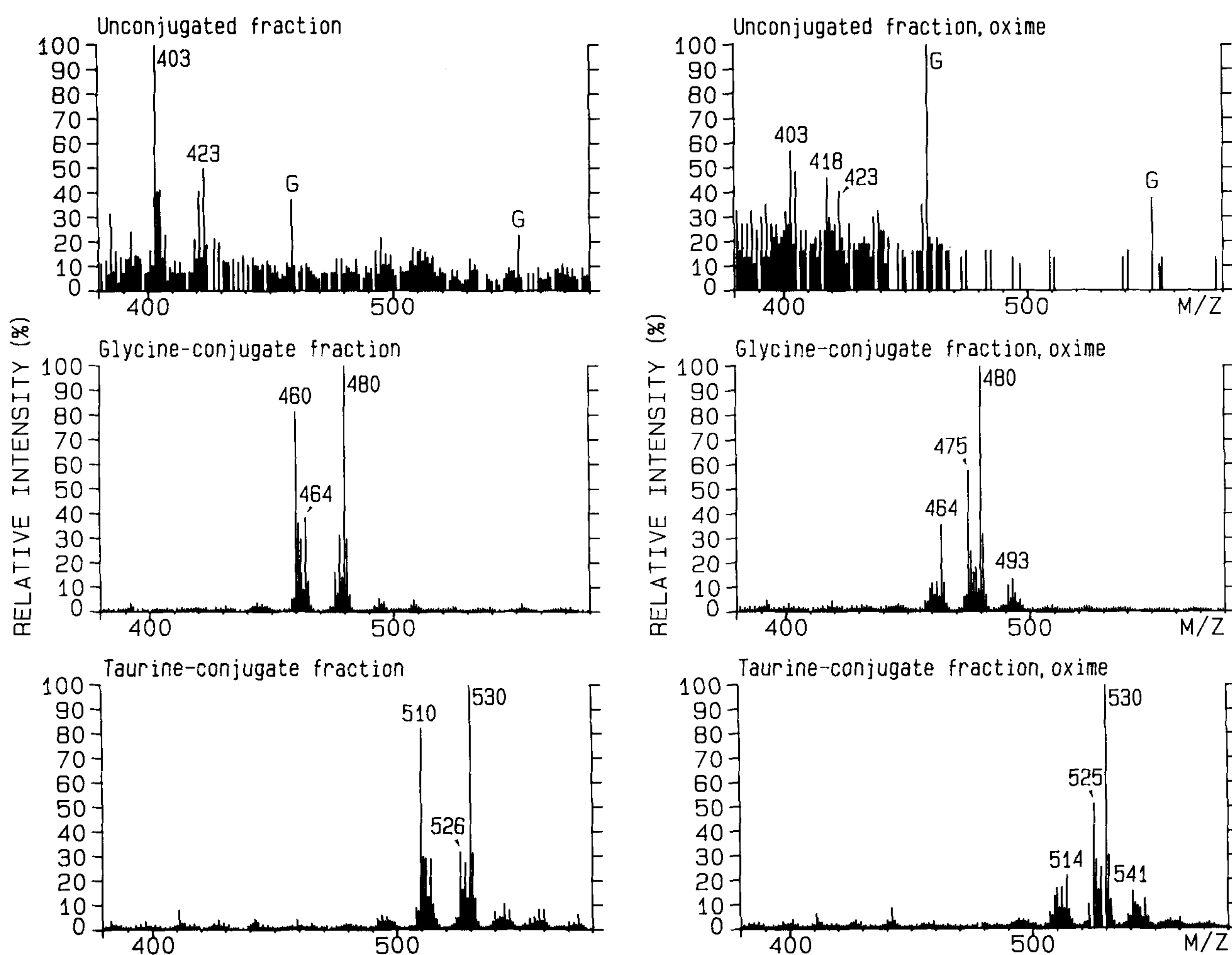

Fig. 2. The high mass regions of the negative ion FAB mass spectra of fractions obtained by chromatography of urine from case 7 on Lipidex-DEAP, before and after reaction with hydroxylammonium chloride. G denotes peaks derived from the glycerol matrix.

analyses of separated and hydrolyzed groups of conjugates (14). Since the yield of quasimolecular ions differs greatly with the structure of the bile acid, quantitative estimates cannot normally be obtained from the FAB spectra.

FABMS analysis of the fractions from Lipidex-DEAP before and after reaction with hydroxylamine proved the presence of ketonic bile acids. The high mass regions of the negative ion FAB mass spectra from the fractions of the sample from case 7H are shown in **Fig. 2**. The unconjugated bile acid fraction gave a predominant peak at *m/z* 403 corresponding to an unsaturated diolone which was converted to an oxime giving a quasimolecular ion at *m/z* 418. The glycine-conjugate fraction gave a corresponding peak of an unsaturated diolone at *m/z* 460 and small peaks for an unsaturated triolone at *m/z* 476 and a saturated triolone at *m/z* 478. These peaks were all shifted by 15 Da after treatment of the sample with hydroxylamine. The taurine conjugate fraction contained unsaturated diolones and triolones giving peaks at *m/z* 510 and *m/z* 526, respectively, shifting by 15 Da after formation of oximes.

Identification of ketonic bile acids

The three fractions of urinary bile acids from each of the three infants were analyzed by GLC before and after reduction or formation of oximes. The relative amounts of compounds in the complex chromatograms varied, but in six out of the nine chromatograms a ketonic bile acid constituted the major peak. Eleven distinct ketonic bile acids were detected. Chromatographic and mass spectrometric data on these are given in **Table 2**. In four of the nine chromatograms the major peak was due to compound I (glycine and taurine conjugates from cases 6 and 7) in one chromatogram to compound II (case 7, unconjugated fraction), and in one chromatogram to compound IV (case 6, unconjugated fraction). Identification of the corresponding oxime was easy when the ketonic bile acid constituted the major compound in the chromatogram, otherwise it was difficult because of the complexity of the chromatograms. The capillary GLC profiles of the methyl ester TMS ethers of the unconjugated bile acids from

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TABLE 2. Chromatographic and mass spectrometric data for ketonic bile acids in urine from newborn infants

	Relative retention time[a]			Retention Index			
	Hi-Eff 8 BP[d]	Methyl Silicone[e]		Methyl Silicone[f]		Characteristic Ions[b]	Suggested Structure[c]
Compound	TMS	TMS	Oxime-TMS	TMS	Oxime-TMS		
I	4.88–5.36	1.23–1.25	1.23–1.24	3309–3323	3336–3345	562,547,472,382,367,357,267,261	B^{1}-7α,12α-diol-3-one
II	6.12–6.55	1.34–1.35	1.27	3349–3364	3336	562,547,472,382,357,267,224	B^{4}-7α,12α-diol-3-one
III	4.42–4.75	1.23–1.25	1.23–1.24	3319–3323	3338	564,549,474,384,269,261,242	B-7α,12α-diol-3-one
IV	5.79–5.98	1.35–1.37	1.10–1.11	3378–3386	3279–3283	564,549,474,456,384,366,359,341, 269,251,175	B-3α,12α-diol-7-one
V	–	1.11–1.12	–	–	–	549,384,269,243,217	B-diolone
VI	–	1.43	–	–	–	564,549,492,474,409,384,366,319, 269,251,243,229,154,121	B-3α,7α-diol-12-one
VII	–	1.38	–	–	–	637,562,547,472,382,357,313,267,261	B-1β,7α,12α-triol-3-one[g]
VIII	2.80–2.85	1.44–1.45	–	–	–	652,562,547,472,457,382,357,267, 249,239,198,183,169,143	B-triolone
IX	4.48	1.59–1.60	–	–	–	637,562,472,454,447,429,382,357, 339,312,249,217,195,182,142	B-1β,3α,12α-triol-7-one[g]
X	3.82–4.05	1.55–1.56	–	3432–3450	–	652,637,562,544,472,447,357,339, 271,249,182,142,129	B-3α,4β,12α-triol-7-one[g]
XI	3.26–3.45	1.45	–	–	–	650,635,560,545,470,455,431,417, 380,365,355,261,274,209,196,193,159	B^{Δ}-triolone

[a]Related to that of the cholic acid derivative.
[b]Given by the methyl ester TMS ether derivative.
[c]B = 5β-cholanoic acid; superscript indicates position of double bond.
[d]Packed column used for quantifications (14).
[e]Capillary column used in GLC-MS analyses.
[f]Capillary column used in GLC analyses.
[g]Tentative structures.

cases 7 and 10 at 3–4 days of life are shown in **Fig. 3**. The GLC-MS analyses showed that compound II was the predominant unsaturated diolone in case 7 and compound I in case 10.

Compound I was usually the predominant ketonic bile acid. The spectrum of the methyl ester TMS ether derivative is shown in **Fig. 4**. A molecular ion was seen at *m/z* 562 and losses of trimethyl-silanols and the side chain gave the main fragment ion at *m/z* 267. The ion at *m/z* 261 was thought to arise by cleavage through the B-ring and to contain the CD-rings, side chain, and C-7 (19). The TMS derivative of the oxime gave a molecular ion at *m/z* 649 (Fig. 4), and the loss of two trimethylsiloxy groups and the side chain produced a base peak at *m/z* 354. The ions at *m/z* 264 and 380 represent the loss of all three trimethylsiloxy groups with and without the side chain, respectively. The fragment ion at *m/z* 195 was thought to arise through cleaveage of the C-9, 10 and C-5, 6 bonds, and to contain an unsaturated A-ring with the TMS-oxime group. The TMS derivative of the reduction product had the same retention time on Hi-Eff 8BP as the derivative of cholic acid, and the spectrum (Fig. 4) had similarities to that of the derivative of 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid, but with absence of the ion at *m/z* 217 (9). The molecular ion (*m/z* 636) was significant and the fragment ions at *m/z* 195 and 182 were thought to arise by cleavage through the B-ring, and to contain the A-ring with and without C-6, respectively. The ion at *m/z* 314 indicated that two hydroxyl groups were present in the BCD-ring part of the molecule (19). On the basis of these results it was assumed that the structure of compound I was 7α,12α-dihydroxy-3-oxo-5β-chol-1-enoic acid. This was confirmed by hydrogenation of the reduction product with hydrogen/platinum(IV) oxide to saturate the double bond, which was shown to give cholic acid as the only product.

The derivative of **compound II** gave a spectrum with a molecular ion at *m/z* 562 and a base peak at *m/z* 267. The TMS-oxime gave a molecular ion and base peak at *m/z* 649, and ABCD-ring fragment ions at *m/z* 354 and 264. The retention times and spectra were identical with those of the corresponding derivatives of 7α,12α-dihydroxy-3-oxo-4-cholenoic acid (cf. 20, 21).

The spectrum of the derivative of **compound III** showed

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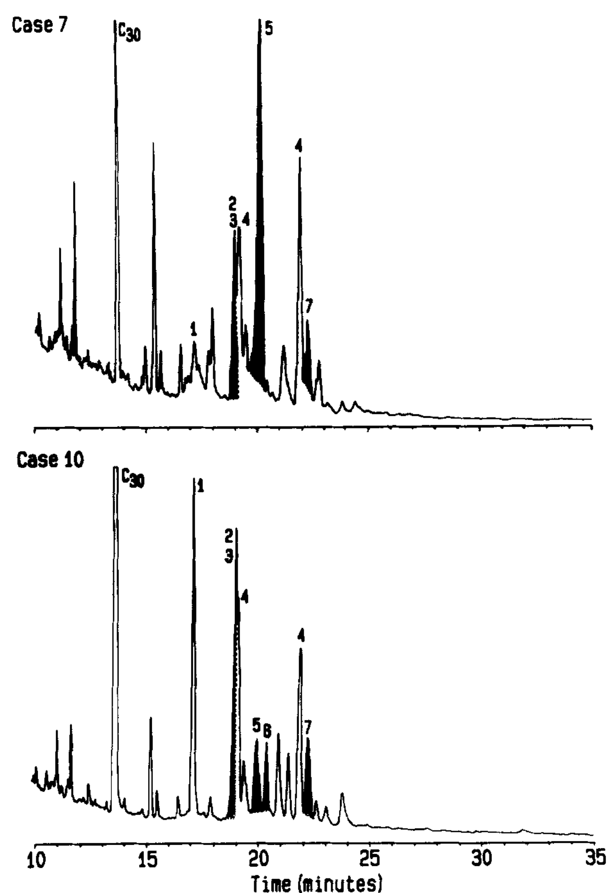

Fig. 3. Capillary GLC profiles of unconjugated urinary bile acids from cases 7 and 10. Mass spectrometry (Table 2) indicated the following bile acid structures. 1. cholic; 2. compound I (7α,12α-dihydroxy-3-oxo-5β-chol-1-enoic); 3. compound III (7α,12α-dihydroxy-3-oxo-5β-cholanoic); 4. tetrahydroxycholanoic; 5. compound II (7α,12α-dihydroxy-3-oxo-4-cholenoic); 6. compound IV (3α,12α-dihydroxy-7-oxo-5β-cholanoic; 7. compound X (tentatively 3α,4β,12α-trihydroxy-7-oxo-5β-cholanoic). Hatched areas indicate ketonic bile acids. C_{30} is the added triacontane.

a molecular ion at *m/z* 564. The ABCD-ring fragment ion at *m/z* 269 was the base peak accompanied by an ion at *m/z* 242 due to additional loss of C-16–C-17. The corresponding oxime gave a molecular ion at *m/z* 651 and an ABCD-ring fragment ion at *m/z* 266. The mass spectra and retention time data corresponded to those given by the derivatives of reference 7α,12α-dihydroxy-3-oxo-5β-cholanoic acid (17).

The derivative of **compound IV** had a retention time and a mass spectrum identical with those of the TMS ether of methyl 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid. Thus, the fragmentation of the molecular ion (*m/z* 564) was extensive with ions due to loss of the 7-oxo group as water (19). The corresponding oxime gave a base peak at *m/z* 562, due to loss of the trimethylsiloxy group of the TMS-oxime from the molecular ion at *m/z* 651. The origin of a major peak at *m/z* 297 (97% relative intensity) is not known.

The derivative of **compound V** gave a peak at *m/z* 549 (M–15) and a base peak at *m/z* 269. The ion due to loss of two trimethylsiloxy groups, *m/z* 384, was very intense. No corresponding oxime could be recognized and the structure of this bile acid is unknown.

The molecular ion of the derivative of **compound VI** at *m/z* 564 lost a fragment of mass 155 characteristic of a 12-oxo bile acid (19). Additional loss of trimethylsilanol yielded ions at *m/z* 319 and 229. This bile acid was identified as 3α,7α-dihydroxy-12-oxo-5β-cholanoic acid. The corresponding oxime could not be localized due to the small amounts present.

The derivative of **compound VII** gave an intense peak at *m/z* 637 (M–15) (**Fig. 5**). Fragment ions due to loss of one to three trimethylsiloxy groups were present, *m/z* 472 (M–2 × 90) being the base peak. Additional loss of the side chain gave peaks at *m/z* 357 and 267. The ion at *m/z* 261 was also intense, indicative of cleavage through the B-ring. The major ion at *m/z* 313 might arise by loss of two trimethylsilanols and the A-ring with its substituents (M–2 × 90–159). Based on these results and on biological grounds the structure 1β,7α,12α-trihydroxy-3-oxo-5β-cholanoic acid is suggested. However, an alternative structure would be 6α,7α,12α-trihydroxy-3-oxo-5β-cholanoic acid. The corresponding oxime could not be found.

Compound VIII gave a molecular ion at *m/z* 652 corresponding to the derivative of a bile acid with one oxo and three hydroxy groups. The base peak at *m/z* 357 was very intense. Fragment ions at *m/z* 169 and 198 were typical. If a 3-oxo-4,7-bistrimethylsiloxy structure is considered, *m/z* 169 could contain C-3–C-7 with substituents minus trimethylsilanol, and *m/z* 198 the A-ring including C-5, C-10, and C-19 with substituents, i.e., corresponding to *m/z* 271 given by 3, 4-bistrimethylsiloxy structures (22).

The derivative of **compound IX** gave a spectrum with peaks characteristic of a 1,3-bistrimethylsiloxy structure, with *m/z* 217 as the base peak and distinct fragment ions at *m/z* 195, 182, and 142. While no molecular ion was seen, the peak at *m/z* 637 (M–15) had a relative intensity of 13% and ions due to losses of trimethylsilanol from a molecular ion of mass 652 were present. Additional loss of the side chain gave a series of peaks at *m/z* 447, 357, and 267 with accompanying peaks at *m/z* 429, 439, and 249 due to loss of the oxo group as water. Based on the mass spectrum and on biological grounds the structure 1β,3α,12α-trihydroxy-7-oxo-5β-cholanoic acid is suggested for this compound. No corresponding oxime could be recognized.

The TMS ether of **compound X** gave a molecular ion at *m/z* 652 (**Fig. 6**). Combinations of losses of trimethylsilanol, water, and the side chain were present as well as a prominent peak at *m/z* 129 and peaks at *m/z* 142, 182, and 271. The latter indicated that two hydroxy groups were lo-

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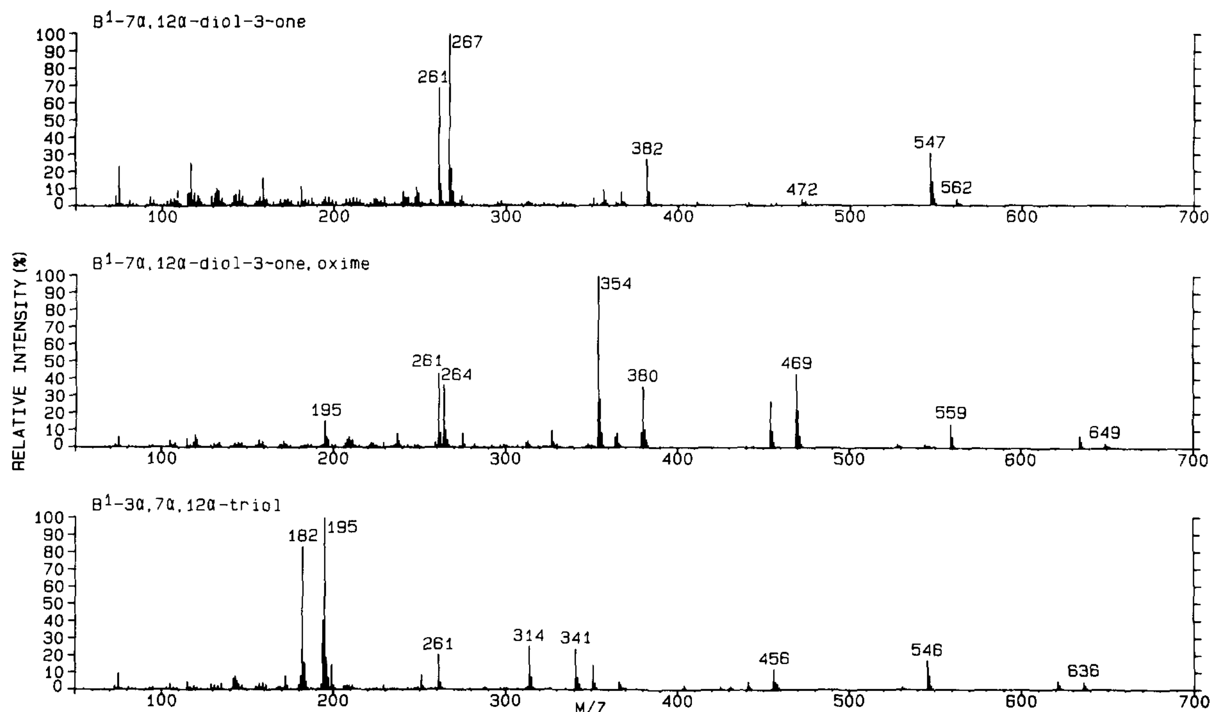

Fig. 4. Mass spectra of the TMS ethers before (upper spectrum) and after (lower spectrum) reduction with sodium borohydride and of the TMS-oxime derivative (middle spectrum) of the methyl ester of compound I shown to be 7α,12α-dihydroxy-3-oxo-5β-chol-1-enoic acid.

cated in the A-ring either at C-2 (23) or C-4 (22) and on biological grounds the structure 3α,4β,12α-trihydroxy-7-oxo-5β-cholanoic acid may be suggested.

The derivative of **compound XI** gave a molecular ion at *m/z* 650 (**Fig. 7**). The peak at *m/z* 355 represents the loss of two trimethylsilanols and the side chain. While the intense fragment ions of mass 159 and 193 are of obvious diagnostic importance, their origin has not been determined and the structure of this unsaturated ketonic bile acid remains unknown.

Daily excretion of ketonic bile acids

The presence of ketonic bile acids in healthy infants was not predicted and the analytical method was adapted for nonketonic bile acids (14). Partial destruction of ketonic bile acids during the alkaline hydolysis later became obvious and the estimated quantities, particularly of 3-oxo acids in the conjugated fractions, are minimum values. Compounds I–IV, VIII, X, and XI were measured separately during the first month. The other compounds occurred only small amounts. The bile acid patterns were very variable, as can also be seen in Figs. 1 and 3. The infants of the slightly cholestatic mother showed no unusual pattern. There was no correlation between patterns and excretion of ketonic bile acids and any available clinical or biochemical parameter.

Ketonic bile acids were present in all samples collected during the first day of life (Table I). **Compound I**

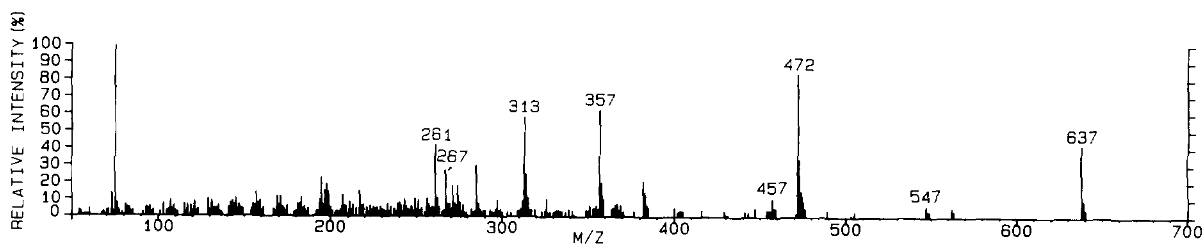

Fig. 5. Mass spectrum of the methyl ester TMS ether of compound VII, tentatively assigned the structure 1β,7α,12α-trihydroxy-3-oxo-5β-cholanoic acid.

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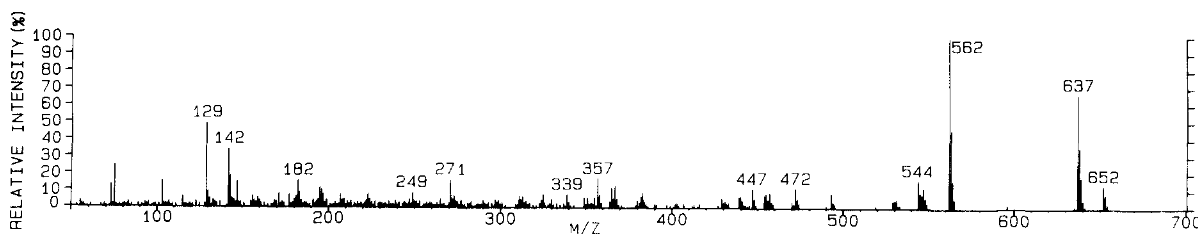

Fig. 6. Mass spectrum of the methyl ester TMS ether of compound X, tentatively assigned the structure 3α,4β,12α-trihydroxy-7-oxo-5β-cholanoic acid.

(7α,12α-dihydroxy-3-oxo-5β-chol-1-enoic acid) was the most common one, mainly found in the unconjugated fraction and present in all infants during the first day of life. It was the predominant ketonic bile acid in seven of the nine samples collected. Case 5 excreted particularly large amounts, 0.5 μmol/24 h, and he also had the largest total excretion of ketonic bile acids. **Compound II** (7α, 12α-dihydroxy-3-oxo-4-cholenoic acid) was found in two of three premature infants and in four of six fullterm infants, mainly in the unconjugated bile acid fraction. The excretion was in all cases lower than that of compound I. **Compound III** (7α,12α-dihydroxy-3-oxo-5β-cholanoic acid) was excreted by two premature and one fullterm infant (case 5). **Compound IV** (3α,12α-dihydroxy-7-oxo-5β-cholanoic acid) was not found in premature but in two fullterm infants. It was present in the unconjugated bile acid fraction. **Compound VIII** (triolone of unknown structure) was found occasionally during the first month and only in the unconjugated fraction. **Compound X** tentatively 3α,4β,12α-trihydroxy-7-oxo-5β-cholanoic acid) was found in minute amounts in one premature infant and it also occurred in three of five fullterm infants. It was present in the unconjugated and taurine-conjugated fractions. **Compound XI** (unsaturated triolone of unknown structure) was found in one premature infant as the predominant bile acid. Two fullterm infants also excreted this acid.

The excretion of ketonic bile acids was in all cases higher during the third than during the first day of life (Table 1). This was true also for the total bile acid excretion (14). The composition of ketonic bile acids was similar in both periods.

At the 6-8th day of life ketonic bile acids were found in all the premature infants. The amounts were lower than during the 3-4th days of life. Compound I was predominant, followed by compound III.

At 1 month of age, ketonic bile acids were found in two of four premature and in two of seven fullterm infants (Table 1). Compound I was the major one in three cases. Compound III was found in three infants, being the only ketonic bile acid excreted by case 7 at this age. Compounds IV and X were detected in one infant each.

There was a significant correlation between the excretion of compound I and total ketonic bile acids in urine during the first month of age, $r = 0.96$, $P < 0.001$. Values from the third day of case 5 were excluded from this statistical analysis. When included, the positive correlation was even stronger. Compound I constituted a mean of 52 ± 27% of the total ketonic bile acids during the first month.

DISCUSSION

The presence and quantitative importance of ketonic bile acids in urine during the neonatal period has not been noted in previous studies (see review by Back (24)). This may be due to the chemical and thermal lability of several of these compounds. Thus, 3-oxo bile acids are reduced during hydrolysis in ethanolic alkali (25), and hydroxy groups can be eliminated to give dienones under acidic or alkaline conditions. Cholylglycine hydrolase does not hydrolyze some ketonic bile acids (26, 27). These analytical difficulties have to be considered in future studies of bile acids in the neonatal period. Direct analysis by

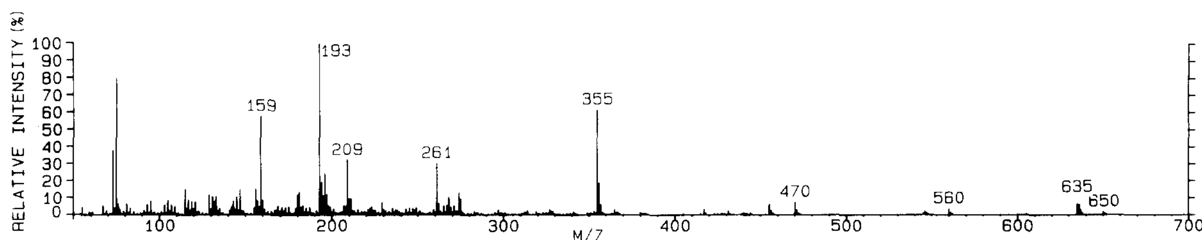

Fig. 7. Mass spectrum of the methyl ester TMS ether of Compound XI assigned to be an unsaturated trihydroxy-oxo bile acid but of unknown configuration.

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FABMS will be important (15, 20, 21) and some problems may be avoided by preparation of oximes as an early step in the analysis (28).

Without correction for analytical errors, ketonic bile acids constituted at least 40% of the unconjugated and a minimum of 16% of all bile acids in urine during the first week of life (14). The most abundant ketonic acid has now been identified as a new bile acid, 7α,12α-dihydroxy-3-oxo-5β-chol-1-enoic acid. Its presence was seen in the FABMS analysis of the crude urine extract, showing that it is not formed as an artefact during the isolation procedure. A mass spectrum of its methyl ester TMS ether was obtained by Clayton, Muller, and Lawson (29) when studying gastric contents of newborns with high intestinal obstruction, but the structure was not determined.

The other unsaturated ketonic bile acid, 7α,12α-dihydroxy-3-oxo-4-cholenoic acid, was previously identified by Clayton et al. (29). Sterols and C_{27} bile acids with a 3-oxo-Δ^4 structure are intermediates in the main pathway from cholesterol to cholic acid (30), and a rate limitation in the 5β-reduction could conceivably lead to excretion of C_{24} bile acids with a 3-oxo-Δ^4 structure. The corresponding intermediate in the synthesis of chenodeoxycholic acid was not detected, which is in agreement with our finding of a low urinary excretion of chenodeoxycholate in the neonatal period (14).

Three of the bile acids may be regarded as products of cholic acid oxidized at C-3, C-7, or C-12. These acids have been found in fetal and neonatal bile (29, 31), and all three are well-known components in feces (5, 32) and bile (3, 6, 33, 34) of healthy adults and in urine of patients with hepatobiliary diseases (35).

Five of the ketonic bile acids had one oxo and three hydroxy groups. None of them was identical with the only reference compound available, 3α,6α,12α-trihydroxy-7-oxo-5β-cholanoic acid. Two of them were tentatively identified as 1β,7α,12α-trihydroxy-3-oxo- and 1β,3α,12α-trihydroxy-7-oxo-5β-cholanoic acids. These structures, supported by the mass spectra, are proposed because of the abundant occurrence of tetrahydroxycholanoates with a 1β-hydroxy group in the fetal and neonatal periods (23, 29, 31, 36–39). Oxidation of the 3α- or 7α-hydroxy group would lead to the triolones. Alternatively, the 1β position of oxidized cholic acid could be hydroxylated. Elimination of water from a 1β-hydroxy-3-oxo structure is a possible mechanism of formation of the Δ^1 bile acid. Such a reaction would be similar to the hepatic 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one (40).

The site(s) of formation of the ketonic bile acids is not known. In the adult, ketonic derivatives of common bile acids are thought to be formed mainly by the intestinal microflora (2, 41). Although intestinal colonization by aerobic bacteria occurs during the first days of life (42), the high proportion of ketonic bile acids already on the first day makes a formation by mammalian enzymes more probable. The liver contains several 3α-hydroxysteroid dehydrogenases (30, 43–45), and the presence of 7α- and 12α-hydroxysteroid dehydrogenases is evident from the reduction of administered 7-oxo and 12-oxo bile acids (10–13). While the intestinal microflora may be of major importance for the oxidoreduction of bile acids at C-7 and C-12 (2, 4), oxidoreduction at C-3 is also extensive in the liver (46, 47). This process may be regulated by the hepatic redox level which could conceivably undergo major adjustments after birth with exposure to higher oxygen tensions and the change from fetal to adult hemoglobin. A higher redox potential during this period could conceivably lead to increased concentrations of oxidized members of redox couples and to rate limitations in reductive reactions, e.g., the 5β-reduction.

A physiological cholestasis has been proposed to exist during the first days of life (36, 48). Bile acids have been suggested to play an etiological role since 3β-hydroxy-5-cholenoic acid, found during this period (49, 50) and in children and adults with cholestatic liver disease (51–53), induces cholestasis in animal models (54). Cholestasis in the mother did not influence the urinary bile acid pattern in the infants in this study. Recently, two conditions have been described in which giant cell hepatitis and cholestasis are associated with the excretion of unusual bile acids in infants. In one of these, all bile acids had a 3β-hydroxy-Δ^5 structure (15). In the other, patients with severe liver disease of different etiology excreted bile acids with a 3-oxo-Δ^4 structure (20, 21). It is therefore of interest that 7α,12α-dihydroxy-3-oxo-4-cholenoic acid is one of the major ketonic bile acids in urine during the first days of life. From a diagnostic point of view, it is important to note that FABMS cannot distinguish between the 3-oxo-Δ^1 and 3-oxo-Δ^4 acids. Thus, direct FABMS analysis has to be combined with chromatographic separation and GLC-MS analysis which produce diagnostically useful fragment ions.

The technical assistance of Ms. B. Emtell and art work of Ms. G. Green are gratefully acknowledged. This work was supported by the Swedish Medical Research Council (grants no. 13X-219 and 19X-4995) and Karolinska Institutet.

Manuscript received 5 January 1989 and in revised form July 1989.

REFERENCES

1. Midtvedt, T., and A. Norman. 1967. Bile acid transformations by microbial strains belonging to genera found in intestinal contents. *Acta Pathol. Microbiol. Scand.* **71:** 629–638.
2. Hylemon, P. B. 1985. Metabolism of bile acids in intestinal microflora. *In* Sterols and Bile acids. H. Danielsson and J. Sjövall, editors. Elsevier Science Publishers B. V., Amsterdam. 331–343.
3. Hepner, G. W., A. F. Hofmann, J. R. Malagelada, P. A. Szczepanik, and P. D. Klein. 1974. Increased bacterial degra-

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ASBMB JOURNAL OF LIPID RESEARCH

dation of bile acids in cholecystectomized patients. *Gastroenterology.* **66:** 556-564.
4. Björkhem, I., L. Liljeqvist, K. Nilsell, and K. Einarsson. 1986. Oxidoreduction of different hydroxyl groups in bile acids during their enterohepatic circulation in man. *J. Lipid Res.* **27:** 177-182.
5. Eneroth, P., B. Gordon, and J. Sjövall. 1966. Characterization of trisubstituted bile acids in human feces. *J. Lipid Res.* **7:** 524-530.
6. Stellaard, F., P. D. Klein, A. F. Hofmann, and J. M. Lachin. 1985. Mass spectrometry identification of biliary bile acids in bile from patients with gallstones before and during treatment with chenodeoxycholic acid. *J. Lab. Clin. Med.* **105:** 504-513.
7. Andersén, E., G. Karlaganis, and J. Sjövall. 1988. Altered bile acid profiles in duodenal bile and urine in diabetic subjects. *Eur. J. Clin. Invest.* **18:** 166-172.
8. Björkhem, I., B. Angelin, K. Einarsson, and S. Ewerth. 1982. Fasting levels of monoketonic bile acids in human peripheral and portal circulation. *J. Lipid Res.* **23:** 1020-1025.
9. Almé, B., A. Bremmelgaard, J. Sjövall, and P. Thomassen. 1977. Analysis of metabolic profiles of bile acids in urine using a lipophilic anion exchanger and computerized gas-liquid chromatography-mass spectrometry. *J. Lipid Res.* **18:** 399-362.
10. Gustafsson, B. E., A. Norman, and J. Sjövall. 1960. Influence of *E. coli* infection on turnover and metabolism of cholic acid in germ-free rats. *Arch. Biochem. Biophys.* **91:** 93-100.
11. Soloway, R. D., A. F. Hofmann, P. J. Thomas, L. J. Schoenfield, and P. D. Klein. 1973. Triketocholanoic (dehydro-cholic) acid. Hepatic metabolism and effect on bile flow and biliary lipid secretion in man. *J. Clin. Invest.* **52:** 715-724.
12. Fromm, H., G. L. Carlson, A. F. Hofmann, S. Farivar, and P. Amin. 1980. Metabolism in man of 7-ketolithocholic acid: precursor of cheno- and ursodeoxycholic acids. *Am. J. Physiol.* **239:** G161-G166.
13. Nakagaki, M., R. G. Danzinger, A. F. Hofmann, and A. Roda. 1983. Hepatic biotransformation of two hydroxy-7-oxo-taurine-conjugated bile acids in the dog. *Am. J. Physiol.* **245:** G411-G417.
14. Strandvik, B., E. Wahlën, and S-Å. Wikström. 1989. Urinary bile acid excretion in healthy premature and fullterm infants in the neonatal period. *Eur. J. Clin. Invest.* Submitted.
15. Clayton, P. T., J. V. Leonard, A. M. Lawson, K. D. R. Setchell, S. Andersson, B. Egestad, and J. Sjövall. 1987. Familial giant cell hepatitis associated with synthesis of $3\beta,7\alpha$-dihydroxy- and $3\beta,7\alpha,12\alpha$-trihydroxy-5-cholenoic acids. *J. Clin. Invest.* **79:** 1031-1038.
16. Karlaganis, G., R. P. Schwarzenback, and G. Paumgartner. 1980. Analysis of serum bile acids by capillary gas-liquid chromatography-mass spectrometry. *J. Lipid Res.* **21:** 377-381.
17. Tandon, R., M. Axelson, and J. Sjövall. 1984. Selective liquid chromatographic isolation and gas chromatographic-mass spectrometric analysis of ketonic bile acids in faeces. *J. Chromatogr.* **302:** 1-14.
18. Shackleton, C. H. L., and K. M. Straub. 1982. Direct analysis of steroid conjugates: the use of secondary ion mass spectrometry. *Steroids.* **40:** 35-51.
19. Sjövall, J., A. M. Lawson, and K. D. R. Setchell. 1985. Mass spectrometry of bile acids. *Methods Enzymol.* **11:** 63-113.
20. Clayton, P. T., E. Patel, A. M. Lawson, R. A. Carruthers, M. S. Tanner, B. Strandvik, B. Egestad, and J. Sjövall. 1988. 3-Oxo-Δ^4 bile acids in liver disease. *Lancet.* **i:** 1283-1284.
21. Setchell, K. D. R., F. J. Suchy, M. B. Welsh, L. Zimmer-Nechemias, J. Heubi, and W. F. Balistreri. 1988. Δ^4-3-Oxosteroid 5β-reductase deficiency described in identical twins with neonatal hepatitis—a new inborn error in bile acid synthesis. *J. Clin. Invest.* **82:** 2148-2157.
22. Setchell, K. D. R., R. Dumaswala, C. Colombo, and M. Ronchi. 1988. Hepatic bile acid metabolism during early development revealed from the analysis of human fetal gallbladder bile. *J. Biol. Chem.* **263:** 16637-16644.
23. Strandvik, B., and S-Å. Wikström. 1982. Tetrahydroxylated bile acids in healthy human newborns. *Eur. J. Clin. Invest.* **12:** 301-305.
24. Back, P. 1988. Urinary bile acids. *In* The Bile Acids. Vol. 4. K. D. R. Setchell, D. Kritchevsky, and P. P. Nair, editors, Plenum Press, New York and London. 405-440.
25. Lepage, G., A. Fontainē, and C. C. Roy. 1978. Vulnerability of keto bile acids to alkaline hydrolysis. *J. Lipid Res.* **19:** 505-509.
26. Beher, W. T., S. Stradnieks, G. R. Beher, and G. J. Lin. 1978. The hydrolysis of bile acid conjugates. *Steroids.* **32:** 355-363.
27. Batta, A. K., G. Salen, and S. Shefer. 1984. Substrate specificity of cholylglycine hydrolase for the hydrolysis of bile acid conjugates. *J. Biol. Chem.* **259:** 15035-15039.
28. Axelson, M., and J. Sjövall. 1985. Studies on the selective analysis of ketonic bile acids and steroids in faeces. *In* Enterohepatic Circulation of Bile Acids and Sterol Metabolism. G. Paumgartner, A. Stiehl, and W. Gerok, editors. MTP Press, Lancaster, UK. 249-258.
29. Clayton, P. T., D. P. R. Muller, and A. M. Lawson. 1982. The bile acid composition of gastric contents from neonates with high intestinal obstruction. *Biochem. J.* **206:** 489-498.
30. Björkhem, I. 1985. Mechanism of bile acid biosynthesis in mammalian liver. *In* Sterols and Bile acids. H. Danielsson and J. Sjövall, editors. Elsevier Science Publishers B. V., Amsterdam. 231-278.
31. Colombo, C., G. Zuliani, M. Ronchi, J. Breidenstein, and K. D. R. Setchell. 1987. Biliary bile acid composition of the human fetus in early gestation. *Pediatr. Res.* **21:** 197-200.
32. Setchell, K. D. R., J. M. Street, and J. Sjövall. 1988. Fecal bile acids. *In* The Bile Acids. Vol. 4. K. D. R. Setchell, D. Kritchevsky, and P. P. Nair, editors. Plenum Press, New York and London. 441-570.
33. Matern, S., J Sjövall, E. W. Pomare, K. W. Heaton, and T. S. Low-Beer. 1975. Metabolism of deoxycholic acid in man. *Med. Biol.* **53:** 107-113.
34. Kikuchi, H. 1972. The metabolic sequence for the occurrence of an anomalous bile acid, 12-ketochenodeoxycholic acid found in the bile of hepatobiliary diseased patients. *J. Biochem.* **72:** 165-172.
35. Amuro, Y., T. Endo, K. Higashino, K. Uchida, and Y. Yamamura. 1981. Urinary and faecal keto bile acids in liver circhosis. *Clin. Chin. Acta.* **114:** 137-147.
36. Back, P., and K. Walter. 1980. Developmental pattern of bile acid metabolism as revealed by bile acid analysis of meconium. *Gastroenterology.* **78:** 671-676.
37. Tohma, M., R. Mahara, H. Takeshita, T. Kurosawa, S. Ikegawa, and H. Nittono. 1985. Synthesis of the 1β-hydroxylated bile acids and identification of $1\beta,3\alpha,7\alpha$-trihydroxy- and $1\beta,3\alpha,7\alpha,12\alpha$-tetrahydroxy-$5\beta$-cholan-24-oic acids in human meconium. *Chem. Pharm. Bull.* **33:** 3071-3073.
38. Tohma, M., R. Mahara, H. Takeshita, T. Kurosawa, and S. Ikegawa. 1986. Synthesis of the 1β-hydroxylated bile acids, unusual bile acids in human biological fluids. *Chem. Pharm. Bull.* **34:** 2890-2899.
39. Shoda, J., R. Mahara, T. Osuga, M. Tohma, S. Ohnishi, H. Miyazaki, N. Tanaka, and Y. Matsuzaki. 1988. Similarity of unusual bile acids in human umbilical cord blood and

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amniotic fluid from newborns and in sera and urine from adult patients with cholestatic liver diseases. *J. Lipid Res.* **29:** 847–858.

40. Skrede, S., M. S. Buchmann, and I. Björkhem. 1988. Hepatic 7α-dehydroxylation of bile acid intermediates, and its significance for the pathogenesis of cerebrotendinous xanthomatosis. *J. Lipid Res.* **29:** 157–164.
41. MacDonald, I. A., V. D. Bokkenheuser, J. Winter, A. M. McLernon, and E. H. Mosbach. 1983. Degradation of steroids in the human gut. *J. Lipid Res.* **24:** 675–700.
42. Rotimi, V. D., and B. I. Duerden. 1981. The development of the bacterial flora in normal neonates. *J. Med. Microbiol.* **14:** 51–62.
43. Elliott, W. H. 1985. Metabolism of bile acids in liver and extrahepatic tissues. *In* Sterols and Bile acids. H. Danielsson and J. Sjövall, editors. Elsevier Science Publishers B. V., Amsterdam. 303–329.
44. Stolz, A., H. Takikawa, Y. Sugiyama, J. Kuhlenkamp, and N. Kaplowitz. 1987. 3α-Hydroxysteroid dehydrogenase activity of the Y' bile acid binders in rat liver cytosol. *J. Clin. Invest.* **79:** 427–434.
45. Amuro, Y., W. Yamade, T. Yamamoto, K. Kudo, M. Fujikura, A. Maebo, T. Hada, and K. Higashino. 1986. Isocholic acid formation from 7α,12α-dihydroxy-3-keto-5β-cholanoic acid with human liver enzyme. *Biochim. Biophys. Acta.* **879:** 362–368.
46. Cronholm, T., I. Makino, and J. Sjövall. 1972. Steroid metabolism in rats given [1-$^2H_2$]ethanol. Oxidoreduction of isomeric 3-hydroxycholanoic acids and reduction of 3-oxo-4-cholenoic acid. *Eur. J. Biochem.* **26:** 251–258.
47. Marcus, S. N., and K. W. Heaton. 1988. Evidence for loss of tritium from 3β-tritiated deoxycholic acid during enterohepatic cycling in man. *Lipids.* **23:** 629–630.
48. Strandvik, B. 1973. Bile acid metabolism in cholestasis of infancy. *Opusc. Med. Suppl.* **29:** 16–17.
49. Makino, I., J. Sjövall, A. Norman, and B. Strandvik. 1971. Excretion of 3β-hydroxy-5-cholenoic and 3α-hydroxy-5α-cholanoic acids in urine of infants with biliary atresia. *FEBS Lett.* **15:** 161–164.
50. Back, P., and K. Ross. 1973. Identification of 3β-hydroxy-5-cholenoic acid in human meconium. *Hoppe-Seyler's Z. Physiol. Chem.* **354:** 83–89.
51. Bremmelgaard, A., and J. Sjövall. 1979. Bile acid profiles in urine of patients with liver diseases. *Eur. J. Clin. Invest.* **9:** 341–348.
52. Back, P. 1973. Identification and quantitative determination of urinary bile acids excreted in cholestasis. *Clin. Chim. Acta.* **44:** 199–207.
53. Németh, A., and B. Strandvik. 1984. Excretion of tetrahydroxylated bile acids in children with alpha-1-antitrypsin deficiency and neonatal cholestasis. *Scand. J. Clin. Lab. Invest.* **44:** 387–392.
54. Javitt, N., and S. Emerman. 1968. Effect of sodium taurolithocholate on bile flow and bile acid excretion. *J. Clin. Invest.* **47:** 1002–1014.

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