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Delta 4-3-oxosteroid 5 beta-reductase deficiency described in identical twins with neonatal hepatitis. A new inborn error in bile acid synthesis.

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Research Article

A new inborn error in bile acid synthesis, manifest in identical infant twins as severe intrahepatic cholestasis, is described involving the delta 4-3-oxosteroid 5 beta-reductase catalyzed conversion of the key intermediates, 7 alpha-hydroxy-4-cholesten-3-one and 7 alpha,12 alpha-dihydroxy-4-cholesten-3-one for chenodeoxycholic and cholic acid synthesis, to the respective 3 alpha-hydroxy-5 beta (H) products. This defect was detected by fast atom bombardment ionization-mass spectrometry from an elevated excretion and predominance of taurine conjugated unsaturated hydroxy-oxo-bile acids. Gas chromatography-mass spectrometry confirmed these to be 7 alpha-hydroxy-3-oxo-4-cholenoic and 7 alpha,12 alpha-dihydroxy-3-oxo-4-cholenoic acids (75-92% of total). Fasting serum bile acid concentrations were greater than 37 mumol/liter; chenodeoxycholic acid was the major bile acid, but significant amounts of allo(5 alpha-H)-bile acids (approximately 30%) were present. Biliary bile acid concentration was less than 2 mumol/liter and consisted of chenodeoxycholic, allo-chenodeoxycholic, and allo-cholic acids. These biochemical findings, which were identical in both infants, indicate a defect in bile acid synthesis involving the conversion of the delta 4-3-oxo-C27 intermediates into the corresponding 3 alpha-hydroxy-5 beta(H)-structures, a reaction that is catalyzed by a delta 4-3-oxosteroid-5 beta reductase enzyme. This defect resulted in markedly reduced primary bile acid synthesis and concomitant accumulation of delta 4-3-oxo-and allo-bile acids. These findings indicate a pathway in bile acid synthesis whereby side chain oxidation can occur despite incomplete alterations to the steroid nucleus, and lend support […]

Δ^4-3-Oxosteroid 5β-Reductase Deficiency Described in Identical Twins with Neonatal Hepatitis

A New Inborn Error in Bile Acid Synthesis

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Abstract

A new inborn error in bile acid synthesis, manifest in identical infant twins as severe intrahepatic cholestasis, is described involving the Δ^4-3-oxosteroid 5β-reductase catalyzed conversion of the key intermediates, 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one for chenodeoxycholic and cholic acid synthesis, to the respective 3α-hydroxy-5β(H) products. This defect was detected by fast atom bombardment ionization-mass spectrometry from an elevated excretion and predominance of taurine conjugated unsaturated hydroxy-oxo-bile acids. Gas chromatography-mass spectrometry confirmed these to be 7α-hydroxy-3-oxo-4-cholenoic and 7α,12α-dihydroxy-3-oxo-4-cholenoic acids (75–92% of total). Fasting serum bile acid concentrations were > 37 μmol/liter; chenodeoxycholic acid was the major bile acid, but significant amounts of *allo*(5α-H)-bile acids ($\sim$ 30%) were present. Biliary bile acid concentration was < 2 μmol/liter and consisted of chenodeoxycholic, *allo*-chenodeoxycholic, and *allo*-cholic acids. These biochemical findings, which were identical in both infants, indicate a defect in bile acid synthesis involving the conversion of the Δ^4-3-oxo-C_{27} intermediates into the corresponding 3α-hydroxy-5β(H)-structures, a reaction that is catalyzed by a Δ^4-3-oxosteroid-5β reductase enzyme. This defect resulted in markedly reduced primary bile acid synthesis and concomitant accumulation of Δ^4-3-oxo-and *allo*-bile acids. These findings indicate a pathway in bile acid synthesis whereby side chain oxidation can occur despite incomplete alterations to the steroid nucleus, and lend support for an active Δ^4-3-oxosteroid 5α-reductase catalyzing the conversion of the Δ^4-3-oxosteroid intermediates to the respective 3α-hydroxy-5α(H)-structures.

Introduction

Bile acids are important organic compounds in bile and are thought to be a major determinant and motive force for the generation of bile secretion at the canalicular level. The postnatal increase in bile secretion that occurs in the human and other species depends largely on the coordinate maturation of hepatic biosynthetic and transport mechanisms for bile acids. Pathways of bile acid synthesis from cholesterol in adults are relatively well defined (1) and involve various modifications of the steroid nucleus, including the conversion of the 3β-hydroxy-Δ^5 structure of this sterol to a 3α-hydroxy-5β(H) structure, which is common to chenodeoxycholic and cholic acids, the principal bile acids in man. Hydroxylations at positions C-7 of the steroid nucleus, and C-12 for cholic acid, and oxidation of the side chain are also required. Studies of human fetal gallbladder bile indicate that while bile acid synthesis is relatively well developed in early gestation (2, 3), alternative pathways in metabolism are operative during human development, including additional hydroxylations of the steroid nucleus (3–9).

Inborn errors involving primary bile acid synthesis from cholesterol are considered uncommon, but studies of affected patients have led to a better understanding of the biochemical pathways. The most well documented inborn errors include cerebrotendinous xanthomatosis and the peroxisomal abnormalities (Zellweger syndrome, pseudo-Zellweger syndrome, infantile Refsums' disease, and neonatal adrenoleukodystrophy), all of which involve an impaired oxidation of the cholesterol side chain (10). More recently a defect involving nuclear alteration of the 3β-hydroxy-Δ^5 structure of 7α-hydroxy-cholesterol was described in an infant with "familial giant cell hepatitis." This condition was characterized by excessive production of C_{24}-bile acids having a 3β-hydroxy-Δ^5 structure (11, 12).

In this report we describe a new inborn error in bile acid synthesis, namely a Δ^4-3-oxosteroid 5β-reductase deficiency, in identical twins with neonatal hepatitis which is reflected by an increased synthesis of bile acids having a 3-oxo-Δ^4-structure and *allo*(5α)-bile acids.

Methods

Case histories. Monochorionic twin male infants S.G. and J.G. presented at birth with cholestatic jaundice. They were the product of a term uncomplicated pregnancy. The parents were not consanguineous. Jaundice, pale stools, and dark urine were noted on the first day of life. Initial liver tests revealed a total bilirubin of 5–7 mg/dl in both infants with a conjugated fraction of 5–6 mg/dl. Serum aminotransferase levels were normal or only slightly increased (serum glutamic oxaloacetic transaminase 103–133, U/liter, normal 27–47 U/liter, and serum glutamic pyruvic transaminase 32–36 U/liter, normal 7–56 U/liter). Serum alkaline phosphatase ranged from 134–166 U/liter (normal 30–360 U/liter). A previous sibling with an identical clinical history had died at age 4 mo of hepatic failure.

Jaundice progressively worsened and both infants grew poorly until referral to our unit at 3 wk of age. The twins were deeply icteric and their lengths, weights, and head circumferences were at or just below

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the 5th percentile for age. In each twin the liver size was small (span 4 cm with the edge palpable at costal margins) and the spleen was not palpable. Total bilirubins were 20–24 mg/dl with direct fractions 14–15 mg/dl. Serum aminotransferase and alkaline phosphatase were slightly elevated. Coagulopathy was present in both infants (prothrombin time 13–15 s, not correctable by parenteral administration of vitamin K). Serum titers for congenital infection including toxoplasmosis, rubella, cytomegalovirus, and herpes simplex were negative. Serum alpha-1-antitrypsin phenotypes were MM. Serum and urine metabolic screening revealed moderate elevation of tyrosine and methionine concentrations consistent with liver disease. Red cell galactose-1-phosphate uridyl transferase assays had normal activities.

Because of coagulopathy and small liver size, both twins underwent open liver biopsy. A cholecystostomy tube was left in place after an operative cholangiogram in the hope of ameliorating the cholestasis (13) in one case (S.G.); in the other (J.G.) bile was obtained by needle aspiration of the gallbladder. Liver biopsies revealed marked lobular disarray as a result of giant cell and pseudoacinar transformation of hepatocytes, hepatocellular and canalicular bile stasis, slight extramedullary hematopoiesis and minimal lobular and portal cellular infiltrate. On electron microscopy bile canaliculi were small, sometimes slit-like in structure, and showed few or absent microvilli. There was a variable amount of electron dense material in the pericanalicular cytoplasm and within the canalicular lumen.

Analysis of bile acids from urine, bile, and serum. Bile acids were extracted and concentrated from urine, bile, and serum samples by liquid-solid extraction using small cartridges of octadecylsilane bonded silica exactly as described in detail previously (14).

A small portion of the urine and bile extracts (equivalent to 50 μl of the original sample) was analyzed directly by fast atom bombardment ionization-mass spectrometry (FAB-MS)[1] (10, 15). The remaining sample was analyzed by gas chromatography-mass spectrometry for total individual bile acids (16). The methanolic extract was evaporated to dryness and bile acid conjugates solvolyzed in a mixture of tetrahydrofuran/1 M trifluoracetic acid in dioxan/methanol (9:0.1:1 by vol) for 2 h at room temperature (17). The volatile reagents were removed by evaporation in a stream of nitrogen gas and the sample was then subjected to enzymic hydrolysis using cholylglycine hydrolase (10 U) in phosphate buffer pH 5.8, overnight at 37°C. Bile acids were extracted from the hydrolysate by passage through a cartridge of octadecylsilane bonded silica. The methanolic extract was then passed through a small column of the lipophilic anion exchange gel, diethylaminohydroxypropyl Sephadex LH-20 (Lipidex DEAP) to remove neutral steroids and sterols. Unconjugated bile acids were recovered from the gel by elution with 5 ml 0.1 M acetic acid in 72% ethanol. After addition of an internal standard (coprostanol 1–10 μg) bile acids were converted to the methyl ester trimethylsilyl (Me-TMS) ether derivatives for analysis by gas chromatography and mass spectrometry (16, 18).

FAB-MS. Negative ion FAB-MS spectra were recorded after placing the equivalent of 50 μl of the urine or the bile extracts onto a small drop of glycerol spotted on a copper target probe. The probe was introduced directly into the ion source of a quadrupole mass spectrometer (Finnigan 4635; Finnigan MAT, San Jose, CA) and a beam of fast atoms of xenon, generated using a saddle-field atom gun (Ion Tech, Teddington, Middlesex, UK) operated at 8 kV and 20 μA was fired at the target containing the sample. Negative ion spectra (scan time 1.5 s) were recorded over the mass range 50–800 D. A list of ions relevant to bile acid conjugates has been published previously (10, 15).

Gas chromatography and mass spectrometry (GC-MS). The Me-TMS ether derivatives were separated by chromatography on a 30 m × 0.4-mm DB-1 (film thickness 0.25 μm) fused silica capillary column (J and W Scientific, Folsom, CA) operated in temperature programing mode from 225°–295°C with increments of 2°C/min and initial and final isothermal periods of 5 and 20 min, respectively. Helium was used as carrier gas at a flow rate of 1.8 ml/min. The sample was introduced by liquid-solid injection using an all glass dropping needle injection system.

GC-MS was carried out using a Finnigan 4635 quadrupole gas chromatograph-mass spectrometer housing an identical GC column and operated under the same chromatographic conditions. Electron impact (70 eV) ionization mass spectra were recorded over the mass range 50–800 D by repetitive scanning (1.5 s/cycle) of the eluting components of the complete GC profile. Data were recorded and processed using the Super Incos Data System (Finnigan MAT).

Quantification and identification of bile acids. Bile acids were quantified by gas chromatography from a comparison of their peak area response with the peak area response obtained for the known amount of added internal standard, coprostanol. The identification of a bile acid was based upon its GC retention index relative to an homologous series of n-alkanes (MU value) and EI mass spectrum compared with reference bile acids (18).

Results

Urinary bile acid analysis

FAB-MS analysis. A typical negative ion FAB-MS spectrum obtained from the urine from a healthy infant, 3 mo of age, and a 3-mo infant with extrahepatic cholestasis is compared in Fig. 1. Since urinary bile acid excretion is extremely low in healthy infants the FAB-MS spectra is unremarkable and shows mainly nonspecific ions from the glycerol and sample matrix. By contrast, patients with marked cholestasis generally excrete elevated quantities of taurine conjugated trihydroxy- (*m/z* 514) and dihydroxy- (*m/z* 498) cholanoates and the respective glycine conjugates (*m/z* 464 and *m/z* 448) as a result of preferential urinary excretion of cholic and chenodeoxycholic acids.

The negative ion FAB-MS spectra of the urine (50 μl) of these twins were essentially identical and revealed the presence of two prominent ions at *m/z* 494 and *m/z* 510, with a minor ion at *m/z* 498 (Fig. 1). These major ions correspond in mass to the quasimolecular ions $[M-1]^-$ of taurine conjugates of unsaturated monooxo-monohydroxy- and monooxo-dihydroxy-cholenoates, respectively. The low intensity ions at *m/z* 444 and *m/z* 460 in urine from infant J.G. correspond in mass to the glycine conjugates of these two unsaturated oxo bile acids, while the ion at *m/z* 498 indicates the presence of lesser amounts of taurine-conjugated dihydroxy-cholanoates in both infant urines. Small amounts of taurine-conjugated trihydroxy bile acids were apparent from the ion at *m/z* 514. There was no indication for the presence of significant concentrations of bile acid sulfates in these samples. The FAB-MS spectra did however, reveal numerous ions in the mass range 300–400 D which arise from metabolites of urinary steroid hormones, many with 3β-hydroxy-Δ^5 structures that are typically found in the urine from infants of this age (19, 20).

GC-MS analysis. After hydrolysis of bile acid conjugates and preparation of the Me-TMS ethers the GC-MS profiles of

1. *Abbreviations used in this paper: allo*-chenodeoxycholic, (3α,7α-dihydroxy-5α-cholanoic); *allo*-cholic, (3α,7α,12α-trihydroxy-5α-cholanoic); hyocholic (3α,6α,7α-trihydroxy-5β-cholanoic); FAB-MS; fast atom bombardment mass spectrometry; GC-MS; gas chromatography-mass spectrometry; Me-TMS; methyl trimethylsilyl; MU, methylene unit; Δ is used to denote unsaturation and the superscript denotes the position of the double bond, e.g., Δ^4 = 4-ene structure.

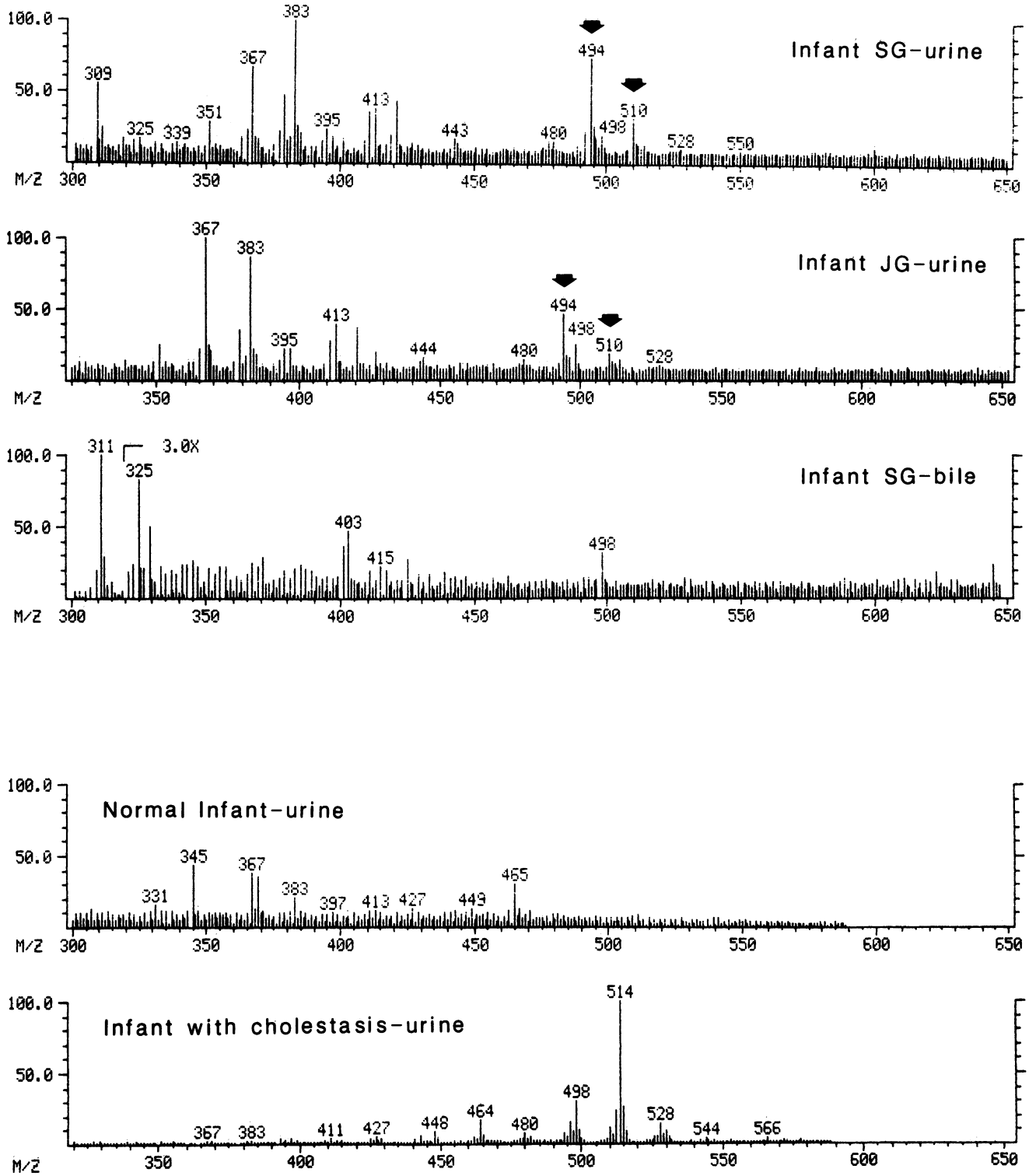

Figure 1. Negative ion FAB-MS spectra obtained from the urine and bile of twins (J.G. and S.G.) with familial giant-cell hepatitis are compared with the spectra typically obtained from the urines of a healthy infant and a patient with extrahepatic cholestasis. The unique ions (arrowed) *m/z* 494 and *m/z* 510 reflect elevated excretion of bile acids having unsaturated oxo-monohydroxy- and unsaturated oxo-dihydroxy structures. The ions *m/z* 498 and *m/z* 514 indicate the presence of taurine conjugated dihydroxy- and trihydroxycholanoates, respectively. For a list of the FAB-MS negative ions corresponding to bile acid conjugates see reference 13.

both urines were strikingly similar, but differed significantly from the profiles typical of healthy infants of 4 mo age (Fig. 2). The profiles of both patients revealed two major components with retention indices of 33.09 and 33.73 MU, respectively, and a number of minor metabolites. The electron impact ionization (70 eV) mass spectra of these two compounds are

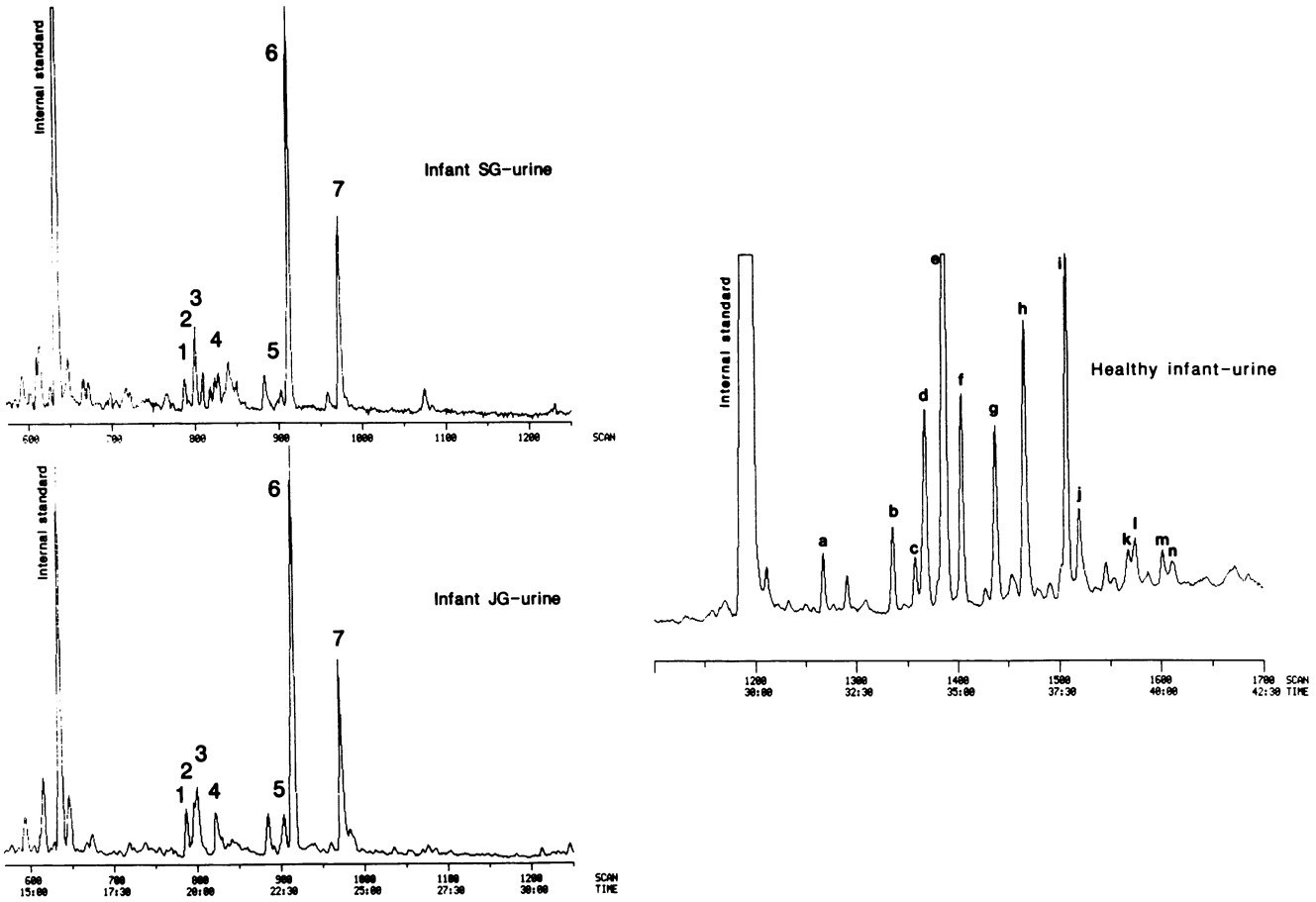

Figure 2. Computer reconstructed total ion current chromatograms from repetitive scanning GC-MS analysis of the Me-TMS ether derivatives of hydrolyzed urine extracts from twins (S.G. and J.G.) with familial giant-cell hepatitis and a healthy infant 4 mo of age. The following bile acids were identified in the patients urine from the GC retention values and mass spectra: (*1*) *allo*-chenodeoxycholic; (*2*) *allo*-cholic; (*3*) chenodeoxycholic; (*4*) trihydroxy bile acid possibly 3β,7α,12α-trihydroxy-5α-cholanoic; (*5*) hyocholic; (*6*) 7α-hydroxy-3-oxo-4-cholenoic; (*7*) 7α,12α-dihydroxy-3-oxo-4-cholenoic; internal standard-coprostanol. Bile acids identified in the chromatograms of healthy infant urine are denoted by letters and identifications listed in Table II.

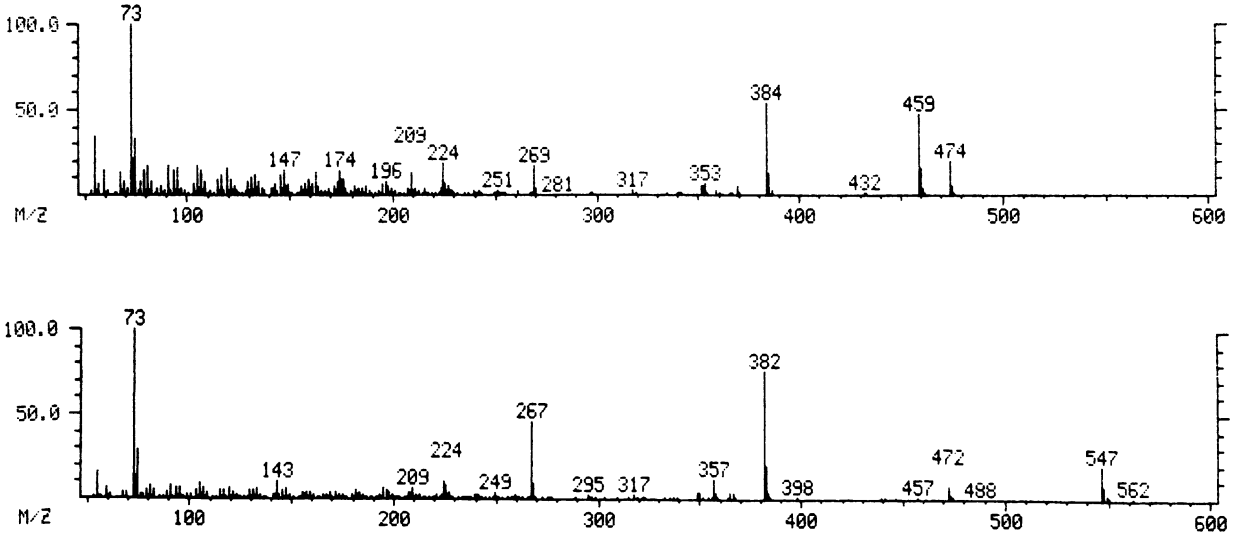

Figure 3. The electron impact (70 eV) ionization mass spectra of the Me-TMS ether derivatives of the two major components in the urine from twins with familial giant-cell hepatitis. These bile acids were identified as 7α-hydroxy-3-oxo-4-cholenoic acid (*upper;* peak 6 in Fig. 2) and 7α,12α-dihydroxy-3-oxo-4-cholenoic acid (*lower;* peak 7 in Fig. 2).

shown in Fig. 3. The mass spectrum of the major bile acid, having retention index 33.09 MU, gave a molecular ion (M^+) at *m/z* 474 consistent with the molecular ion of a Me-TMS ether derivative of a monohydroxy-oxo-cholenoic acid structure. The presence of the single derivatized hydroxy function is apparent from the ion at *m/z* 384 (M-90), which arises from the loss of trimethylsilanol, while the loss of the 5 carbon side chain (−115 D) gives rise to the ion at *m/z* 269. The ions at *m/z* 224 and 209 are indicative of a 7-hydroxy-3-oxo-4-ene structure and arise from a fragment consisting of the A/B rings, with and without the angular methyl group that is formed following cleavage of the C-7,8 and C-9,10 bonds. The mass spectrum and GC retention index of this compound was found to be identical to the mass spectrum of the Me-TMS ether derivative of 7α-hydroxy-3-oxo-4-cholenoic acid (18).

The electron impact ionization mass spectrum of the Me-TMS ether of the bile acid with retention index 33.73 MU showed a similar fragmentation pattern to that of the earlier eluting peak, except that the molecular ion (*m/z* 562) was higher by 88 D due to the presence of an additional derivatized hydroxy group. The presence of two derivatized hydroxy groups in the molecule is evident from the ions at *m/z* 472 (M-90) and *m/z* 382 (M-[2 × 90]) and the side chain loss (−115) gives rise to the ion at *m/z* 267. The ions at *m/z* 224 and *m/z* 209 discussed above, are also present in this spectrum, which was shown to be identical to the mass spectrum of the Me-TMS ether derivative of 7α,12α-dihydroxy-3-oxo-4-cholenoic acid (18).

Of the minor peaks in the GC-MS profile, chenodeoxycholic acid and *allo*-chenodeoxycholic acid (3α,7α-dihydroxy-5α-cholanoic acid), *allo*-cholic acid (3α,7α,12α-trihydroxy-5α-cholanoic acid) and 3α,6α,7α-trihydroxy-5β-cholanoic (hyocholic acid) were identified from their mass spectra and GC retention indices. A number of minor compounds remain unidentified. Quantitatively, total urinary bile acid concentrations were elevated (Table I) compared with a control group (Table II) of six healthy 4-mo old infants (0.63–2.25 μmol/liter). The two Δ^4-3-oxo bile acids accounted for up to 92% of the total bile acids excreted in the random samples of urine collected from both infants while being undetectable in the urine from healthy infants.

Biliary bile acid analysis

The negative ion FAB-MS spectra (Fig. 1) of the gallbladder bile (50 μl) from one of these infants (S.G.) indicated the presence of relatively low concentrations of tauro-dihydroxy-cholanoic acids. In contrast to urine, there was no evidence for the presence of Δ^4-3-oxo bile acids in bile, and there was also no detectable trihydroxycholanoates or glycine and sulfate-conjugated bile acids.

GC-MS analysis of the bile samples confirmed the FAB-MS data, indicating the presence of low concentrations of chenodeoxycholic acid (< 2.0 μmol/liter), and traces of *allo*-chenodeoxycholic and allo-cholic acids (Table I).

Serum bile acid analysis

Serum bile acids were not determined by the FAB-MS technique. Total individual serum bile acids were determined by GC and GC-MS after solvolysis/hydrolysis, purification, and derivatization. The GC profiles of serum from both infants were virtually identical and the chromatogram obtained from infant S.G. is shown in Fig. 4. The major bile acid in serum was identified by mass spectrometry to be chenodeoxycholic acid. GC-MS analysis however, revealed the presence of *allo*-cholic acid, which co-eluted with chenodeoxycholic. The retention indices of the Me-TMS ether derivatives of these two bile acids are almost identical (32.14 MU for *allo*-cholic; 32.16 MU for chenodeoxycholic acid) and they were incompletely resolved under the GC conditions employed, but under the GC-MS scanning conditions a relatively pure mass spectrum could be obtained Fig. 5 (lower trace). This mass spectrum shows all of the ions found in the Me-TMS ether derivative of cholic acid (although there is a trace of the chenodeoxycholic acid derivative present), but the relative intensities of the fragments produced under electron impact ionization conditions differ significantly from cholic acid. The origins of these ions have been discussed previously (21) and the spectrum is identical to previously published spectra of the Me-TMS ether derivative of *allo*-cholic acid (18, 21). A large chromatographic peak with a shorter retention index than chenodeoxycholic acid was present in the serum from both infants. The mass spectrum of the Me-TMS ether derivative of this compound is shown in Fig. 5 (upper panel). This 3,7-dihydroxy bile acid has

Table I. Bile Acid Concentrations (μmol/liter) in Biological Fluids of Twins (S.G. and J.G.) with Familial Neonatal Hepatitis Measured by Gas Chromatography and Mass Spectrometry

	Bile		Urine*		Plasma	
Bile acid	S.G.	J.G.	S.G.	J.G.	S.G.	J.G.
allo-chenodeoxycholic	0.16	—	0.92	1.74	8.71	6.00
Chenodeoxycholic and *allo*-cholic‡	1.35	—	1.25	2.26	31.84	25.10
3β,7α,12α-trihydroxy-5α-cholenoic§	0.16	—	0.53	1.02	2.71	1.70
7α-hydroxy-3-oxo-4-cholenoic	ND	—	26.24	11.0	7.26	4.00
7α,12α-dihydroxy-3-oxo-4-cholenoic	ND	—	16.20	6.36	1.66	0.46
Hyocholic	ND	—	0.92	0.64	ND	ND
Total	1.67	—	46.06	23.02	52.18	37.26
% Composition of 3-oxo-Δ^4 bile acids	0%	—	92.1%	75.4%	17.1%	12.0%
Estimated % composition of *allo*-bile acids	25.7%	—	2.6%	9.5%	28.9%	29.6%

ND, not detected. Bile sample from patient J.G. not available for analysis. * Normal values for urinary bile acids are given in Table II.
‡ *Allo*-cholic acid was present at ~ 20% of the level of chenodeoxycholic acid. § Tentative identification, may be traces of cholic acid present.

Table II. Values for Urinary Bile Acid Concentrations (μmol/liter) for Six Healthy Infants of 4 mo Age

Peak*	Bile acid	1	2	3	4	5	6	Mean±SD
a	3α,7α,12α-trihydroxy-5β-norcholan-23-oic (norcholic acid)	0.159	0.053	0.095	0.100	0.044	0.027	0.080±0.044
b	3β,7α-dihydroxy-5β-cholenoic	0.088	0.033	0.108	0.089	0.046	0.095	0.077±0.027
c	3α,12α-dihydroxy-5β-cholenoic (deoxycholic)	0.040	0.017	0.060	0.038	0.026	0.119	0.050±0.034
d	3β-hydroxy-5-cholenoic	0.059	0.035	0.260	0.106	0.034	0.075	0.095±0.078
e	3α,7α-dihydroxy-5β-cholenoic (chenodeoxycholic acid)	0.466	0.180	0.560	0.469	0.235	0.640	0.425±0.165
f	3α,7α,12α-trihydroxy-5β-cholenoic (cholic acid)	0.130	0.064	0.210	0.176	0.075	0.180	0.135±0.061
g	3α,7β-dihydroxy-5β-cholenoic (ursodeoxycholic acid)	0.177	0.047	0.140	0.139	0.052	0.095	0.108±0.048
h	3α,7β,12α-trihydroxy-5β-cholenoic	0.320	0.070	0.220	0.146	0.100	0.114	0.161±0.079
i	3α,6α,7α-trihydroxy-5β-cholenoic + 1β-tetrahydroxy-cholenoic	0.093	0.026	0.046	0.097	0.038	—	0.060±0.029
j	1β,3α,7α,12α-tetrahydroxy-5β-cholenoic	0.498	0.080	0.320	0.714	0.235	0.088	0.323±0.226
k	Trihydroxy bile acid	0.039	—	0.046	0.049	0.013	—	0.037±0.014
l	1,3,X-trihydroxy-cholenoic	0.074	0.018	0.055	0.082	0.024	—	0.051±0.026
m	Unknown bile acid	0.052	—	0.044	0.047	0.018	—	0.040±0.013
n	1,3,7-trihydroxy-cholenoic	—	—	—	—	—	—	—
	Total	2.195	0.623	2.160	2.250	0.940	1.43	1.60±0.65

* Letters refer to peaks indicated in chromatogram of healthy infant urine shown in Fig. 2.

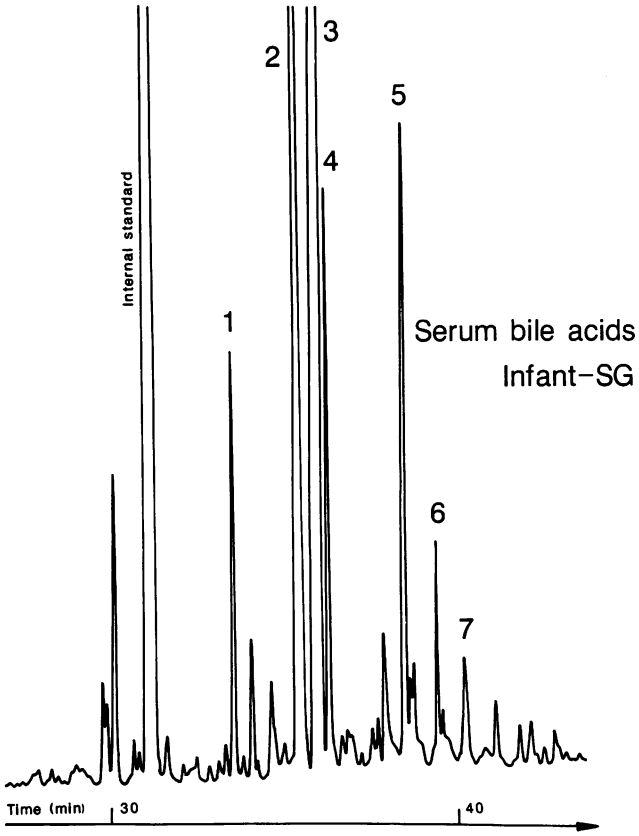

Figure 4. The capillary column GC profile of the total serum bile acids of patient SG. The Me-TMS ether derivatives of bile acids were separated on a 30-meter DB-1 fused silica capillary column using temperature programmed conditions from 225°–295°C with increments of 2°C/min after initial and final isothermal periods of 5 and 20 min, respectively. The following components were identified by GC-MS analysis: (*1*) cholesterol; (*2*) *allo*-chenodeoxycholic; (*3*) *allo*-cholic and chenodeoxychoic (unresolved); (*4*) 3β,7α,12α-trihydroxy-5α-cholanoic; (*5*) 7α-hydroxy-3-oxo-4-cholenoic; (*6*) dihydroxy bile acid (possibly 3β,7β-dihydroxy-5β-cholanoic); (*7*) 7α,12α-dihydroxy-3-oxo-4-cholenoic; internal standard-coprostanol.

a similar fragmentation pattern to that of chenodeoxycholic acid, however the relative intensities of the individual fragment ions are significantly different. The mass spectrum and GC retention index was found to be identical to the Me-TMS ether derivative of an authentic standard of *allo*-chenodeoxycholic (18).

A trihydroxy bile acid was also present in low concentrations in the serum (and in small amounts in urine) of both infants that had a retention index similar to the Me-TMS ether derivative of cholic acid, however the relative intensities of the major fragments in the mass spectrum differed. Although the authentic reference bile acid was unavailable, we tentatively suggest on the basis of retention values (18, 22) that this trihydroxy bile acid may be either 3β,7α,12α-trihydroxy-5α-cholanoic acid, or a mixture of this compound coeluting with small amounts of cholic acid. The serum of both infants also contained 7α-hydroxy-3-oxo-4-cholenoic and 7α,12α-dihydroxy-3-oxo-4-cholenoic acids, and several other unidentified minor components.

The total bile acid concentrations determined from a single sample from each infant were 52.18 μmol/liter (S.G.) and 37.26 μmol/liter (J.G.), respectively (Table I); significantly greater than for bile acid concentrations of similarly aged healthy infants (23) or healthy adults (24). Because of the coelution of *allo*-cholic acid and chenodeoxycholic it was not possible to accurately determine the individual concentrations of these two bile acids, however from the relative ion-abundances it was estimated that chenodeoxycholate exceeded *allo*-cholic acid by approximately four- to fivefold. The Δ^4-3-oxo bile acids accounted for up to 17% of the total serum, bile acids, while the *allo*-bile acids made up ~ 30% of the total.

Discussion

By the application of FAB-MS, the urines of two twin infants with familial neonatal hepatitis were screened for possible inborn errors in the cholesterol-bile acid biosynthetic pathway (10). This technique, because of its simplicity and speed, af-

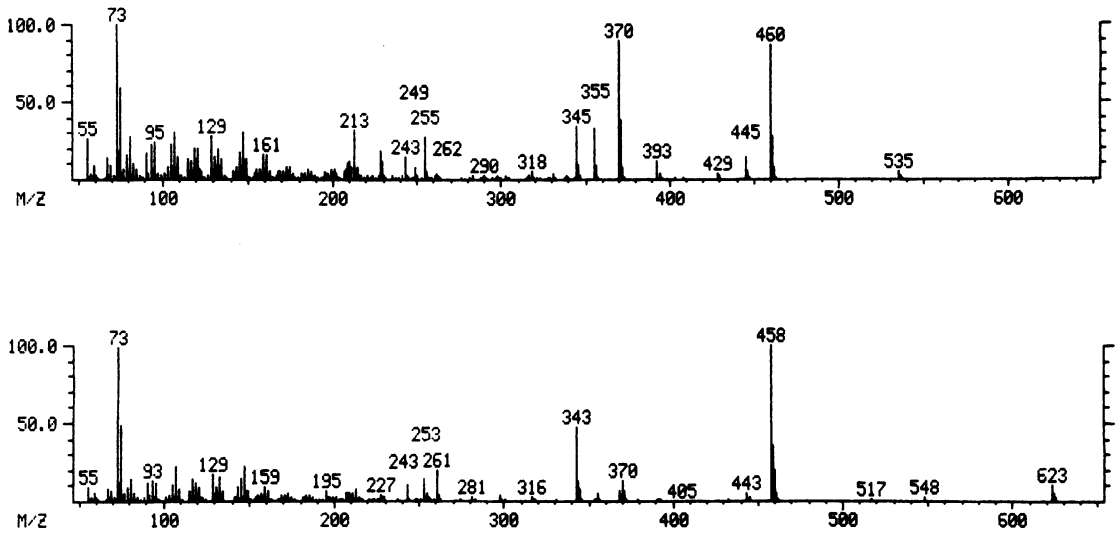

Figure 5. The electron impact (70 eV) ionization mass spectrum of the Me-TMS ether derivatives of two 5α-bile acids present in the serum of twins with familial giant-cell hepatitis and identified as *allo*-chenodeoxycholic acid (*upper*) and *allo*-cholic acid (*lower*) contaminated with traces of chenodeoxycholic because of incomplete chromatographic resolution of these two bile acids).

fords the most appropriate and definitive screening procedure for the detection of inborn errors in the cholesterol-bile acid biosynthetic pathway (10, 25, 26). Cholestasis can be confirmed by the finding of an elevated urinary bile acid excretion and a qualitative profile of the types of bile acid conjugates that are present may be obtained on samples of urine as small as 10–50 μl in volume. Once a defect in bile acid synthesis is suspected, confirmation may be obtained by GC-MS analysis and characterization of increased excretion of intermediates (and/or their metabolites) in the pathway. Using the above approach, a defect in bile acid synthesis, involving a deficiency of the 3β-hydroxysteroid dehydrogenase/isomerase enzyme was recently identified for the first time in an infant with a familial giant cell hepatitis (11, 12).

The negative ion FAB-MS spectra of the urine samples (Fig. 1) of the twins reported here were unique, and not typical of FAB-MS spectra for previously described inborn errors in bile acid synthesis [e.g., see reference 10]. The FAB-MS spectra revealed two major bile acid structures that were exclusively taurine conjugated and had molecular ions consistent with unsaturated oxo-hydroxy- and unsaturated oxo-dihydroxy-C_{24} bile acids. These two compounds were definitively identified by GC-MS as 7α-hydroxy-3-oxo-4-cholenoic and 7α,12α-dihydroxy-3-oxo-4-cholenoic acids and quantitatively accounted for between 75 and 92% of the total bile acids in urine and 12 and 17% of the total serum bile acids (Table I). This markedly contrasts urinary (Fig. 1), and serum bile acid composition of healthy infants of a similar age where Δ^4-3-oxo bile acids were not detected by these techniques.

Biliary bile acid concentrations measured in one of the infants was extremely low (1.67 μmol/liter) and bile contained mainly chenodeoxycholic acid and *allo*-chenodeoxycholic acid. There was no biliary excretion of these Δ^4-3-oxo bile acids.

Approximate estimates of daily bile acid synthesis can be made on the assumption that in these patients urinary excretion represented the sole route of bile acid loss. Daily urine volumes for the two patients were estimated from daily fluid intake to be 320 ml (J.G.) and 375 ml (S.G.), respectively; therefore total bile acid synthesis approximates to 4.6 μmol/d (1.8 mg/d, J.G.) and 9.3 μmol/d (3.7 mg/d, S.G.), which is markedly reduced compared to published figures for cholate synthesis rates in healthy infants (18–27 mg/d [27]).

These results point to a defect in bile acid synthesis affecting the conversion of the Δ^4-3-oxo intermediates into the 3α-hydroxy-5β(H) products. In the classical pathways for cholic and chenodeoxycholic acid synthesis (Fig. 6), cholesterol is first 7α-hydroxylated to give rise to 7α-hydroxy-cholesterol (28), and this step is rate limiting (29). 7α-Hydroxycholesterol is oxidized to 7α-hydroxy-4-cholesten-3-one by the action of 3β-hydroxysteroid dehydrogenase/isomerase enzyme (30). For cholic acid synthesis, 7α-hydroxy-4-cholesten-3-one requires 12α-hydroxylation, and 7α,12α-dihydroxy-4-cholesten-3-one is formed (31). These two Δ^4-3-oxo intermediates are normally converted to cholestane-3α,7α-diol and cholestane-3α,7α,12α-triol, respectively, in two steps; (*a*) by reduction of the Δ^4 bond, a reaction which is catalyzed by a NADPH dependent Δ^4-3-oxosteroid 5β-reductase enzyme (32, 33) to give rise to the 7α-hydroxy-5β-cholestan-3-one and 7α,12α-dihydroxy-5β-cholestan-3-one; and (*b*) by reduction of the 3-oxo group to a 3α-hydroxy group under the action of a 3α-hydroxysteroid dehydrogenase enzyme (32, 33).

Following these changes to the steroid nucleus, side chain hydroxylation and oxidation occur to reduce the C_{27}-sterol to a C_{24}-bile acid (1). In the major pathway for bile acid synthesis in the adult it is generally accepted that side chain oxidation occurs only after the necessary modifications to the steroid nucleus have taken place.

Increased production of Δ^4-3-oxo bile acids may be explained on the basis of a deficiency in the Δ^4-3-oxosteroid 5β-reductase enzyme (Fig. 6). Under circumstances of inadequate primary bile acid formation or secretion as occurs in other inborn errors in the cholesterol-bile acid pathway (10), a relative increase in the production of intermediates (or metabolites of these intermediates) takes place. In these infants, the increased levels of 7α-hydroxy-4-cholesten-3-one and 7α,12α-

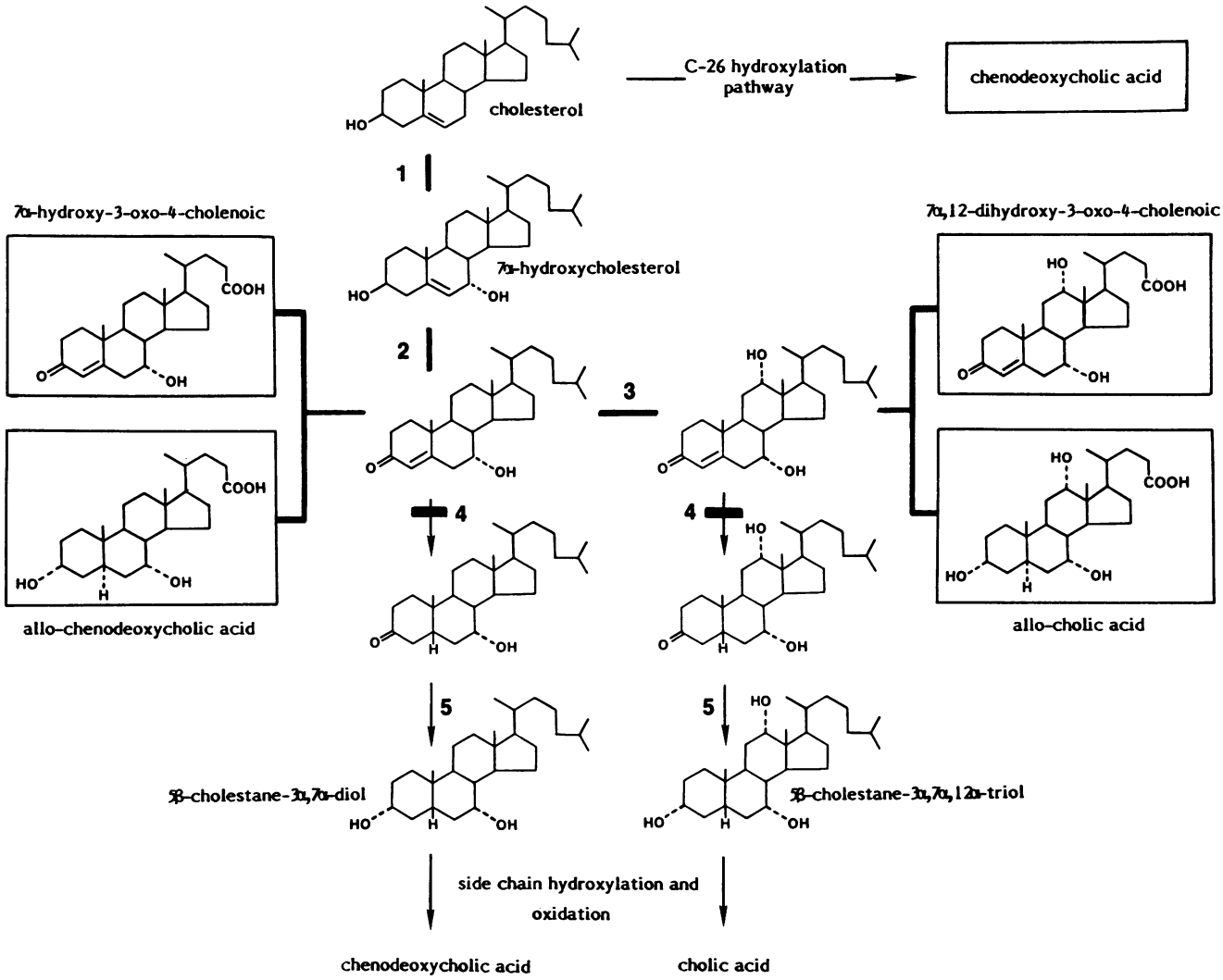

Figure 6. Biosynthetic pathways for bile acid synthesis from cholesterol in man indicating the point of the defect in synthesis in twins with familial giant-cell hepatitis and the resulting metabolism of the accumulated precursors. The numbers indicate the key enzymes involved in the early stages of the pathway: (*1*) cholesterol 7α-hydroxylase; (*2*) 3β-hydroxysteroid dehydrogenese/isomerase; (*3*) 12α-hydroxylase; (*4*) Δ^4-3-oxosteroid-5β-reductase; (*5*) 3α-hydroxysteroid dehydrogenase. The defect in Δ^4-3-oxosteroid 5β-reductase activity led to an increased production of Δ^4-3-oxosteroids with subsequent metabolism of these precursors by side chain oxidation to Δ^4-3-oxo bile acids and *allo*-bile acids (shown in boxes).

dihydroxy-4-cholesten-3-one that are formed, are subsequently converted by side chain oxidation to the respective Δ^4-3-oxo-cholenoic acids. The finding of significant levels of these Δ^4-3-oxo bile acids therefore provides evidence for a pathway in early life in which side chain oxidation takes place despite incomplete modifications to the C_{27}-steroid nucleus.

The presence of relatively high proportions of *allo*(5α-H)-bile acids further supports a deficiency or inactivity in hepatic Δ^4-3-oxosteroid 5β-reductase activity in these infants. Normally *allo*-bile acids are minor constituents in mammals, although in some lower vertebrates they are major bile acids (34). Several different pathways have been proposed for the formation of *allo*-bile acids in mammals (1). One of these includes the action of intestinal micro-organisms. This pathway is unlikely in these infants because there is negligible biliary secretion of bile acids into the gastrointestinal tract and because of the relative immaturity in bacterial colonization of the gut at this age of life (35). Formation of *allo*-bile acids has been demonstrated by a series of reactions using 5α-cholestanol as the precursor (36, 37), but in these patients cholestanol levels were not determined. It has been shown that this sterol may be formed from 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one by a novel pathway involving hepatic 7α-dehydroxylation in patients with cerebrotendinous xanthomatosis (38). In these infants however, *allo*-chenodeoxycholic and *allo*-cholic acid are most probably formed from 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one, the normal substrates for the Δ^4-3-oxosteroid 5β-reductase. Björkhem and Einarsson have demonstrated that in rats these two precursors, in the presence of NADPH, can be converted to the corresponding 3-oxo-5α analogues by liver microsomes (39), and these can then be efficiently converted to 3α-hydroxy-5α-steroids in the cytosol. In rats, the capacity of the microsomal Δ^4-3-oxosteroid 5α-reductase is high, but the quantitative importance of this pathway in man is unclear. It is probable that in the absence of an active Δ^4-3-

oxosteroid 5β-reductase as appears the case in these infants, the Δ^4-3-oxosteroid 5α-reductase becomes quantitatively important in the metabolic handling of the increased amounts of C_{27} Δ^4-3-one intermediates formed, thereby leading to significant levels of *allo*-bile acids.

The presence of high concentrations of chenodeoxycholic in plasma, requires explanation. Chenodeoxycholic acid is the major bile acid formed in the fetus during early gestation (2, 3), but at term in the healthy neonate, cholic acid concentrations exceed those of chenodeoxycholic acid (40). This may be due to an immaturity in the separate pathway for cholic acid synthesis involving a microsomal 12α-hydroxylase and/or because of a suppression of the activity of this enzyme by other bile acids that accumulate in this condition. An alternative possibility is that chenodeoxycholic is formed via the pathway, proposed by Yamasaki et al. (41), which involves initial formation of 26-hydroxycholesterol (42–44). If this were the case, significant amounts of lithocholate and 3β-hydroxy-5-cholenoate might be anticipated, but this was not observed in these infants. In the early studies on the isolation and partial purification of the Δ^4-3-oxosteroid 5β-reductase from rat liver it was suggested that the reduction of the double bond in 7α,12α-dihydroxy-cholest-4-en-3-one and 7α-hydroxy-cholest-4-en-3-one may be catalyzed by separate enzymes (32). Furthermore, the activity of this enzyme preparation towards C_{19} and C_{21} steroids indicates either its heterogeneity or broad specificity. If there are several Δ^4-3-oxosteroid 5β-reductases in man then this could explain the preferential formation of chenodeoxycholic acid in these infants.

The FAB-MS analysis indicated that the principal mode of hepatic conjugation of bile acids was with taurine, which is also a characteristic feature of bile acid synthesis in the human fetus (3). There were small quantities of glycine conjugates but negligible amounts of bile acids sulphates in urine and bile. The lack of significant sulphation of bile acids is consistent with observations for urinary bile acid excretion in healthy infants (8), but contrary to the general findings in cholestatic liver disease where increase proportions of bile acid sulphates are found in serum (45) and urine (46, 47). In these patients, this observation is not surprising, since 75–92% of the total urinary bile acids possess a Δ^4-3-oxo structure thus making them unsuitable substrates for sulphate conjugation.

The mechanism by which these infants developed cholestasis is a matter of conjecture. Primary bile acids are essential to the development of bile flow. Both infants, however, were unable to synthesize adequate amounts of chenodeoxycholic acid, and cholic acid. Perhaps the increased production and/or accumulation of unsaturated Δ^4-3-oxo bile acids led to the cholestasis. Unsaturated bile acids with a 3β-hydroxy-Δ^5 configuration have been shown to be hepatoxic in animal models (48), but to our knowledge bile acids with a Δ-3-oxo structure have not been examined. The solubility of these oxo bile acids is likely to be less than the respective hydroxy analogues with the potential for precipitation and accumulation within the hepatocyte.

Early diagnosis of inborn errors of this type may be important in the clinical management of these patients. Irrespective of the exact pathogenesis of the cholestasis we suggest that oral bile acid therapy be carefully evaluated as a stimulus for bile flow and improved elimination of toxic substances, including bilirubin. Bile acid therapy may also serve to inhibit cholesterol 7α-hydroxylation, the rate-limiting step in endogenous bile acid synthesis thereby preventing the accumulation of potentially toxic Δ^4-3-oxo bile acid intermediates in a similar manner to the response seen in cerebrotendinous xanthomatosis (49, 50).

In conclusion, we describe here a new inborn error in bile acid synthesis detected in twins presenting with familial giant-cell hepatitis. It is of particular relevance that an inborn error involving a different, but early step in the synthesis of bile acids from cholesterol was recently reported in an infant with familial giant cell hepatitis (15–17) and unsaturated oxo bile acids have been reported to occur in infants with fatal liver disease (51).

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