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B F Kase, … , B Strandvik, I Björkhem

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Research Article

The last step in bile acid formation involves conversion of 3 alpha,7 alpha,12 alpha-trihydroxy-5 beta-cholestanoic acid (THCA) into cholic acid and 3 alpha,7 alpha-dihydroxy-5 beta-cholestanoic acid (DHCA) into chenodeoxycholic acid. The peroxisomal fraction of rat and human liver has the highest capacity to catalyze these reactions. Infants with Zellweger syndrome lack liver peroxisomes, and accumulate 5 beta-cholestanoic acids in bile and serum. We recently showed that such an infant had reduced capacity to convert a cholic acid precursor, 5 beta-cholestane-3 alpha,7 alpha,12 alpha-triol into cholic acid. 7 alpha-Hydroxy-4-cholesten-3-one is a common precursor for both cholic acid and chenodeoxycholic acid. Intravenous administration of [3H]7 alpha-hydroxy-4-cholesten-3-one to an infant with Zellweger syndrome led to a rapid incorporation of 3H into biliary THCA but only 10% of 3H was incorporated into cholic acid after 48 h. The incorporation of 3H into DHCA was only 25% of that into THCA and the incorporation into chenodeoxycholic acid approximately 50% of that in cholic acid. The conversion of intravenously administered [3H]THCA into cholic acid in another infant with Zellweger syndrome was only 7%. There was a slow conversion of THCA into 3 alpha,7 alpha,12 alpha-trihydroxy-5 beta-C29-dicarboxylic acid. The pool size of both cholic- and chenodeoxycholic acid was markedly reduced. Preparations of liver from two patients with Zellweger syndrome had no capacity to catalyze conversion of THCA […]

In Vivo and In Vitro Studies on Formation of Bile Acids in Patients with Zellweger Syndrome

Evidence That Peroxisomes Are of Importance in the Normal Biosynthesis of Both Cholic and Chenodeoxycholic Acid

Bengt Frode Kase, Jan I. Pedersen, Birgitta Strandvik, and Ingemar Björkhem

Institute for Nutrition Research, School of Medicine, University of Oslo, Oslo 3, Norway; Departments of Pediatrics, Clinical Chemistry, and Research Center, Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge, Sweden

Abstract

The last step in bile acid formation involves conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) into cholic acid and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) into chenodeoxycholic acid. The peroxisomal fraction of rat and human liver has the highest capacity to catalyze these reactions.

Infants with Zellweger syndrome lack liver peroxisomes, and accumulate 5β-cholestanoic acids in bile and serum. We recently showed that such an infant had reduced capacity to convert a cholic acid precursor, 5β-cholestane-3α,7α,12α-triol into cholic acid.

7α-Hydroxy-4-cholesten-3-one is a common precursor for both cholic acid and chenodeoxycholic acid. Intravenous administration of [^{3}H]7α-hydroxy-4-cholesten-3-one to an infant with Zellweger syndrome led to a rapid incorporation of ^{3}H into biliary THCA but only 10% of ^{3}H was incorporated into cholic acid after 48 h. The incorporation of ^{3}H into DHCA was only 25% of that into THCA and the incorporation into chenodeoxycholic acid ~ 50% of that in cholic acid.

The conversion of intravenously administered [^{3}H]THCA into cholic acid in another infant with Zellweger syndrome was only 7%. There was a slow conversion of THCA into 3α,7α,12α-trihydroxy-5β-C_{29}-dicarboxylic acid. The pool size of both cholic- and chenodeoxycholic acid was markedly reduced.

Preparations of liver from two patients with Zellweger syndrome had no capacity to catalyze conversion of THCA into cholic acid. There was, however, a small conversion of DHCA into chenodeoxycholic acid and into THCA. It is concluded that liver peroxisomes are important both for the conversion of THCA into cholic acid and DHCA into chenodeoxycholic acid.

Introduction

The last step in the biosynthesis of cholic acid is generally believed to be the oxidative cleavage of the side chain of 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA)[1] in the liver (Fig. 1, Reference 1). In rat (2, 3) and human liver (unpublished observation), this conversion is most efficiently catalyzed by the peroxisomal fraction. Infants with the rare and fatal, inherited cerebrohepatorenal syndrome of Zellweger have an apparently complete lack of peroxisomes in the liver (4). High levels of 5β-cholestanoic acids in bile and serum (5, 6) indicate impaired liver peroxisomal function in bile acid formation (2, 3, 6). Because the defect is within an excretory pathway, it can be studied by the use of radioactive precursors. This provides a unique opportunity to evaluate the role of liver peroxisomes in vivo. We recently showed that administration of a labeled cholic acid precursor, 5β-cholestane-3α,7α,12α-triol, to an infant with Zellweger syndrome led to a rapid incorporation of label into THCA and a slow incorporation into cholic acid (6). The pool size of cholic acid was also found to be markedly reduced (6).

Similarly to the conversion of THCA into cholic acid, 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) has been shown to be efficiently converted into chenodeoxycholic acid in vivo (7–9). We have recently found that the peroxisomal fraction has a high capacity to catalyze this reaction also in vitro (unpublished observation). In patients with Zellweger syndrome the concentration of DHCA in serum is lower than that of THCA (6), and the concentration of chenodeoxycholic acid is often higher than that of cholic acid (6, 10). This raises the question of whether there is a more efficient conversion of DHCA into chenodeoxycholic acid than of THCA into cholic acid in these patients.

7α-Hydroxy-4-cholesten-3-one is a common precursor to both cholic and chenodeoxycholic acid (1, 11–13). In the present work we administered labeled 7α-hydroxy-4-cholesten-3-one to a patient with Zellweger syndrome to study whether there is also a defective side-chain cleavage in the biosynthesis of chenodeoxycholic acid in this disease. The rate of incorporation of label into DHCA, THCA, chenodeoxycholic acid, and cholic acid was measured. THCA is the main bile acid intermediate accumulated in patients with Zellweger syndrome, and in a second patient we therefore studied the conversion of labeled THCA into cholic acid. Finally, we studied the conversion of THCA into cholic acid and that of DHCA into chenodeoxycholic acid in liver biopsies from two patients with Zellweger syndrome and a control infant.

Methods

Patient I. This infant was a first-born, fullterm female with muscular hypotonia, convulsions, and dysmorphic features characteristic of Zellweger syndrome. Phenobarbital (3–5 mg/kg) was instituted in the neonatal period. Hepatomegaly, elevated liver transaminase activities in serum, and gradually decreasing coagulation test values were found. There was diffuse liver fibrosis, but no cirrhosis, nor signs of bile stasis. Electron microscopy did not show normal peroxisomal structures, and peroxidase-

Address reprint requests to Dr. Björkhem, Department of Clinical Chemistry, Huddinge University Hospital, S-141 86 Huddinge, Sweden.

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1. *Abbreviations used in this paper:* DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; GC-MS, gas chromatography-mass spectroscopy; HPLC, high pressure liquid chromatography; TEA, triethylamine; TFA, trifluoroacetic acid; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid.

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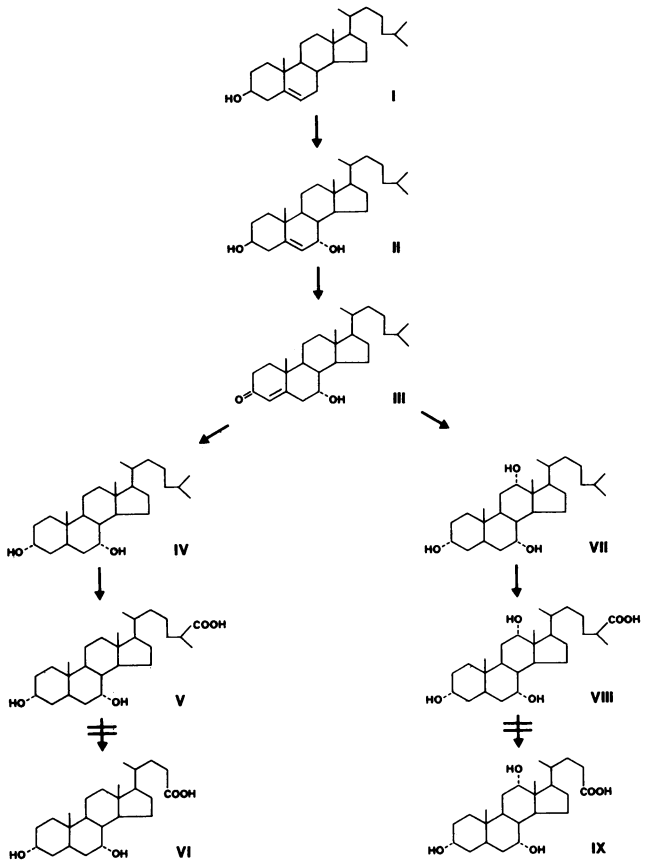


Figure 1. Simplified scheme showing the sequence of reactions in the formation of bile acids from cholesterol. The partial metabolic block in patients with the Zellweger disease is indicated. (I) cholesterol; (II) 7α-hydroxycholesterol; (III) 7α-hydroxy-4-cholesten-3-one; (IV) 5β-cholestane-3α,7α-diol; (V) DHCA; (VI) chenodeoxycholic acid; (VII) 5β-cholestane-3α,7α,12α-triol; (VIII) THCA; (IX) cholic acid.

positive organelles could not be demonstrated. Cultured fibroblasts showed accumulation of saturated long-chain fatty acids.

5β-Cholestanoic acids were determined in hydrolyzed samples of serum, bile, and urine as previously described (14, 15). As shown in Table I, high levels typical of Zellweger syndrome (6, 10) were detected. At the age of 5 mo the infant died.

Patient II. This infant was born asphyctic. At the time hepatomegaly developed, his muscular hypotonia, convulsions, and dysmorphic features were connected with Zellweger syndrome (16).

From the age of 7 wk the convulsions were treated with phenobarbital (5 mg/kg). The liver was slightly enlarged but no signs of cholestasis were present. High levels of liver transaminases in serum were detected whereas serum bilirubin was within normal range. In light microscopy of a liver biopsy specimen no signs of cirrhosis, nor bile stasis were seen. Normal peroxisomal structures could not be recognized by electron microscopy of liver biopsies after catalase staining with diaminobenzidine (17).

The same pattern of bile acid intermediates and abnormal bile acids was detected in serum, bile, and urine as in patient I (Table I). The infant died at the age of 6 mo. Renal cortical cysts, slight liver steatosis, and a slight atrophy of the adrenals were found at autopsy.

Patient III. This patient was a first-born fullterm female with Zellweger syndrome. The case history of this patient has been reported in detail in our previous work (6).

Control subjects. Normal human liver material (65 mg) was made available during laparotomy for pyloric stenosis in a 3-mo-old female.

Ethical aspects. The studies were approved by the ethical committee of the Karolinska Institute at Huddinge University Hospital as well as by the medical staff at the Department of Child Surgery, National Hospital, University of Oslo.

Preparation of labeled steroids. 7β-^{3}H-7α-hydroxy-4-cholesten-3-one (7 Ci/mol), 7β-^{3}H-3α,7α,12α-trihydroxy-5β-cholestanoic acid (200 Ci/mol), and 7β-^{3}H-3α,7α-dihydroxy-5β-cholestanoic acid (50 Ci/mol) were prepared as described (1, 3, 6, 7). [24-^{14}C]cholic acid (50 Ci/mol) was from Amersham International, Amersham, England. The compounds were purified by high pressure liquid chromatography (HPLC) (see below) before use. Both THCA and DHCA contained the 25*R* and 25*S* isomers. The experiments were performed with both C-25 stereoisomers because both forms are converted equally well into cholic and chenodeoxycholic acid (Reference 3, unpublished observations).

In vivo administration of labeled steroids and collection of bile and serum samples. [^{3}H]7α-hydroxy-4-cholesten-3-one (~15.5 × 10^{6} dpm) and [^{14}C]cholic acid (~1.2 × 10^{6} dpm) were dissolved in 0.5 ml of ethanol, filtered, mixed with 5 ml of 20% human serum albumin, and administered to patient I by use of the same procedure as previously described (6). Duodenal contents were collected at least 2 h after each meal, 6, 12, 24, and 48 h after infusion. Venous blood samples were drawn after 24 and 48 h, and urine was collected from 18 to 24 h after infusion.

[^{3}H]THCA (~26.1 × 10^{6} dpm) and [^{14}C]cholic acid (~0.8 × 10^{6} dpm) were dissolved in ethanol, filtered, and given to patient II in the

Table I. Bile Acid Concentration in Serum, Duodenal Aspirate, and Urine from Two Patients with the Cerebrohepatorenal Syndrome of Zellweger

	Serum		Bile		
	Patient I	Patient II	Patient I	Patient II	Urine of patient I
	μmol/liter	*μmol/liter*	*%*	*%*	*μmol/liter*
Cholic acid	1.2	5.3	34	59	0.7
Chenodeoxycholic acid	4.1	3.8	47	38	0.6
3α,7α,12α-Trihydroxy-5β-cholestanoic acid	6.2	5.0	17	3	0.4
3α,7α-Dihydroxy-5β-cholestanoic acid	2.2	0.2	3		<0.1
3α,7α,12α,24-Tetrahydroxy-5β-cholestanoic acid	0.5	0.5			<0.1
3α,7α,24-Trihydroxy-5β-cholestanoic acid	0.2				<0.1
3α,7α,12α-Tetrahydroxy-5β-cholestanoic acid	0.8				0.2
3α,7α,12α-Trihydroxy-27-carboxymethyl-5β-cholestan-26-oic acid	6.4	2.4			0.1
Deoxycholic acid	0	0			0

same way as for patient I. Duodenal contents were sampled 3, 4, 8, 16, 24, 48, 68, and 72 h after infusion. Venous blood samples were drawn after 24, 49, and 72 h. Urine was collected in two portions: 5–17 h and 17–29 h after the infusion.

Analyses of serum, bile, and urine. The serum samples (0.25 ml) were hydrolyzed and extracted as described previously (14, 15). The alkaline hydrolysis used cleaves conjugates with amino acids and glucuronic acid. The major part of the sulfates (if present) are cleaved in connection with the acid extraction step. To the serum samples used for assay by isotope dilution–mass spectrometry, deuterium-labeled cholic acid, chenodeoxycholic acid, and deoxycholic acid were added prior to hydrolysis (14, 15). The methyl ester trimethylsilyl ether derivatives were prepared prior to combined gas-liquid chromatography–mass spectrometry (GC-MS). An LKB 9000 instrument (LKB Produkter, Bromma, Sweden) equipped with a multiple ion detector and an 1.5% SE-30 column was used (14, 15). The amount of cholic acid, chenodeoxycholic acid, and THCA was measured by isotope dilution–mass spectrometry as described previously (6, 15). The approximate concentration of 24-OH-THCA and C_{29}-dicarboxylic acid was determined from the tracing at m/e 253 (cf. Reference 6) (Table I). For further identification, see below.

The bile, serum, and urine samples collected after the in vivo administration of the radioactive compounds were hydrolyzed and extracted as above. Aliquots of the extracts were chromatographed on HPLC by using a Zorbax ODS column (5 × 250 mm). Trifluoroacetic acid (TFA), 30 mM, adjusted to pH 2.9 with triethylamine (TEA) in concentrations of 25%, 19%, or 10% in methanol was used as solvent (TFA/TEA) at a flow rate of 1 ml/min. Aliquots of the fractions were evaporated, and assayed for ^3H and ^{14}C as described previously (6). The recovery from the HPLC columns was essentially complete. Fractions containing radioactivity were extracted with ethyl acetate after acidification (3). The extracts were converted to the methyl ester trimethylsilyl ether derivatives (14, 15) and analyzed by combined GC-MS as described above. The relatively small amounts of serum available made it impossible to record full mass spectra. The identity of the different C_{27} and C_{24} bile acids and the C_{29}-dicarboxylic acid was established by selected ion monitoring as described previously (6, 15). In vivo conversion of [^3H]7α-hydroxy-4-cholesten-3-one and [^3H]THCA into cholic acid was based on the ratio between $^3H/^{14}C$ in biliary cholic acid and the corresponding ratio in the infused mixture of ^3H-labeled precursor and [^{14}C]cholic acid (6). In the present work we were able to compare this method with that described by Hanson and Williams (18). The two methods were in good agreement (cf. Results). The cholic acid pool size was determined by the Lindstedt technique (19).

In a separate experiment the different conjugates of the urinary bile acids in unhydrolyzed urine were separated according to the method of Almé et al. (20). After enzymatic solvolysis and alkaline hydrolysis of the taurine and glycine conjugates, the different bile acids in each fraction were identified and quantitated as described by Almé et al. (20).

Preparation of subcellular fractions of liver biopsies. From patient III needle biopsy samples (total 180 mg) were taken half an hour after death. In order to establish the diagnosis, an open liver biopsy (275 mg) was taken from patient II at the age of 4 mo. The major part of the biopsy material (small parts were used for electron microscopy) was immediately put on ice-cold 0.25 M sucrose, 1 mM EDTA, 15 mM Hepes, adjusted to pH 6.5 with trizma base, and homogenized in a Potter-Elvehjem homogenizer. In patient III part of the whole homogenate was used for incubation. From the remainder of this homogenate, a mitochondrial (M) fraction (4,900 g_{av} for 10 min) and a light mitochondrial (L) fraction (24,200 g_{av} for 10 min) were prepared (3).

The homogenates of the biopsy specimens from patient II and the control were centrifuged at 600 g_{av} for 10 min to remove nuclei and debris. The pellets were rehomogenized and the suspensions recentrifuged. From the combined supernatants (E fraction), subcellular fractions were prepared as above in addition to a microsomal (P) fraction (75,000 g_{av} for 60 min).

Because of the small amounts of liver material available from the control, the mitochondrial fractions (M + L) were combined. All pellets were resuspended in 0.25 M sucrose, 15 mM Hepes, pH 7.4. The protein concentrations were determined by the method of Lowry et al. (21) using bovine serum albumin as standard.

Incubations, extractions, and chromatographic procedures. The incubations with THCA and DHCA were performed with all the subcellular fractions as described (3) except that the reaction volume was reduced to either 0.5 or 0.75 ml. The M fraction and the L fraction were incubated both in the absence and presence of 1 mM potassium cyanide in the reaction mixtures. The reactions were terminated after 1 h, hydrolyzed, and extracted with ethylacetate as described (3).

Aliquots of the extracts were analyzed by HPLC using a Zorbax ODS column and assayed for radioactivity as described (3). The extracts of the DHCA and THCA incubations were eluted in respectively 17% and 24% of 30 mM TFA adjusted to pH 2.9 with TEA in methanol.

Aliquots of the HPLC fractions were converted into the methyl ester trimethylsilyl ether derivatives and analyzed by combined GC-MS as described above. The identity of cholic acid, chenodeoxycholic acid, and THCA was established by monitoring of the same selected ions as above and as in previous work (3, 6, 14, 15).

Results

Conversion in vivo of 7β-^{3}H-7α-hydroxy-4-cholesten-3-one into bile acids in patient I. After infusion of the mixture of [^3H]7α-hydroxy-4-cholesten-3-one and [^{14}C]cholic acid, serum, urine, and bile samples were hydrolyzed and extracted as described in Methods. The ratio of ^3H to ^{14}C activity of infused material measured after extraction as with the samples was 10.4. In the serum extracts this ratio was 14.9 and 8.7 (24- and 48-h samples, respectively) indicating an early retention of the ^3H activity in serum. The ratio was 4.9 in the urine extract and varied between 2.1 and 3.7 in the bile extracts.

The bile extracts were analyzed by HPLC using 19% TFA/TEA in MeOH as eluent and the product peaks were identified as described in Methods. The chromatogram of the extract of the sample collected 6 h after the infusion is shown in Fig. 2. Four major radioactive peaks were detected. The most nonpolar splitted peak was identified as DHCA. The largest peak contained THCA. The material in the peak that eluted at 16 ml was identified as chenodeoxycholic acid. The peak that eluted at 10 ml contained both ^3H and ^{14}C activity and was identified as cholic acid.

To enhance the chromatographic resolution of the polar products, aliquots of the bile extracts were analyzed by using 25% TFA/TEA in methanol as eluent. In this chromatographic system cholic acid eluted after 15 ml. A minor peak that eluted

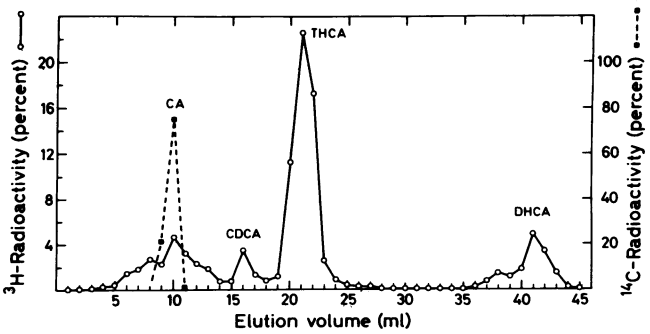


Figure 2. Reversed-phase HPLC of a duodenal bile extract 6 h after infusion of 7β-^{3}H-7α-hydroxy-4-cholesten-3-one and [^{14}C]cholic acid. 19% TFA/TEA was used as eluent as described in Methods. The main peaks were identified as shown. CA, cholic acid; CDCA, chenodeoxycholic acid.

after 12 ml contained material identified by GC-MS as derivative of 3α,7α,12α-trihydroxy-5β-cholestanoic acid with a fourth hydroxyl group in the steroid nucleus. Selected ion monitoring showed the presence of an ion at m/e 251 (loss of four trimethylsilyl groups from the steroid nucleus) as well as presence of ions at m/e 498 (M-3 × 90) and m/e 588 (M-2 × 90). Attempts to prepare an acetonide (cf. Reference 22) of the compound for tentative localization of the fourth hydroxyl group failed, excluding hydroxylation in the 6α position (6). A small peak that eluted at 17 ml was identified as 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid.

The $^3H/^{14}C$ ratio in the cholic acid isolated increased from 0.1 in the 6-h sample to 1.1 in the 48-h sample. After 48 h, the $^3H/^{14}C$ ratio was thus 10% of the ratio in the infused mixture, indicating that ~10% of the [^{3}H]7α-hydroxy-4-cholesten-3-one had been converted into cholic acid.

The specific activities of biliary THCA and DHCA remained considerably higher than those of cholic acid and chenodeoxycholic acid during the sampling period (Fig. 3). Between 6 and 24 h the specific activity of THCA decreased by 45% and that of DHCA by 300%.

The specific ^3H activities of cholic acid were about three times higher than those of [^{3}H]chenodeoxycholic acid (Fig. 3) whereas the concentrations of the same bile acids in the duodenal aspirates were nearly the same (cf. Discussion). From the specific radioactivity-decay curve of [^{14}C]cholic acid (Fig. 3), it was calculated (23) that the pool size of cholic acid in patient I was only 2.2 mg (11 mg/m^2) (Table II). The synthesis rate of cholic acid was estimated to 1.9 mg/d (9.5 mg/m^2·d). In contrast, Watkins et al. (24) found a mean pool size of ~40 mg (290 mg/m^2) and a mean synthesis rate of 20 mg/d (110 mg/m^2·d) in five healthy newborns. The approximate size of the pool of chenodeoxycholic acid could be calculated from the ratio between cholic acid and chenodeoxycholic acid in bile and the pool size of cholic acid. The pool size of chenodeoxycholic acid was found to be 3.0 mg (15 mg/m^2) (Table II).

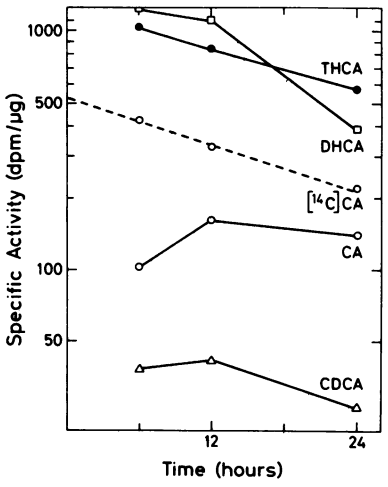


Figure 3. Specific activity decay curves of biliary [^{3}H]THCA, [^{3}H]DHCA, [^{3}H]cholic acid (CA), [^{3}H]chenodeoxycholic acid (CDCA), and [^{14}C]cholic acid (o-----o) after infusion of 7β-^{3}H-7α-hydroxy-4-cholesten-3-one and [^{14}C]cholic acid. The specific activity of [^{14}C]cholic acid at the time of administration used to calculate the cholic acid pool was obtained by extrapolation of the curve to the ordinate.

Table II. Bile Acid Pool Size and Synthetic Rate in Patients with Zellweger Syndrome

	Cholic acid		Pool size	
	Pool size	Synthesis	Chenodeoxycholic acid	THCA
	mg/m²	*mg/m² × 24 h*	*mg/m²*	*mg/m²*
Patient I	11	9.5	15	
Patient II	35	15		70
Patient III*	24	9		
Normal newborn infants (n = 5)‡	290±36	110±20		

* Data from Reference 6.
‡ Data from Reference 24.

Because a considerable fraction of the bile acids in the infants are present in the serum, the above calculations are valid only if the specific radioactivity is the same in serum as in bile. This was found to be the case, both with cholic acid and THCA.

The serum extracts from samples drawn 24 and 48 h after the infusion of [^{3}H]7α-hydroxy-4-cholesten-3-one and [^{14}C]-cholic acid were chromatographed on HPLC (Fig. 4). The material in the tritium-containing peaks was analyzed by GC-MS as above. The least polar double peak contained 25*S* and 25*R* THCA. The compound corresponding to the peak that eluted at 28 ml was identified as 3α,7α,12α-trihydroxy-5β-C_{29}-dicarboxylic acid. The peak that eluted at 30 ml contained an unidentified compound in addition to small amounts of C_{29}-dicarboxylic acid. The material in the main polar peak that eluted in front of cholic acid (12–14 ml) was identified as a tetrahydroxylated 5β-cholestanoic acid, with all hydroxyl groups in the steroid nucleus.

The minor peak at 18 ml contained both ^3H- and ^{14}C-labeled cholic acid and the trace of ^3H activity at 37 ml was identified as chenodeoxycholic acid.

After elution of THCA, the chromatographic solvent was changed from 25% to 10% TFA/TEA in methanol in order to

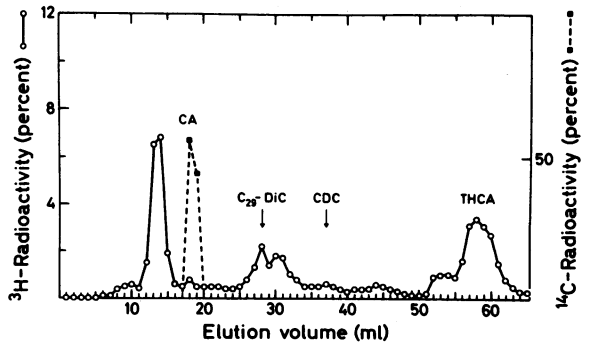


Figure 4. Reversed-phase HPLC of serum extract 24 h after infusion of 7β-^{3}H-7α-hydroxy-4-cholesten-3-one. 24% TFA/TEA was used as eluent (cf. Methods). The main polar peak at 13–14 ml contained THCA with a fourth hydroxyl group in the steroid nucleus. The material in the other peaks are as labeled in the figure: CA, cholic acid; C_{29}-DiC, 3α,7α,12α-trihydroxy-27-carboxymethyl-5β-cholestan-26-oic acid; CDC, chenodeoxycholic acid.

elute fewer polar compounds than THCA. One major peak was seen at 11–12 ml containing DHCA. This peak comprised 7.4% of the total radioactivity in the chromatogram.

The ^3H activity corresponding to THCA amounted to 20% of the total activity recovered from the 24-h serum sample and decreased to 10% in the 48-h serum sample. The activity corresponding to C_{29}-dicarboxylic acid increased from 4% to 7%, and the bile acid with four hydroxyl groups in the steroid nucleus increased from 17% to 29% of the total activity recovered in the 24- and 48-h serum sample, respectively.

The urine sample collected between 18 and 24 h after the infusion of [^{3}H]7α-hydroxy-4-cholesten-3-one and [^{14}C]cholic acid contained 8,600 dpm ^3H and 1,800 dpm ^{14}C per ml.

Peaks corresponding to THCA, cholic acid, and two more polar compounds were obtained. A major peak in front of cholic acid was identified as tetrahydroxylated 5β-cholestanoic acid with all hydroxyl groups in the steroid nucleus (cf. below). Only trace amounts of [^{3}H]chenodeoxycholic acid were detected.

Conversion in vivo of 7β-^{3}H-3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid in patient II. After infusion of the mixture of [^{3}H]THCA and [^{14}C]cholic acid, serum, urine, and bile samples were hydrolyzed and extracted as described in Methods. The $^3H/^{14}C$ ratio of the infused material was 32 (after extraction as with the samples). In the serum extracts there were only trace amounts of ^{14}C activity. In the urine samples the ratio was ~35, and in the extracts of the duodenal aspirates the ratio fell from 16 to 10 3–72 h after the infusion.

The bile extracts were analyzed by HPLC using 24% TFA/TEA in methanol as eluent. The chromatogram of the extract of the duodenal aspirate collected 8 h after the infusion is shown in Fig. 5. The most nonpolar splitted peak contained the *R*- and the *S* form of THCA. The peak that eluted between 31 and 34 ml was identified as a side-chain hydroxylated THCA derivative not identical with 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid. The minor peak (25–26 ml) contained 3α,7α,12α-trihydroxy-C_{29}-dicarboxylic acid and small amounts of a compound with still longer retention time than C_{29}-dicarboxylic bile acid on gas chromatography. This unknown compound did not have other ions than that at m/e 253 in common with the C_{29}-dicarboxylic acid. The shoulder of the main peak (20 ml) contained 24-OH-THCA, while the main peak contained cholic acid. The two most polar peaks contained bile acids with a tetrahydroxylated steroid nucleus (ions at m/e 251 and m/e 498).

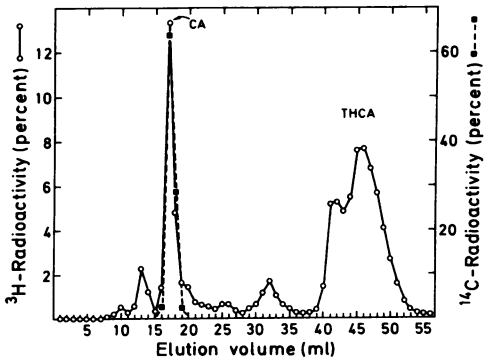
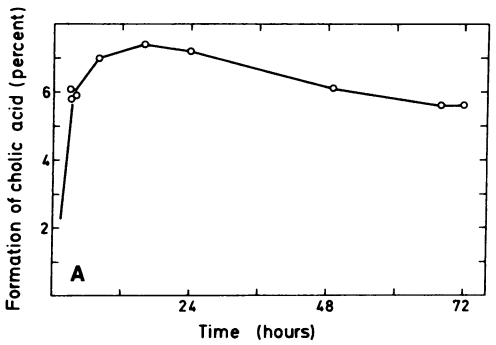


Figure 5. Reversed-phase HPLC of extract of duodenal bile collected 9 h after the administration of [^{3}H]THCA. 24% TFA/TEA was used as eluent. THCA was eluted at 45 ml, side-chain hydroxylated THCA at 32 ml (see Results), cholic acid at 17 ml and 5β-cholestanoic acids with four hydroxyl groups in the steroid nucleus at 13 and 10 ml.

The ratio of ^3H activity to that of ^{14}C activity recovered in cholic acid increased from 2.0 in the 3-h sample to 2.4 in the 16-h sample and then slowly declined to 1.8 in the 72-h sample. At 16 h the $^3H/^{14}C$ ratio was 7.4% of that in the infused mixture, indicating that only 7.4% of the THCA had been converted into cholic acid in this patient (Fig. 6 *A*). To calculate conversion of a putative precursor to cholic acid, Hanson and Williams divided the area beneath the specific activity curve of [^{3}H]cholic acid (derived from the precursor) by the specific activity curve produced by the injected [^{14}C]cholic acid (18). When this method was applied to our results (Fig. 6 *B*), a conversion of THCA to cholic acid of 7.2% was found. Thus, there is a good agreement between the two methods.

From the specific radioactivity decay curve of [^{3}H]THCA, the pool size of THCA could be estimated to 15 mg (70 mg/m^2). From the specific radioactivity curve of [^{14}C]cholic acid, it could be calculated that the pool size of cholic acid in patient II was 7.3 mg (35 mg/m^2) and that the synthetic rate of cholic acid was 3.1 mg/d (15 mg/m^2 · d) (Table II) (cf. Reference 24).

The HPLC profiles of the 24- and 72-h serum extracts are shown in Fig. 7. The least polar peak (elution volume 39–50) contained THCA. The 3α,7α,12α-trihydroxy-C_{29}-dicarboxylic



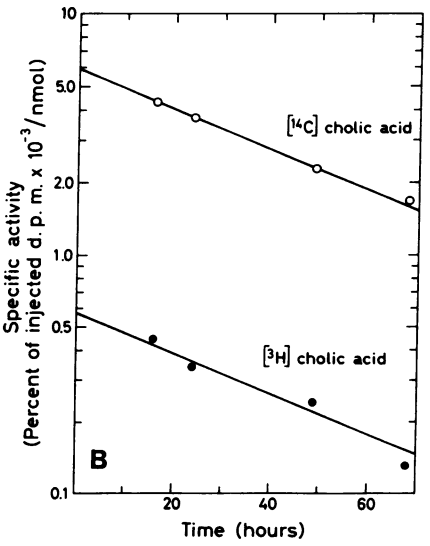


Figure 6. (*A*) Time course of cholic acid formation after infusion of [^{3}H]THCA and [^{14}C]cholic acid. The values plotted were derived from chromatograms as shown in Fig. 5. The ratio ^3H to ^{14}C in the cholic acid peak fraction was expressed in percent of the $^3H/^{14}C$ ratio in the infused material. (*B*) Specific activity decay curves of [^{3}H]cholic acid derived from [^{3}H]THCA and [^{14}C]cholic acid.

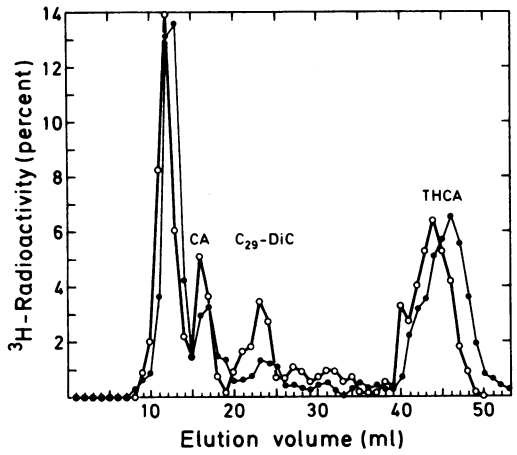


Figure 7. Reversed-phase HPLC of serum extracts 24 h (● —— ●) and 72 h (○ —— ○) after infusion of [^{3}H]THCA. 25% TFA/TEA was used as eluent. The main radioactivity peaks correspond to THCA (44 and 46 ml), C_{29}-dicarboxylic bile acid (23 ml), cholic acid (16–17 ml) and 5β-cholestanoic acid with four hydroxyl groups in the steroid nucleus (12–13 ml).

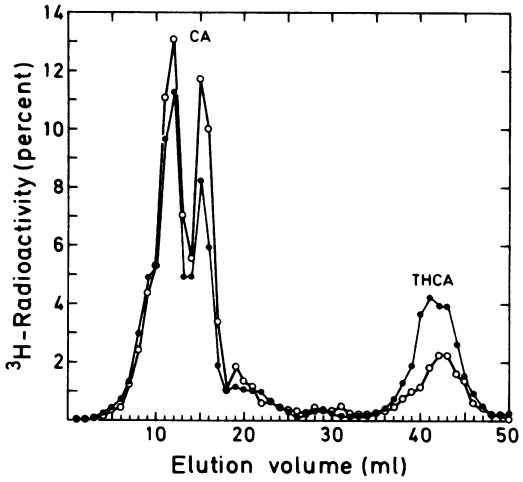


Figure 8. Reversed-phase HPLC of urine extracts 5–17 h (● —— ●) and 17–29 h (○ —— ○) after infusion of [^{3}H]THCA and [^{14}C]cholic acid. The main radioactive peaks correspond to THCA (40–44 ml), cholic acid (15 ml) and 5β-cholestanoic acids with tetrahydroxylated steroid nucleus (12 ml). The minor peak at 19 ml contained 24-OH-THCA. Trace amounts of C_{29}-dicarboxylic bile acid was found at 23 ml.

acid was eluted between 22 and 25 ml and cholic acid between 16 and 17 ml. The most polar major peak (elution volume 10–14 ml) contained the tetrahydroxylated 5β-cholestanoic acid with all hydroxyl groups in the steroid nucleus.

The ^{3}H activity in the peak corresponding to the C_{29}-dicarboxylic acid increased slowly from 3.3 to 7.7% in the 24- and 72-h serum samples. The high retention of ^{3}H in THCA in all the serum samples is consistent with an accumulation of THCA in the circulation (see also Fig. 4).

The urine samples collected between 5 and 17 h after the infusion contained 7,880 dpm ^{3}H and 250 dpm ^{14}C per milliliter and that collected between 17 and 29 h contained 6,900 dpm ^{3}H and 210 dpm ^{14}C per milliliter. The HPLC profiles of the extract of the two urine samples are shown in Fig. 8. THCA was eluted between 37 and 47 ml and cholic acid between 14 and 17 ml. The main polar peak contained the C_{27} steroid with tetrahydroxylated nucleus, indicating a rapid renal excretion of this metabolite. The minor peak that eluted after 19 ml contained 24-OH-THCA. The small material that eluted at 23 ml contained 3α,7α,12α-trihydroxy-C_{29}-dicarboxylic acid, but only trace amounts of radioactivity indicating a slow renal excretion of this metabolite.

Pattern of urinary bile acids. The total amount of C_{24} and C_{27} bile acids in urine was determined in two 24-h samples from patient I and in one sample from patient II and was found to be 1.0, 1.1, and 2.2 μmol, respectively. The corresponding excretion in healthy infants of the same age (n = 3) was found to vary between 0.2 and 0.7 μmol (mean, 0.4 μmol). The percentage of bile acids with an incompletely oxidized side chain in the three urine samples was found to be 35%, 45%, and 49%, respectively. No C_{27} or C_{29} bile acid could be detected in the urine samples from the healthy infants. The composition of the different bile acids in one of the urine samples from patient I is given in Table I. The percent distribution of the different bile acids in the unconjugated, taurine, glycine, and sulfate fractions was within normal limits (results not shown).

Conversion of [^{3}H]THCA to cholic acid in vitro. Incubation of THCA with the homogenate or the light mitochondrial fraction of the control liver resulted in the formation of at least three product peaks more polar than the substrate (Fig. 9 *A*). The most prominent polar peak had the same elution volume as cholic acid and the identity of this product was confirmed by GC-MS. The presence of 1 mM potassium cyanide (KCN) in the incubation medium stimulated the rate of cholic acid formation about twofold, and the rates reported in Table III were those obtained in the presence of KCN. The light mitochondrial fraction also exhibited the highest specific catalase activity (not shown). The microsomal fraction did not catalyze formation of cholic acid but the formation of the two smaller less polar peaks was highest with this fraction. The identity of these products was never established. Rates obtained with fractions from an adult liver were of the same magnitude. None of the subcellular liver fraction from the patients catalyzed formation of cholic acid from THCA (Fig. 9 *B* and Table III).

Conversion of [^{3}H]3α,7α-dihydroxy-5β-cholestanoic acid. Incubation of [^{3}H]DHCA with the homogenate or the light mitochondrial fraction both from the control and from patient II resulted in the formation of a product peak with an elution volume identical to that of chenodeoxycholic acid (Fig. 10 *A* and *B*). The identity of this product was confirmed by GC-MS in both cases (cf. Methods). A product peak slightly less polar than chenodeoxycholic acid had the same elution volume as THCA, and the identity of this product was confirmed by GC-MS. The rates of conversion of DHCA to chenodeoxycholic acid were considerably higher with fractions from the control than from the patient (Table III). In particular with the light mitochondrial fraction the rate was seven times higher in the control than in the patient. The rates of THCA formation from DHCA were of the same order of magnitude in both cases (Table III). A conversion to chenodeoxycholic acid was also detected

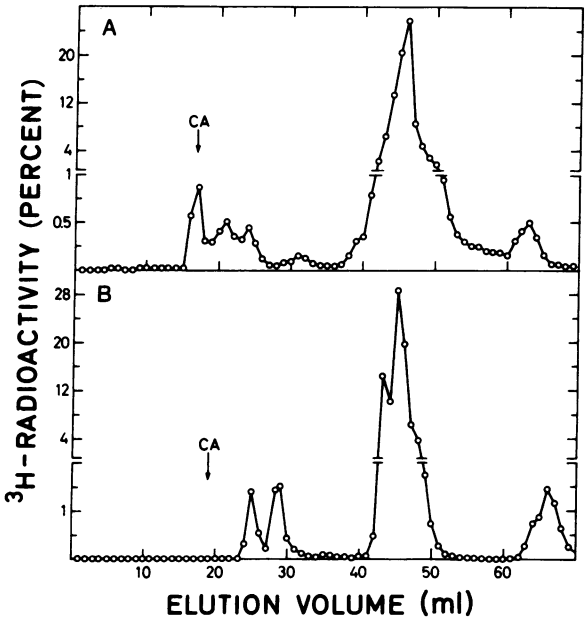


Figure 9. Reversed-phase HPLC of the extracts of incubations with 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and the light mitochondrial fraction from the liver of the control subject (*A*) and of a patient with Zellweger syndrome (*B*). The incubations were as described in Methods with the addition of 1 mM KCN. The chromatographic conditions were as described in Methods. The most polar product peak in *A* was identified as cholic acid (CA). The less polar product peaks in both *A* and *B* were trihydroxylated steroid nucleus derivatives.

in the light mitochondrial fraction from the liver of patient III. In this case, however, the unidentified material that eluted in front of chenodeoxycholic acid was not separated from the latter, thus making calculation of conversion obsolete.

Discussion

Suitability of the present in vivo model for studies on the importance of peroxisomes for biosynthesis of bile acids. The validity of the present model for studying the importance of the peroxisomes for biosynthesis of bile acids in vivo is dependent upon the absolute lack of peroxisomes in the liver. In the ideal model the mitochondrial function should be normal, in that a minor mitochondrial conversion of DHCA into chenodeoxycholic acid and THCA into cholic acid cannot be excluded. Under in vitro conditions, however, the peroxisomal fraction has a much higher specific activity than the mitochondrial fraction, and it is possible that the small activity obtained in the mitochondrial fraction may be due to contamination by peroxisomes (2, 3).

In spite of considerable efforts, no peroxisomes could be detected by electron microscopy of catalase-stained liver biopsies from our patients. The mitochondria observed in the liver biopsies were essentially normal (cf. Reference 25).

Absence of peroxisomal structures may not necessarily mean that the peroxisomal enzymes involved in side-chain cleavage of bile acid intermediates are absent. Thus, it was recently reported that catalase, normally found in the peroxisomes, was present in the cytosol of fibroblasts from patients with Zellweger

Table III. Conversion of THCA to Cholic Acid and of DHCA to Chenodeoxycholic Acid and to THCA by Liver Subcellular Fractions from Two Patients with Zellweger Syndrome and from One Control Subject

Fraction	Formation of cholic acid from THCA	Formation of chenodeoxycholic acid from DHCA	Formation of THCA from DHCA
	nmol × mg^{-1} × h^{-1}	**Percent conversion × mg^{-1} × h^{-1}*	
Whole homogenate			
Patient III (0.35 mg/ml)	n.d.		
Combined supernatant (E) fraction			
Patient II (0.9 mg/ml)	n.d.	4.3	5.4
Control subject (1.1 mg/ml)	0.7	13.5	6.3
Light mitochondrial (L) fraction‡			
Patient II (0.53 mg/ml)	n.d.	7.3	9.5
Patient III (0.56 mg/ml)	n.d.		
Mitochondrial (M + L) fractions‡			
Control subject (0.26 mg/ml)	2.7	52.3	15.4
Microsomal (P) fraction			
Patient II (1.6 mg/ml)	n.d.	0.6	4.2
Control subject (0.24 mg/ml)	n.d.	7.5	22.5

Incubation conditions were as described in Methods. Protein concentrations used in incubations are given in parentheses. n.d., not detectable; the detection limit corresponds to 0.01 nmol × mg^{-1} × h^{-1}.

* Given as percent conversion because after the completion of the experiments it was found that the unlabeled substrate was to some extent contaminated by THCA.

‡ The rates given are those in the presence of 1 mM KCN.

syndrome (26). We have confirmed this finding also in a liver biopsy from patient II (unpublished observation). In order to exclude presence of side-chain cleavage enzyme(s) elsewhere than in the peroxisomes in the patients with Zellweger syndrome, we studied the rate of conversion of DHCA and THCA into the corresponding bile acids in liver biopsies from two of the patients. There was no conversion of THCA, whereas a small conversion of DHCA could be demonstrated. Presumably the mitochondria were not responsible for the latter small activity because it was insensitive to KCN. It is a characteristic of the peroxisomal β-oxidation of long-chain fatty acids to be insensitive to cyanide (27). It should be pointed out that the low or absent side-chain cleavage activity in the liver biopsies from the patients was not due to a general inactivation. Thus both preparations contained normal or even higher levels of microsomal 25-hydroxylase activity and mitochondrial 26-hydroxylase activity towards 5β-cholestane-3α,7α,12α-triol (results not shown). The results of our in vitro experiments are in accordance with the contention that the patients with Zellweger syndrome are suitable for the present studies in vivo.

It should be emphasized that there were no signs of liver cirrhosis or cholestasis in any of our patients. With the exception of increased levels of THCA, the concentration of bile acids was normal in the liver biopsies as measured by isotope dilution–mass spectrometry (results not shown). That the accumulation of C_{27} bile acids with an incompletely oxidized side chain is not

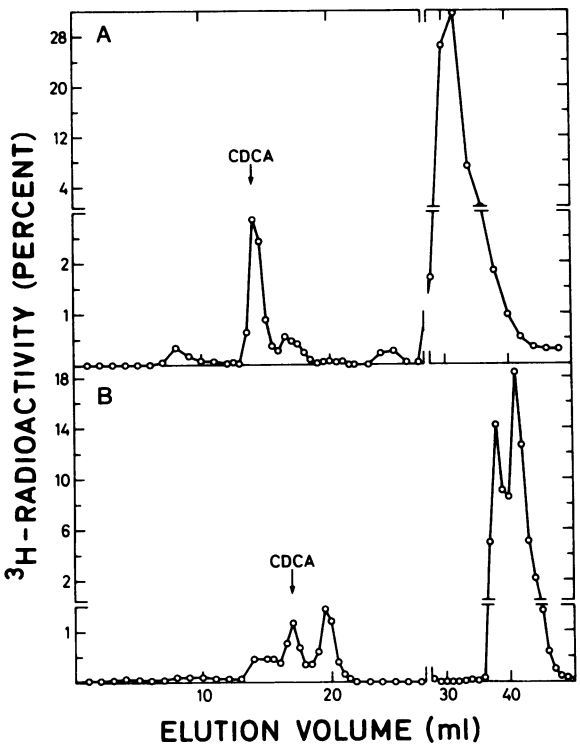


Figure 10. Reversed-phase HPLC of the extracts of incubations with 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) and the light mitochondrial fraction from the liver of the control subject (*A*) and of a patient with Zellweger syndrome (*B*). The incubation conditions were identical in *A* and *B* and as described in Methods with the addition of 1 mM KCN. The chromatographic conditions were as described in Methods. Two different Zorbax ODS columns were used in *A* and *B*. The most polar product peak was identified as chenodeoxycholic acid (CDCA). The least polar product peak (elution volume 19 ml) was identified as THCA.

due to a more general liver damage is also supported by the very rapid conversion of ^{3}H-labeled 7α-hydroxy-4-cholesten-3-one into DHCA and THCA in patient I. It is evident that all the microsomal, cytosolic, and mitochondrial enzymes involved in these conversions were intact. The deficiency is thus restricted to the side-chain cleavage.

The present patients were treated with phenobarbital, a known inducer of drug-metabolizing enzymes. We have shown, however, that the pattern of bile acid precursors and bile acids in serum of patients with Zellweger syndrome is similar before and during phenobarbital treatment. There is no reason to believe that the peroxisomal enzymes studied here should be markedly affected by the treatment.

Studies with ^{3}H-labeled 7α-hydroxy-4-cholesten-3-one: in vivo evidence for a block in the side-chain cleavage of DHCA and THCA in Zellweger syndrome. The maximal conversion of ^{3}H-labeled 7α-hydroxy-4-cholesten-3-one into cholic acid was only 10% (after 48 h), and the conversion after 12 h was only ~4%. The exact conversion into chenodeoxycholic acid could not be calculated, but was similar to or even less than the conversion into cholic acid. This is in marked contrast to the results by Hanson et al. (11), who studied conversion of labeled 7α-hydroxy-4-cholesten-3-one into the primary bile acids in adults. After administration of the labeled steroid to a patient with a bile fistula, ~85% of the administered isotope was recovered in bile as cholic acid and chenodeoxycholic acid after 12 h. This must mean that the side-chain cleavage is essentially complete after only one passage through the normal liver. Similarly, when the precursor was given to a subject with an intact enterohepatic circulation, there was an essentially complete conversion to primary bile acids (12).

The rapid conversion of labeled 7α-hydroxy-4-cholesten-3-one into DHCA and THCA and the subsequent slow conversion into the primary bile acids must mean that there is a deficiency in the side-chain cleavage of both DHCA and THCA. The specific activity in DHCA decreased however faster with time than did the specific radioactivity in THCA. Part of the explanation for this may be a conversion of DHCA into THCA. A previous study in vivo (8) as well as the present in vitro studies have shown that DHCA can be 12α-hydroxylated to yield THCA in human liver. Under conditions where DHCA accumulates, a larger fraction of DHCA may thus be converted into THCA.

The marked difference in specific radioactivity between DHCA and chenodeoxycholic acid as well as between THCA and cholic acid in the experiment with labeled 7α-hydroxy-4-cholesten-3-one (Fig. 3) would suggest that the major part of the primary bile acids in our patients are synthesized in a pathway bypassing DHCA and THCA as intermediates. This should, however, be evaluated in relation to the finding that the overall biosynthesis of bile acids was reduced by a factor of about 10.

It is evident that most of the DHCA and THCA formed are either excreted or transformed to products other than primary bile acids (cf. below). Our data do not allow us to draw conclusions concerning the quantitative importance of any alternative pathways.

The only alternative pathway for side-chain cleavage known at present is the 25-hydroxylase pathway described by Shefer et al. (28). This pathway is however specific for formation of cholic acid, and cannot be utilized for biosynthesis of chenodeoxycholic acid. Attempts to demonstrate accumulation of 25-hydroxylated C_{27} steroids (28) failed. In view of the small amounts of C_{24} bile acids formed in patients with Zellweger syndrome (cf. below), there are no reasons to believe that the alternative mechanism for side-chain cleavage is of importance under normal conditions.

Because the side-chain cleavage reaction is not rate-limiting under normal conditions, a reduction in the rate of this reaction in vitro and in vivo may not necessarily mean that the overall rate of biosynthesis of C_{24} bile acids is reduced. In consonance with the results of our previous work (6), we found, however, that both our patients had a markedly reduced pool size and rate of synthesis of cholic acid. Also the pool size of chenodeoxycholic acid was reduced (Table II). The higher urinary excretion of [^{3}H]cholic acid than of [^{3}H]chenodeoxycholic acid may explain the higher serum concentration of the latter (Table I).

Metabolism of THCA. In view of the finding that THCA and its metabolites were the major accumulated labeled compounds after administration of labeled 7α-hydroxy-4-cholesten-3-one (present work) and 5β-cholestane-3α,7α,12α-triol (6), labeled THCA was administered to patient II. There was a slow incorporation of label into cholic acid and after 16 h only ~7.4% had been converted into cholic acid. The same magnitude of conversion into cholic acid from THCA was also found in the

patient with Zellweger syndrome described in Reference 6, while the conversion of 5β-cholestane-3α,7α,12α-triol into cholic acid was two times higher (6). This difference may be due to an alternative pathway bypassing THCA under the pathological conditions. Individual differences cannot be excluded, however, and newly formed THCA from 5β-cholestane-3α,7α,12α-triol may be more efficiently converted to cholic acid than exogenous THCA. From the specific activity decay curve of THCA, it could be calculated that the pool of THCA was 15 mg. Thus the pool of THCA was greater than the total amount of C_{24} bile acids. Because the concentration of THCA in the bile of this patient was low, it is evident that the hepatic clearance of THCA is low as compared with the corresponding clearance of the C_{24} bile acids. The concentration of THCA in urine was also rather low, indicating a limited capacity for renal excretion.

A tetrahydroxylated 5β-cholestanoic acid with all the hydroxyl groups in the steroid nucleus was found to be an important metabolite of THCA both in the present and the previous (6) work. The position of the fourth hydroxyl group was never established. The 1- and 2-positions were excluded by the mass spectrum (cf. Reference 6), and it is evident from the present work that the fourth hydroxyl group cannot form an acetonide with the 3α-, the 7α- or the 12α-hydroxyl groups. Both in the experiment with labeled 7α-hydroxy-4-cholesten-3-one and THCA, the tetrahydroxylated 5β-cholestanoic acid was the major labeled metabolite in serum and urine. From the pattern of radioactive metabolites in urine, it is obvious that the more polar bile acids in general have the highest renal clearance. The 3α,7α,12α-trihydroxy-C_{29}-dicarboxylic acid is an exception, however, and in spite of a high concentration of this compound in serum there was only a relatively low urinary excretion and practically no biliary excretion at all. As judged from the experiment with labeled THCA, the rate of chain elongation of THCA to yield this dicarboxylic acid is a relatively slow process, and the accumulation in serum is thus mainly due to the inefficient elimination. The accumulation of very long chain fatty acids in patients with Zellweger disease (29) may to some extent also be due to increased chain elongation of long-chain fatty acids. Why the lack of peroxisomes should lead to an increased rate of chain elongation of THCA and possibly also fatty acids is not known.

We conclude from the results of the present and the previous (6) works that peroxisomes are of importance for side-chain cleavage of both DHCA and THCA and that the normal peroxisomal conversion of DHCA and THCA into bile acids is defective in patients with Zellweger syndrome. Microsomal hydroxylations of both the steroid side chain and the steroid nucleus are alternative pathways that promote excretion of the accumulated DHCA and THCA. Chain elongation of the accumulated THCA leads to 3α,7α,12α-trihydroxy-C_{29}-dicarboxylic acid, which is accumulated in serum owing to a relatively small renal clearance.

Acknowledgments

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