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Bile acid N-acetylglucosaminidation. In vivo and in vitro evidence for a selective conjugation reaction of 7 beta-hydroxylated bile acids in humans.

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Research Article

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Bile Acid *N*-Acetylglucosaminidation

In Vivo and In Vitro Evidence for a Selective Conjugation Reaction of 7β-Hydroxylated Bile Acids in Humans

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Abstract

The aim of this study was to define whether *N*-acetylglucosaminidation is a selective conjugation pathway of structurally related bile acids in humans. The following bile acids released enzymatically from *N*-acetylglucosaminides were identified: 3α,7β-dihydroxy-5β-cholanoic (ursodeoxycholic), 3β,7β-dihydroxy-5β-cholanoic (isoursodeoxycholic), 3β,7β-dihydroxy-5α-cholanoic (alloisoursodeoxycholic), 3β,7β-dihydroxy-5-cholenoic, 3α,7β,12α-trihydroxy-5β-cholanoic, and 3α,6α,7β-trihydroxy-5β-cholanoic acids. The selectivity of conjugation was studied by administration of 0.5 g ursodeoxycholic (UDCA) or hyodeoxycholic (HDCA) acids, labeled with ^{13}C, to patients with extrahepatic cholestasis, and of 0.5 g of ^{13}C-labeled chenodeoxycholic acid (CDCA) to patients with extra- or intrahepatic cholestasis. After administration of [24-^{13}C]-CDCA, labeled glucosides, and the glucuronide of CDCA were excreted in similar amounts. Labeled *N*-acetylglucosaminides of UDCA and isoUDCA were also formed. When [24-^{13}C]-UDCA was given, ^{13}C-label was detected in the *N*-acetylglucosaminide, the glucosides, and the glucuronide of UDCA, and in the *N*-acetylglucosaminide of isoUDCA. In the patient studied, 32% of the total UDCA excreted in urine was conjugated with *N*-acetylglucosamine. In contrast, 96% of the excreted amount of [24-^{13}C]HDCA was glucuronidated, and ^{13}C-labeled glucosides but no *N*-acetylglucosaminide were detected. The selectivity of *N*-acetylglucosaminidation towards bile acids containing a 7β-hydroxyl group was confirmed in vitro using human liver and kidney microsomes and uridine diphosphate glucose (UDP)-*N*-acetylglucosamine. These studies show that *N*-acetylglucosaminidation is a selective conjugation pathway for 7β-hydroxylated bile acids. (*J. Clin. Invest.* 1992. 89:1981–1987.) Key words: ursodeoxycholic acid • glucosidation • glucuronidation • gas chromatography-mass spectrometry • stable isotope labeling

Introduction

The metabolism of bile acids in humans includes various conjugation reactions. Besides aminoacyl amidation with glycine or taurine (1) and sulfation (2), three glycosidic conjugation pathways have been established both in vivo and in vitro during the last years: glucuronidation (3, 4), glucosidation (5, 6), and *N*-acetylglucosaminidation (7, 8). Semiquantitative estimates indicated a similar urinary excretion rate for the three glycosidic conjugates, at least in healthy humans (6, 7, 9, 10).

The present paper gives data on urinary bile acid glycosides after oral administration of ^{13}C-labeled chenodeoxycholic (CDCA),[1] ursodeoxycholic (UDCA), and hyodeoxycholic (HDCA) acids to patients with cholestatic liver diseases. The results illustrate the structural preferences of the different glycosidation pathways. Determinations of structures of bile acids conjugated in vivo together with enzymatic data in vitro provide evidence for a selective conjugation of 7β-hydroxylated bile acids like UDCA with *N*-acetylglucosamine. This selective metabolic pathway may help to explain the mechanism of the beneficial effect of UDCA in patients with cholestatic liver diseases.

Methods

Materials. The sources and the purity of most materials are described elsewhere (10, 11). Alloursodeoxycholic acid and methyl-3,7-diketo-5α-cholanoate were kind gifts of Drs. H. Iida (Nihon University, Fukushima, Japan) and W. H. Elliott (St. Louis University Medical School, St. Louis, MO), respectively. Isoursodeoxycholic acid synthesized by Dr. F. C. Chang was obtained from Dr. Bader's Library of Rare Chemicals (Aldrich Chemical Co., Milwaukee, WI). [24-^{13}C]CDCA, [24-^{13}C]UDCA, and [24-^{13}C]HDCA acids were synthesized in a modification (12) of the method by Tserng and Klein (13), giving a ^{13}C excess of 89±2 atom %, calculated (14) from the main fragment ions containing the side chain.

Structure analysis of bile acid N-acetylglucosaminides. Extraction, purification, and characterization of bile acid *N*-acetylglucosaminides was performed using modifications of previously described methods (7, 11). A flow scheme is shown in Fig. 1. In short, total bile acids were extracted from multiple 24 h collections of urine with Sep-Pak octadecylsilane-bonded silica (C_{18}) cartridges and separated by anion exchange chromatography on Lipidex-diethylaminohydroxypropyl (DEAP). A known amount of [24-^{14}C]cholic acid was added to urine to monitor separations and recoveries. The first fraction containing unconjugated bile acids and nonamidated bile acid glucosides and *N*-ace-

Preliminary data of this study were presented at the 32nd IUPAC Congress, 2–7 August, 1989, Stockholm, Sweden, at the XI International Bile Acid Meeting, 11–13 October, 1990, Freiburg, Germany, and at the 41st Annual Meeting of the AASLD, 3–6 November, 1990, Chicago, IL.

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1. *Abbreviations used in this paper:* alloisoUDCA, alloisoursodeoxycholic acid; alloUDCA, alloursodeoxycholic acid; CDCA, chenodeoxycholic acid; C_{18}, octadecylsilane-bonded silica; DEAP, diethylaminohydroxypropyl; FABMS, fast atom bombardment mass spectrometry; GC/MS, gas chromatography-mass spectrometry; HDCA, hyodeoxycholic acid; isoUDCA, isoursodeoxycholic acid; M/Z, mass/charge ratio; RI, retention index; 7-epiCA, 7-epicholic acid; 7-epiHCA, 7-epihyocholic acid; TMS, trimethylsilyl; UDCA, ursodeoxycholic acid; UDP, uridine diphosphate glucose.

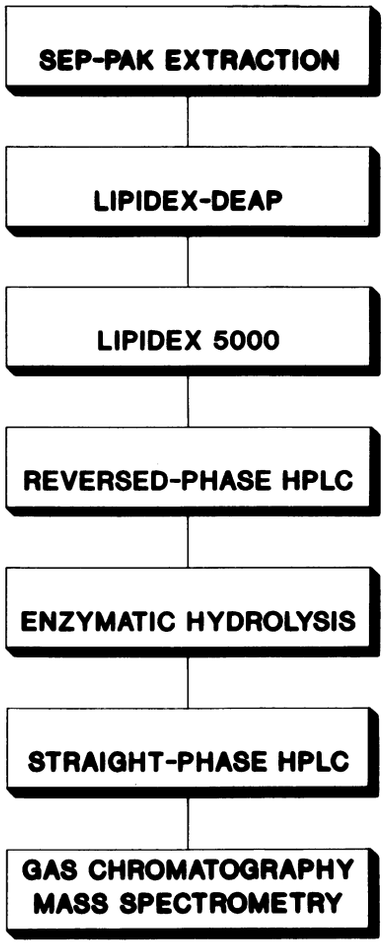

Figure 1. Flow scheme for the characterization of bile acid *N*-acetylglucosaminides from human urine.

tylglucosaminides was eluted with 0.1 M acetic acid in 70% ethanol and methylated with diazomethane. Methyl esters of unconjugated bile acids were removed on Lipidex 5000 yielding a fraction containing bile acid glucosides and *N*-acetylglucosaminides. These conjugates were fractionated by reversed-phase HPLC on a steel column (μBondapak C_{18}; Waters Assoc., Milford, MA), using a methanol/water gradient (11) without acetic acid. The methyl esters of ^{14}C-labeled tracers and bile acid glycosides eluted ~ 10 ml later than the respective free acids (7, 11). Bile acid glycosides were located by gas-liquid chromatography (GLC), using column *A* (see below) after conversion into trimethylsilyl (TMS) ether derivatives. The methyl esters of bile acid *N*-acetylglucosaminides were dissolved in 100 μl methanol and hydrolyzed with 1 U/ml *N*-acetylglucosaminidase (Boehringer Mannheim, Mannheim, Germany) in 50 mM sodium citrate, pH 4.5, in a total volume of 50 ml at 37°C for 16 h. Liberated bile acid methyl esters were further separated by normal-phase HPLC on a silanol column (LiChrosorb DIOL; E. Merck, Darmstadt, Germany). The equipment was the same as for reversed-phase HPLC, elution conditions were as follows: isocratic elution for 40 min with hexane/2-propanol (95:5, vol/vol), followed by a linear gradient of 5–45% 2-propanol in hexane over a 40-min period. Fractions of 1 ml were collected at a flow rate of 1 ml/min. The bile acid methyl esters were located by GLC and were analyzed by gas chromatography-mass spectrometry (GC/MS) using columns *A* and *B* (see below) after converting into TMS ether derivatives. In addition, unsaturated bile acid methyl esters were hydrogenated with H_2/PtO_2 in 200 μl glacial acetic acid (15). Hydroxyl groups were oxidized with chromic acid (Jones reagent) in acetone at 0°C (16). The methyl esters of the oxo bile acids obtained were dissolved in acetone and analyzed by GLC and GC/MS using column *A* (see below).

Patients studied. Clinical and relevant routine laboratory data are given in Table I. One patient (*A*) with intrahepatic cholestasis due to alcoholic liver disease and another patient (*B*) with extrahepatic cholestasis were given 0.5 g of [24-^{13}C]CDCA. Two other patients with extrahepatic cholestasis obtained 0.5 g of [24-^{13}C]UDCA (*C*), and [24-^{13}C]-HDCA (*D*), respectively. Informed consent was given by all patients. The study was carried out in accordance with the Helsinki Declaration II and was approved by the local ethics committee.

Analysis of bile acids in urine after oral administration of ^{13}C-labeled bile acids. Total bile acids were extracted from 24-h collections of urine by repetitive use of Sep-Pak C_{18} cartridges. Group separation of bile acids by anion-exchange chromatography on Lipidex-DEAP was performed by a modification of the original method of Almé et al. (17) for the estimation of glycosidic bile acid conjugates (10). Three fractions are obtained: (*a*) unconjugated bile acids and nonamidated bile acid glucosides and *N*-acetylglucosaminides; (*b*) glycine- or taurine-conjugated bile acids and glycine- or taurine-conjugated bile acid glucosides; and (*c*) bile acid glucuronides. In addition, a fraction (*d*) containing bile acid mono- and disulfates, was collected by elution with 15 ml 0.3 M ammonium acetate, pH 9.6, in 70% ethanol (17). Fraction (*b*) was treated with cholylglycine hydrolase yielding deamidated bile acids and bile acid glucosides (10), and fraction (*c*) was hydrolyzed with cholylglycine hydrolase and the digestive juice of *Helix pomatia* yielding deamidated and deglucuronidated bile acids (GlcA in Table II). Fraction (*d*) was solvolysed with trifluoroacetic acid in tetrahydrofuran (18) and treated with cholylglycine hydrolase (Sulfates in Table II). After extraction with Sep-Pak C_{18} and methylation, fractions (*a*) and (*b*) were further subfractionated on Lipidex 5,000 with chloroform/hexane 1:4–1:1 (vol/vol) (19), yielding unconjugated bile acids, bile acid glucosides, and *N*-acetylglucosaminides from fraction (*a*) (Uncon, Glc, and GlcNAc in Table III), and deamidated bile acids and deamidated bile acid glucosides from fraction (*b*) (G/T and G/T-Glc in Table II). The methyl esters of bile acid *N*-acetylglucosaminides were hydrolyzed with *N*-acetylglucosaminidase as described above. After conversion to TMS ether derivatives, nonamidated and amidated glucosides (Glc and G/T-Glc) were analyzed by GLC and GC/MS using column *A*, while both columns *A* and *B* were used for all other fractions (Uncon, GlcNAc, G/T, GlcA, and Sulfates).

GLC and mass-spectrometry. GLC, GC/MS, and fast atom bombardment mass spectrometry (FABMS) were performed using the equipment and conditions previously described (11). Column *A* was a 24 m × 0.32 mm ID fused silica capillary coated with crosslinked

Table I. Clinical and Relevant Routine Laboratory Data Obtained Immediately Before Oral Administration of ^{13}C-labeled Bile Acids to the Patients Studied

Patient	Disease	Bilirubin	Alkaline phosphatase	γ-Glutamyl transpeptidase
		mg/dl	*U/liter*	*U/liter*
		(0.1–1.2)	(30–115)	(<30)
A	Alcoholic liver cirrhosis	3.0	181	40
B	Carcinoma of the head of the pancreas	12.7	786	288
C	Carcinoma of the ampulla of vater	21.7	304	125
D	Carcinoma of the gallbladder	14.0	420	289

The normal ranges are given in parentheses.

Table II. Urinary Excretion (μg/24 h) of Bile Acids during the First 24 h after Oral Administration of 0.5 G of ^{13}C-labeled Bile Acids

Patient	Bile acid	Urinary bile acid excretion (μg · 24 h^{-1})						
		Uncon	Glc	GlcNAc	G/T	G/T-Glc	GlcA	Sulfates
A*	Total	272	176	459	7065	935	653	10184
	(% of total)	(1.4)	(0.9)	(2.3)	(35.8)	(4.7)	(3.3)	(51.6)
	CDCA	28	88	ND	1051	552	451	6926
	(% ^{13}C)	(19)	(7)		(5)	(5)	(7)	(6)
	HDCA	ND	ND	ND	14	7	39	40
	UDCA	4	45	53	578	11	3	173
	(% ^{13}C)	(19)	(49)	(47)	(45)	(63)	(63)	(55)
B	Total	92	76	741	3235	609	1259	18838
	(% of total)	(0.4)	(0.3)	(3.0)	(13.0)	(2.5)	(5.1)	(75.8)
	CDCA	2	18	ND	538	137	451	14887
	(% ^{13}C)	(46)	(9)		(5)	(5)	(3)	(5)
	HDCA	ND	22	ND	20	3	168	63
	UDCA	4	4	23	113	9	8	822
	(% ^{13}C)	(9)	(5)	(12)	(15)	(5)	(12)	(19)
C	Total	97	194	1785	7025	1482	1419	10610
	(% of total)	(0.3)	(0.9)	(7.9)	(31.1)	(6.6)	(6.3)	(46.9)
	CDCA	2	40	ND	384	352	744	6257
	HDCA	ND	26	ND	21	3	34	157
	UDCA	7	113	1517	1647	207	212	1062
	(% ^{13}C)	(90)	(60)	(65)	(72)	(72)	(72)	(62)
D	Total	379	1729	456	4176	1764	46177	15369
	(% of total)	(0.5)	(2.5)	(0.7)	(6.0)	(2.5)	(65.9)	(21.9)
	CDCA	5	7	ND	157	215	1902	6537
	HDCA	79	1418	ND	244	18	42483	17
	(% ^{13}C)	(89)	(87)		(80)	(86)	(90)	(79)
	UDCA	ND	127	42	40	4	115	150

The extent of ^{13}C-labeling is given as atom percent excess. Abbreviations: [^{13}C]CDCA, [^{13}C]HDCA, [^{13}C]UDCA, ^{13}C-labeled chenodeoxycholic, hyodeoxycholic, and ursodeoxycholic acids, respectively; Uncon, unconjugated bile acids; Glc, GlcNAc, GlcA, bile acid glucosides, *N*-acetylglucosaminides and glucuronides, respectively; G/T, glycine- or taurine-conjugated bile acids; G/T-Glc, glycine- or taurine-conjugated bile acid glucosides; ND, not detected. * Patient A and B received [^{13}C]CDCA, patient C 0.5 g [^{13}C]UDCA, and patient D 0.5 g [^{13}C]HDCA.

methyl silicone (film thickness 0.25 μm; Quadrex Corp., New Haven, CT). Column *B* was a 25 m × 0.32 mm ID polar fused silica capillary (Unicoat UC-1625) coated with silicone gum containing 25% 4-phenoxyphenyl, 2% vinyl, and 73% methyl groups (KSV Chemicals, Helsinki, Finland). For GLC, the samples were injected on column in 1 μl hexane at 60°C. The temperature of column *B* was then taken to 280°C at 30°C/min in analyses of liberated bile acids, while column *A* was taken to 300°C at 30°C/min in analyses of nonamidated and amidated bile acid glucosides and *N*-acetylglucosaminides. Bile acid derivatives were identified by comparisons with authentic compounds and by typical mass spectra. They were quantitated from peak areas as related to that given by the internal standard cholestane. Derivatives of bile acid glucosides were identified by comparison with the derivatives of enzymatically prepared reference compounds (6, 7) and were quantitated from peak areas using the TMS derivative of deoxycorticosterone glucoside as standard (kindly donated by Dr. R. Neher, Ciba Corp., Basel, Switzerland). Retention indices were calculated in relation to the *n*-alkanes C_{30} and C_{36} at 280°C, and C_{40}, C_{44}, and C_{46} at 300°C, respectively. For GC/MS, a falling needle injection system was used and the samples were analyzed isothermally at 280°C (column *B*) and 300°C (column *A*), respectively. The ^{13}C content of dihydroxy bile acid glycosides was calculated as described (12). The ^{13}C content of TMS ethers of liberated methyl dihydroxy cholanoates was determined from m/z 369, 370, 371, and 372. Negative ion FAB spectra of *N*-acetylglucosaminides of UDCA and isoUDCA synthesized in vitro were recorded as described (7, 11).

Synthesis of bile acid N-acetylglucosaminides in vitro. Activities of *N*-acetylglucosaminyltransferase from human liver and kidney microsomes were determined as previously described (8). The following modifications were used for the synthesis of *N*-acetylglucosaminides of UDCA and isoUDCA in amounts suitable for structural analysis: ~ 4 mg of microsomal protein prepared as described (20) were incubated for 1 h at 37°C in 18 ml reaction mixture containing 3 mM UDP-*N*-acetyl-D-glucosamine, 0.1 M sodium acetate (pH 6.2), 2 mM $MgCl_2$, 0.002% Brij, 0.05 mM ADP, and 0.1 mM UDCA and isoUDCA, respectively. After extraction with Sep-Pak C_{18} cartridges, the *N*-acetylglucosaminides of UDCA and isoUDCA were purified by chromatography on Lipidex-DEAP and reversed-phase HPLC, and analyzed by GLC, GC/MS, and negative-ion mode FABMS (7, 11).

Results

Structure analysis of bile acid N-acetylglucosaminides

Bile acid *N*-acetylglucosaminides from urine of three healthy subjects were purified on Lipidex-DEAP and, after methylation, on Lipidex 5000. They were localized and partially characterized by GC/MS giving the typical fragments m/z 173 and 186 from the derivatized *N*-acetylglucosamine moiety, m/z 461 and 371 from saturated dihydroxy, m/z 459 and 369 from unsaturated dihydroxy, and m/z 549, 459, and 369 from saturated trihydroxy bile acid derivatives (6, 7). Table III gives the

Table III. Chromatographic and Structural Characteristics of the Intact Bile Acid N-Acetylglucosaminides from Human Urine and of the Bile Acids Liberated by N-Acetylglucosaminidase

Bile acid *N*-acetylglucosaminides			Bile acids liberated by *N*-acetylglucosaminidase			
RP-HPLC	GLC-RI column	Bile acid GlcNAc	SP-HPLC	GLC-RI column		Bile acid structure
ml	*A*		*ml*	*A*	*B*	
50–51	4402	B-diol	64–65	3260	3350	7-epiCA
42–44	4408	B-triol	27–32	3230	3405	isoUDCA
48–50	4436	B-diol	31–32	3230	3449	UDCA
48–52	4438	B-triol	64–65	3291	3449	B-triol
49–50	4463	B^{Δ}-diol	36–37	3282	3485	B-diol
49–52	4488	B^{Δ}-diol	41–42	3282	3485	B^{Δ}-diol
45–47	4502	B-triol	42–43	3288	3507	B^{Δ}-diol
42–44	4512	B-triol	41–42	3289	3518	$3\beta,7\beta$-$^{\Delta 5}$
46–47	4528	B^{Δ}-diol	64–65	3407	3549	7-epiHCA
50–51	4535	B-diol	35–39	3346	3572	alloisoUDCA
49–52	4552	B-diol	64–65	3365	3609	B-triol

B, cholan-24-oic acid; $^{\Delta}$, double bond; GlcNAc, *N*-acetylglucosamine; RP-HPLC and SP-HPLC, reversed-phase and normal-phase HPLC; GLC-RI, retention indices in GLC as methyl ester TMS ether derivatives on columns *A* and *B* (see Methods); $3\beta,7\beta$-$^{\Delta 5}$, $3\beta,7\beta$-dihydroxy-5-cholenoic acid.

retention indices and proposed structures for derivatized bile acid *N*-acetylglucosaminides isolated by reversed-phase HPLC. These conjugates were quantitatively cleaved with *N*-acetylglucosaminidase giving rise to four major peaks at RI:s 3,287, 3,350, 3,356, and 3,397 representing the methyl ester TMS ether derivatives of two saturated and two unsaturated dihydroxy bile acids (7). Using the same column at a final temperature of 280°C the retention index (RI):s were 3,230, 3,282, 3,288, and 3,346, respectively. This modification shifted the RI of derivatized cholic acid from 3,275 (7) to 3,210 and permitted a better comparison with data from a comprehensive study on the separation of isomeric bile acid derivatives on different GLC columns (Ichimiya, I., M. Axelson, and J. Sjövall, manuscript submitted for publication). Detailed GC/MS studies of the four major peaks indicated the presence of more than four compounds incompletely separated on the methyl silicone column. The derivatives of ursodeoxycholic and isoursodeoxycholic acids, which were eluted together at RI 3,230 on the methyl silicone column separated on UC-1,625. The derivatives of two unsaturated bile acids coeluted at RI 3,288 on the methyl silicone column but at RI 3,507 and 3,518, respectively, on UC-1,625. On the polar column the peak of derivatized UDCA at RI 3,449 contained a derivatized trihydroxy bile acid, and the peak at RI 3,485 also represented a mixture. For this reason, an additional separation of the bile acid methyl esters by normal-phase HPLC was performed. Table III gives the fractions of elution upon normal-phase HPLC, the retention indices on UC-1,625, and the proposed structures for the bile acids obtained after hydrolysis with *N*-acetylglucosaminidase. Three saturated and one unsaturated dihydroxy and two saturated trihydroxy bile acids conjugated with *N*-acetylglucosamine were identified.

$3\beta,7\beta$-dihydroxy-5β-cholanoic and $3\alpha,7\beta$-dihydroxy-5β-cholanoic acids (isoursodeoxycholic and ursodeoxycholic acids). The isoursodeoxycholic and ursodeoxycholic acids were identified by comparison with authentic bile acids. In addition, the configuration at C-5 was determined by chromic acid oxidation and comparison of the dioxocholanoates formed with authentic methyl 3,7-dioxo-5β-cholanoate (RI 3,326 on methyl silicone). IsoUDCA and UDCA constituted ~ 5–10% and 10–15%, respectively, of the total bile acid *N*-acetylglucosaminides.

$3\beta,7\beta$-dihydroxy-5α-cholanoic acid (alloisoursodeoxycholic acid). The derivative of $3\beta,7\beta$-dihydroxy-5α-cholanoic acid (Fig. 2) had RI:s of 3,346 on the methyl silicone and 3,572 on the UC-1625 columns. Chromic acid oxidation yielded methyl 3,7-dioxo-5α-cholanoate, with an RI of 3403 on methyl silicone (Fig. 3). The mass spectrum of this compound was identical to that of authentic methyl 3,7-dioxo-5α-cholanoate (21). The $3\beta,7\beta$-positions of the hydroxyl groups were characterized by the shift of RI:s in comparison with the RI:s of the derivatives of authentic $3\alpha,7\alpha$- and $3\alpha,7\beta$-dihydroxy-5α-cholanoic acids (3,184 and 3,297 on the methyl silicone, and 3,243 and 3,456 on the UC-1,625 column, respectively) as well as the derivatives of authentic 3α-hydroxy-5α-cholanoic and 3β-hydroxy-5α-cholanoic acids (3,120 and 3,170 on the methyl silicone, and 3,364 and 3,494 on the UC-1,625 column, respectively). AlloisoUDCA constituted 20–25% of the total bile acid *N*-acetylglucosaminides.

$3\beta,7\beta$-dihydroxy-5-cholenoic acid. The TMS ether of methyl $3\beta,7\beta$-dihydroxy-5-cholenoate gave a mass spectrum very similar to that of the derivative of $3\beta,7\alpha$-dihydroxy-5-cholenoic acid (15). However, the retention indices of the $3\beta,7\beta$-dihydroxy-5-cholenoic acid derivative were about 80 and 120

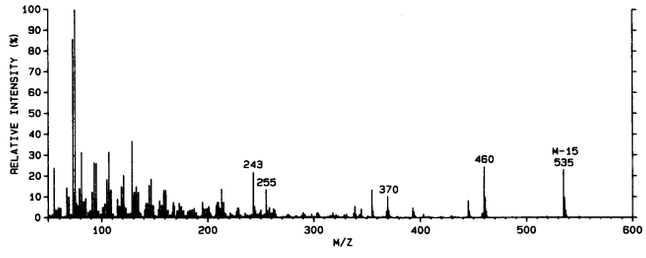

Figure 2. Electron impact mass spectrum of the methyl ester TMS ether derivative of alloisoursodeoxycholic acid liberated from urinary bile acid *N*-acetylglucosaminides.

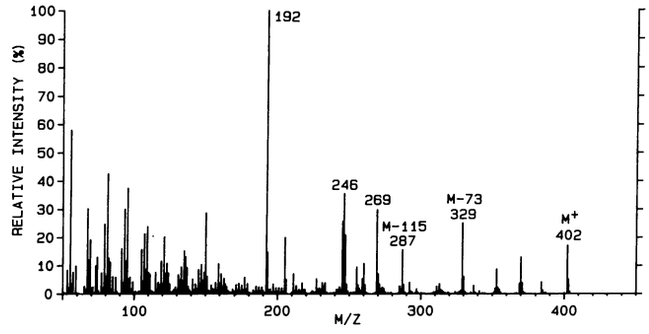

Figure 3. Electron impact mass spectrum of the methyl 3,7-dioxocholanoate obtained by chronic acid oxidation of alloisoursodeoxycholic acid liberated from urinary bile acid *N*-acetylglucosaminides.

U higher on methyl silicone and UC-1,625 columns, respectively. These RI differences are identical to those between the derivatives of 3β,7α- and 3β,7β-dihydroxy-5β-cholanoic acids confirming the 3β,7β-configuration. Hydrogenation of 3β,7β-dihydroxy-5-cholenoate gave three products: 3β-hydroxy-5β-cholanoic (isolithocholic acid), 3β-hydroxy-5α-cholanoic, and 3β,7β-dihydroxy-5α-cholanoic acids, analogous to the hydrogenation products of 3β,7α-dihydroxy-5-cholenoic acid (15). The RI:s of the derivatized hydrogenation products were 3,335, 3,507, and 3,572, respectively, on UC-1,625. The 3β,7β-dihydroxy-5-cholenoic acid and its derivatives and probably also the *N*-acetylglucosamine conjugate are very labile. Water may be eliminated during enzymatic hydrolysis and derivatization yielding a monohydroxy bile acid containing two double bonds. The methyl ester TMS ether derivative of such a product had RI:s of 3,181 on methyl silicone and 3,332 on UC-1,625, and gave a mass spectrum with a base peak at m/z 458. The hydroxyl group was lost upon hydrogenation, yielding methyl 5α-cholanoate with an RI of 3,241 on UC-1,625 identified from its typical mass spectrum (22). The 3β,7β-dihydroxy-5-cholenoic acid constituted ~ 15% of the total bile acid *N*-acetylglucosaminides.

3α,7β,12α-trihydroxy-5β-cholanoic (7-epicholic acid) and 3α,6α,7β-trihydroxy-5β-cholanoic (7-epihyocholic acid) acids. The 7-epicholic and 7-epihyocholic acids were identified by comparisons with the retention indices (see Table III) of authentic bile acids both on methyl silicone and UC-1,625 columns (Ichimiya, I., M. Axelson, and J. Sjövall, manuscript submitted for publication), the mass spectra of the authentic compounds (23) and in the case of 7-epicholic acid by chromic acid oxidation to methyl 3,7,12-trioxo-5β-cholanoate. These trihydroxy bile acids constituted 5–10% of the total bile acid *N*-acetylglucosaminides.

Unidentified bile acids. A saturated and an unsaturated dihydroxy bile acids constituting ~ 20% of the total bile acid *N*-acetylglucosaminides coeluted at RI 3,485 on UC-1,625. These compounds seem to carry hydroxyl groups in the same positions since hydrogenation of the unsaturated compound isolated by normal-phase HPLC yielded the saturated dihydroxy compound. The hydrogenation also gave a saturated monohydroxy compound, the derivative of which eluted at RI 3,231 on UC-1,625. The fragment ion m/z 243, indicative of TMS ethers of 3,7-dihydroxycholanoates (22), was particularly intense in the spectrum of the unknown saturated dihydroxycholanoate. The presence of two trimethylsiloxy groups in this ion (22, 23) was supported by the relative intensities of the isotope peaks at m/z 244 and 245. Oxidation of the saturated dihydroxy acid yielded a dioxocholanoate, the mass spectrum of which was different from available reference spectra. Thus, the structures of these two acids remain unknown.

The methyl ester TMS ether derivatives of the trihydroxy bile acids with RI:s of 3,449 and 3,609 on UC-1,625 showed structural relationships giving similar mass spectra with fragments at m/z 195, 208, 243, and 253. The three former ions were also seen in the mass spectrum of the derivatized dihydroxy bile acid with an RI of 3,485 on UC-1,625, indicative of a structural relationship. The derivatized trihydroxy bile acid with an RI of 3,325 on UC-1,625 gave a mass spectrum with m/z 217 as base peak, very similar to that of the derivative of 1β,3α,7β-trihydroxycholanoic acid (24).

Analysis of bile acid glycosides in urine after oral administration of ^{13}C-labeled bile acids

Table II shows the urinary excretion of total bile acids and of CDCA, UDCA, and HDCA during the first 24 h after oral administration of ^{13}C-labeled bile acids. The total excretion of bile acids in urine was 20–25 mg in patients A, B, and C, and about 70 mg in patient D.

[24-^{13}C]CDCA. Sulfates and glycine or taurine conjugates comprised about 90% of the urinary bile acids in the patients with intrahepatic (patient A) and extrahepatic (patient B) cholestasis. Total glycosidic conjugates constituted about 11% in both patients with similar excretion rates of nonamidated and amidated glucosides, *N*-acetylglucosaminides, and glucuronides. In patient A, CDCA constituted 50% of the total nonamidated glucosides, 60% of the total glycine- or taurine-conjugated glucosides, and 70% of the total glucuronides. In patient B, it contributed to 20% of the total nonamidated glucosides, 20% of the total glycine- or taurine-conjugated glucosides, and 70% of the glucuronides. Only minor amounts (below 10%) of ^{13}C-labeled CDCA were present in these conjugate fractions in both patients. An *N*-acetylglucosaminide of CDCA was not detectable either in patient A or in patient B. In both patients, however, the conjugates of UDCA, including the *N*-acetylglucosaminide, had a higher content of ^{13}C than the CDCA conjugates. Additionally, isoUDCA *N*-acetylglucosaminide (12 μg/24 h) with a ^{13}C excess of 42 atom % was found in the urine from patient A.

[24-^{13}C]UDCA. Sulfates and glycine- or taurine-conjugates constituted 78% and glycosides 22% of the urinary bile acids in patient C. UDCA was excreted as glycine or taurine conjugates (35%) and sulfates (22%). Only 2% of UDCA was excreted as glucoside (60% of total nonamidated glucosides), 4% as glycine- or taurine-conjugated glucoside (15% of total glycine- or taurine-conjugated glucosides), and 4% as a glucuronide (15% of total glucuronides). 32% of UDCA was excreted as *N*-acetylglucosaminide constituting 85% of the total *N*-acetylglucosaminides. The ^{13}C excess among the UDCA conjugates was 60–72 atom % for amidated and nonamidated glucosides, 52 atom % for the *N*-acetylglucosaminide, and 33 atom % for the glucuronide. The isoUDCA *N*-acetylglucosaminide (21 μg/24 h) had a ^{13}C excess of 45 atom % and small amounts of labeled glycine and taurine conjugates of isoUDCA were also detected.

[24-^{13}C]HDCA. Glycosidic conjugates constituted 72% of the urinary bile acids in patient D. Sulfates and glycine or taurine conjugates constituted 28% of the urinary bile acids. 96% of HDCA was excreted as a glucuronide (90% of the total glucuronides) and 3.2% was glucosidated (82% of total nonamidated glucosides). The ^{13}C excess of the glucosides and the gluc-

uronide of HDCA were 86–90 atom %, and not significantly different from that of the administered compound. An *N*-acetylglucosaminide of HDCA was not detected.

Bile acid N-acetylglucosaminides synthesized in vitro

Bile acid *N*-acetylglucosaminides were synthesized in vitro by incubation of human kidney or liver microsomes with UDP-*N*-acetylglucosamine and bile acids containing 7β-hydroxyl groups. Bile acid *N*-acetylglucosaminyltransferase activity was not detectable towards common primary and secondary bile acids, with the exception of hyodeoxycholic acid which was converted at a rate of less than 2 pmol · min^{-1} · mg $protein^{-1}$ (8). The highest activities were observed with isomers of ursodeoxycholic acid (Table IV).

The *N*-acetylglucosaminides of UDCA and isoUDCA were synthesized enzymatically in amounts sufficient for analysis by GLC and GC/MS. The methyl ester TMS ether derivatives of the *N*-acetylglucosaminides of UDCA and isoUDCA had the same RI of 4,438 as the *N*-acetylglucosaminide of [24-^{13}C]-UDCA from the urine of patient C, and showed the typical fragmentation pattern for derivatized dihydroxy bile acid *N*-acetylglucosaminides in GC/MS (7). The molecular weights of these compounds were confirmed by FABMS, showing a quasimolecular negative ion at m/z 594.

Discussion

Since the first report in 1975 (25), UDCA has been used successfully for the dissolution of cholesterol gallstones (26). More recently, UDCA has been shown to significantly improve the clinical and biochemical indices in patients with primary biliary cirrhosis (27). Beneficial effects have also been observed in other cholestatic diseases like sclerosing cholangitis, chronic hepatitis, biliary atresia, and cystic fibrosis [recently reviewed in 28]. However, little is known about the mechanism(s) of improvement of the liver function by UDCA. The lowered pool size of possibly hepatotoxic bile acids like CDCA has been suggested as an explanation (29), as well as a choleretic effect of UDCA (30). A decrease of primary bile acids in serum (31–33) and urinary excretion (32, 33) has been demonstrated in patients with primary biliary cirrhosis during treatment with UDCA. However, in a recent study, UDCA did not lower the pool size of CDCA in serum of cholestatic patients (34). Displacement of hydrophobic, more hepatotoxic bile acids by the hydrophilic UDCA (35) in bile has been discussed as an explanation, but this theory has also been questioned (36). In vitro, a direct hepatoprotective effect of UDCA, has been observed (37).

Table IV. Bile Acid Substrate Specificity of N-Acetylglucosaminyltransferases

Bile acid	Enzyme activity (pmol · min^{-1} × mg $protein^{-1}$)	
	Kidney	Liver
3β,7β-dihydroxy-5β-cholanoic acid (isoUDCA)	67.5	45.9
3α,7β-dihydroxy-5α-cholanoic acid (alloUDCA)	22.3	26.3
3α,7β-dihydroxy-5β-cholanoic acid (UDCA)	13.5	8.1
3α,7β-dihydroxy-5β-cholanoyltaurine (TUDCA)	6.8	1.6
3α,7β-dihydroxy-5β-cholanoylglycine (GUDCA)	2.6	0.4

The data from this and a previous (7) study show that UDCA is among those few bile acids that are conjugated with *N*-acetylglucosamine. Additional bile acids released from the *N*-acetylglucosaminide conjugates were identified as isoursodeoxycholic, alloisoursodeoxycholic, 3β,7β-dihydroxy-5-cholenoic, 7-epicholic, and 7-epihyocholic acids, all containing a 7β-hydroxyl group. Other common primary or secondary bile acids were not detected, but two major dihydroxy acids remain unidentified. Use of both polar and nonpolar GLC columns was important for the elucidation of the complexity of the mixture. The need for polar GLC columns to separate UDCA and isoUDCA has recently been demonstrated in studies of bile acid profiles in plasma or urine after oral administration of UDCA (24, 33, 38–40).

The labeling of urinary bile acids after oral administration of ^{13}C-labeled CDCA confirm the epimerization of CDCA to UDCA during the enterohepatic circulation (41–44). The low content of ^{13}C in the conjugates of CDCA, compared with the conjugates of UDCA, is probably due to an epimerization of the orally given CDCA before equilibration with the pool of CDCA, and to the different sizes of the CDCA and UDCA pools. Oral administration of ^{13}C-labeled UDCA confirmed the isomerization of UDCA to isoUDCA (24, 33, 38–40). In the patient with intrahepatic cholestasis the ^{13}C-labeled CDCA was converted via UDCA to isoUDCA *N*-acetylglucosaminide. An intact enterohepatic circulation seems to be necessary for this two-step isomerization since ^{13}C-labeled isoUDCA *N*-acetylglucosaminide was not found in the urine of the patient with extrahepatic cholestasis given ^{13}C-labeled CDCA.

An important observation in the present study is the high excretion rate of *N*-acetylglucosaminides of isoUDCA and UDCA. In the patient given UDCA, it exceeded the rate of excretion of the corresponding sulfates. This indicates that the urinary excretion of UDCA and isoUDCA is higher than previously realized, since the presence of glucosides or *N*-acetylglucosaminides has not been considered in published studies. However, large amounts of nonsulfated, nonglucuronidated urinary UDCA have been detected in all cases (33, 36, 39).

Our study further emphasizes the specificity in conjugation reactions. It is well established that the major part of the bile acid glucuronides in urine are 6-hydroxylated (9), and that HDCA and other 6-hydroxy bile acids are efficiently glucuronidated at position C-6 (6, 16, 45, 46). The glucuronide of orally administered ^{13}C-HDCA represented almost 70% of the total urinary bile acids, which confirms the previous observations using ^{14}C-labeled HDCA (47). Thus, glucuronidation is a conjugation pathway with preference for 6-hydroxylated bile acids. The structures of the bile acids identified in the present study indicate that conjugation with *N*-acetylglucosamine is a selective pathway for 7β-hydroxylated bile acids. This is supported by the in vitro formation of *N*-acetylglucosaminides of 7β- but not 7α-hydroxylated bile acids using both kidney and liver microsomes. The possibility that this conjugation reaction is related to the physiological and therapeutical effects of UDCA observed in patients with primary biliary cirrhosis remains to be studied. It could be speculated that UDCA, by competing for the hepatic UDP-*N*-acetylglucosamine, could decrease the availibility of this intermediate for other reactions, such as for-

mation of glycoproteins, and thereby affect important biological functions, e.g., of cell membranes.

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References

1. Killenberg, P. G., and J. T. Jordan. 1978. Purification and characterization of bile acid-CoA:amino acid *N*-acyltransferase from rat liver. *J. Biol. Chem.* 253:1005–1010.

2. Palmer, R. H. 1967. The formation of bile acid sulfates: a new pathway of bile acid metabolism in humans. *Proc. Natl. Acad. Sci. USA.* 58:1047–1050.

3. Back, P., K. Spaczynski, and W. Gerok. 1974. Bile salt glucuronides in urine. *Hoppe-Seyler's Z. Physiol. Chem.* 355:749–752.

4. Matern, H., S. Matern, Ch. Schelzig, and W. Gerok. 1980. Bile acid UDP-glucuronosyltransferase from human liver. Properties and studies on aglycone substrate specificity. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 118:251–254.

5. Matern, H., S. Matern, and W. Gerok. 1984. Formation of bile acid glucosides by a sugar nucleotide-independent glucosyltransferase isolated from human liver microsomes. *Proc. Natl. Acad. Sci. USA.* 81:7036–7040.

6. Marschall, H.-U., B. Egestad, H. Matern, S. Matern, and J. Sjövall. 1987. Evidence for bile acid glucosides as normal constituents in human urine. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 213:411–414.

7. Marschall, H.-U., B. Egestad, H. Matern, S. Matern, and J. Sjövall. 1989. *N*-Acetylglucosaminides. A new type of bile acid conjugate in man. *J. Biol. Chem.* 264:12989–12993.

8. Matern, H., R. Bolz, H.-U. Marschall, J. Sjövall, and S. Matern. 1990. Bile acid *N*-acetylglucosaminides. Formation by microsomal *N*-acetylglucosaminyltransferases in human liver and kidney. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 270:11–14.

9. Almé, B., and J. Sjövall. 1980. Analysis of bile acid glucuronides in urine. Identification of 3α,6α,12α-trihydroxy-5β-cholanoic acid. *J. Steroid Biochem.* 13:907–91.

10. Wietholtz, H., H.-U. Marschall, R. Reuschenbach, H. Matern, and S. Matern. 1991. Urinary excretion of bile acid glucosides and glucuronides in extrahepatic cholestasis. *Hepatology.* 13:656–662.

11. Marschall, H.-U., G. Green, B. Egestad, and J. Sjövall. 1988. Isolation of bile acid glucosides and *N*-acetylglucosaminides from human urine by ion-exchange chromatography and reversed-phase high-performance liquid chromatography. *J. Chromatogr.* 452:459–468.

12. Matern, S., H.-U. Marschall, A. Schill, B. Schumacher, W. Lehnert, J. Sjövall, and H. Matern. 1991. Synthesis of ^{13}C labeled chenodeoxycholic, hyodeoxycholic, and ursodeoxycholic acids for the study of bile acid metabolism in liver disease. *Clin. Chim. Acta.* 203:77–90.

13. Tserng, K.-Y., and P. D. Klein. 1977. An improved synthesis of 24-^{13}C-labeled bile acids using formyl esters and a modified lead tetraacetate procedure. *J. Lipid Res.* 18:400–403.

14. Biemann, K. 1962. Mass Spectrometry. Organic Chemical Applications. McGraw-Hill Inc., New York. 223–227.

15. Clayton, P. T., J. V. Leonhard, A. M. Lawson, K. D. R. Setchell, S. Anderson, B. Egestad, and J. Sjövall. 1987. Familial giant cell hepatitis associated with synthesis of 3β,7α-dihydroxy- and 3β,7α,12α-trihydroxy-5-cholenoic acids. *J. Clin. Invest.* 79:1031–1038.

16. Marschall, H.-U., H. Matern, B. Egestad, S. Matern, and J. Sjövall. 1987. 6α-Glucuronidation of hyodeoxycholic acid by human liver, kidney and small bowel microsomes. *Biochim. Biophys. Acta.* 921:392–397.

17. 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.* 18:339–362.

18. Hirano, Y., H. Miyazaki, S. Higashidate, and F. Nakayama. 1987. Analysis of 3-sulfated and nonsulfated bile acids by one-step solvolysis and high performance liquid chromatography. *J. Lipid Res.* 28:1524–1529.

19. Bremmelgaard, A., and J. Sjövall. 1980. Hydroxylation of cholic, chenodeoxycholic, and deoxycholic acids in patients with intrahepatic cholestasis. *J. Lipid Res.* 21:1072–1081.

20. Matern, S., H. Matern, E. H. Farthmann, and W. Gerok. 1984. Hepatic and extrahepatic glucuronidation of bile acids in man. Characterization of bile acid uridine 5'-diphosphate-glucuronosyltransferase in hepatic, renal, and intestinal microsomes. *J. Clin. Invest.* 74:402–410.

21. Mitra, M. N., and W. H. Elliott. 1968. Bile acids. XXIV. Raney nickel in the preparation of allocholanoic acids. *J. Org. Chem.* 33:2814–2818.

22. Lawson, A. M., and K. D. R. Setchell. 1988. Mass spectrometry of bile acids. *In* The Bile Acids. Chemistry, Physiology, and Metabolism. Vol. 4. K. D. R. Setchell, D. Kritchevsky, and P. P. Nair, editors. Plenum Press, New York. 167–267.

23. Sjövall, J., A. M. Lawson, and K. D. R. Setchell. 1985. Mass spectrometry of bile acids. *Methods Enzymol.* 111:63–113.

24. Koopman, B. J., B. G. Wolthers, J. C. van der Molen, G. T. Nagel, and W. Kruizinga. 1987. Abnormal urinary bile acids in a patient suffering from cerebrotendinous xanthomatosis during oral administration of ursodeoxycholic acid. *Biochim. Biophys. Acta.* 917:238–246.

25. Makino, I., K. Shinozaki, K. Yoshino, and S. Nakagawa. 1975. Dissolution of cholesterol gallstones by ursodeoxycholic acid. *Jpn. J. Gastroenterol.* 72:690–702.

26. Bachrach, W. H., and A. F. Hofmann. 1982. Ursodeoxycholic acid in the treatment of cholesterol cholelithiasis: a review. *Dig. Dis. Sci.* 27:833–856.

27. Poupon, R., R. Poupon, Y. Calmus, Y. Chrétien, F. Ballet, and F. Darnis. 1987. Is ursodeoxycholic acid an effective treatment for primary biliary cirrhosis? *Lancet.* i:834–836.

28. James, O. F. W. 1990. Ursodeoxycholic acid treatment for chronic cholestatic liver disease. *J. Hepatol.* 11:5–8.

29. Hofmann, A. F., and H. Popper. 1987. Ursodeoxycholic acid for primary biliary cirrhosis. *Lancet.* ii:398–399. (Corresp.)

30. Hofmann, A. F. 1990. Bile acid secretion, bile flow and biliary lipid secretion in humans. *Hepatology.* 12:17S–25S.

31. Chrétien, Y., R. Poupon, M. F. Gherardt, O. Chazouilleres, D. Labbe, A. Myara, and F. Trivin. 1989. Bile acid glycine and taurine conjugates in serum of patients with primary biliary cirrhosis: effect of ursodeoxycholic treatment. *Gut.* 30:1110–1115.

32. Batta, A. K., G. Salen, R. Arora, S. Shefer, G. S. Tint, J. Abroon, D. Eskreis, and S. Katz. 1989. Effect of ursodeoxycholic acid on bile acid metabolism in primary biliary cirrhosis. *Hepatology.* 10:414–419.

33. Stiehl, A., G. Rudolph, R. Raedsch, B. Möller, U. Hopf, E. Lotterer, J. Bircher, U. Fölsch, J. Klaus, R. Endele, and M. Senn. 1990. Ursodeoxycholic acid-induced changes of plasma and urinary bile acids in patients with primary biliary cirrhosis. *Hepatology.* 12:492–497.

34. Beuers, U., U. Spengler, F. M. Zwiebel, J. Pauletzki, S. Fischer, and G. Paumgartner. 1992. Effects of ursodeoxycholic acid on the kinetics of the major hydrophobic bile acids in health and in chronic cholestatic liver disease. *Hepatology* 15, In press.

35. Schölmerich, J., M. S. Becher, K. Schmidt, R. Schubert, B. Kremer, S. Feldhaus, and W. Gerok. 1984. Influence of hydroxylation and conjugation of bile salts on their membrane-damaging properties. Studies on isolated hepatocytes and lipid membrane vesicles. *Hepatology.* 4:661–666.

36. Nakagawa, M., C. Colombo, and K. D. R. Setchell. 1990. Comprehensive study of the biliary bile acid composition of patients with cystic fibrosis and associated liver disease before and after UDCA administration. *Hepatology.* 12:322–334.

37. Galle, P. R., L. Theilmann, R. Raedsch, G. Otto, and A. Stiehl. 1990. Ursodeoxycholate reduces hepatotoxicity of bile salts in primary human hepatocytes. *Hepatology.* 12:486–491.

38. Maeda, M., H. Öhama, H. Takeda, M. Yabe, M. Nambu, and T. Namihisa. 1984. Identification of 3β,7β-dihydroxy-5β-cholan-24-oic acid in serum from patients treated with ursodeoxycholic acid. *J. Lipid Res.* 25:14–26.

39. Batta, A. K., R. Arora, G. Salen, G. S. Tint, D. Eskreis, and S. Katz. 1989. Characterization of serum and urinary bile acids in patients with primary biliary cirrhosis by gas-liquid chromatography-mass spectrometry: effect of ursodeoxycholic acid treatment. *J. Lipid Res.* 30:1953–1962.

40. Beuers, U., S. Fischer, U. Spengler, and G. Paumgartner. 1991. Formation of iso-ursodeoxycholic acid during administration of ursodeoxycholic acid in man. *J. Hepatol.* 13:97–103.

41. Fedorowski, T., G. Salen, G. S. Tint, and E. Mosbach. 1979. Transformation of chenodeoxycholic acid and ursodeoxycholic acid by human intestinal bacteria. *Gastroenterology.* 77:1068–1073.

42. Hofmann, A. F. 1976. The enterohepatic circulation of bile acids in man. *Adv. Intern. Med.* 21:501–534.

43. Carey, M., and M. J. Calahane. 1988. Enterohepatic circulation. *In* The Liver: Biology and Pathobiology, 2nd ed. I. M. Arias, W. B. Jacoby, H. Popper, D. Schachter, and D. A. Shafritz, editors. Raven Press, New York. 573–616.

44. 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.

45. Radominska-Pyrek, A., P. Zimniak, Y. M. Irshaid, R. Lester, T. R. Tephly, and J. St. Pyrek. 1987. Glucuronidation of 6α-hydroxy bile acids by human liver microsomes. *J. Clin. Invest.* 80:234–241.

46. Parquet, M., M. Pessah, E. Saquet, C. Salvat, and A. Raizman. 1988. Effective glucuronidation of 6α-hydroxylated bile acids by human hepatic and renal microsomes. *Eur. J. Biochem.* 171:329–334.

47. Saquet, E., M. Parquet, M. Riottot, A. Raizman, P. Jarrige, C. Huguet, and R. Infante. 1983. Intestinal absorption, excretion, and biotransformation of hyodeoxycholic acid in man. *J. Lipid Res.* 24:604–613.