Importance of Peroxisomes in the Formation of Chenodeoxycholic Acid in Human Liver. Metabolism of $3\alpha,7\alpha$ -Dihydroxy- 5β -cholestanoic Acid in Zellweger Syndrome

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ABSTRACT. Infantile Zellweger syndrome belongs to the group of peroxisomal disorders that lack peroxisomes. Both trihydroxycoprostanic acid (THCA), the precursor to cholic acid, and dihydroxycoprostanic acid (DHCA), the precursor to chenodeoxycholic acid, accumulate in this disease. In previous studies, we have shown that liver peroxisomes are required for the conversion of THCA into cholic acid both *in vitro* and *in vivo* by measuring a defective conversion in infants with Zellweger syndrome. In our present study, the conversion of DHCA into chenodeoxycholic acid has been measured in an infant with Zellweger syndrome to evaluate the importance of liver peroxisomes for the formation of chenodeoxycholic acid. Coprostanic acidemia was present from the second day of life with high levels of THCA and only trace amounts of DHCA. The conversion of i.v. administered [^3H]DHCA into chenodeoxycholic acid was only 7% compared with the 80% conversion in an analogous study in an adult. There was, however, a rapid incorporation of ^3H into biliary THCA and, after a lag phase, the ^3H was incorporated into biliary cholic acid. After 72 h, 15% of [^3H]DHCA was converted to cholic acid. The pool size of DHCA was 1.2 mg/m² and the pool size of both cholic acid and chenodeoxycholic acid was markedly reduced. The renal excretion of cholic acid was more efficient than that of the less polar chenodeoxycholic acid. More polar metabolites of DHCA and THCA are formed in alternative metabolic pathways facilitating renal excretion of these toxic intermediates. We conclude that liver peroxisomes are essential for a normal conversion of DHCA into chenodeoxycholic acid. (*Pediatr Res* 29: 64–69, 1991)

Abbreviations

DHCA, $3\alpha,7\alpha$ -dihydroxycoprostanic acid (or $3\alpha,7\alpha$ -dihydroxy- 5β -cholestanoic acid)
THCA, $3\alpha,7\alpha,12\alpha$ -trihydroxycoprostanic acid
C₂₅-dicarboxylic acid, $3\alpha,7\alpha,12\alpha$ -trihydroxy-27-carboxymethylcoprostanic acid
GC-MS, gas chromatography-mass spectrometry

Cholesterol is oxidized to cholic acid and chenodeoxycholic acid by enzymes localized to several subcellular compartments of the human hepatocyte (1). Recently *in vitro* and *in vivo* studies in man have indicated that intact liver peroxisomes are required for the oxidative cleavage of the side chain of THCA, the last step in the biosynthesis of cholic acid (2–5). The relative importance of liver peroxisomes in the formation of chenodeoxycholic acid is less well established. We have shown that the *in vitro* formation of chenodeoxycholic acid from DHCA is most efficiently catalyzed by the peroxisomal fraction of rat and human liver homogenates (3, 6). The accumulation of DHCA in the circulation of infants with Zellweger syndrome that are lacking liver peroxisomes (7, 8) is, however, often reported to be considerably lower than that of THCA (4, 5, 9, 10). In addition, serum levels of chenodeoxycholic acid are often higher than those of cholic acid in the first weeks of life (4, 5, 9, 11). In a liver biopsy from a patient with Zellweger syndrome, there was at least a small formation of chenodeoxycholic acid from DHCA (5). In contrast, there was no detectable conversion of THCA into cholic acid (5).

We have shown that the tritium label of [^3H]7 α -hydroxy-4-cholesten-3-one, a common precursor to both cholic and chenodeoxycholic acid, was rapidly incorporated into both THCA and DHCA in an infant with Zellweger syndrome (5). There was a very slow incorporation of radioactivity into both cholic acid and chenodeoxycholic acid. Due to the experimental design, however, no detailed quantitative information could be obtained with respect to formation of labeled chenodeoxycholic acid, but it was concluded that peroxisomes are important also in the normal biosynthesis of this bile acid. To more specifically study the role of peroxisomes in the biosynthesis of chenodeoxycholic acid *in vivo*, we administered [7β - ^3H]DHCA together with [^{14}C]chenodeoxycholic acid and [^{14}C]cholic acid to an infant with Zellweger syndrome. We also measured the pool sizes of DHCA, chenodeoxycholic acid, and cholic acid.

PATIENT AND METHODS

Clinical. Three pregnancies preceded the present one. The first two resulted in two healthy girls. After the second pregnancy, the mother developed hypothyroidism and was treated with thyroxine. In 1982, a third girl was born with severe neurologic dysfunction, bilateral pes equino-varus, and stigmata similar to those in trisomy 21. She died when she was 2½ mo old. At autopsy, neuronal migration arrest, liver fibrosis, and microcysts in renal cortex were found. A frozen sample of urine stored from this daughter contained high amounts of coprostanic acids, con-

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firming 2 y *post-mortem* the assumed diagnosis of Zellweger syndrome. The mother aborted spontaneously once before she delivered the infant described here.

The boy was delivered in 1984 at term by cesarean section because of maternal bleeding. He weighed 2350 g, was dysmature, hypotonic, and had craniofacial dysmorphic features as described for Zellweger syndrome. Bilateral pes equino-varus and corneal opacity with partial detachment of iris to cornea in the right eye were also observed in the perinatal period.

At 1 mo of age, he got convulsions and was treated with diazepam. Clinical signs of bile stasis occurred at the age of 6 wk with decoloring of the feces and darkening of the urine, but icterus was not visible. No other clinical signs of hepatic failure were observed. At the age of 3 mo, the infant died.

At autopsy, bilateral bronchopneumonia and microcysts in renal cortex were found. The liver was enlarged with a greenish surface. Bile plugs in bile canaliculi and intracellular bile pigment accumulation were widespread. There were no signs of cell necrosis in the liver parenchyma, no cirrhosis, and only slight fibrosis. In the CNS, heterotopias of both pyramidal cells and Purkinje cells were seen. Nucleus olivarius lacked its natural foldings.

Laboratory findings in serum (reference data in parentheses) at the age of 4 d and 4 wk, respectively, were as follows: Hb, 22.6 and 11.4 g/dL; bilirubin, 139 and 12 μ mol/L; alanine aminotransferase, 31 U/L and 380 U/L (0–40 U/L); aspartate aminotransferase, 79 U/L and 646 U/L (0–40 U/L); γ -glutamyltranspeptidase, 62 and 169 U/L (5–14 U/L); total cholesterol, 2.5 and 3.0 mmol/L (2.6–5.5 mmol/L); total protein, 5.6 g/dL (6.0–8.0 g/dL); and albumin, 3.8 g/dL (3.5–5.0 g/dL). Coagulation tests were low normal. There was no hypoglycemia; 2.6 mmol/L was the lowest blood glucose level detected. Phytanic acid oxidase activity in cultured fibroblasts was 1 pmol/h/mg protein (45–130 pmol/h/mg protein). The amounts of very long chain fatty acids in cultured fibroblasts were determined: C:22 was 0.201 μ g fatty acid/mg protein (0.90 ± 0.40 μ g/mg) and C:26 was 0.394 μ g fatty acid/mg protein (0.07 ± 0.04 μ g/mg), giving a C:26/C:22 of 1.960 (0.08 ± 0.03). Bile acids in serum were identified and quantified by isotope dilution GC-MS (see Results).

Ethical aspects. The *in vivo* studies were approved by the Ethical Committee of the Central Hospital of Akershus, Norway.

Preparation of labeled steroids. [^3H]DHCA (50 Ci/mol) was prepared as described (12). [^{14}C]cholic acid (50 Ci/mol) and [^{14}C]chenodeoxycholic acid (50 Ci/mol) were from Amersham International, Amersham, England. The compounds were purified by HPLC before use. DHCA contained the 25-R and 25-S isomers. The study was performed with both C-25 stereoisomers, inasmuch as both forms are converted equally well into chenodeoxycholic acid *in vitro* (3).

***In vivo* administration of labeled steroids and collection of bile, serum, and urine samples.** [^3H]DHCA (10.4×10^6 dpm), [^{14}C]cholic acid (0.36×10^6 dpm) and [^{14}C]chenodeoxycholic acid (0.42×10^6 dpm) were dissolved in 0.5 mL of ethanol, filtered, and slowly mixed into 5.5 mL of 20% human serum albumin. At the age of 8 wk, 5 mL of the mixture were given i.v. during 15 min. Duodenal intubation was performed and duodenal contents were sampled at least 2 h after each meal, at 2, 6, 12, 24, 48, and 72 h after the infusion. The amount of aspirated duodenal fluid was only 1–2 mL to avoid disturbances in the pools of the bile acids. Venous blood samples were drawn after 24, 48, and 72 h. Urine was collected in four periods: 0–6, 6–24, 24–48, and 48–72 h.

Analyses of serum, bile, and urine. The serum samples, (0.25 mL) were hydrolyzed under alkaline conditions to deamidate the bile acids. The acidic extraction desulfates the bile acids as described previously (5, 13, 14). To the serum samples used for assay by isotope dilution-mass spectrometry, ^3H -labeled cholic acid, chenodeoxycholic acid, and deoxycholic acid were added before hydrolysis (13, 14). The methyl ester trimethylsilyl ether derivatives were prepared before combined GC-MS. An LKB

2091-051 GC-MS (LKB Instruments, Inc., Gaithersburg, MD) operating at 70 eV equipped with a four-channel multiple ion detector (LKB 2091-710) was used. The capillary fused silica column (obtained from JW Scientific Inc., Folsom, CA) was 30 m $\times$ 0.25 mm and was coated with the midpolarity DB-1301 phase. Initial temperature was 180°C for 2 min followed by an increase of 8°C/min up to 290°C. The above conditions were used for all identifications. For quantitations of the different bile acids, isothermal conditions were used under the same conditions as previously described in detail (4, 14). The approximate concentration of C₂₉-dicarboxylic acid was determined from the tracing at m/z 253 (4, 5). The concentration of DHCA was determined by tracing the ion at m/z 412 (from DHCA) and the ion at m/z 374 (from $^3\text{H}_4$ -labeled chenodeoxycholic acid) with use of an appropriate standard curve.

The bile, serum, and urine samples, collected after the *in vivo* administration of radioactive compounds, and an aliquot of the infusate were hydrolyzed as above and extracted with Sep-Pak C 18 cartridges (Waters Associates, Millipore Corp., Milford, MA) after acidification. The cartridges were washed with 5–10 mL H₂O before elution with 10 mL methanol (15, 16). Aliquots of the extracts were chromatographed on HPLC with a Zorbax octadecylchlorosilane column (5 $\times$ 250 mm, DuPont de Nemours & Co., Inc., Wilmington, DE) and 20% 30 mM trifluoroacetic acid adjusted to pH 2.9 with triethylamine in methanol as solvent (trifluoroacetic acid/triethylamine) at a flow rate of 0.5–1 mL/min. Aliquots of the fractions were evaporated and assayed for ^3H and ^{14}C as described previously (4). The recovery from the HPLC column was essentially complete. Fractions containing radioactivity were extracted on Sep-Pak C 18 cartridges after acidification as described. The extracts were converted to the methyl ester trimethylsilyl ether derivatives and analyzed by combined GC-MS as described above. The identity of the different C₂₇ and C₂₄ bile acids and the C₂₉-dicarboxylic bile acid was

Table 1. Serum bile acid concentrations in infant with Zellweger syndrome

Bile acid (μ mol/L)*	Age (d)	
	2	30
Cholic acid	1.7	2.2
Chenodeoxycholic acid	0.8	1.1
THCA	5.5	22.8
DHCA	<0.2	<0.5
C ₂₉ -dicarboxylic acid	0.7	15.9

* Serum concentrations of bile acids in 10 healthy 1-mo-old infants: cholic acid, 4.18 ± 2.31 μ mol/L; chenodeoxycholic acid, 7.09 ± 3.01 μ mol/L (Ref. 19). THCA, DHCA, and C₂₉-dicarboxylic acid are normally not detected in infant serum (Ref. 4).

Fig. 1. Reversed-phase HPLC of extract of serum collected 24 h after infusion of [^3H]DHCA. Twenty percent trifluoroacetic acid in methanol was used as eluent (see Materials and Methods). The peak that eluted at 12–14 mL contained 3 α ,7 α ,12 α -tri-hydroxy-C₂₉-dicarboxylic acid and the minor peaks at 3–4 mL and 7–8 mL contained C₂₇-steroid acids with four hydroxyl groups in the steroid nucleus. The material in the other peaks are as labeled in the figure: CA, cholic acid; CDCA, chenodeoxycholic acid.

Fig. 2. Reversed-phase HPLC of extracts of duodenal bile collected 6 and 48 h after the administration of $[^3\text{H}]\text{DHCA}$, $[^{14}\text{C}]\text{chenodeoxycholic acid (CDCA)}$, and $[^{14}\text{C}]\text{cholic acid (CA)}$. Twenty percent trifluoroacetic acid in methanol was used as eluent (see Materials and Methods). The main radioactive peaks correspond to DHCA (34–37 mL), THCA (19–23 mL), CDCA (15–17 mL), and CA (9–10 mL). The peak at 6 mL contained a 5β -cholestanoic acid with four hydroxyl groups in the steroid nucleus as judged by the mass spectrum.

Fig. 3. Time course of the formation of chenodeoxycholic acid (CDCA) and cholic acid (CA) after infusion of $[^3\text{H}]\text{DHCA}$, $[^{14}\text{C}]\text{CDCA}$, and $[^{14}\text{C}]\text{CA}$. The values plotted were derived from chromatograms as shown in Figure 2. The approximate CDCA formation (O—O) was calculated by expressing the ratio of ^3H to ^{14}C in the biliary CDCA peak as per cent of the $[^3\text{H}]\text{DHCA}/[^{14}\text{C}]\text{CDCA}$ ratio in the infused material. To calculate the CA formation (●—●) the ratio of ^3H to ^{14}C in the biliary CA peak was expressed as per cent of the $[^3\text{H}]\text{DHCA}/[^{14}\text{C}]\text{CA}$ ratio in the infused material.

established by selected ion monitoring as described previously (4, 5).

In vivo conversions of $[^3\text{H}]\text{DHCA}$ into chenodeoxycholic acid and cholic acid were measured as follows: The activities of biliary $[^3\text{H}]\text{chenodeoxycholic acid}$ and $[^3\text{H}]\text{cholic acid}$ formed *in vivo*

Fig. 4. Sp act decay curves of biliary $[^3\text{H}]\text{DHCA}$, $[^3\text{H}]\text{cholic acid (CA)}$, and $[^3\text{H}]\text{chenodeoxycholic acid (CDCA)}$ after infusion of $[^3\text{H}]\text{DHCA}$. By extrapolating the $[^3\text{H}]\text{DHCA}$ decay curve to the ordinate, the hypothetical sp act at time 0 h was obtained. The latter value was used to calculate the DHCA pool. The same method (18) was used for the $[^3\text{H}]\text{CA}$ and $[^3\text{H}]\text{CDCA}$ decay curves to calculate the pool sizes of cholic and chenodeoxycholic acids (not shown).

were divided with the activities of biliary $[^{14}\text{C}]\text{chenodeoxycholic acid}$ and $[^{14}\text{C}]\text{cholic acid}$, respectively. The $^3\text{H}/^{14}\text{C}$ ratio of the biliary chenodeoxycholic acid as per cent of the $[^3\text{H}]\text{DHCA}/[^{14}\text{C}]\text{chenodeoxycholic acid}$ ratio in the infusate gives an approximate measure of the *in vivo* conversion of DHCA into chenodeoxycholic acid (4). The calculation of the per cent conversion of DHCA into cholic acid was based on the $^3\text{H}/^{14}\text{C}$ ratio of biliary cholic acid to that of the $[^3\text{H}]\text{DHCA}/[^{14}\text{C}]\text{cholic acid}$ in the infusate in the same way. The conversions were also calculated according to the method described by Hanson and Williams (17). The two methods were in good agreement (5). The pool sizes of cholic acid, chenodeoxycholic acid, and DHCA were determined by the Lindstedt technique (18).

Urine sample. Urine (2 L) collected before, during, and after the *in vivo* study, was mixed and extracted on Sep-Pak C 18 cartridges after acidification (15). Full recovery of radioactivity was achieved. The extract was hydrolyzed and reextracted as above. Aliquots were chromatographed on HPLC using a 5- μm Beckman Ultrasphere octadecylchlorosilane column (10 $\times$ 250 mm) and eluted as above. The more polar metabolites that appeared before cholic acid were reextracted and rechromatographed for better separation. Radioactive fractions occurring in the HPLC were extracted and derivatized before combined GC-MS.

RESULTS

Serum bile acids. The clinical diagnosis of Zellweger syndrome was supported by measuring high levels of THCA in a blood sample drawn on the 2nd day of life (Table 1) (4). At this early stage, the serum level of C_{29} -dicarboxylic acid was very low. After 4 wk of age, however, the level of the C_{29} -dicarboxylic acid had increased considerably and was in fact the dominating coprostanic acid in serum together with THCA. The serum levels of the primary bile acids in man, cholic acid and chenodeoxycholic acid, were low on the 2nd day of life and continued to be low compared to levels of age-matched controls (19) and to levels we have measured in other infants with the Zellweger syndrome (4

Table 2. Bile acid pool sizes and synthesis rate in patients with Zellweger syndrome

	Cholic acid		Chenodeoxycholic acid		THCA (pool size, mg/m ²)	DHCA (pool size, mg/m ²)
	Pool size* (mg/m ²)	Synthesis (mg/m ² × 24 h)	Pool size* (mg/m ²)	Synthesis (mg/m ² × 24 h)		
Patient I†	11	9.5	15			
Patient II†	35	15	6	0.6	70	
Patient III‡	24	9				
Patient IV§	1.5	1.1	5.7	3.2		12

* Pool sizes in a healthy 7-wk-old infant: cholic acid, 696 mg/m²; chenodeoxycholic acid, 174 mg/m² (Ref. 23).

† Data from Ref. 5.

‡ Data from Ref. 4.

§ Present study.

Fig. 5. Reversed phase HPLC of extracts of urine obtained after infusion of [³H]DHCA, [¹⁴C]chenodeoxycholic acid, and [¹⁴C]cholic acid. Twenty percent trifluoroacetic acid in methanol was used as eluent (see Materials and Methods). A, 0–6-h urine sample; B, 6–24-h urine sample; C, 24–48-h urine sample. The chromatogram of the 48–72-h urine sample extract was similar to C. The main radioactive peaks correspond to DHCA (39–46 mL), THCA (20–25 mL), 3 α ,7 α ,12 α -trihydroxy-C₂₉-dicarboxylic acid (14–15 mL), and 5 β -cholestanic acids with four hydroxyl groups in the steroid nucleus (6 and 9 mL).

5). The concentrations of DHCA were very low compared to those of THCA (Table 1).

After the *in vivo* administration of [³H]DHCA, [¹⁴C]cholic acid, and [¹⁴C]chenodeoxycholic acid, serum samples were hydrolyzed, extracted, and subjected to HPLC. The HPLC profile of the 24-h serum extract is shown in Figure 1. The least polar split peak (elution volume 41–49 mL) was shown to contain DHCA (R- and S-form). In this extract, [³H]DHCA accounted for 23% of the total recovered activity. In the 48-h extract it accounted for 18% and, after 72 h, 12% of the total tritium activity in serum was retained in DHCA.

The material in the main split peak that eluted after 21–25 mL was identified as THCA by GC-MS (Fig. 1). Still, after 48 h and 72 h, the peak containing THCA accounted for the main tritium activity, 34 and 30%, respectively. The minor peak at 17 mL contained both ³H- and ¹⁴C-labeled chenodeoxycholic acid and that at 10 mL, ³H- and ¹⁴C-labeled cholic acid. The compound corresponding to the peak that eluted at 13 mL was identified as 3 α ,7 α ,12 α -trihydroxy-C₂₉-dicarboxylic acid. The peaks that eluted at 7–8 mL and 3–4 mL contained coprostanic acids with a tetrahydroxylated steroid nucleus (m/z 251 and

m/z 498). The amount of tritium in each of these polar fractions increased about two-fold in the 72-h serum extract.

Biliary excretion of metabolites of [³H]DHCA. The main part of the bile acid pool is kept within the enterohepatic circulation, and bile contains the immediate products of bile acid synthesis. Thus, samples of duodenal bile (see Materials and Methods) are most suitable to measure the conversion of [³H]DHCA into chenodeoxycholic and cholic acid. Bile extracts analyzed by HPLC from samples collected 6 and 48 h after the infusion are shown in Figure 2.

The least polar split peak in the chromatogram contained DHCA. During the first 24 h, the tritium activity in the DHCA peak was mainly incorporated into the split peak at 21 mL containing THCA and into the peak at 16 mL containing chenodeoxycholic acid. Later, two more polar peaks occurred: the peak at 9.5 mL contained cholic acid and the peak at 6 mL contained a not yet identified compound. The peak that eluted at 12 mL contained 3 α ,7 α ,12 α ,24-tetrahydroxycoprostanic acid and an unidentified compound. In the 72 h bile extract, the [³H]DHCA was still detectable (3% of total tritium activity of the radiogram). At that time, the major part of the ³H-activity appeared in the peaks that contained THCA and the unidentified compound at 6 mL and, to a lesser extent, in the peaks corresponding to chenodeoxycholic acid and cholic acid.

Both ³H- and ¹⁴C-activities were measured in the peaks containing chenodeoxycholic acid and cholic acid. The ³H/¹⁴C ratio in these peaks was correlated to the ³H/¹⁴C ratio in the infusate. The ratio of ³H-activity from [³H]DHCA to that of ¹⁴C-activity from [¹⁴C]chenodeoxycholic acid in the infusate was approximately 22. The corresponding [³H]DHCA/[¹⁴C]cholic acid ratio of the infusate was approximately 25.

The ratio of ³H-activity to that of ¹⁴C-activity recovered in the biliary chenodeoxycholic acid increased from 0 after 2 h to 1.6 after 72 h. At 72 h, the ³H/¹⁴C ratio was 7% of that in the infused material, indicating that only about 7% of the DHCA had been converted into chenodeoxycholic acid in this patient (Fig. 3). When the area under the sp act curve of [³H]chenodeoxycholic acid (derived from precursor) was divided by the area under the sp act curve of the [¹⁴C]chenodeoxycholic acid (17), the conversion of [³H]DHCA into [³H]chenodeoxycholic acid was calculated to be only 2%. In a similar study, an 80% conversion of DHCA into chenodeoxycholic acid after 12 h was found in an adult with bile fistula (20).

The conversion of [³H]DHCA into [³H]cholic acid was more difficult to estimate due to the fact that [³H]cholic acid was still synthesized after 72 h (Fig. 3). At 72 h, the ³H/¹⁴C ratio had increased to 3.9, corresponding to 15% of the [³H]DHCA/[¹⁴C]cholic acid ratio in the infused material. A sp act curve of [³H]cholic acid was constructed by drawing a line through the point giving the sp act at 72 h and with the same decay as the sp act curve of [¹⁴C]cholic acid. The conversion of [³H]DHCA into [³H]cholic acid was then calculated to be about 12% (17).

From the specific radioactivity decay curve of biliary [³H]DHCA, the pool size of DHCA in the patient could be estimated to be 0.23 mg (1.2 mg/m²), with a synthesis rate of 0.9 mg/m²/

Fig. 6. Proposed metabolism of DHCA (I) in the Zellweger patient. The DHCA is 12 α -hydroxylated to THCA (II). Both DHCA and THCA accumulate as a consequence of impaired peroxisomal formation of chenodeoxycholic acid (III) and cholic acid (IV). THCA accumulates more than DHCA due to an efficient 12 α -hydroxylase. The side chain of THCA is slowly elongated to a C₂₉-dicarboxylic acid, which accumulates as a consequence of a slow renal excretion. The steroid nucleus and the side chain of THCA are efficiently hydroxylated. The tetrahydroxylated coprostanic acids formed are efficiently excreted to urine and thus accumulate to a small extent.

24 h (Fig. 4). From the specific radioactivity decay curve of biliary [¹⁴C]chenodeoxycholic acid and [¹⁴C]cholic acid (not shown), the pool size of chenodeoxycholic acid was calculated to be 1.1 mg (5.7 mg/m²) and the synthesis rate 0.6 mg/d (3.2 mg/m²/24 h). The pool size of cholic acid was about 0.3 mg (1.5 mg/m²) and the synthesis rate only about 0.2 mg/24 h (1.1 mg/m²/24 h) (Table 2).

Renal excretion of ³H- and ¹⁴C-labeled bile acids. The urine samples collected between 0 and 6 h after infusion contained 1100 dpm of ³H and 960 dpm of ¹⁴C per mL. The relative recovery of ¹⁴C radioactivity to that of ³H decreased in the subsequent urine samples, and the 48–72 h sample contained 3700 dpm of ³H and 370 dpm of ¹⁴C per mL.

The HPLC profiles of the extracts of the three urine samples are shown in Figure 5. DHCA was eluted between 40 and 50 mL, THCA between 20 and 25 mL, chenodeoxycholic acid between 16 and 17 mL, the 3 α ,7 α ,12 α -trihydroxy-C₂₉-dicarboxylic acid at 14 mL, 3 α ,7 α ,12 α ,24-tetrahydroxycoprostanic acid at 11 mL as a shoulder to the peak containing cholic acid at 10 mL (Fig. 5B). The two most polar peaks at 9.5 mL and 6 mL both contained tetrahydroxylated C₂₇-steroid acids with four hydroxyl groups in the steroid nucleus.

In the urine samples collected during the first 6 h after infusion, [³H]DHCA and [³H]THCA were the main peaks. Throughout the study, [³H]THCA continued to be the main peak, indicating an efficient excretion of this metabolite. There was also a high incorporation of ³H activity into the tetrahydroxylated C₂₇ steroid with all hydroxyl groups in the steroid nucleus. The peak that eluted at 14 mL was small and unchanged throughout the study, indicating a slow renal excretion of the 3 α ,7 α ,12 α -trihydroxy-C₂₉-dicarboxylic acid.

The activity from [¹⁴C]cholic acid accounted for almost all of the ¹⁴C activity detected in the urine samples. Only trace amounts of [¹⁴C]chenodeoxycholic acid could be detected in the samples.

DISCUSSION

Zellweger syndrome was diagnosed clinically on the 1st day of our patient's life. The high levels of C₂₉-dicarboxylic acid in

serum and the high C:26/C:22 ratio in cultured fibroblasts were considered sufficient to exclude "Pseudo-Zellweger" and to confirm the diagnosis.

Zellweger syndrome with its lack of peroxisomes represents an attractive model to estimate the role of liver peroxisomes *in vivo* (5, 21). High serum levels of THCA and to a lesser extent DHCA are constant findings. About 10% of THCA has been found to be converted into cholic acid under this pathologic condition (5). This contrasts with the very efficient conversion obtained in previous analogous *in vivo* studies in adults (80–90% conversion) (17) and explains the very low pool sizes of cholic acid in infants with Zellweger syndrome compared to age-matched controls (22, 23).

In our present *in vivo* study on the conversion of DHCA into primary bile acids in an infant with Zellweger syndrome, a 2-h bile sample contained ³H-labeled DHCA and THCA and ¹⁴C-labeled cholic and chenodeoxycholic acid, indicating a rapid hepatic uptake and biliary excretion despite clinical signs of bile stasis. As time passed, only a minor part of the tritium in the administered [³H]DHCA was incorporated into chenodeoxycholic acid. In addition, the course of incorporation was very slow, only about 3% after 12 h. This finding is in contrast to the 80% conversion of DHCA into chenodeoxycholic acid after 12 h in an adult with bile fistula (20). The very low pool size of chenodeoxycholic acid, calculated from the sp act decay curve of biliary [¹⁴C]chenodeoxycholic acid, supports the low formation of chenodeoxycholic acid. The pool size in this infant (5.7 mg/m²) was in fact only 3% of that of a 7-wk-old healthy infant in a previous study (23). These findings strongly suggest that, in man, intact liver peroxisomes are required for a normal formation of chenodeoxycholic acid from DHCA.

Although serum and urine levels of bile and coprostanic acids vary with age and within the Zellweger population, the main part of the coprostanic acids, including the C₂₉-dicarboxylic acid and the tetrahydroxylated acids, is 12 α -hydroxylated. Serum levels of DHCA may vary considerably (24), and probably reflect individual differences in activity of, or access of DHCA to, the 12 α -hydroxylase. It is evident from the present and previous studies (5), however, that the 12 α -hydroxylation of DHCA into

THCA is highly effective and represents the first step in the main metabolic pathway for DHCA in peroxisome deficiency (Fig. 6). Consequently, the conversion of DHCA into cholic acid is at least two-fold higher than its conversion into chenodeoxycholic acid. In contrast, the patient with a bile fistula incorporated only 2% of the tritium activity from administered [^3H]DHCA into cholic acid (20). In the metabolic defect found in our patient, the cholic acid formation pathway is relatively more important, allowing an increased excretion of toxic metabolites (25, 26).

The course of cholic acid formation showed a lag phase of about 12 h, and the conversion was most rapid after 48 h when the formation of chenodeoxycholic acid leveled off (Fig. 3). This may be due to a competition between DHCA and THCA for the same enzymes. A slow formation of THCA cannot explain the finding, inasmuch as THCA was detectable already in the 2-h bile sample and was the major labeled compound in the 6-h bile sample.

After 72 h, about 25% of the tritium label of [^3H]DHCA had been incorporated into primary bile acids. Despite the ongoing cholic acid formation, it is evident that primary bile acids were only minor metabolites of [^3H]DHCA.

Bile stasis has been observed previously in infants with Zellweger syndrome. In our patient, the bile stasis may explain the small pool size of cholic acid compared with those measured in previously studied infants with this syndrome (Table 2) (4, 5). The uptake of bile acids from the portal vein to the hepatocytes is most probably impaired in bile stasis, allowing more of the bile acids to pass into the systemic circulation. According to the analyses of the urine samples, the renal clearance of [^{14}C]cholic acid seemed to be considerably higher than that of [^{14}C]chenodeoxycholic acid. This may contribute to the very low pool size of cholic acid compared with that of chenodeoxycholic acid.

To summarize, the present *in vivo* study has shown that the side-chain cleavage of DHCA into chenodeoxycholic acid is almost completely blocked in a patient with Zellweger syndrome. As a consequence, the pool size of chenodeoxycholic acid is markedly reduced. The major part of the accumulated DHCA is converted to THCA. The latter compound and unusual hydroxylated metabolites of this acid accumulate due to impaired conversion into cholic acid.

We can conclude from this and previous *in vivo* studies (4, 5) that liver peroxisomes are essential for a normal conversion of coprostanic acids into primary bile acids in man.

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REFERENCES

1. Björkhem I 1985 Mechanism of bile acid biosynthesis in mammalian liver. In: Danielsson H, Sjövall J (eds) *Comprehensive Biochemistry*. Elsevier, Amsterdam, pp 231–278
2. Kase F, Björkhem I, Pedersen JI 1983 Formation of cholic acid from $3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholestanoic acid by rat liver peroxisomes. *J Lipid Res* 24:1560–1567
3. Kase BF, Prydz K, Björkhem I, Pedersen JI 1986 *In vitro* formation of bile acids in human liver peroxisomes. *Biochim Biophys Acta* 877:37–42
4. Kase BF, Björkhem I, Hågå P, Pedersen JI 1985 Defective peroxisomal cleavage of the C_{27} -steroid side chain in cerebro-hepato-renal syndrome of Zellweger. *J Clin Invest* 75:427–435
5. Kase BF, Pedersen JI, Strandvik B, Björkhem I 1985 *In vivo* and *in vitro* studies on formation of bile acids in patients with the Zellweger syndrome. Evidence that peroxisomes are of importance in the normal biosynthesis of both cholic and chenodeoxycholic acid. *J Clin Invest* 76:2393–2402
6. Prydz K, Kase BF, Björkhem I, Pedersen JI 1986 Formation of chenodeoxycholic acid from $3\alpha,7\alpha$ -dihydroxy- 5β -cholestanoic acid by rat liver peroxisomes. *J Lipid Res* 27:622–628
7. Goldfischer S, Moore C, Johnson A, Spiro A, Valsamis A, Wisniewski H, Ritch R, Norton W, Rapin I, Gartner L 1973 Peroxisomal and mitochondrial defects in the cerebro-hepato-renal syndrome. *Science* 182:62–64
8. Wanders RJA, van Roermund CWT, Schutgens RBH, Barth PG, Heymans HSA, van den Bosch H, Tager JM 1990 The inborn errors of peroxisomal β -oxidation: a review. *J Inher Metab Dis* 13:4–36
9. Hanson R, Szczepanik-van Leeuwen P, Williams G, Grabowski G, Sharp H 1979 Defects of bile acid synthesis in Zellweger's syndrome. *Science* 203:1107–1108
10. Monnens L, Bakkeren J, Parmentier G, van Haelst U, Trijbels J, Eyssen H 1980 Disturbance in bile acid metabolism of infants with the Zellweger (cerebro-hepato-renal) syndrome. *Eur J Pediatr* 133:31–35
11. Parmentier GG, Janssen GA, Eggermont EA, Eyssen HJ 1979 C_{27} bile acids in infants with coprostanic acidemia and occurrence of a $3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β - C_{29} dicarboxylic bile acid as a major component in their serum. *Eur J Biochem* 102:173–183
12. Gustafsson J 1979 Metabolism of $3\alpha,7\alpha$ -dihydroxy- 5β -cholestanoic acid by rat liver *in vivo* and *in vitro*. *J Lipid Res* 20:265–270
13. Angelin B, Björkhem I, Einarsson E, Ewerth S 1982 Hepatic uptake of bile acids in man. Fasting and postprandial concentrations of individual bile acids in portal venous and systemic blood serum. *J Clin Invest* 70:724–731
14. Björkhem I, Falk O 1983 Assay of the major bile acids in serum by isotope dilution-mass spectrometry. *Scand J Clin Lab Invest* 43:163–170
15. Kase BF, Prydz K, Björkhem I, Pedersen JI 1986 Conjugation of cholic acid with taurine and glycine by rat liver peroxisomes. *Biochem Biophys Res Commun* 138:167–173
16. Kase BF, Björkhem I 1989 Peroxisomal bile acid-CoA:amino acid N-acyltransferase in rat liver. *J Biol Chem* 264:9220–9223
17. Hanson RF, Williams GC 1977 Metabolism of $3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholestanoic acid in normal subjects with an intact enterohepatic circulation. *J Lipid Res* 18:656–659
18. Lindstedt S 1957 The turnover of cholic acid in man. *Acta Physiol Scand* 40:1–9
19. Sinikka H, Similä S, Finni K, Mäentausta O, Jänne O 1980 Cholic acid and chenodeoxycholic acid concentrations in serum during infancy and childhood. *Acta Paediatr Scand* 69:659–662
20. Hanson RF 1971 The formation and metabolism of $3\alpha,7\alpha$ -dihydroxy- 5β -cholestan-26-oic acid in man. *J Clin Invest* 50:2051–2055
21. Kase BF 1989 Role of liver peroxisomes in bile acid formation: inborn error of C_{27} -steroid side chain cleavage in peroxisome deficiency (Zellweger syndrome). *Scand J Clin Lab Invest* 49:1–10
22. Watkins JB, Ingall D, Szczepanik P, Klein PD, Lester R 1973 Bile salt metabolism in the newborn. Measurement of pool size and synthesis by stable isotope technique. *N Engl J Med* 288:431–434
23. Heubi JE, Balistreri WF, Suchy FJ 1982 Bile salt metabolism in the first year of life. *J Lab Clin Med* 100:127–136
24. Eyssen H, Eggermont E, Van Eldere J, Jaeken J, Parmentier G, Janssen G 1985 Bile acid abnormalities and the diagnosis of the cerebro-hepato-renal syndrome (Zellweger syndrome). *Acta Paediatr Scand* 74:539–544
25. Hanson RF, Williams GC, Hachey D, Sharp HL 1977 Hepatic lesions and hemolysis following administration of $3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholestan-26-oic acid to rats. *J Lab Clin Med* 90:536–548
26. Lee MJ, Whitehouse MW 1965 Inhibition of electron transport and coupled phosphorylation in liver mitochondria by cholanic (bile) acids and their conjugates. *Biochim Biophys Acta* 100:317–328