Subcellular organization of bile acid amidation in human liver: a key issue in regulating the biosynthesis of bile salts

Karianne Solaas,* Anne Ulvestad,* Odd Söreide ,† and Bengt Frode Kase[1,*]

Department of Pediatric Research* and Department of Surgery,† Rikshospitalet, N-0027 Oslo, Norway

Abstract **To extend our knowledge of how the synthesis of free bile acids and bile salts is regulated within the hepatocyte, bile acid-CoA:amino acid *N*-acyltransferase and bile acid-CoA thioesterase activities were measured in subcellular fractions of human liver homogenates. Some bile acids, both conjugated and unconjugated, have been reported to be natural ligands for the farnesoid X receptor (FXR), an orphan nuclear receptor. The conversion of [^{14}C]choloyl-CoA and [^{14}C]chenodeoxycholoyl-CoA into the corresponding tauro- and glyco-bile acids or the free bile acids was measured after high-pressure liquid radiochromatography. There was an enrichment of the *N*-acyltransferase in the cytosolic and the peroxisomal fraction. Bile acid-CoA thioesterase activities were enriched in the cytosolic, peroxisomal, and mitochondrial fractions. The highest amidation activities of both choloyl-CoA and chenodeoxycholoyl-CoA were found in the peroxisomal fraction (15–58 nmol/mg protein/min). The K_m was higher for glycine than taurine both in cytosol and the peroxisomal fraction. These results show that the peroxisomal de novo synthesis of bile acids is rate limiting for peroxisomal amidation, and the microsomal bile acid-CoA synthetase is rate limiting for the cytosolic amidation. The peroxisomal location may explain the predominance of glyco-bile acids in human bile. Both a cytosolic and a peroxisomal bile acid-CoA thioesterase may influence the intracellular levels of free and conjugated bile acids.**—Solaas, K., A. Ulvestad, O. Söreide, and B. F. Kase. **Subcellular organization of bile acid amidation in human liver: a key issue in regulating the biosynthesis of bile salts.** ***J. Lipid Res.*** **2000.** 41: **1154–1162.**

Supplementary key words bile acid-CoA:amino acid *N*-acyltransferase • peroxisomes • choloyl-CoA • chenodeoxycholoyl-CoA • taurine • glycine • bile acid-CoA thioesterase • farnesoid X receptor

Excretion of bile by the liver is of fundamental biological importance in vertebrates. In humans, bile acids conjugated with glycine and taurine are the major solutes in bile, and unconjugated bile acids are almost nondetectable in normal bile (1). Conjugated bile acids are less toxic and are more efficient promoters of intestinal absorption of dietary lipid than unconjugated bile acids (2).

Current knowledge of the synthesis of bile acid–amino acid conjugates in human liver is the result of two independent enzymatic reactions with a bile acid-coenzyme A thioester intermediate (3, 4). An ATP-dependent microsomal enzyme, bile acid-CoA synthetase (EC 6.2.1.7), catalyzes the intermediate formation and is considered the rate-limiting step in bile acid amidation (5). In the second reaction, the thioester bond is cleaved, and an amide bond is formed between the bile acid and the amino acids glycine or taurine (6). The bile acid-CoA:amino acid *N*-acyltransferase (EC 2.3.1.65) catalyzes this reaction. In human liver this enzyme has been localized to cytosol (5, 7, 8) and shown to be a 50-kDa monomeric protein catalyzing the formation of both taurine and glycine conjugates of bile acids (8).

Significant differences have been observed in the relative amounts of taurine- and glycine-conjugated bile acids among species (9). In humans, taurine conjugation dominates in the newborn. In adulthood the ratio of glycine– to taurine–bile acid conjugates in bile is about 3.5:1, dependent on dietary intake of amino acids (10).

The role of a high bile acid-CoA-cleaving activity in cytosol on the microsomal–cytosolic two-step amidation model has not been considered. However, a bile acid-CoA thioesterase activity has been studied in human liver homogenate (11).

Another bile acid-CoA-forming enzyme system exists within the hepatocyte. The primary bile acids, cholic acid and chenodeoxycholic acid, are metabolites of cholesterol and formed by several enzymatic steps within the liver cell. The final oxidative step takes place in the peroxisomes (12). This β-oxidative cleavage of the cholesterol side chain is catalyzed by the tri- and dihydroxy coprostanoyl-CoA thiolase, with the formation of choloyl-CoA and chenodeoxycholoyl-CoA, respectively.

In the present study, high *N*-acyltransferase activities were measured in the peroxisomal and cytosolic fractions of human liver homogenate. In vitro, the peroxisomal *N*-acyltransferase has the capacity to amidate all de novo-synthesized bile acids. Both high cytosolic and peroxisomal bile acid-CoA thioesterase activities may influence the intracellular levels of free and conjugated bile acids.

Abbreviations: ATP, adenosine triphosphate; CA, cholic acid; CDCA, chenodeoxycholic acid; CoASH, coenzyme A; FXR, farnesoid X receptor; HPLC, high-pressure liquid chromatography; MWCO, molecular weight cutoff.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

[24-^{14}C]choloyl-CoA and [24-^{14}C]chenodeoxycholoyl-CoA, with a specific radioactivity of 1.48–2.22 GBq/mmol, as well as the corresponding unlabeled esters were synthesized by the mixed anhydride procedure (13) from labeled and unlabeled cholic acid or chenodeoxycholic acid. Labeled and unlabeled compounds were mixed and purified shortly before use by high-pressure liquid chromatography (HPLC) (see below).

Taurine, glycine, cholic acid, chenodeoxycholic acid, and coenzyme A (CoASH) were obtained from Sigma (St. Louis, MO). [24-^{14}C]cholic acid and [24-^{14}C]chenodeoxycholic acid were purchased from DuPont-New England Nuclear (Boston, MA). Optiprep, Nycodenz, and Maxidenz were purchased from Nycomed Pharma AS (Oslo, Norway). Other chemicals were commercial products of high purity.

Liver material

Liver tissue from three patients (liver cyst [1] and colorectal metastases [2]) was sampled immediately after completion of liver resection. Homogenates were prepared from macroscopically normal tissue. The liver resections were carried out under inflow occlusion (20 min) interrupted by 5 min of reperfusion.

Preparation of human liver subcellular fractions

The liver homogenate was prepared as described (14), and centrifuged for 10 min at 2,200 rpm (500 g_{av}) in the 8750 rotor of a Heraeus (Hanau, Germany) Christ Cryofuge 20-3. The pellet was rehomogenized and the suspension was recentrifuged, the pellet giving the crude nuclear fraction (N) and the combined supernatants the postnuclear supernatant (E). The mitochondrial fraction (M) was pelleted by centrifugation of fraction E for 10 min in the same rotor at 6,000 rpm (4000 g_{av}) and the light mitochondrial fraction (L) by centrifugation of the 4,000 g_{av} supernatant at 12,000 rpm (15,500 g_{av}) for 10 min. The 15,500 g_{av} supernatant was centrifuged at 32,000 rpm (76,000 g_{av}) for 1 h in the Ti 70 rotor of a Beckman (Fullerton, CA) L8-55 ultracentrifuge to obtain the microsomal pellet (P). The supernatant was used as the cytosolic fraction (S).

The L fraction (48 mg of protein in 1–2 mL of 0.25 M sucrose, 15 mM HEPES, pH 7.4), prepared from the homogenate of the cystic liver, was layered on top of a linear Nycodenz gradient (13–45%, w/v). The gradient tubes contained 32 mL of Nycodenz with a 3-mL Maxidenz cushion and were centrifuged at 20,000 rpm for 75 min in the VTi50 rotor of a Beckman L8-55 ultracentrifuge. Gradient fractions of 2.5 mL were collected. Nycodenz inhibited the amidation reaction and a less polar ^{14}C-labeled product was formed from the bile acid-CoA thioester, eluting as a main peak 15 mL after the free ^{14}C-labeled bile acids in the radiochromatograms. This less polar product remains unidentified. After dialysis of the gradient fractions with Slide-A-Lyzer dialysis cassettes (molecular weight cutoff [MWCO] of 10,000; Pierce, Rockford, IL) in sucrose buffer overnight, a single polar ^{14}C-labeled product eluted.

An aliquot of the L fraction prepared from the homogenate of one of the metastatic livers was layered on top of a 13–45% (w/v) Optiprep density gradient centrifuged as the Nycodenz gradient. Gradient fractions were collected from the bottom of the gradient. No dialysis was performed.

To characterize the subcellular fractions, the mitochondrial marker cytochrome *c* oxidase (15), the microsomal marker *o*-nitrophenylacetate esterase (16), the peroxisomal marker D-amino acid oxidase (17), and the cytosolic marker lactate dehydrogenase (18) were measured. Protein concentration was determined by the method of Lowry et al. (19), or by a modified method (20), in fractions containing Nycodenz or Optiprep.

Bile acid-CoA thioesterase assay

Under standard conditions the incubation mixture contained the following in 250 μL of 50 mM potassium phosphate buffer, pH 8 (11): 200–300 μM choloyl-CoA or chenodeoxycholoyl-CoA (50,000–100,000 cpm), and 50–150 μg of protein from the main subcellular fractions or 25–50 μl of the gradient fractions. After a 10-min preincubation at 37°C, the reaction was started by addition of the bile acid-CoA ester dissolved in 10 μL of filtrated water. The reactions were terminated by adding 50 μL of 0.6 M $HClO_4$ to the reaction mixture. Addition of 300 μL of 2-propanol before centrifugation, allowed detection of the entire radioactivity in the supernatant. Control incubations were performed either with boiled fraction proteins or by omission of protein to detect nonenzymatic cleavage of the thioesters. Blank activities were subtracted from the detected enzyme activities in all cases.

Bile acid-CoA:amino acid *N*-acyltransferase assay

All incubations were performed in triplicate. The amidations of the bile acid-CoA esters were measured under the same conditions as described for the thioesterase assay with the addition of 20 mM glycine or taurine. The reactions were terminated by addition of 45 μL of 1 M KOH. After 30 min of hydrolysis at 70°C to cleave the thioester, the mixture was acidified by HCl and applied to a Sep-Pak C_{18} cartridge. The radioactivity was eluted with 10 mL of 2-propanol. Control incubations were performed and blank activities were subtracted from the detected enzyme activities in each case.

Bile acid-CoA synthetase assay

The incubation mixture contained the following in 250 μl of 50 mM potassium phosphate buffer, pH 7.4: 2 mM CoASH, 7 mM ATP, 10 mM $MgCl_2$, 5 mM dithiothreitol, bovine serum albumin (0.67 mg/mL), and 54 μM [^{14}C]cholic acid (200,000 cpm) in 3.5 μL of ethanol. Fifty to 100 μg of protein of the microsomal fraction and the peroxisomal fraction were incubated per reaction. The reaction was started by the addition of cholic or chenodeoxycholic acid. After 60 min of incubation at 37°C, the reaction was terminated as for the thioesterase assay (see above). Blank activities of incubations containing denatured protein were subtracted.

Radiochromatography

Aliquots of the incubation extracts of the *N*-acyltransferase assay were analyzed by reversed-phase HPLC, using a Beckman ODS 5μ (4.6 mm × 25 cm) column. Cholic acid was separated from its conjugates by eluting with 25% 30 mM trifluoroacetic acid (adjusted to pH 2.9 with triethylamine) in methanol, and chenodeoxycholic acid was separated from its conjugates with 20% 30 mM trifluoroacetic acid, pH 2.9 with triethylamine, in methanol.

Aliquots from the supernatants of the thioesterase assay were analyzed by HPLC with a Beckman ODS 5μ (10 mm × 25 cm) column. [^{14}C]cholic acid was separated from [^{14}C]choloyl-CoA by eluting with 38% 2-propanol in 50 mM potassium phosphate buffer, pH 7.0. The amount of 2-propanol was increased to 44% to achieve a baseline separation of [^{14}C]chenodeoxycholic acid and [^{14}C]chenodeoxycholoyl-CoA. The same chromatographic systems were used to purify the bile acid-CoA esters before incubations. Separation of bile acid-CoA from amino acid conjugates of bile acids for studying the competition between the thioesterase and the *N*-acyltransferase was achieved by mixing phosphate buffer, pH 4.0, with 2-propanol.

The eluent was assayed for ^{14}C radioactivity by a Beckman 171 radioisotope detector coupled to the HPLC system. The scintilla-

TABLE 1. Subcellular distribution of marker enzyme activities in human liver[a]

Fraction	Protein			Cytochrome *c* Oxidase (%)	D-Amino Acid Oxidase (%)	*o*-Nitrophenyl Acetate Esterase (%)	Lactate Dehydrogenase (%)
	mg/ml	mg	%				
E + N		1,606	100	100 (1.0)	100 (1.0)	100 (1.0)	100 (1.0)
N	21.5	558	35	41 (1.2)	20 (0.6)	38 (1.1)	28 (0.8)
M	35.0	168	11	37 (3.5)	8 (0.8)	4 (0.3)	5 (0.5)
L	28.9	139	9	32 (3.6)	23 (2.5)	7 (0.8)	6 (0.7)
P	35.6	214	13	17 (1.3)	16 (1.2)	57 (4.3)	11 (0.8)
S	3.6	408	25	ND (ND)	21 (0.8)	6 (0.2)	45 (1.8)
Recoveries			93	127	88	112	95

[a] The enzyme activities were measured in triplicate by the method described in Materials and Methods. The activities are expressed as percentages. The relative specific activities (in parentheses) are expressed as percent activity/percent protein; ND, not detectable.

tion cocktail Flo-Scint III (Packard, Meriden, CT) was used for radiochromatography in the thioesterase assay and Ultima Flo M (Packard) was used for radiochromatography in the *N*-acyltransferase assay.

RESULTS

Liver tissue samples

Highest bile acid *N*-acyltransferase activity was detected in the soluble fraction (S) and the light mitochondrial fraction (L) from the homogenate of the liver tissue. The light mitochondrial fraction from the homogenate of the second liver sample (with the cyst) was further fractionated on a Nycodenz gradient (not shown) as described in Materials and Methods. The main *N*-acyltransferase activity cosedimented with the peroxisomal marker enzyme, D-amino acid oxidase, to the bottom of the gradient. A competitive inhibition of the *N*-acyltransferase activity with Nycodenz present in the reaction mixture was detected. Optiprep, the dimer of Nycodenz, was tested and showed no inhibition of the *N*-acyltransferase activity when added to the reaction mixtures with the main subcellular fractions. The light mitochondrial fraction of the homogenate from the surrounding liver tissue of the second resectate with metastasis was fractionated in an Optiprep gradient. The results from this liver homogenate are presented below. The distribution of marker enzyme activities, shown in **Table 1**, indicates intact organelles after liver resection carried out under 20-min periods of inflow occlusion (see Materials and Methods).

Subcellular distribution of *N*-acyltransferase activity

In extracts from incubations containing ^{14}C-labeled choloyl-CoA or chenodeoxycholoyl-CoA, together with protein from the main subcellular fractions and taurine or glycine, only one polar product peak appeared in the radiochromatograms. The identity of the product as tauro- and glycoconjugates of cholic acid has been previously established by mass spectrometry (21). **Table 2** shows the subcellular distributions of taurocholic acid and glycocholic acid formation as well as those of taurochenodeoxycholic and glycochenodeoxycholic acid formation. The highest specific *N*-acyltransferase activity was always obtained in the soluble (S) fraction. A significant part of the total *N*-acyltransferase activity was also particle bound and located to the mitochondrial fractions (M + L), of which the highest activity was in the light mitochondrial fraction (L).

In the fractionated Optiprep gradient containing the separated organelles of the L fraction, most of the *N*-acyl-

TABLE 2. Subcellular distribution of bile acid-CoA thioesterase and bile acid-CoA:amino acid *N*-acyltransferase in human liver[a]

Fraction	Formation of											
	Cholic Acid		Chenodeoxycholic Acid		Taurocholic Acid		Glycocholic Acid		Taurocheno-deoxycholic Acid		Glycocheno-deoxycholic Acid	
	nmol/mg/min	Relative Specific Activity	nmol/mg/min	Relative Specific Activity	nmol/mg/min	Relative Specific Activity	nmol/mg/min	Relative Specific Activity	nmol/mg/min	Relative Specific Activity	nmol/mg/min	Relative Specific Activity
E	2.0		2.1		3.5		3.6		2.4		4.0	
E + N		1.0		1.0		1.0		1.0		1.0		1.0
N	1.5	0.8	1.1	0.7	2.2	0.7	2.5	0.8	0.9	0.4	2.1	0.6
M	1.9	1.1	2.0	1.1	3.0	1.0	2.8	0.9	1.3	0.6	2.7	0.8
L	3.1	1.7	2.5	1.4	3.0	1.0	3.8	1.2	2.5	1.2	4.8	1.4
P	1.4	0.8	1.3	0.7	1.1	0.4	1.1	0.4	0.9	0.4	1.2	0.9
S	2.4	1.3	2.7	1.6	7.2	2.4	8.7	2.7	5.8	2.8	6.8	2.0

[a] Thioesterase activities against choloyl-CoA and chenodeoxycholoyl-CoA and formation of taurocholic, glycocholic, taurochenodeoxycholic, and glycochenodeoxycholic acid in subcellular fractions. The subcellular fractions were isolated and the incubations were performed in triplicate by the method described in Materials and Methods. Relative specific activity is expressed as percent activity/percent protein. Recoveries of activities were from 99 to 119%.

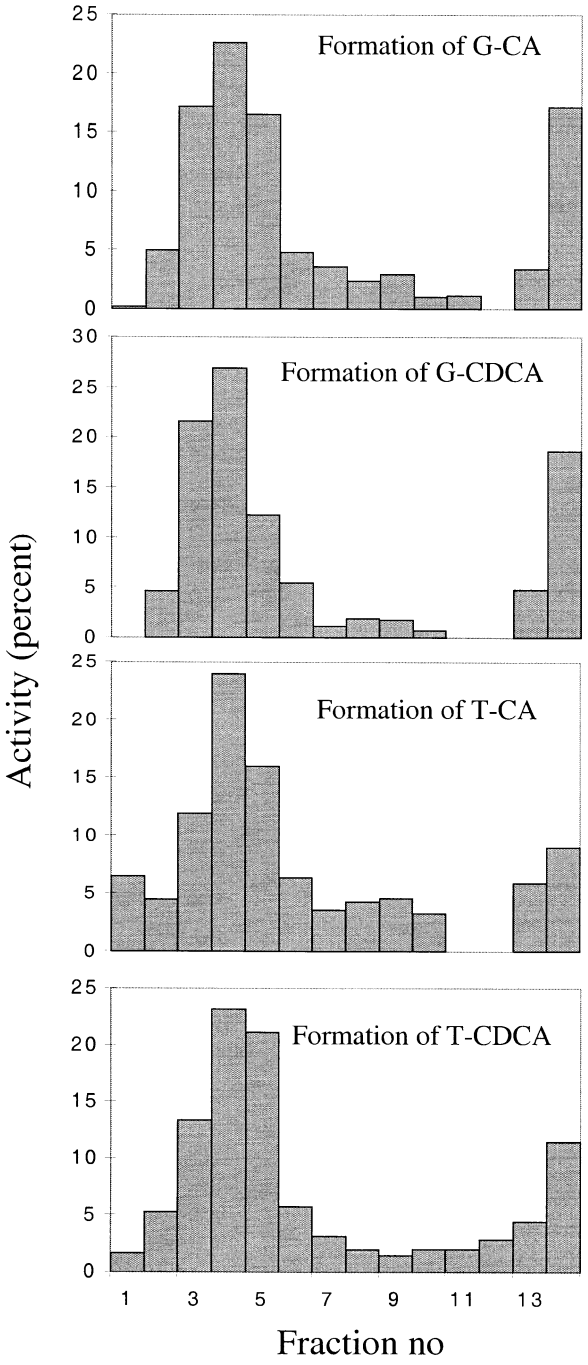

Fig. 1. Activities of bile acid-CoA:amino acid *N*-acyltransferase in fractions after centrifugation of a light mitochondrial (L) fraction of human liver on an Optiprep density gradient (13–45% w/v). The gradient was centrifuged, fractions were collected, and assays performed as described in Materials and Methods. Fraction 1 represents the bottom and fraction 14 the top of the gradient. Enzyme activities are expressed as a percentage of the amount of the whole gradient. Specific activities in the gradient fractions are given in Table 3. G-CA, glycocholic acid; G-CDCA, glycochenodeoxycholic acid; T-CA, taurocholic acid; T-CDCA, taurochenodeoxycholic acid.

transferase activity was obtained in fractions 3, 4, and 5 from the bottom of the gradient (**Fig. 1**), cobanding with the peroxisomal marker, D-amino acid oxidase activity (**Fig. 2**). A minor *N*-acyltransferase activity was detected on top of the gradient in fraction 14, cobanding with the cytosolic marker, lactate dehydrogenase (Fig. 2). The microsomal marker, *o*-nitrophenylacetate esterase, and the mitochondrial marker, cytochrome *c* oxidase, did not cosediment with the *N*-acyltransferase activity.

A 6- to 16-fold enrichment of enzyme activity was achieved when fractionating the liver homogenate (E) into peroxisomal fraction 4 of the Optiprep gradient (**Table 3**). Under standard in vitro conditions with saturating levels of amino acids, the highest specific *N*-acyltransferase activities were measured for the conjugation of glycine with choloyl-CoA, and the lowest activity for the conjugation of taurine with chenodeoxycholoyl-CoA.

Subcellular distribution of bile acid-CoA thioesterase activity

The activities of choloyl-CoA thioesterase and chenodeoxycholoyl-CoA thioesterase in the main fractions show a multiorganelle distribution (Table 2). Highest specific acitivies were detected in the light mitochondrial fraction (L) and the soluble fraction (S). In the Optiprep gradient the thioesterase activity cobanded with the D-amino acid oxidase activity in the lower fractions 3–5, with the cytochrome *c* oxidase activity in fraction 10 and with the lactate dehydrogenase activity in the top fraction 14 (Fig. 2). The distribution and the specific thioesterase activities against the two bile acid-CoA esters were similar.

The highest specific cleaving activity against choloyl-CoA and chenodeoxycholoyl-CoA was detected in the peroxisomal fraction (Table 3). Both in the soluble fraction (Table 2) and the peroxisomal fraction the *N*-acyltransferase activity was about twice that of the corresponding thioesterase activity. An exception was the formation of taurochenodeoxycholic acid in the peroxisomal fraction, which was in the same range as for the peroxisomal cleavage rate of chenodeoxycholoyl-CoA (Table 3).

Kinetic properties of *N*-acyltransferase

Enzyme kinetics of the *N*-acyltransferase were studied in the cytosolic and the peroxisomal fractions. In the presence of saturating amounts of choloyl-CoA and chenodeoxycholoyl-CoA, *N*-acyltransferase activity varied with increasing taurine and glycine concentrations to conform to Michaelis–Menten kinetics in both compartments (**Fig. 3**). In the peroxisomal fraction the K_m for glycine was about 10 times higher than the K_m for taurine. The V_{max} values of taurine and glycine conjugations with primary bile acid CoAs were higher in the peroxisomal fraction than in the cytosolic fraction (**Table 4**).

To estimate the influence of the bile acid-CoA thioesterase activity on the amino acid–bile acid conjugation under the in vitro conditions described, the formation of free ^{14}C-labeled bile acid was measured in incubations with 20 mM taurine and 20 mM glycine present (**Fig. 4**). The thioesterase activities against both the bile acid-CoAs were only slightly reduced with the presence of glycine or taurine in the reaction mixture. After a 30-min incubation with cytosol, 14% of the chenodeoxycholoyl-CoA was cleaved without amino acids in the reaction mixture,

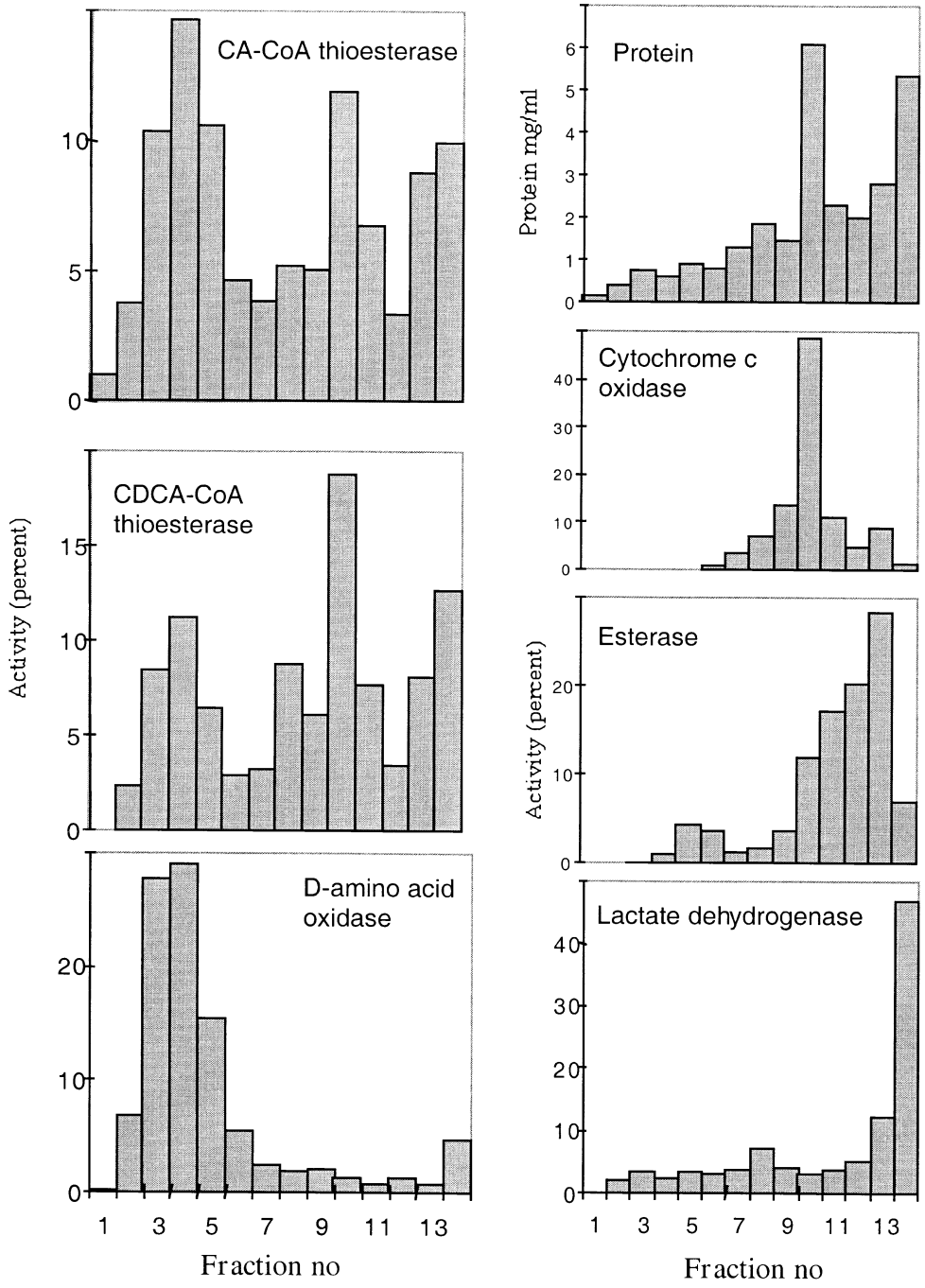

Fig. 2. Activities of bile acid-CoA thioesterase and marker enzymes, and protein concentrations in fractions of the Optiprep density gradient (see legend to Fig. 1). Enzyme activities are expressed as a percentage of the amount in the whole gradient. Specific activities of CA-CoA thioesterase and CDCA-CoA thioesterase in the gradient fractions are given in Table 3. CA-CoA, choloyl-coenzyme A; CDCA-CoA, chenodeoxycholoyl-coenzyme A.

and 11% with taurine or glycine present. For the choloyl-CoA the corresponding figures were 8 and 7%. In the 30-min incubation with peroxisomes, 15% of choloyl-CoA was cleaved without amino acid, and 10% was cleaved with taurine or glycine present. For the chenodeoxycholoyl-CoA the corresponding figures were 10 and 7%.

Bile acid-CoA synthetase activity was not detected in the peroxisomal fraction. However, in parallel incubations with protein from the microsomal fraction, a mean activity of 0.34 nmol/mg of protein per minute was measured.

DISCUSSION

Several research groups have shown that bile acid–amino acid conjugates must be formed by two successive enzymatic reactions in mammalian liver, including human liver (3, 5, 6, 22). Initially, the rate-limiting microsomal synthetase catalyzes the formation of the bile acid-CoA thioester, followed by conjugation with taurine or glycine catalyzed by the bile acid-CoA:amino acid *N*-acyltransferase in cytosol.

TABLE 3. Specific activities of bile acid-CoA:amino acid *N*-acyltransferase and bile acid-CoA thioesterase in Optiprep gradient fractions 1–14, compared with human liver homogenate fraction E and light mitochondrial fraction L[a]

Fraction	Formation of					
	Cholic Acid	Chenodeoxycholic Acid	Taurocholic Acid	Glycocholic Acid	Taurochenodeoxycholic Acid	Glycochenodeoxycholic Acid
	nmol/mg/min					
E	2.0	2.1	3.5	3.6	2.4	4.0
L	3.1	2.1	3.0	3.8	2.5	4.8
1	4.2	0	23.4	2.2	5.8	0
2	6.0	3.9	10.2	18.5	5.0	9.3
3	8.7	7.7	14.1	33.6	6.6	22.8
4[b]	16.0*	13.3†	37.3‡	57.8§	15.1‖	37.2#
5	7.6	5.0	16.2	27.6	9.0	11.1
6	3.8	2.6	7.3	9.1	2.8	5.6
7	1.9	1.7	2.5	6.1	0.9	0.7
8	1.8	3.3	2.1	2.0	0.4	0.9
9	2.2	2.9	2.9	3.1	0.4	1.0
10	1.3	2.1	0.5	0.3	0.1	0.1
11	1.8	2.2	0	0.8	0.3	0
12	1.1	1.2	0	0	0.5	0
13	2.0	2.0	1.9	1.8	0.6	1.4
14	1.2	1.6	1.5	3.2	0.8	2.8

[a] The E and L fractions and the gradient fractions were isolated by the method described in Materials and Methods. The incubation conditions were the same for the E and L fractions and the gradient fractions.

[b] Ratio of specific activities between fraction 4 of the Optiprep gradient and the homogenate (E): * 8.0; † 6.3; ‡ 10.7; § 161.; ‖ 6.3; # 9.3.

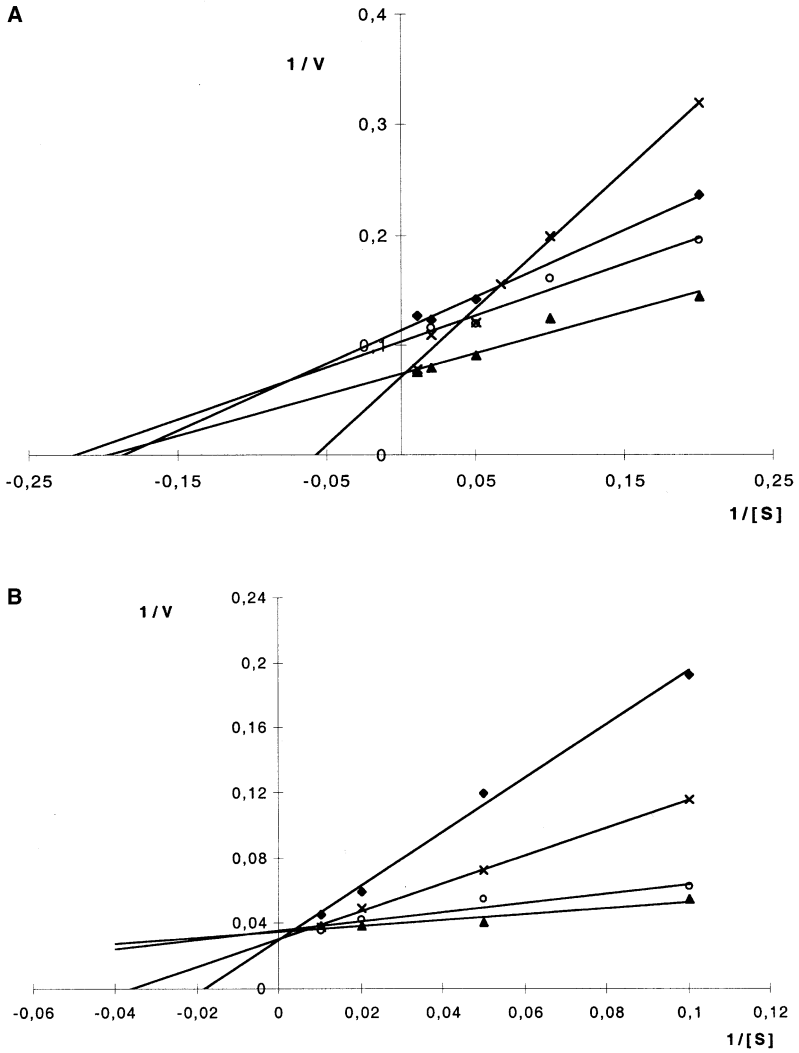

Fig. 3. Kinetic properties of bile acid-CoA:amino acid *N*-acyltransferase in cytosol (A) and the peroxisomal fraction (B). Double-reciprocal plots of reaction velocity versus concentrations of glycine and taurine in the reaction mixtures with saturating levels of choloyl-CoA and of chenodeoxycholoyl-CoA. The *N*-acyltransferase activities are average values of triplicate incubations performed with 50 μg of cytosolic protein and 30 μg of peroxisomal protein as described in Materials and Methods. Aliquots of the supernatants were radiochromatographed by reversed-phase HPLC. ▲–▲ Formation of taurocholic acid; ×–× formation of glycocholic acid (multiplication symbols); ◆–◆ formation of glycochenodeoxycholic acid; ○–○ formation of taurochenodeoxycholic acid (open circles).

TABLE 4. Enzyme kinetics of bile acid-CoA amino acid *N*-acyltransferase in cytosol and the peroxisomal fraction of human liver homogenate[a]

Bile Acid-CoA Amino Acid *N*-acyltransferase	K_m(app.)	V_{max}(app.)
	M	*nmol/mg/min*
Cytosol fraction		
CA-CoA + G → G-CA	2.0×10^{-2}	15.3
CDCA-CoA + G → G-CDCA	5.0×10^{-3}	8.9
CA-CoA + T → T-CA	5.0×10^{-3}	13.7
CDCA-CoA + T → T-CDCA	4.3×10^{-3}	9.3
Peroxisomal fraction		
CA-CoA + G → G-CA	2.9×10^{-2}	34.0
CDCA-CoA + G → G-CDCA	4.8×10^{-2}	30.3
CA-CoA + T → T-CA	2.2×10^{-3}	24.4
CDCA-CoA + T → T-CDCA	5.0×10^{-3}	23.3

Abbreviations: CA-CoA, choloyl-coenzyme A; CDCA-CoA, chenodeoxycholoyl-coenzyme A; G, glycine; T, taurine; G-CA, glycocholic acid; G-CDCA, glycochenodeoxycholic acid; T-CA, taurocholic acid; T-CDCA, taurochenodeoxycholic acid.

[a] The incubations were performed as described in Fig. 3.

The results of the present investigation conclusively show that the peroxisomal fractions of human liver homogenates most efficiently catalyze the conjugation of choloyl-CoA and chenodeoxycholoyl-CoA with taurine and glycine. Compartmentalization of *N*-acyltransferase in human liver to peroxisomes is in agreement with the terminal tripeptide sequence of the enzyme, a variant of the type 1 peroxisomal targeting sequence Ser-Lys-Leu (8, 23).

More than 30 years ago, the *N*-acyltransferase in human liver was shown in a single report to be particle bound and sedimentable to the light mitochondrial fraction (5). The present results demonstrate that peroxisomes were the particle involved, in accordance with our previous findings in rat liver (24). Unlike in rat liver, however, a considerable *N*-acyltransferase activity was measured in the cytosolic fraction of the human liver homogenates. It remains unclear whether the cytosolic activity was due to leakage of peroxisomal *N*-acyltransferase during homogenization and fractionation of the liver tissue, or whether newly synthesized *N*-acyltransferase is active during translocation from free ribosomes to peroxisomes. Both the high cytosolic specific activities and the high relative specific activities of the *N*-acyltransferase (Table 2), compared with D-amino acid oxidase (Table 1) in cytosol, provide some evidence of a functional cytosolic *N*-acyltransferase. Proteins may be translocated into the peroxisomal matrix in a folded or oligomerized state. Thus the same *N*-acyltransferase(s) catalyzing the conjugation with primary bile acids may be present in both cytosol and peroxisomes. However, we cannot disregard the possibility of two different enzymes located in the cytosol and the peroxisomes, respectively.

An efficient peroxisomal *N*-acyltransferase in human liver implies that a major part of bile acids is amidated in the peroxisomes. Evidently all newly formed primary bile acid-CoA esters within the peroxisomes are instantly conjugated in situ with glycine or taurine before secretion into bile canaliculi. A variable but minor part of enteral bile acids is deconjugated by microbes and recirculated back to liver via the portal vein in the enterohepatic circulation. The microsomal–cytosolic two-step conjugation system may be of importance for reconjugation of recycled primary and secondary bile acids. If the cytosolic *N*-acyltransferase activity is due to leakage from the peroxisomes during fractionation and amidation is exclusively peroxisomal, CoA-activated bile acids must be transported from endoplasmic reticulum to the peroxisomes. Such a transport route is also considered for the coprostanoyl-CoA, precursors for the primary bile acids. A transport competition between coprostanoyl-CoAs and bile acid-CoAs may in this case exist, and the cytosolic bile acid-CoA thioesterase may turn out to be important for regulation of the intracellular level of free bile acids. So far, a bile acid-CoA synthetase activity has not been detected in the peroxisomal fraction, but a physiologic activity may be masked by the peroxisomal choloyl-CoA thioesterase.

A peroxisomal *N*-acyltransferase also implies that the peroxisomal synthesis of bile acid-CoA thioesters is the

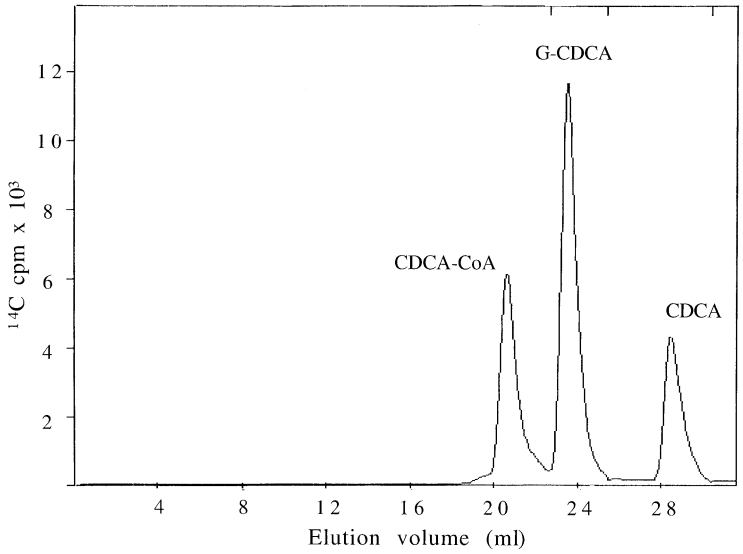

Fig. 4. Reversed-phase high-pressure liquid radiochromatography. [^{14}C]Chenodeoxycholoyl-CoA (CDCA-CoA), glyco-[^{14}C]chenodeoxycholic acid (G-CDCA), and [^{14}C]chenodeoxycholic acid (CDCA) were eluted with 40% 2-propanol in 50 mM potassium phosphate, pH 4.0, and separated by a Beckman ODS 5μ (10 mm × 25 cm) column.

main source of substrate for the amidation reaction within the hepatocyte, and not the microsomal bile acid-CoA synthetase. In a peroxisomal fraction of human liver homogenate the rate of formation of cholic acid from 3α,7α,12α-trihydroxy coprostanic acid was 1.3 nmol/mg of protein per minute, and that of chenodeoxycholic acid from 3α,7α-dihydroxy coprostanic acid was 3.2 nmol/mg of protein per minute (25). Consequently, the peroxisomal β-oxidation of the coprostanic acid may be an important rate-limiting step for bile acid amidation.

Over the last 4 to 5 decades all activities reported for bile acid-CoA:amino acid *N*-acyltransferase have been measured without considering a bile acid-CoA thioesterase. In human liver homogenate, multiorganelle bile acid-CoA-cleaving activities have been demonstrated (11). These activities were much higher relative to the *N*-acyltransferase activity, to the bile acid-CoA synthetase in endoplasmic reticulum, and to the bile acid-CoA formation in the peroxisomes than in rat liver (24). We have no information about the specificity of this activity so far, but high activities in cytosol and the peroxisomal fractions indicate a regulation of bile acid amidation at the level of free CoASH and unconjugated primary bile acids within the hepatocyte. The activity of the peroxisomal thioesterase should be tested with 5β-cholestanoyl-CoAs, and a competition study with bile acid-CoAs should be carried out.

The bile acid-CoA thioesterase activities detected in human liver homogenates may be related to the long chain acyl-CoA thioesterases. Cytosolic acyl-CoA thioesterase (CTE-I) is a peroxisome proliferator-induced enzyme (26), closely related to its mitochondrial and peroxisomal counterparts MTE-I (27) and PTE-I (28) in rat and mouse. These enzymes contain an active site common to serine esterases. Sequence analysis of cytosolic acyl-CoA thioesterase from rat and mouse liver, and searches of databanks, revealed sequence similarity to rat, mouse, and human bile acid-CoA:amino acid *N*-acyltransferase (28). The *N*-acyltransferase contains a catalytic triad similar to that of the serine esterases, except that the serine is replaced by a cysteine. If bile acid-CoAs are substrates for one or more of these thioesterases, these enzymes may be involved in regulation of bile acid amidation and of the level of free bile acids within the hepatocyte. The bile acid-CoA-cleaving activity may, however, be more complex. A bile acid-CoA-cleaving activity was detected in *Eubacterium* sp. strain VPI 12708, catalyzed by an enzyme without the characteristic thioesterase active site, and with an amino acid sequence different from those of the other thioesterases (29). A human peroxisomal acyl-CoA thioesterase has been identified and is suggested to have a role in fatty acid oxidation (30).

We have demonstrated that the thioesterase activity against the primary bile acid-CoAs is only slightly inhibited by the presence of taurine or glycine. This indicates a competition between thioesterase activity and the *N*-acyltransferase activity both in cytosol and peroxisomal fractions of human liver homogenate (Fig. 4). The regulation of this competition may be important for the optimal production of potent activators for the farnesoid X receptor (FXR) (31, 32). Activation of this receptor represses the synthesis of bile acids in liver and increases the synthesis of transport proteins in the intestine. Conjugated mono- and dihydroxylated bile acids are believed to represent natural FXR ligands in liver and ileal enterocytes that express bile acid transporters. Hence, the hepatocytic levels of conjugated and unconjugated bile acids may influence bile acid synthesis via the FXR.

Kinetic experiments have clearly shown that taurine is preferred as the amino acid substrate for the *N*-acyltransferase both in the cytosolic and peroxisomal fractions (Fig. 3; ref. 3). In humans, however, glycine conjugates predominate, with the ratio of glycine to taurine bile acid conjugates in bile usually being 3.5:1. These apparently conflicting results have previously been explained by higher concentrations of glycine than taurine in human liver. The concentrations of free amino acids have been measured in human liver tissue from biopsies obtained during laparoscopic surgery. The mean concentration of taurine was more than twice that of glycine per kilogram of liver (wet weight) (33). From these data we should expect greater taurine conjugation in humans, similar to the rat. If glycine and taurine conjugation of bile acids is catalyzed by a single enzyme (8), a reasonable explanation for the high degree of glycine conjugation may be that human alanine:glyoxylate aminotransferase (AGT) is targeted to liver peroxisomes. This enzyme catalyzes the conversion of glyoxylate to glycine with alanine as an amino donor. In a peroxisomal fraction of human liver homogenate a specific activity of AGT was measured at 13 nmol/mg of protein per minute (34). With a high production of glyoxylate from glycolate, a high glycine concentration may be compartmentalized in peroxisomes. Thus glycine formation from glyoxylate in peroxisomes may influence the glycine–taurine conjugate ratio in human bile. This is also in agreement with results from isolated hepatocytes showing that glycine conjugation of bile acids is regulated by hepatocellular biosynthesized glycine, not by transported glycine (35). In contrast, the rate of taurine conjugation was shown to be dependent on transported taurine. The high degree of glycine-conjugated bile acids may reflect the relative importance of the peroxisomal *N*-acyltransferase under physiologic conditions (**Fig. 5**).

Human liver peroxisomes seem to combine, in an elegant manner, the detoxification of glyoxylate and free bile acids by converting glyoxylate to glycine followed by conjugation with bile acid before secretion into bile. Further examination of the peroxisomal and cytosolic bile acid-CoA thioesterases, providing free chenodeoxycholic acid for activation of the FXR, will extend our understanding of how bile acids and bile salts regulate their biosynthesis and their enterohepatic recirculation.

The skilled technical assistance of Rigmor J. Sletta is appreciated. This work was supported by the Medinnova Foundation.

Manuscript received 7 January 2000 and in revised form 3 April 2000.

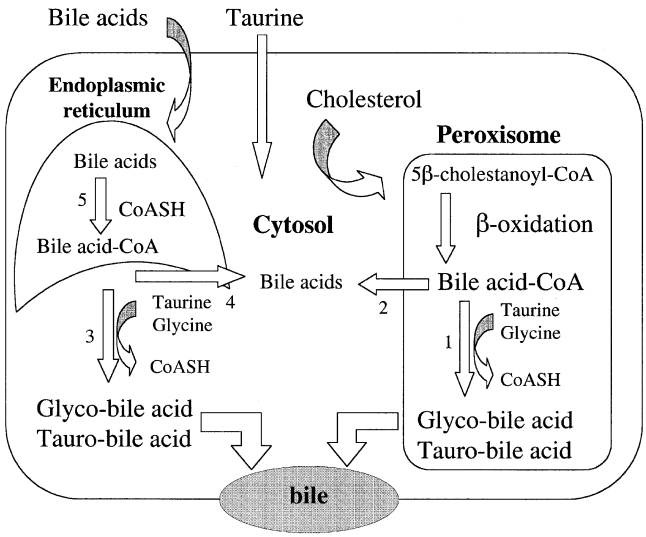

Fig. 5. Proposed compartmentalization of bile acid conjugation with taurine and glycine in the human liver cell. In cells located in the periportal regions the left side pathway may dominate, and in cells located in the pericentral regions the right side pathway may dominate. 1, Peroxisomal bile acid-CoA:amino acid *N*-acyltransferase; 2, peroxisomal bile acid-CoA thioesterase; 3, cytosolic bile acid-CoA:amino acid *N*-acyltransferase; 4, cytosolic bile acid-CoA thioesterase; 5, microsomal bile acid-CoA synthetase.

REFERENCES

1. Björkhem, I. 1985. Mechanism of bile acid biosynthesis in mammalian liver. *In* Steroids and Bile Acids. H. Danielsson and J. Sjövall, editors. Elsevier Science, New York. 231–260.
2. Vessey, D. A., M. H. Crissey, and D. Zakim. 1977. Kinetic studies of the enzymes conjugating bile acids with taurine and glycine in bovine liver. *Biochem. J.* **163:** 181–183.
3. Siperstein, M. D., and A. W. Murray. 1955. Enzymatic synthesis of cholyl-CoA and taurocholic acid. *Science.* **138:** 377–378.
4. Bremer, J. 1956. Cholyl-S-CoA as an intermediate in the conjugation of cholic acid with taurine by rat liver microsomes. *Acta Chem. Scand.* **10:** 56–71.
5. Scherstén, T., P. Björntorp, P-H. Ekdahl, and S. Björkerud. 1967. The synthesis of taurocholic and glycocholic acids by preparation of human liver. II. Analysis of the stimulating effect of the L fraction. *Biochim. Biophys. Acta.* **141:** 155–163.
6. Czuba, B., and D. A. Vessey. 1981. Purification and characterization of cholyl-CoA:taurine *N*-acyltransferase from the liver of domestic fowl. *Biochem. J.* **195:** 263–266.
7. Kimura, K., E. Okuno, J. Inada, H. Ohyama, and R. Kido. 1983. Purification and characterization of amino-acid *N*-choloyltransferase from human liver. *Hoppe-Seyler's Z. Physiol. Chem.* **364:** 637–645.
8. Falany, C. N., M. R. Johnson, S. Barnes, and R. B. Diasio. 1994. Glycine and taurine conjugation of bile acids by a single enzyme. *J. Biol. Chem.* **269:** 19375–19379.
9. Johnson, M. R., S. Barnes, J. B. Kwakye, and R. B. Diasio. 1991. Purification and characterization of bile acid-CoA:amino acid *N*-acyltransferase from human liver. *J. Biol. Chem.* **266:** 10227–10233.
10. Hardison, W. G. M., and S. Grundy. 1983. Effect of bile acid conjugation pattern on bile acid metabolism in normal humans. *Gastroenterology.* **84:** 617–620.
11. Solaas, K., R. Sletta, O. Søreide, and B. F. Kase. 2000. The presence of choloyl- and chenodeoxycholoyl–coenzyme A thioesterase activity in human liver. *Scand. J. Clin. Lab. Invest.* **60**: 91–102.
12. Kase, B. F., J. I. Pedersen, B. Strandvik, and I. Björkhem. 1985. In vivo and in vitro studies on formation of bile acids in patients with Zellweger syndrome. *J. Clin. Invest.* **76:** 2393–2402.
13. Shah, P. P., and E. Staple. 1968. Synthesis of coenzyme A esters of some bile acids. *Steroids.* **12:** 571–575.
14. Prydz, K., B. F. Kase, I. Björkhem, and J. I. Pedersen. 1986. Formation of chenodeoxycholic acid from 3α,7α-dihydroxy-5β-cholestanoic acid by rat liver peroxisomes. *J. Lipid Res.* **27:** 622–628.
15. Slinde, E., and T. Flatmark. 1973. Determination of sedimentation coefficients of subcellular particles of rat liver homogenates. *Anal. Biochem.* **56:** 324–340.
16. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thines-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. *J. Cell Biol.* **61:** 188–200.
17. Baudhuin, P., H. Beaufay, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, P. Jacques, and C. de Duve. 1964. Intracellular distribution of monoamino oxidase, aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. *Biochem. J.* **92:** 179–184.
18. Wanders, R. J. A., M. Kos, B. Roest, A. J. Meijer, G. Schrakamp, H. S. A. Heymans, W. H. H. Tegelaers, H. van den Bosch, R. B. H. Schutgens, and J. M. Tager. 1984. Activity of peroxisomal enzymes and intracellular distribution of catalase in Zellweger syndrome. *Biochem. Biophys. Res. Commun.* **123:** 1054–1061.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
20. Bensadoun, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering material. *Anal. Biochem.* **70:** 241–250.
21. Kase, B. F., K. Prydz, I. Björkhem, and J. I. Pedersen. 1986. Conjugation of cholic acid with taurine and glycine by rat liver peroxisomes. *Biochem. Biophys. Res. Commun.* **138:** 167–173.
22. Killenberg, P. G. 1978. Measurement and subcellular distribution of choloyl-CoA synthetase and bile acid-CoA:amino acid *N*-acyltransferase activities in rat liver. *J. Lipid Res.* **19:** 24–31.
23. McNew, J. A., and J. M. Goodman. 1996. The targeting and assembly of peroxisomal proteins: some old rules do not apply. *Trends Biochem. Sci.* **21:** 54–58.
24. Kase, B. F., and I. Björkhem. 1989. Peroxisomal bile acid-CoA:amino acid *N*-acyltransferase in rat liver. *J. Biol. Chem.* **264:** 9220–9223.
25. Kase, B. F., K. Prydz, I. Björkhem, and J. I. Pedersen. 1986. In vitro formation of bile acids from di- and trihydroxy-5β-cholanoic acid in human liver peroxisomes. *Biochim. Biophys. Acta.* **877:** 37–42.
26. Lindquist, P. J., L. T. Svensson, and S. E. H. Alexson. 1998. Molecular cloning of the peroxisome proliferator-induced 46-kDa cytosolic acyl-CoA thioesterase from mouse and rat liver. *Eur. J. Biochem.* **251:** 631–640.
27. Svensson, L. T., S. E. H. Alexson, and J. K. Hiltunen. 1995. Very long chain and long chain acyl-CoA thioesterases in rat liver mitochondria. Identification, purification, characterization, and induction by peroxisome proliferators *J. Biol. Chem.* **270:** 12177–12183.
28. Svensson, L. T., M. Wilke, and S. E. H. Alexson. 1995. Peroxisome proliferators differentially regulate long-chain acyl-CoA thioesterases in rat liver. *Eur. J. Biochem.* **230:** 813–820.
29. Ye, H-Q., D. H. Mallonee, J. E. Wells, I. Björkhem, and P. B. Hylemon. 1995. The bile acid-inducible *baiF* gene from *Eubacterium* sp. strain VPI 12708 encodes a bile acid-coenzyme A hydrolase. *J. Lipid Res.* **40:** 17–23.
30. Jones, J. M., K. Nau, M. T. Geraghty, R. Erdmann, and S. J. Gould. 1999. Identification of peroxisomal acyl-CoA thioesterases in yeast and humans. *J. Biol. Chem.* **274:** 9216–9223.
31. Makishima, M., A. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf, and B. Shan. 1999. Identification of a nuclear receptor for bile acids. *Science.* **284:** 1362–1365.
32. Parks, D. J., S. G. Blanchard, R. K. Bledsoe, G. Chandra, T. G. Consler, S. A. Kliewer, J. B. Stimmel, T. M. Willson, A. M. Zavacki, D. D. Moore, and J. M. Lehmann. 1999. Bile acids: natural ligands for an orphan nuclear receptor. *Science.* **284:** 1365–1368.
33. Barle, H., B. Ahlman, B. Nyberg, K. Andersson, P. Essén, and J. Wernerman. 1996. The concentrations of free amino acids on human liver tissue obtained during laparoscopic surgery. *Clin. Phys.* **16:** 217–227.
34. Danpure, C. J., and P. R. Jennings. 1986. Peroxisomal alanine: glyoxylate aminotransferase deficiency in primary hyperoxaluria type I. *FEBS Lett.* **201:** 20–24.
35. Ohkuma, S., J. Tamura, and K. Kuriyama. 1984. Roles of endogenous and exogenous taurine and glycine in the formation of conjugated bile acids: analyses using freshly isolated and primary cultured rat hepatocytes. *Jpn. J. Pharmacol.* **35:** 347–358.