Defective Peroxisomal Cleavage of the C_{27}-Steroid Side Chain in the Cerebro-Hepato-Renal Syndrome of Zellweger

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Abstract

Based on in vitro work with rat liver, we recently suggested that the peroxisomal fraction is most important for the oxidation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) into cholic acid. The cerebro-hepato-renal syndrome of Zellweger is a fatal recessive autosomal disorder, the most characteristic histological feature of which is a virtual absence of peroxisomes in liver and kidneys. This disease offers a unique opportunity to evaluate the relative importance of peroxisomes in bile acid biosynthesis. A child with Zellweger syndrome was studied in the present work. In accordance with previous work, there was a considerable accumulation of THCA, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid (24-OH-THCA), 3α,7α,12α-trihydroxy-27-carboxymethyl-5β-cholestan-26-oic acid (C_{29}-dicarboxylic acid), and 3α,7α-dihydroxy-5β-cholestanoic acid in serum. In addition, a tetrahydroxylated 5β-cholestanoic acid with all the hydroxyl groups in the steroid nucleus was found. ^{3}H-Labeled 5β-cholestane-3α,7α,12α-triol was administered intravenously together with ^{14}C-labeled cholic acid. There was a rapid incorporation of ^{3}H in THCA and a slow incorporation into cholic acid. The specific radioactivity of ^{3}H in THCA was about one magnitude higher than that in cholic acid. The conversion was evaluated by following the increasing ratio between ^{3}H and ^{14}C in biliary cholic acid. The rate of incorporation of ^{3}H in cholic acid was considerably less than previously reported in experiments with healthy subjects, and the maximal conversion of the triol into cholic acid was only 15–20%. About the same rate of conversion was found after oral administration of ^{3}H-THCA. Both in the experiment with ^{3}H-5β-cholestane-3α,7α,12α-triol and with ^{3}H-THCA, there was an efficient incorporation of ^{3}H in the above unidentified tetrahydroxylated 5β-cholestanoic acid. There was only slow incorporation of radioactivity into 24-OH-THCA and into the C_{29}-dicarboxylic acid. From the specific activity decay curve of ^{14}C in cholic acid obtained after intravenous injection of ^{14}C-cholic acid, the pool size of cholic acid was calculated to be 24 mg/m^2 and the daily production rate to 9 mg/m^2 per d. This corresponds to a reduction of ~85 and 90%, respectively, when compared with normal infants. It is concluded that liver peroxisomes are essential in the normal conversion of THCA to cholic acid. In the Zellweger syndrome this conversion is defective, and as a consequence the accumulated THCA is either excreted as such or transformed into other metabolites by hydroxylation or side chain elongation. The accumulation of THCA, as well as the similar rate of conversion of 5β-cholestane-3α,7α,12α-triol and THCA into cholic acid, support the contention that the 26-hydroxylase pathway with intermediate formation of THCA is the most important pathway for formation of cholic acid in man.

Introduction

The cerebro-hepato-renal syndrome of Zellweger is an autosomal recessive disorder that is fatal within the first year of life (1). The virtual absence of peroxisomes in liver and kidney has been described as the most consistent histologic finding (2).

Increased amounts of C_{27}-bile acid intermediates have been detected in bile, serum, and urine of the patients (3–6). These intermediates include 3α,7α,dihydroxy-5β-cholestan-26-oic acid (DHCA),[1] 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA), and 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (24-OH-THCA) (4–6). The accumulation of bile acid precursors with a partially oxidized side chain has been attributed to a mitochondrial defect in the cleavage of the steroid side chain (4, 6).

According to current concepts, the degradation of the steroid side chain in the biosynthesis of cholic acid starts with a mitochondrial 26-hydroxylation and subsequent oxidation to THCA (for review see reference 7). The further conversion seems to involve 24-OH-THCA and 3α,7α,12α-trihydroxy-24-on-5β-cholestanoic acid as intermediates, and propionic acid is cleaved off in the final step. An alternative pathway has been proposed involving microsomal 25- and 24*S*-hydroxylation of 5β-cholestane-3α,7α,12α-triol. Subsequent oxidation in this pathway yields 3α,7α,12α,25-tetrahydroxy-5β-cholestan-24-one which is cleaved by cytosolic enzymes to cholic acid and acetone (8).

Accumulation of THCA in patients with Zellweger syndrome would lend support to the contention that the major pathway in cholic acid formation involves 26-hydroxylation and intermediate formation of THCA.

We have recently shown that in rat liver the formation of cholic acid from THCA is most efficiently carried out by the peroxisomal fraction (9, 10).

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1. *Abbreviations used in this paper:* DHCA, 3α,7α-dihydroxy-5β-cholestan-26-oic acid; GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography; 24-OH-THCA, 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; C_{29}-dicarboxylic acid, 3α,7α,12α-trihydroxy-27-carboxymethyl-5β-cholestan-26-oic acid.

Zellweger syndrome appears to offer a unique opportunity to evaluate the importance of liver peroxisomes in bile acid formation. In addition, the degree of conversion of different bile acid intermediates into THCA in such patients may give information with respect to the relative importance of the two alternative pathways described for biosynthesis of cholic acid in man.

We here report a study on the conversion in vivo of 5β-cholestane-3α,7α,12α-triol and THCA into cholic acid in a patient with Zellweger syndrome. No peroxisomes could be found in the liver of this patient. The limited capacity for conversion of these precursors into cholic acid in vivo suggests that the metabolic defect resides at the peroxisomal level and that under normal conditions the major part of cholic acid formation proceeds via the peroxisomal pathway.

Methods

Clinical. The child was a fullterm female of healthy nonconsanguineous parents. In mother's family several members had epilepsy. Muscular hypotonia prevailed from birth. She displayed a craniofacial dysmorphy characteristic of Zellweger syndrome (1). There was pes equino varus and four finger line bilaterally. With time, hepatomegaly developed and convulsions appeared which increased in frequency and duration. She was treated with 5 mg/kg phenobarbital. This floppy infant died at 4-mo-old.

Cerebral computer tomography was normal while EEG was pathologic. The levels of alanine aminotransferase and aspartate aminotransferase were highest shortly after birth (aspartate aminotransferase 1,164 U/L [normal range 26–75 U/L], alanine aminotransferase 423 U/L [14–84 U/L]) and declined towards the final stage. Gamma GT (600 U/L [10–35 U/L]) and alkaline phosphatases (1,700–2,000 U/L [275–1,050 U/L]) remained high. Total bilirubin in serum was normal. Serum protein electrophoresis was normal. Normotest values gradually decreased, but were almost normal at the time of the in vivo study (60% [70–130%]). Serum cholesterol was 6.7 mmol/ml (2.2–4.2 mmol/ml) while triglycerides were in the normal range. No pipecolate was detectable in urine. Cultured fibroblasts contained high amounts of saturated long chain fatty acids (11). At autopsy the liver was found enlarged with normal extrahepatic bile ducts. The liver surface was uneven, and microscopically marked interlobular septae and large periportal areas were seen. The lobular architecture was intact and cirrhosis was not detected. By electron microscopy the cellular architecture appeared normal with increased amounts of glycogen. Normal peroxisomal structures could not be recognized. In the central nervous system neuronal migration arrest typical for Zellweger syndrome (12) was observed.

The ethical aspects of the present study were approved by the ethical board of the Norwegian Council for Science and the Humanities.

Preparation of labeled steroids. 7β-^{3}H-5β-cholestane-3α,7α,12α-triol (7 Ci/mol) and 7β-^{3}H-THCA (200 Ci/mol) were prepared as described (13, 14). Separation on high pressure liquid chromatography (HPLC) of the latter compound (10) showed that it contained 28% of the 25*R* and 72% of the 25*S* isomer. 24-^{14}C-Cholic acid (50 Ci/mol) was from Amersham International plc, Amersham, England. The compounds were purified by HPLC (see below) before use.

In vivo administration of labeled steroids and collection of bile and serum samples. The tritium-labeled triol (13.4×10^6 dpm) and the ^{14}C-labeled cholic acid (1.5×10^6 dpm) were dissolved in 0.5 ml of ethanol. The solution was passed through a Millex 0.22-μm filter (Millipore Co., Bedford, MA) and slowly added under shaking to 5 ml of sterile human albumin solution (albumin 20%, Kabi AB, Stockholm, Sweden). The mixture was slowly infused intravenously during 30 min. Duodenal intubation was performed under X-ray visualization. Duodenal contents were sampled at least 2 h after each meal (mother's milk), 3, 6, 24, and 48 h after the infusion. The amount of aspirated duodenal fluid was small, each sample ~1 ml to avoid disturbances in the pools of the bile acids. Venous blood samples were collected after 24 and 48 h. Urine was collected after 15 h.

1 mo later the patient received ^{3}H-labeled THCA, 13×10^6 dpm, and ^{14}C-labeled cholic acid, 1.5×10^6 dpm, dissolved in 180 μl ethanol, mixed into the milk, and given through a gastric tube. Blood samples were collected 6, 12, and 24 h after the meal and duodenal content was aspirated 11 h later.

Extraction, chromatography, and analytical procedures. The serum samples (0.25 ml) were hydrolyzed and extracted as described previously (15, 16). In this extraction procedure, most but not all cholesterol is removed. To the serum samples used for assay by isotope dilution-mass spectrometry, deuterium-labeled cholic acid, chenodeoxycholic acid, and deoxycholic acid were added before hydrolysis (15, 16). The methyl ester trimethylsilyl ether derivatives were prepared before combined gas chromatography-mass spectrometry (15, 16). An LKB 9000 instrument equipped with a multiple ion detector and an 1.5% SE-30 column was used (15, 16). The amount of cholic acid was measured by use of the ions at *m/e* 623 and 628 (corresponding to the M-15 ion in the mass spectrum of derivative of unlabeled and $^2H_5$-labeled cholic acid, respectively [16]). The amount of chenodeoxycholic acid was measured by use of the ions at *m/e* 370 and 374 (corresponding to the M-2 × 90 ion in the mass spectrum of derivative of unlabeled and ^{2}H-labeled chenodeoxycholic acid, respectively). The amount of THCA was measured by use of the ions at *m/e* 410 and *m/e* 374 (corresponding to the M-3 × 90 ion in the mass spectrum of derivative of unlabeled THCA and the M-2 × 90 ion in the mass spectrum of the derivative of $^2H_4$-chenodeoxycholic acid).

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 separated by HPLC using a Zorbax ODS column (50 × 250 mm, particle size 5 μm). Phosphoric acid/potassium dihydrogen phosphate, 25 mM, pH 3.4, 24% in methanol was used as solvent at a flow rate of 1 ml/min. (Several columns were used. The retention times shown in the figures below may therefore vary from one chromatogram to another.) 1-ml fractions were collected. Parts of the fractions were evaporated and after addition of counting solution counted at an efficiency of 82% for ^{14}C and 58% for ^{3}H in a liquid scintillation spectrometer (Packard Tri-Carb 300C). External standardization was used for determination of quenching and the energy region settings were automatically optimized. The data were corrected for ^{14}C activity counted in the tritium channel. Fractions containing radioactivity were extracted twice with 9 ml ethyl acetate after acidification with HCl. The ethyl acetate was washed twice with water, taken to dryness under N_2, and the residue dissolved in a small volume of methanol. The extracts were converted to the methyl ester trimethylsilyl ether derivatives (15, 16) and analyzed by combined gas chromatography-mass spectrometry (GC-MS) as described above. For identification of the different steroids, characteristic ions were followed by multiple ion detection. In a few cases in which sufficient amounts of material could be obtained, a full mass spectrum was recorded (refer above and to Results).

Assumptions and calculations. It was assumed that no loss of ^{3}H occurred during the metabolism of 7β-^{3}H-labeled precursors of cholic acid. The justification of this assumption is supported by a recent study with a mixture of 7β-^{3}H- and 24-^{14}C-labeled cholic acid. There was only a negligible loss of ^{3}H from the cholic acid during the enterohepatic circulation in man (Björkhem, I., K. Einarsson, and K. Nilsell, unpublished observation).

The conversion of the ^{3}H-labeled triol and the ^{3}H-labeled THCA into cholic acid was based on the ratio between ^{3}H and ^{14}C in the mixture injected into the patient, which was compared with the ratio between ^{3}H and ^{14}C in the cholic acid isolated. It is then assumed that 100% conversion of the ^{3}H-labeled precursor should yield cholic acid with a ratio ^{3}H/^{14}C, identical with that in the material injected. The validity of this experimental approach is evident from results of a similar study by Hanson et al. (17, 18). It was reported that after

intravenous administration of ^{3}H-THCA or ^{3}H-5β-cholestane-3α,7α,12α,26-tetrol together with ^{14}C-cholic acid to normal subjects, the ^{3}H-cholic acid specific activity decay curves were within 81–91% of the ^{14}C-cholic acid specific activity decay curves. Furthermore, in an in vivo study on another Zellweger patient that was performed after the completion of this work, we obtained a larger number of bile samples and were thus able to compare our method with that used by Hanson and Williams (17). The percent conversion calculated by our method was 20% higher than calculated by the method of Hanson and Williams (17). From the decay of the specific radioactivity of ^{14}C in cholic acid in bile isolated after the intravenous administration of ^{14}C-cholic acid, the pool size and half life of cholic acid was determined according to the Lindstedt technique (19).

Results

Serum bile acids. Fig. 1 *A* shows multiple ion recordings of the ion at *m/e* 253 (corresponding to the trihydroxycoprostanoic nucleus) and at *m/e* 255 (corresponding to the dihydroxycoprostanoic nucleus) obtained in analysis of a hydrolyzed and derivatized sample of a serum extract from the patient. From the pattern obtained, it is evident that there was a predominance of trihydroxy bile acids. In the recording of the ion at *m/e* 253, peaks occurred with retention times identical to derivatives of cholic acid, THCA, and 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (24-OH-THCA). In addition there was a prominent peak with a retention time typical for that of the 3α,7α,12α-trihydroxy-27-carboxy-methyl-5β-cholestan-26-oic acid (C_{29}-dicarboxylic acid) described by Parmentier et al. (5). Evidence has been given that the structure of this acid is 3α,7α,12α-trihydroxy-27-carboxymethyl-5β-cholestan-26-oic acid (20). In addition, a peak occurred with a retention time slightly longer than that of 24-OH-THCA in the recording of the ion at *m/e* 253. The identity of this compound could never be established.

The peak occurring in the tracing of *m/e* 255, just in front of the chenodeoxycholic acid peak, corresponds to cholesterol. The trimethylsilyl ether of methyl chenodeoxycholic acid partly cochromatographed with deoxycholic acid under the conditions employed. Under slightly different chromatographic conditions it was shown that there was no derivative of deoxycholic acid. There were significant amounts of DHCA but only traces of 24-OH-DHCA. In a separate recording of the ion at *m/e* 372 (corresponding to the M-90 ion in the mass spectrum of derivative of lithocholic acid) it was shown that there were only traces of lithocholic acid.

In view of the relatively small amounts of serum available, it was not possible to record full mass spectra. The identity of cholic acid was established, however, by selected recording of the ions at *m/e* 623 (M-15) and *m/e* 368 (M-3 × 90). The identity of chenodeoxycholic acid was also confirmed by the tracing of the ion at *m/e* 370 (M-2 × 90). The identity of THCA was confirmed by the tracing of the ions at *m/e* 500 and *m/e* 410 (M-2 × 90 and M-3 × 90 in the mass spectrum of derivative of THCA), and that of 24-OH-THCA by the ions at *m/e* 588 and *m/e* 498 (M-2 × 90 and M-3 × 90 in the mass spectrum of derivative of 24-OH-THCA). The identity of the C_{29}-dicarboxylic acid was established by use of the ions at *m/e* 482 (M-3 × 90) and *m/e* 737 (M-15) (5).

The concentrations of cholic acid, chenodeoxycholic acid, and THCA were determined by isotope dilution-mass spectrometry using $^{2}H_{5}$-labeled cholic acid and chenodeoxycholic acid as internal standards (16). The approximate concentration of 24-OH-THCA and C_{29}-dicarboxylic acid was determined from the tracing at *m/e* 253. It was assumed that the relative

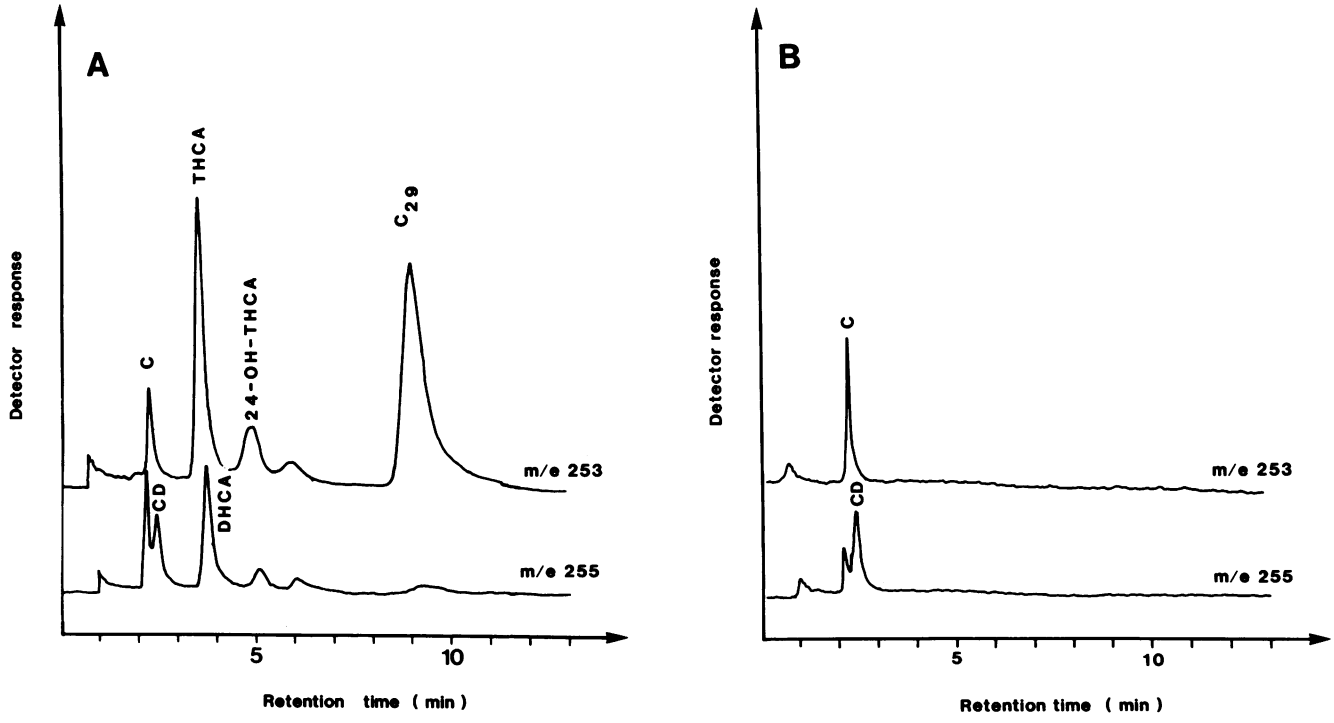

Figure 1. (*A*) Multiple ion detector recording of trimethylsilyl ether methyl ester derivative of an extract of serum from the patient at the age of 10 wk. C, cholic acid; CD, chenodeoxycholic acid. (*B*) Similar recording from a healthy infant at the same age.

intensity of the ion at *m/e* 253 was similar in the mass spectrum of THCA, 24-OH-THCA, and C_{29}-dicarboxylic acid. The results of these determinations of serum samples taken at different occasions are summarized in Table I.

For the reasons of comparison, a selected ion monitoring analysis of a serum extract from a healthy infant of the same age is shown in Fig. 1 *B*. As expected, only peaks corresponding to cholic acid and chenodeoxycholic acid were seen. When compared with normal fasting reference values (21, 22), the serum concentrations of cholic acid and chenodeoxycholic acid could be regarded as normal. During the further progression of the disease, both chenodeoxycholic and cholic acid remained within normal limits (Table I). Deoxycholic acid was not detected in most samples. The precursors of the primary bile acids and the C_{29}-dicarboxylic acid that were measured in high concentrations are normally not detected in infant serum.

Biliary bile acids. Using the same isotope dilution technique as in the analyses of the serum bile acids, it was shown that the composition of bile acids in a sample of bile from the patient at the age of 10 wk was the following: cholic acid 42%, chenodeoxycholic acid 53%, THCA 4%, 24-OH-THCA 1%, and C_{29}-dicarboxylic acid 0%. Normally, only cholic acid and chenodeoxycholic acid can be expected to occur in the bile of an infant of this age, and the relative amount of cholic acid can be expected to vary between 20 and 60% (23, Björkhem, I., unpublished observation).

Conversion in vivo of 7β-^{3}H-5β-cholestane-3α,7α,12α-triol into bile acids. After administration in vivo of ^{3}H-labeled 5β-cholestane-3α,7α,12α-triol and ^{14}C-cholic acid, bile, serum, and urine samples were hydrolyzed and extracted as described in Methods. The ratio of ^{3}H to ^{14}C activity of the infused material (in dpm) was 8.9. In the serum extracts this ratio was 13.3 in the 24-h sample and 15.8 in the 48-h sample. In bile extracts this ratio fell from 3.8 to 2.9.

The bile extracts were analyzed by HPLC, and the chromatogram of the extract of the sample collected 3 h after the infusion is shown in Fig. 2. Two major peaks of radioactivity

Table I. Serum Bile Acid Concentrations from the Age Diagnosis Established Until the Child with Zellweger Syndrome Died

	Age (*wk*)			
Bile acids	4	10*	13	16
	μmol/L	*μmol/L*	*μmol/L*	*μmol/L*
Cholic acid‡	3.8	3.2	2.8	1.6
Chenodeoxycholic acid‡	10.4	7.0	7.3	3.7
Deoxycholic acid‡	0.0	0.2	0.0	0.0
THCA	11.5	2.8	2.9	2.1
DHCA	1.2	1.2	2.3	2.5
24-OH-THCA	1.0	0.4	0.8	1.0
3α,7α,24-Trihydroxy-5β-cholestanoic acid	0.3	0.1	0.8	0.8
3α,6α,7α,12α-Tetrahydroxy-5β-cholestanoic acid§	0.5			1.6
C_{29}-dicarboxylic acid	3.2	5.1	2.7	3.3

* At this age, 7β-^{3}H-5β-cholestane-3α,7α,12α-triol was infused.
‡ Normal values for cholic acid, chenodeoxycholic acid, and deoxycholic acid in healthy infants are 0.6–18 μmol/L, 1.9–21 μmol/L, and <0.2 μmol/L, respectively, at the age of 1 mo, and 0.3–20 μmol/L, 1.9–16 μmol/L, and <0.1 μmol/L, respectively, at the age of 3 mo (22).
§ Tentatively identified (see text).

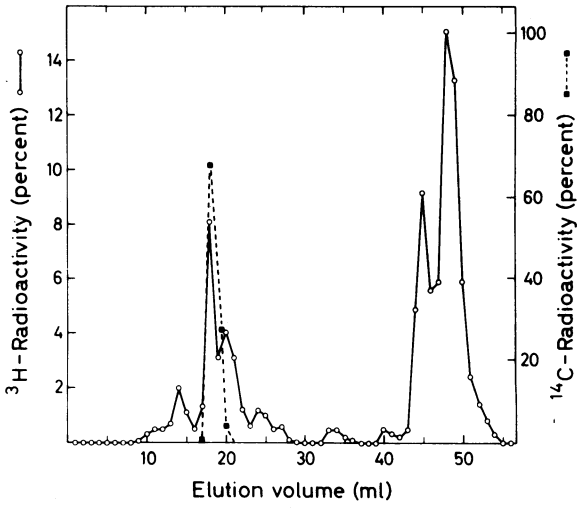

Figure 2. Reversed phase high pressure liquid chromatogram of a duodenal bile extract 3 h after infusion of ^{3}H-5β-cholestane-3α,7α,12α-triol and ^{14}C-cholic acid. Hydrolysis, extraction, and HPLC procedures are given in Methods. The main peaks correspond to the following: 3α,6α,7α,12α-tetrahydroxy-5β-cholestanoic acid (tentatively identified, see text), 14 ml, cholic acid, 18 ml, 24-OH-THCA, 20 ml, 25*R*-THCA, 45 ml, and 25*S*-THCA, 48 ml. For additional information see text.

were detected. The more polar of these, that eluted at 18 ml, contained both ^{3}H and ^{14}C activity. The retention time was identical to that of cholic acid. The presence of cholic acid in this material was confirmed by GC-MS using selected ion monitoring of the ions at *m/e* 253, *m/e* 368, and *m/e* 623. The largest and most nonpolar splitted peak that eluted from 44 to 53 ml had the same retention time as 25*R* and 25*S* THCA. Only tritium activity was detected in this peak. The presence of THCA in this material was confirmed by GC-MS, using the ion at *m/e* 253, *m/e* 410, and *m/e* 500. Several smaller tritium-containing peaks were also detected in the chromatograms. The peak that eluted at 14 ml contained material identified by GC-MS as a derivative of THCA with a 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). (For tentative identification see below.) A small peak that eluted at 20 ml contained material that was identified as 24-OH-THCA using the ions at 253, *m/e* 498 and *m/e* 588. The material corresponding to the small peak that eluted at 33–34 ml was never identified. Recovery from the column was essentially complete in this chromatographic system and no unmetabolized ^{3}H-5β-cholestane-3α,7α,12α-triol was detected.

The chromatogram in Fig. 2 showed that ^{3}H-THCA accounted for 65% of total recovered activity in the 3-h bile sample. This relative activity declined almost linearly to 35% after 48 h. This finding reflected rapid conversion of ^{3}H-triol into ^{3}H-THCA and a high degree of retention of the formed ^{3}H-THCA.

After 24 and 48 h, the ^{3}H/^{14}C ratio in cholic acid was 15% of the infused material (Fig. 3). The value of 20% found after 35 h was based on very low activity (~200 dpm for tritium) and might therefore be uncertain.

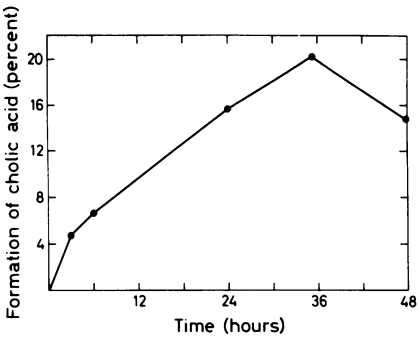

Figure 3. Time course of cholic acid formation after infusion of ^{3}H-5β-cholestane-3α,7α,12α-triol and ^{14}C-cholic acid. The values plotted are derived from chromatograms as shown in Fig. 2. The ratio of ^{3}H/^{14}C in the cholic acid peak fraction (elution volume 18 ml) has been expressed as percentage of the ^{3}H/^{14}C ratio of the infused ^{3}H-triol and ^{14}C-cholic acid tritium activity.

Fig. 4 shows the specific radioactivity decay curves of ^{14}C-cholic acid, ^{3}H-cholic acid, and ^{3}H-THCA. From the specific radioactivity decay curve of ^{14}C-cholic acid, it could be calculated that the pool size of cholic acid in the patient was only 24 mg/m^2. The synthetic rate of cholic acid (19, 24) was 9 mg/m^2/d. The specific radioactivity of ^{3}H in cholic acid increased between 3 and 6 h and then decreased. Due to the small amounts of radioactivity, the accuracy in the latter measurement may be low, and no specific information can therefore be obtained from the decay of ^{3}H specific radioactivity between 6 and 24 h. The specific radioactivity of ^{3}H-THCA was about one magnitude higher than that of ^{3}H-cholic acid and remained constant between 3 and 24 h. The most likely explanation of this fairly constant specific activity is a continuous formation of ^{3}H-THCA from an intrahepatic pool of ^{3}H-triol.

The serum samples drawn 24 and 48 h after the infusion of ^{3}H-5β-cholestane-3α,7α,12α-triol and ^{14}C-cholic acid were hydrolyzed and extracted as described in Methods. The extracts were separated on HPLC and the material in the tritium-containing peaks was analyzed by GC-MS as above. The HPLC profile of the extract of the serum sample taken 24 h after the infusion is shown in Fig. 5. The least polar splitted peak (elution volume 34–40 ml) was shown to contain THCA. A major tritium-containing peak eluted in front of cholic acid (elution volume 12 ml). The material had the same elution volume relative to cholic acid as that in the corresponding chromatogram obtained in the analysis of the bile samples (refer above). Smaller radioactive peaks were shown to contain cholic acid (elution volume 15 ml) and 24-OH-THCA (elution volume 19 ml). The material that eluted at 20 ml was identified as the C_{29}-dicarboxylic acid. A full mass spectrum was thus identical with that reported by Parmentier et al. (5) and contained prominent peaks at 253, 281, 343(M-2 × 90-229), 482(M-3 × 90), 572(M-2 × 90), and 662(M-90). The peak that eluted at 5 ml was not identified. A small amount of the injected radioactivity was not recovered from the column in this chromatographic system. When the samples were analyzed on the same Zorbax ODS column but eluted with 19% acetate (pH 4.37) in methanol (8), an additional peak amounting to 15% of the tritium activity was obtained. The retention time was identical to that of 5β-cholestane-3α,7α,12α,26-tetrol. The material in this peak was not sufficient for complete identifi-

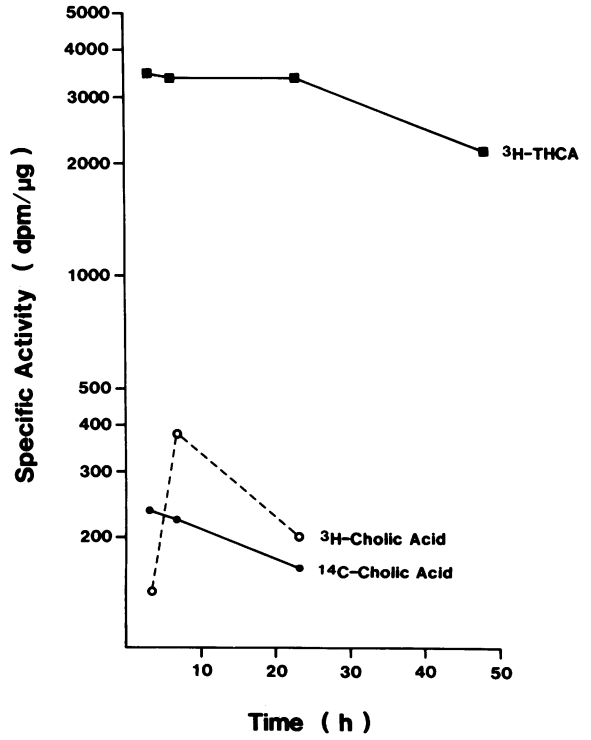

Figure 4. Specific radioactivity decay curves of ^{3}H-THCA, ^{3}H-cholic acid and ^{14}C-cholic acid obtained after intravenous administration of a mixture of ^{3}H-5β-cholestane-3α,7α,12α-triol and ^{14}C-cholic acid.

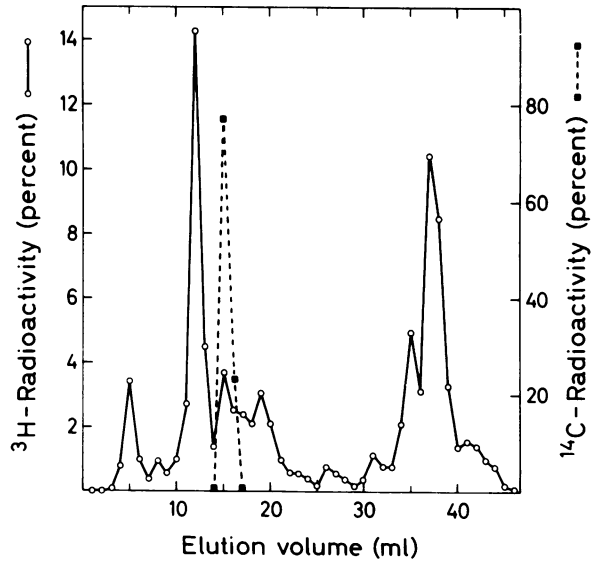

Figure 5. Reversed phase high pressure liquid chromatogram of serum extract 24 h after infusion of ^{3}H-5β-cholestane-3α,7α,12α-triol. Hydrolysis, extraction, and HPLC procedures are given in Methods. The most polar peak with elution volume 5 ml was not identified. Other peaks correspond to the following: 3α,6α,7α,12α-tetrahydroxy-5β-cholestanoic acid (tentatively identified, see text), 12 ml, cholic acid, 15 ml, 24-OH-THCA, 19 ml, 25*R* and 25*S*-THCA, 35 and 37 ml, respectively. C_{29}-dicarboxylic acid was identified in the fraction that eluted at 20 ml. For additional information see text.

cation, but the presence of a trihydroxy cholestane nucleus was confirmed by selected ion monitoring of the ion at *m/e* 253. The tritium activity corresponding to THCA amounted to 35 and 26% of the total activity recovered after 24 and 48 h, respectively.

In the overnight urine sample collected 15 h after the infusion the ^{3}H activity was 5,500 dpm/ml. After extraction and chromatography, this activity could entirely be accounted for as THCA or more polar products. In particular, 55% of the activity was recovered in fractions corresponding to tetrahydroxylated 5β-cholestanoic acid with all hydroxyl groups in the steroid nucleus (see below).

Conversion in vivo of 7β-^{3}H-THCA into bile acids. After administration per os of ^{3}H-THCA and ^{14}C-labeled cholic acid, bile and serum samples were hydrolyzed, extracted, and analyzed by HPLC as described in Methods. The material in the tritium-containing peaks was identified by GC-MS as described above. The HPLC profile of the extract of the bile collected 11 h after administration of the radioactive compounds is shown in Fig. 6. About 50% of the tritium activity was recovered in the peak corresponding to THCA (elution volume 39–48 ml), and most of the remaining was recovered in the cholic acid fraction. The material in the small peak that eluted in front of cholic acid (elution volume 11–12 ml) was not identified, but corresponded to the similar peak observed in the chromatogram of the serum extract (see below). The ratio of ^{3}H to ^{14}C in the cholic acid peak was 7.7% of that in the radioactive mixture given per orally. This shows that after 11 h the formation of cholic acid from THCA is considerably reduced compared with what is found when ^{3}H-THCA is administered to normal subjects (17).

In the chromatogram of the extract of the pooled serum samples (Fig. 7), a peak corresponding to THCA was identified as above (elution volume 46–53 ml). The major tritium-containing peak that eluted in front of cholic acid (elution volume 12–14 ml) was identified as a tetrahydroxylated 5-β-cholestanoic acid, with all hydroxyl groups in the steroid nucleus. A full mass spectrum of this compound contained

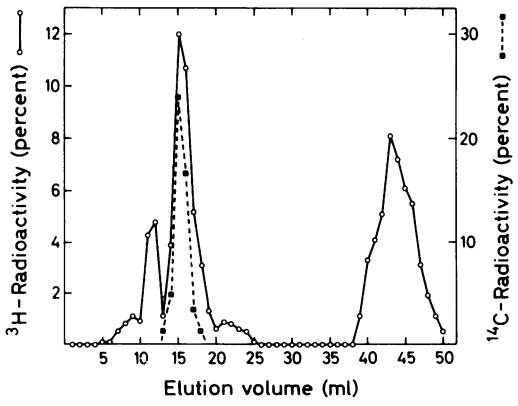

Figure 6. Reversed phase high pressure liquid chromatogram of duodenal bile extract 11 h after the administration of ^{3}H-THCA per os. Hydrolysis, extraction, and HPLC procedures are given in Methods. The main radioactive peaks correspond to the following: 3α,6α,7α,12α-tetrahydroxy-5β-cholestanoic acid (tentatively identified, see text), 11–12 ml, cholic acid, 15 ml, 24-OH-THCA, 21 ml, and THCA, 43 ml. For additional information see text.

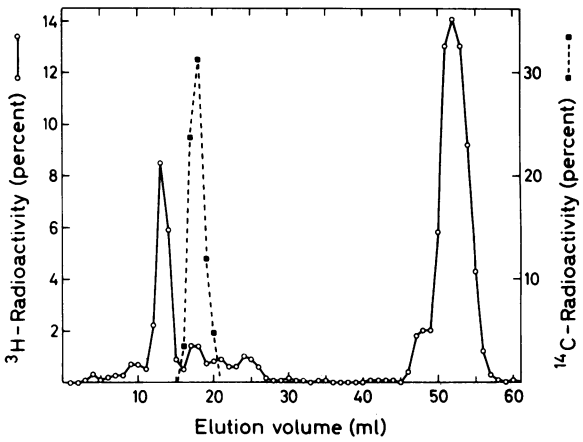

Figure 7. Reversed phase high pressure liquid chromatogram of pooled serum extracts 6, 12, and 24 h after the administration of ^{3}H-THCA per os. Hydrolysis, extraction, and HPLC procedures are given in Methods. The main radioactive peaks correspond to the following: 3α,6α,7α,12α-tetrahydroxy-5β-cholestanoic acid (tentatively identified, see text), 13 ml, cholic acid, 17–18 ml, 24-OH-THCA, 21 ml, C_{29}-dicarboxylic acid, 21 ml, 25*R*- and 25*S*-THCA, 48 and 51 ml.

prominent ions at *m/e* 251 (loss of four trimethylsilyl groups from the steroid nucleus), 341(251 + 90), 393(M-4 × 90-15), 408(M-4 × 90), 498(M-3 × 90), and 588(M-2 × 90). The compound is thus a tetrahydroxylated 5β-cholestanoic acid with all the hydroxyl groups in the steroid nucleus. Smaller peaks were identified as cholic acid (elution volume 17–18 ml), 24-OH-THCA (elution volume 21 ml), and the C_{29}-dicarboxylic acid (elution volume 24–25 ml).

Discussion

Increased amounts in serum and bile of C_{27}-steroids with a partially oxidized side chain is a characteristic finding in Zellweger syndrome (4–6). This was confirmed in the present work (Fig. 1 and Table I). A defective mitochondrial side chain cleavage has been suggested as an explanation for this accumulation (4, 6). Abnormal structure and function of liver mitochondria have been described in Zellweger syndrome (2), but such a finding does not seem to be obligate (25). More remarkable, however, is that no peroxisomes can be recognized in liver and kidneys of the patients (2), and the possibility has been discussed that the mitochondrial changes are secondary to the lack of peroxisomes (25, 26). Recently, we could demonstrate that in the rat liver the peroxisomal fraction was by far the most active in catalyzing conversion of THCA into cholic acid (9, 10), and the same seems to be the case in human liver (unpublished experiments), In view of this, it seems likely that the accumulation of the C_{27}-steroid bile acid precursors, in particular of THCA in Zellweger syndrome, is due to a reduced capacity for peroxisomal β-oxidation of the steroid side chain.

The results of our in vivo experiments showed that radioactivity given as 5β-cholestane-3α,7α,12α-triol rapidly appeared in more polar products with partially oxidized C_{27}-steroid side chain, mainly as THCA (Fig. 2). This shows that the mitochondrial 26-hydroxylation is functioning in Zellweger syndrome and supports the previous suggestion that the metabolic

defect is localized at the final side chain cleavage reaction (4). In addition, the rapid conversion of 5β-cholestane-3α,7α,12α-triol into THCA seems to exclude the possibility that microsomal 25- and 24*S*-hydroxylation of 5β-cholestane-3α,7α,12α-triol is an important pathway for formation of cholic acid in man (8, 27). If this had been the case, there would be no reason for accumulation of THCA. The results thus support the contention that the 26-hydroxylase pathway is the most important one in the normal biosynthesis of cholic acid (28, 29).

The block in bile acid synthesis was not complete, since tritiated cholic acid was detected after in vivo administration both of ^{3}H-5β-cholestane-3α,7α,12α-triol and ^{3}H-THCA. The conversion into cholic acid was, however, very slow and incomplete compared with what has been observed when bile acid precursors have been administered in vivo to adult humans. When such labeled precursors are administered to either bile fistula patients or to normal subjects, there is a rapid (within minutes) and almost complete conversion to bile acids (17, 18, 30–32). When ^{3}H-5β-cholestane-3α,7α,12α-triol was given to bile fistula patients, ~75% was converted to cholic acid (31). The peak specific activity in cholic acid occurred already after 30 min and the tritium activity corresponding to the precursor was completely eliminated after 13 h (31). Similarly, when ^{3}H-THCA was administered intravenously to either normal subjects (17) or to bile fistula patients (32), there was a rapid and nearly complete (81–97%) conversion into cholic acid. These results contrast with our finding of a maximal conversion of 5β-cholestane 3α,7α,12α-triol to cholic acid of ~15–20% (Fig. 3). 11 h after administration, the degree of conversion to cholic acid was only 9% for ^{3}H-5β-cholestane-3α,7α,12α-triol and 8% for ^{3}H-THCA (Figs. 3 and 6). In the evaluation of these results it should be kept in mind that the THCA experiment was performed only a few weeks before the patient died.

β-Oxidation of the steroid side chain is not a limiting step under normal conditions, and there is likely to be a considerable overcapacity of enzyme activity in the peroxisomes. The low conversion of ^{3}H-THCA into cholic acid observed here may be due to residual peroxisomal enzyme activity, or to the presence of alternative pathways to cholic acid bypassing the peroxisomal system. The similar rate of conversion of labeled 5β-cholestane-3α,7α,12α-triol and of THCA into cholic acid during the first 11 h (refer above) makes it unlikely that the 25-hydroxylase pathway (see Introduction) plays an important role in the Zellweger syndrome as has been suggested (4). If alternative pathways exist from the triol, it is therefore more likely that these involve metabolic modifications of THCA.

The very low specific radioactivity of ^{3}H in the cholic acid as compared with that of ^{3}H-THCA may suggest that part of the cholic acid in the patient had been formed by an unknown pathway bypassing both 5β-cholestane-3α,7α,12α-triol and THCA. More likely, the low ratio of specific activities may be explained by a very slow influx of tritium from THCA into a much larger cholic acid pool in bile (refer to Results). Differences in slopes of the decay curves could tell if alternative pathways are involved. Such information would require a longer collection period and more data points than we were able to obtain in Fig. 4. Also, Hanson et al. (33), who studied the metabolism of labeled THCA in two siblings with cholestasis due to intrahepatic bile duct anomalies, reported similar findings as ours. Also, in that case, there was a higher specific radioactivity in THCA than in cholic acid isolated from urine. The differences were, however, less marked than in the present study.

The pool size of cholic acid (24 mg/m^2) was drastically reduced in our patient. Watkins et al. (23) reported a pool size of ~300 mg/m^2 for newborn healthy full-term infants (24) and ~90 mg/m^2 for premature infants. The daily production rate of cholic acid corresponds to ~$^1/_{10}$ of that formed in normal newborns (24), which indicates that the deficient conversion of THCA to cholic acid is not compensated for by alternative pathways.

A main product in serum and urine from both 5β-cholestane-3α,7α,12α-triol and THCA was identified as a tetrahydroxylated 5β-cholestanoic acid with all hydroxyl groups in the steroid nucleus. 1β and 6α-hydroxylated cholic acid has been identified in human neonatal urine (34) and 2β- and 6α-hydroxylated cholic acid has been found in duodenal aspirates from neonates with high intestinal obstruction (35). It thus appears likely that the tetrahydroxylated THCA could be hydroxylated in 1β, 2β, or 6α position. Since a characteristic ion at *m/e* 217 or *m/e* 243 was lacking, the first two possibilities may be excluded. We are left with the possibility that the structure corresponds to 3α,6α,7α,12α-tetrahydroxy-5β-cholestanoic acid, but further work is needed to establish this. Since this compound is relatively polar, it may represent an important route for urinary excretion of accumulated THCA. The retention time of the hydroxylated THCA on gas chromatography is very similar to that of 24-OH-THCA, and by mass fragmentography they share several fragments. This may have caused overestimation of the amount of accumulated 24-OH-THCA in Zellweger syndrome in a previous report (4). After injection of 5β-cholestane-3α,7α,12α-triol, and after administration of THCA per os, we could detect only small amounts of tritium activity in the peak corresponding to 24-OH-THCA (Figs. 5 and 7). Accumulation of 24-OH-THCA has been taken as evidence for a metabolic block in the conversion of 24-OH-THCA into cholic acid in mitochondria (4). Another possibility is that the 24-OH-THCA identified may be a product of hydroxylation in the endoplasmic reticulum. Determination of the stereoisomeric structure of the compound may differentiate between these possibilities.

It should be emphasized that our patient was treated with phenobarbital, and it cannot be excluded that the hydroxylase activity observed here in part may have been induced by the treatment. Very recently, however, we were able to analyze serum from another infant with the Zellweger syndrome that had never been treated with phenobarbital. The pattern of bile acids in serum from that infant was almost identical to that obtained here.

After administration of the cholic acid precursors, we could detect only small amounts of radioactivity that corresponded to the C_{29}-dicarboxylic acid. This appeared surprising in view of the high concentration of this compound in serum. The explanation may be that this metabolite is formed very slowly by chain elongation of THCA. Since there is very little excretion of this compound in bile and urine (5) it accumulates in plasma. The chain elongation of THCA may be analogous to the elongation of long-chain fatty acids in fibroblasts cultured from skin biopsies from patients with Zellweger syndrome (36). Chain shortening of very long-chain fatty acids is one

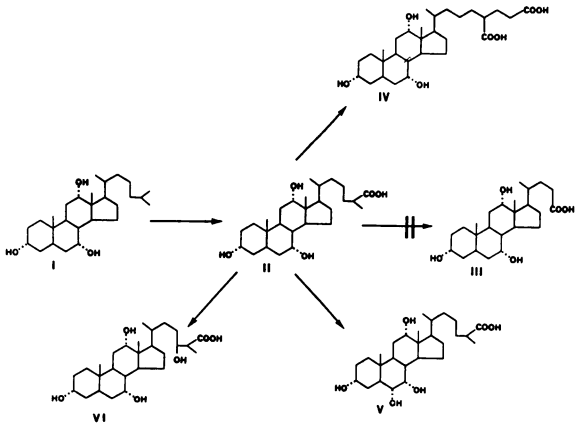

Figure 8. Proposed metabolism of 5β-cholestane-3α,7α,12α-triol (*I*) in Zellweger patient. The triol is oxidized to THCA (*II*) via 5β-cholestane-3α,7α,12α,26-tetrol (not shown). THCA accumulates as a consequence of impaired peroxisomal formation of cholic acid (*III*) and is hydroxylated in the 24-position (*VI*) and in a nuclear position, possibly 6α (*V*), or side chain elongated to C_{29}-dicarboxylic acid (*IV*).

important function of peroxisomes (37). Accumulation of C_{29}-dicarboxylic acid (5) and very long-chain fatty acids in Zellweger syndrome (36) may thus both be the expression of compensating mechanisms to restricted peroxisomal metabolic processes.

Adrenal leucodystrophy is another clinical entity with accumulation of very long-chain fatty acids and depletion of peroxisomes (11, 36). Thus, both this disease and Zellweger syndrome probably represent a new class of peroxisomal diseases with different clinical expressions.

We conclude from the present findings that liver peroxisomes are essential in the normal formation of cholic acid from THCA. In Zellweger syndrome, there is a defective conversion of THCA to cholic acid (Fig. 8), and as a consequence the accumulated THCA is either excreted as such or transformed to other metabolites by hydroxylation or by chain elongation.

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