

# The metabolism of 3alpha, 7alpha, 12alpha-trihydroxy-5beta-cholestan-26-oic acid in two siblings with cholestasis due to intrahepatic bile duct anomalies. An apparent inborn error of cholic acid synthesis.

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## Research Article

Studies were carried out in a family in which two children with cholestasis due to intrahepatic bile duct anomalies were shown to have increased amounts of the cholic acid precursor, 3alpha, 7alpha, 12alpha-trihydroxy-5beta-cholestan-26-oic acid (THCA). The metabolism of THCA was studied in one of these patients after an intravenous injection of (3H)THCA, and the cause of the increased amounts of THCA in this condition was found to be due to a metabolic defect in the conversion of this compound into cholic acid. A small amount of (3H)cholic acid was also identified after (3H)THCA administration, confirming that this metabolic defect was incomplete. Varanic acid (3alpha, 7alpha, 12alpha, 24xi-tetrahydroxy-5beta-cholestan-26-oic acid), a metabolite of THCA, could not be identified in either of these patients. By assuming that this compound would be conjugated and excreted if the metabolic block occurred after the formation of varanic acid, the defect in these patients appears to be due to a deficiency of a 24-hydroxylating enzyme system required to convert THCA into varanic acid. This condition appears to be transmitted in an autosomal recessive fashion, because the two affected patients were of opposite sex, and neither a normal sibling nor the two parents have increased amount of THCA in their bile.

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# The Metabolism of $3\alpha$ , $7\alpha$ , $12\alpha$ -Trihydroxy- $5\beta$ -Cholestan-26-Oic Acid in Two Siblings with Cholestasis Due to Intrahepatic Bile Duct Anomalies

## AN APPARENT INBORN ERROR OF CHOLIC ACID SYNTHESIS

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**ABSTRACT** Studies were carried out in a family in which two children with cholestasis due to intrahepatic bile duct anomalies were shown to have increased amounts of the cholic acid precursor,  $3\alpha,7\alpha,12\alpha$ -trihydroxy- $5\beta$ -cholestan-26-oic acid (THCA). The metabolism of THCA was studied in one of these patients after an intravenous injection of [ $^3\text{H}$ ]THCA, and the cause of the increased amounts of THCA in this condition was found to be due to a metabolic defect in the conversion of this compound into cholic acid. A small amount of [ $^3\text{H}$ ]cholic acid was also identified after [ $^3\text{H}$ ]THCA administration, confirming that this metabolic defect was incomplete. Varanic acid ( $3\alpha,7\alpha,12\alpha,24\beta$ -tetrahydroxy- $5\beta$ -cholestan-26-oic acid), a metabolite of THCA, could not be identified in either of these patients. By assuming that this compound would be conjugated and excreted if the metabolic block occurred after the formation of varanic acid, the defect in these patients appears to be due to a deficiency of a 24-hydroxylating enzyme system required to convert THCA into varanic acid.

This condition appears to be transmitted in an autosomal recessive fashion, because the two affected patients were of opposite sex, and neither a normal

sibling nor the two parents have increased amount of THCA in their bile.

## INTRODUCTION

Recently Eyssen, Parmentier, Compernolle, Boon, and Eggermont reported the identification of large quantities of  $3\alpha,7\alpha,12\alpha$ -trihydroxy- $5\beta$ -cholestan-26-oic acid (THCA)<sup>1</sup> in the bile of two unrelated infants with intrahepatic bile duct anomalies (1). This compound has been isolated previously from human bile (2), demonstrated to arise from cholesterol (3, 4), and shown to be efficiently metabolized to cholic acid (3). On the basis of this evidence, THCA is considered to be an intermediate in cholic acid synthesis, as shown in Fig. 1. Because of rapid conversion to cholic acid, THCA is normally found in only trace quantities in human bile (2).

We have recently carried out studies in a family in which the syndrome described by Eyssen et al. appears to have a genetic origin. This report describes studies designed to (a) define further the nature of the metabolic defect that leads to increased THCA excretion, and (b) determine if the presumed carrier

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<sup>1</sup>Abbreviations used in this paper: CI, chemical ionization; DHCA,  $3\alpha,7\alpha$ -dihydroxy- $5\beta$ -cholestan-26-oic acid; EI, electron impact; MS, mass spectrometry, THCA,  $3\alpha,7\alpha,12\alpha$ -trihydroxy- $5\beta$ -cholestan-26-oic acid.

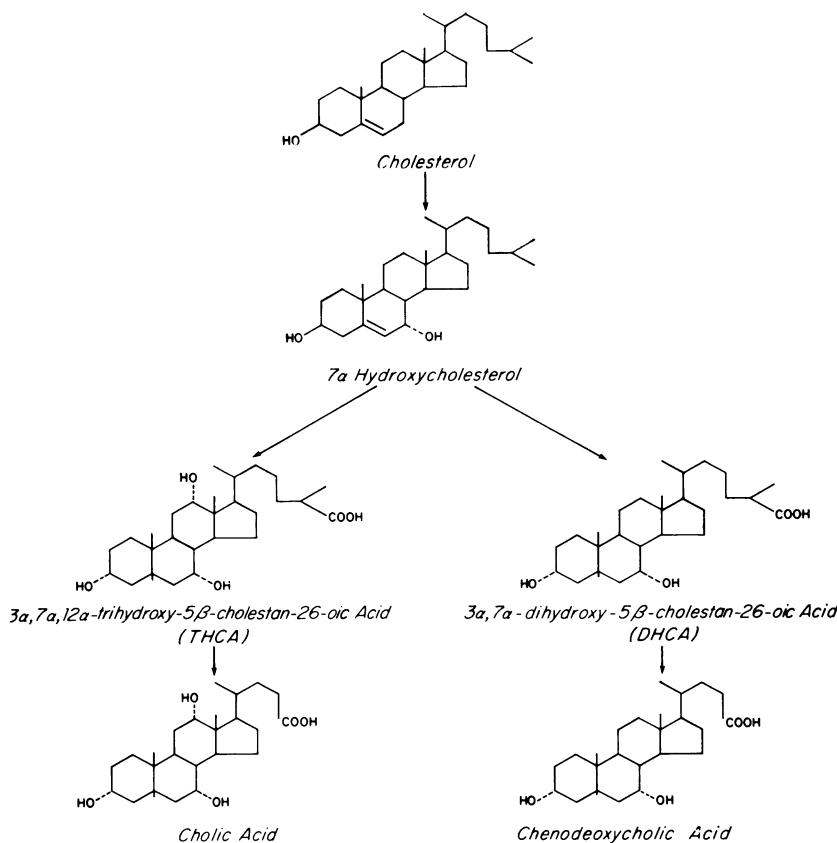


FIGURE 1 Pathway of cholic acid and chenodeoxycholic acid synthesis involving THCA and DHCA, respectively.

state for this condition results in increased THCA excretion.

## METHODS

### Reference and labeled compounds

Authentic *3α,7α*-dihydroxy-5β-cholest-26-oic acid (DHCA) and THCA were isolated from *Alligator mississippiensis* gallbladder bile in the following fashion: 20 ml of gallbladder bile, diluted with an equal amount of ethanol, were filtered, evaporated to dryness, and hydrolyzed with 4.5 N NaOH at 130°C for 24 h. After acidification, the bile acids were extracted, and DHCA and THCA were purified by Celite partition chromatography (Johns-Manville Products Corp., Celite Div., Denver, Colo.) (2). Their structures were confirmed by mass spectrometry.

[2,4-<sup>3</sup>H]THCA ([<sup>3</sup>H]THCA) was prepared as follows. Methyl-7α,12α-dihydroxy-3-keto-5β-cholest-26-oate, prepared by Oppenauer oxidation of methyl THCA, was labeled with tritium by adsorption chromatography on alumina (5). The labeled compound was reduced with NaBH<sub>4</sub>, and the 3α and 3β epimers were separated on a thin-layer chromatography (TLC) plate prepared from equal parts (by weight) of silicic acid and magnesium silicate (Woelm, Aluphar Chemicals, New Orleans, La.). A constant specific activity was observed when 140,000 dpm of methyl [<sup>3</sup>H]THCA were added to 50 mg of purified methyl THCA

and the mixture was recrystallized with benzene: petroleum ether (BP 60–70°C) ( $2.94 \times 10^8$  dpm/mg); benzene: petroleum ether ( $2.82 \times 10^8$  dpm/mg); benzene: petroleum ether ( $2.82 \times 10^8$  dpm/mg); and ethyl acetate: petroleum ether ( $2.86 \times 10^8$  dpm/mg).

To determine the radiochemical stability of [2,4-<sup>3</sup>H]-THCA and if THCA isolated from the alligator is metabolized to cholic acid in mammals, 2 μCi of [<sup>3</sup>H]THCA dissolved in 0.1 ml of absolute ethanol, was injected intravenously into a bile fistula rat. 97% of the radioactivity was recovered in the bile during the first 24 h after the injection, and after hydrolysis, extraction, and TLC, 95% of the radioactivity was identified as cholic acid.

Varanic acid (*3α,7α,12α,24E*-tetrahydroxy-5β-cholest-26-oic acid) was isolated from gallbladder bile of the water monitor, *Varanus salvator*. The bile was diluted with ethanol, filtered, evaporated to dryness, and hydrolyzed with 4.5 N NaOH at 130°C for 24 h. After acidification, the bile acids were extracted, methylated, and separated on TLC with benzene, isopropanol, acetic acid (30: 10: 1, vol/vol) as developing solvents. A compound was identified that had identical TLC and gas liquid chromatographic (GLC) mobilities as a sample of methyl varanate generously supplied by Prof. G. A. D. Haslewood, Guy's Hospital, London. Chemical ionization mass spectrometry of methyl varanate, with methane as the reagent gas, disclosed the presence of a protonated molecular ion at *m/e* 481 (MH<sup>+</sup>)

as well as characteristic ions at  $m/e$  463 ( $MH^+ - H_2O$ ),  $m/e$  445 ( $MH^+ - 2H_2O$ ),  $m/e$  427 ( $MH^+ - 3H_2O$ ), and  $m/e$  409 ( $MH^+ - 4H_2O$ ). In addition, there are several minor ions stemming from the loss of  $CH(CH_3)-CO_2CH_3$  from the side chain, resulting from  $\beta$ -fission of the methyl ester. This series of ions begins at  $m/e$  393 ( $MH^+ - R$ ) and by successive loss of four molecules of water produces ions at  $m/e$  375, 357, 339, and 321. The ions comprising this spectrum are consistent with the structure assigned to methyl varanate.

### Mass spectrometry (MS)

Electron impact (EI) mass spectra were obtained on a Perkin-Elmer model 270 mass spectrometer at an ion source temperature of 150°C and an ionization potential of 70 eV (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Bile acids were analyzed as the methyl ester-acetate derivative and were introduced into the mass spectrometer through the GLC inlet system. GLC-MS analysis were performed on a 1.8 m  $\times$  1-mm ID glass column packed with 0.25% SP-525 on Gas Chrom Q (Applied Science Labs, Inc., State College, Pa.) at 270°C with a helium carrier gas. Chemical ionization (CI) mass spectra were obtained on a Biospect CI mass spectrometer (Scientific Research Instruments, G. D. Searle & Co., Baltimore, Md.), with methane as the reagent gas and a source temperature of 165°C. The methylated and acetylated bile acids were introduced through the GLC inlet system under the conditions noted above. The methylated, unacetylated bile acids were introduced through the solid inlet system in silanized quartz probe tubes.

Electron impact ionization of bile acids is a highly energetic process that results in mass spectra characterized by extensive C-C fragmentation of the steroid nucleus. Under EI conditions, the bile acids seldom exhibit a molecular ion. Since the acetate group used in the GLC-MS analysis is lost in the ionization process, one can only determine the total number of functional groups present in the molecule. CI, however, is a very mild ionization process in which a proton is transferred from an ionized reagent gas to the compound of interest. Since the amount of energy transferred is low (typically less than C-C bond dissociation energies), very little fragmentation of the steroid nucleus occurs. CI spectra of the bile acid methyl esters frequently exhibit a protonated molecular ion and are characterized by sequential loss of each hydroxyl. In this respect, the CI mass spectrum allows one to determine the total number as well as type of functional groups present.

### Bile acid analysis

**Bile.** Bile samples, collected by duodenal aspiration after contraction of the gallbladder with cholecystokinin,<sup>2</sup> were diluted with an equal volume of ethanol and stored at 3°C. An aliquot was filtered, evaporated to dryness, and hydrolyzed with cholylglycine hydrolase (6), after which the solution was acidified and extracted twice with diethyl ether. An aliquot was methylated and acetylated (7), and the bile acid derivatives were measured with GLC. THCA was quantitated in bile by measuring the amount of THCA isolated after Celite column partition chromatography. The fractions containing THCA (last half of the 40% benzene and the first quarter of the 60% benzene fractions) were combined and the THCA was measured by GLC.

<sup>2</sup>Gastrointestinal Hormone Research Unit, Chemistry Department, Karolinska Institute, Stockholm, Sweden.

**Urine.** 60-ml urine samples from jaundiced patients were passed through an Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, Pa.) column (1.5  $\times$  20 cm), after which the column was washed with 60 ml of distilled water and the bile salts were eluted with 100 ml of methanol. The methanol was evaporated, and the bile salts were subjected to solvolysis (8) and hydrolyzed in 4.5 N NaOH at 130°C for 24 h. After acidification, the bile acids were extracted with diethyl ether, methylated, acetylated, and quantitated by GLC.

**Serum.** Serum bile acids were measured as described by Carey (9), except that solvolysis was carried out before hydrolysis. After methylation and acetylation, the individual bile acids were quantitated by GLC.

**Stool.** A stool sample from patient T. H. (see case reports), collected while he was receiving cholestyramine and stored at -5°C, was extracted initially by refluxing with 0.1 N  $NH_4CO_3$  in 50% methanol for 8 h. The extract was evaporated, and the residue was extracted with petroleum ether (BP 60-70°C), after which the aqueous fraction was hydrolyzed with 4.5 N NaOH at 130°C for 24 h. After acidification and extraction with diethyl ether, the bile acids were separated on a Celite partition column, methylated, acetylated, and quantitated by GLC.

### Gas liquid chromatography (GLC)

GLC was carried out on a Barber Colman gas chromatograph (Searle Analytic Inc., Des Plaines, Ill.), equipped with a hydrogen flame detector. A 1.8 m  $\times$  4-mm ID U-

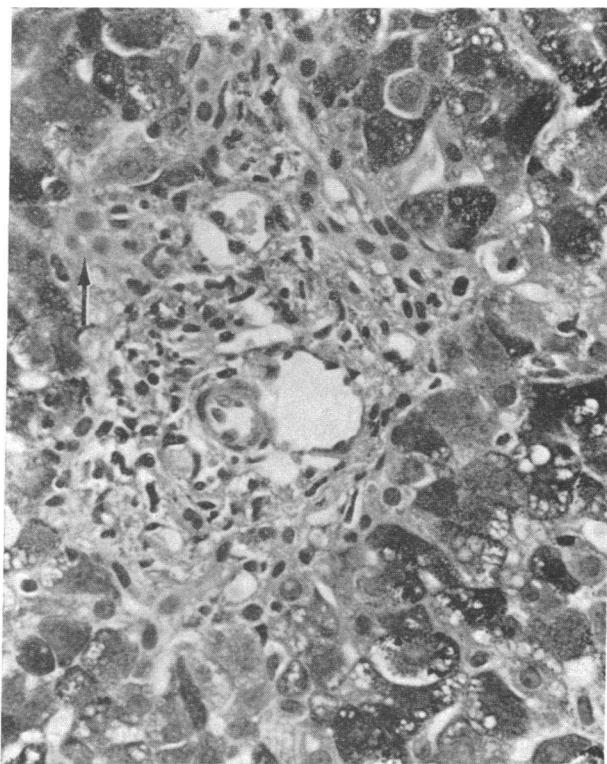


FIGURE 2 Representative portal area from the initial percutaneous liver biopsy of patient J. H. Arrow indicates a bile duct structure. ( $\times 250$ ) Periodic acid Schiff stain.

TABLE I  
Bile Acids Present in Patients and Subjects Studied

Subject	Age	Diagnosis	Source of specimen	Treatment	Total amount	Cholic acid	Cheno-deoxy-cholic acid	Deoxy-cholic acid	THCA
					mg/100 ml		relative %		
T. H.	8 mo	Intrahepatic paucity of bile ducts	Serum Stool	Cholestyramine Cholestyramine	0.9 0.3	0 0	28 0	0	72 100
J. H.	4 mo	Intrahepatic paucity of bile ducts	Bile Serum	None None	9.5 0.9	6 5	22 30	0 0	72 65
	9 mo	Intrahepatic paucity of bile ducts	Urine Bile	None Phenobarbital	0.3 13.4	26 34	18 55	0 0	55 11
	15 mo	Intrahepatic paucity of bile ducts	Bile	Phenobarbital and cholestyramine	10.3	63	27	0	10
	18 mo	Intrahepatic paucity of bile ducts	Bile	Phenobarbital and cholestyramine	10.4	34	21	0	45
	23 mo	Intrahepatic paucity of bile ducts	Urine	Phenobarbital and cholestyramine	0.3	19	0	0	81
R. H.	38 yr	Father	Bile	None Cholestyramine	130 536	48 84	37 15	15 1	0.40 0.01
S. H.	36 yr	Mother	Bile	None Cholestyramine	334 150	30 83	34 14	36 3	0.03 0.01
S. H.	6 yr	Sister	Bile	None	254	60	38	2	0.03
H. R.	34 yr	Normal	Bile	None Cholestyramine	157 928	44 90	42 8	14 2	0.03 0.10
I. N.	32 yr	Normal	Bile	None Cholestyramine	132 630	60 90	21 7	19 3	0.15 0

shaped column packed with 0.5% QF-1 on Gas Chrom Q was used. The following temperatures were used: column, 240°C; detector, 260°C; injector, 240°C. Comparisons were made with standards of known concentrations.

#### Bile acid kinetics

The metabolism of THCA was studied in patient J. H. (see case reports) after informed parental consent when she was 8 mo of age. 6  $\mu$ Ci of [<sup>3</sup>H]THCA dissolved in 1.5 ml of sterile ethanol was injected intravenously through a saline infusion. Urine was collected continuously, initially in 8-h fractions for the first day, and then in 12-h fractions for 5 more days. The specific activities of [<sup>3</sup>H]THCA and [<sup>3</sup>H]cholic acid were determined in urine samples collected at 0.3, 0.6, 1, 2, 3, 4, 5, and 6 days. [<sup>3</sup>H]THCA and [<sup>3</sup>H]cholic acid were isolated by Celite partition chromatography as described above, and the specific activities were determined, with GLC to measure the mass of THCA, and cholic acid and liquid scintillation counting to measure the radioactivity. A Fluor alloy TLA counting mixture (Beckman Instruments, Inc., Fullerton, Calif.) was used in the counting vials, and quenching was estimated by adding an internal standard of [<sup>3</sup>H]toluene to the counting vials.

Computer analysis, by the method of least squares (10), was used to determine the best fit of the [<sup>3</sup>H]THCA spe-

cific activity data to the equation  $\ln A = \ln B - \alpha t$ , in which  $A$  equals specific activity at time  $t$ ,  $B$  equals specific activity at the time of injection, assuming instantaneous mixing, and  $\alpha$  equals the slope of the specific activity decay curve. The pool size of THCA equals dpm injected/specific activity at  $t_0$  and the daily synthesis rate of THCA equals pool size  $\times$  slope of the specific activity decay curve.

#### Case reports

T. H. was the 5-lbs boy, product of the first pregnancy of nonconsanguineous parents. Neither parent has evidence of liver disease on physical examination or by standard liver function tests. Jaundiced from birth, T. H. was found to have hepatomegaly at 4 mo of age. Galactosemia and cystic fibrosis were excluded by the appropriate diagnostic tests. At laparotomy, patency of the extrahepatic biliary system was demonstrated by T-tube cholangiography, with visualization of the common bile duct and several first-order intrahepatic branches. Only trace amounts of bile drained from the T-tube after the operation. Small bile ducts were infrequently identified in the otherwise unremarkable portal areas of the wedge biopsy specimen from the liver.

At 6 mo of age, hepatosplenomegaly and clubbing were prominent, but no xanthomas or cardiac murmurs were

noted. A percutaneous liver biopsy showed a marked increase in portal fibrosis. Rachitic changes were noted on bone roentgenograms. At 8 mo of age an acanthocytic hemolytic anemia developed, and he died from liver failure. Advanced cirrhosis and evidence of portal hypertension were noted at autopsy.

A second pregnancy resulted in a girl, S. H., who is presently 6 yr of age and has no evidence of liver disease.

J. H., a girl, was the 5½-lbs term product of the third pregnancy. Scleral icterus was first noted at 4 mo of age. Her height and weight were less than normal but within 2 SD of the mean. Pruritus had not been observed. Hepatomegaly was the only abnormal finding on physical examination, and subsequent audiometry supported the clinical impression of impaired hearing.

The initial liver function studies were: bilirubin, direct 2.0, total 3.9 mg/100 ml; ornithine carbamyl transferase,

52 (nl < 45 IU/liter); alkaline phosphatase, 632 (nl < 395 IU/liter). The serum cholesterol and triglycerides were within normal limits. A [<sup>131</sup>I]rose bengal clearance of 39% was consistent with intrinsic liver disease (11). In 21 portal areas noted on a percutaneous liver biopsy specimen, only 15 bile duct-like structures could be identified (see Fig. 2). A mild increase in connective tissue was seen with a reticulin strain. Cystic fibrosis, galactosemia, and alpha-1-antitrypsin deficiency were excluded, and screening tests for toxoplasmosis, rubella, cytomegalic inclusion disease, herpes virus, hepatitis-associated antigen, and varicella were negative.

At 4 mo of age phenobarbital in a dosage of 3 mg/kg was started and increased to 12 mg/kg, in an attempt to increase bile flow. Plasma phenobarbital levels ranged between 25 and 45  $\mu$ g/ml. During the next 5 mo there was no change in serum bilirubin and bile acid concentrations,

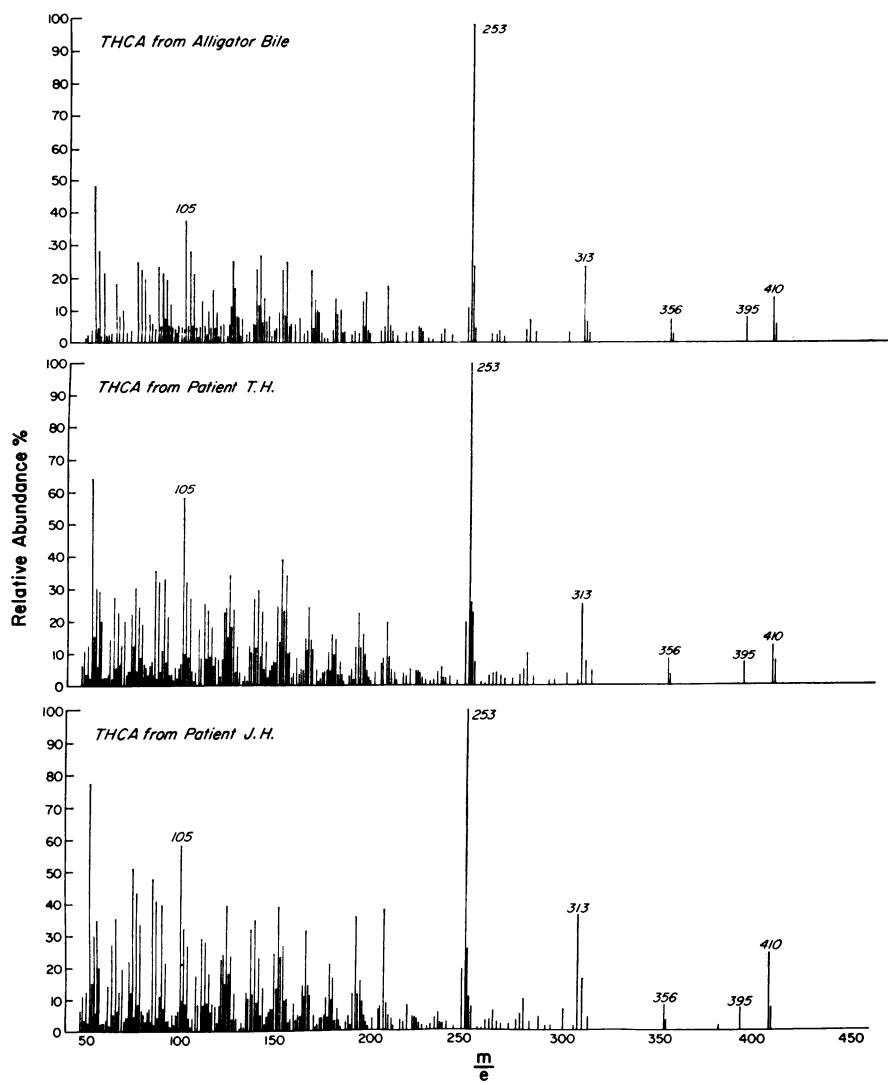


FIGURE 3 Mass spectra of THCA isolated from alligator bile, patients T. H. and J. H. The characteristic ion  $m/e$  410 ( $M^+ - 30Ac$ ) for methyl THCA acetate was observed in each sample.

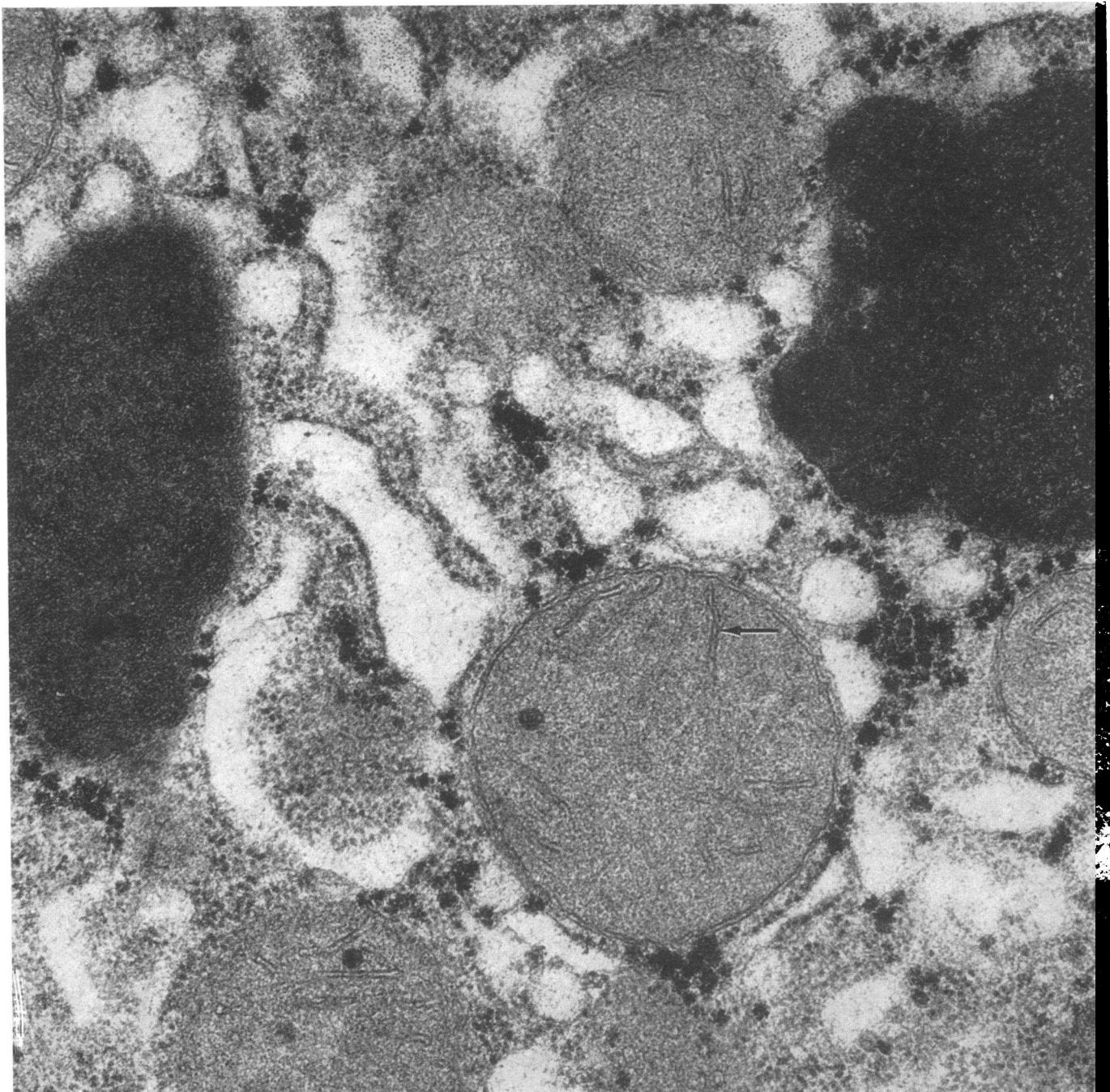


FIGURE 4 Electron micrograph of a small section of hepatic cell cytoplasm from liver biopsy obtained from J. H. In the center is a typical normal mitochondrion. This double-membraned structure has normal irregularly distributed membranous cristae (→). The homogeneous, finely granular matrix of the mitochondrion is interrupted only by the cristae and one dense granule. The two dark bodies on either side of the electron micrograph represent bile accumulation in single-membrane structures. (× 58,000).

and no weight gain. At 9 mo of age a second percutaneous liver biopsy disclosed increased portal fibrosis, and bile duct structures were now more readily identified. Cholestyra-

mine was added to the treatment regimen, and over the subsequent 6-mo period her serum bilirubin and bile acid concentrations returned to normal. Despite the apparent im-

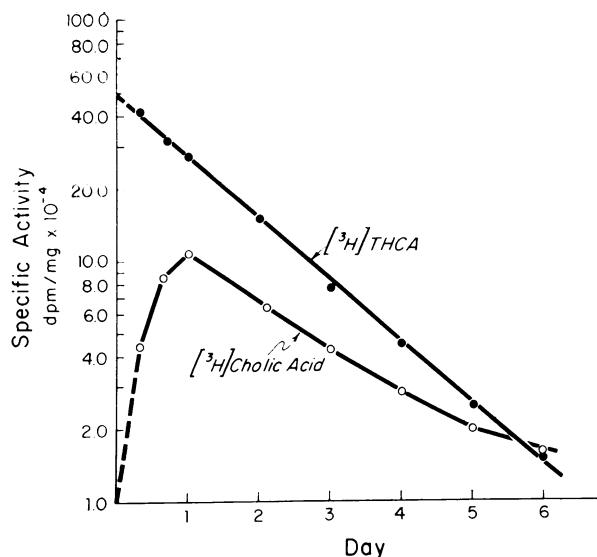


FIGURE 5 Specific activity decay curves of  $[^3\text{H}]$ THCA and  $[^3\text{H}]$ cholic acid after the administration of  $[^3\text{H}]$ THCA to J. H.

provement in hepatic function, growth was minimal, and hypophosphatemic rickets developed and persisted despite large oral (50,000 U/day) or intramuscular (5,000,000 U) supplements of vitamin D and oral calcium (1 g/day). At 23 mo of age the serum bilirubin concentration began to increase, and acanthocytes were prominent on a smear of her peripheral blood. The patient expired of liver failure several weeks after a patent extrahepatic biliary system was demonstrated by operative cholangiography. At autopsy advanced cirrhosis and evidence of portal hypertension were noted.

## RESULTS

*Identification of THCA in patients T. H. and J. H.* Large amounts of THCA were tentatively identified by GLC in the serum, bile, and urine of patient J. H. and in the serum and stool of her sibling, T. H. (See Table I). The identity of THCA in these two patients was confirmed by mass spectroscopy (Fig. 3). THCA was first identified in J. H. at 4 mo of age before initiation of therapy, and it was present on four other occasions until her death at 24 mo of age (Table I). In contrast, THCA could not be identified in urine samples from five other children with similar anomalies of the intrahepatic biliary tract, six children with other types of cholestatic liver disease, and three adults with advanced alcoholic cirrhosis in liver failure. Neither DHCA nor varanic acid, an intermediary metabolite in the  $\beta$ -oxidation of THCA (12), could be identified by GLC in specimens collected from either T. H. or J. H.

THCA is metabolized to cholic acid by mitochondrial enzymes (12); thus to determine if an alteration of hepatic mitochondria structure was present in patient

J. H., a liver biopsy specimen was prepared, as previously described (13), and examined under a Philips 300 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.). As shown in Fig. 4, the mitochondria appear normal.

Only trace amounts of THCA were present in bile samples obtained from both parents and the normal 6-yr-old sister, as shown in Table I; similar trace amounts of THCA were present in the bile of two normal subjects. To determine if an increase in the synthesis of cholic acid would result in an increase in the percentage of THCA in bile, both parents and two normal subjects were fed cholestyramine, 16 g/day, for 2 wk. As depicted in Table I, the percentage of THCA in bile did not increase with cholestyramine feeding.

*Metabolism of THCA in patient J. H.* After the intravenous administration of  $[^3\text{H}]$ THCA to patient J. H., the specific activities of  $[^3\text{H}]$ THCA and  $[^3\text{H}]$ cholic acid were measured in the urine for the next 6 days. Approximately 70% of the administered isotope was excreted in the urine during this 6-day period. Specific activity curves of  $[^3\text{H}]$ THCA and  $[^3\text{H}]$ cholic acid are shown in Fig. 5. The calculated pool size and daily synthesis rate of THCA were  $95 \text{ mg/m}^2$  and  $55.7 \text{ mg/m}^2$  per day, respectively.

The following study was performed to determine the fractions of the urinary bile acids excreted as free, conjugated, or sulfated acids. A 60-ml urine sample collected from J. H. was passed through an XAD-2 column ( $1.5 \times 30 \text{ cm}$ ), after which the bound sterols were eluted with methanol. The eluent was divided into three equal aliquots, and the free unsulfated bile acids were quantitated in the first aliquot by GLC after methylation and acetylation. The conjugated, unsulfated bile acids in the second aliquot were quantitated by GLC after hydrolysis, methylation, and acetylation.

TABLE II  
Free, Conjugated, and Sulfated Bile Acids  
in the Urine of J. H.

	Free	Conjugated	Sulfated
	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$
Cholic acid	12	37	0
THCA	18	79	8
Chenodeoxycholic acid	0	0	100

A 60-ml sample of urine was passed through an XAD-2 column and the methanol eluate was divided into three aliquots. Bile acids were then quantitated as the methyl ester acetate derivatives on GLC before (a) and after hydrolysis (b), and after solvolysis and hydrolysis (c). Free = a, conjugated = b - a, sulfated = c - b.

The third aliquot was first solvolyzed and then hydrolyzed, methylated, and acetylated, and the bile acids were quantitated by GLC. As shown in Table II, 75% and 76% of the THCA and cholic acid, respectively, were excreted in the urine as conjugates, while all of the urinary chenodeoxycholic acid was sulfated.

## DISCUSSION

The present report describes two siblings with neonatal, cholestatic liver disease associated with excretion of markedly increased quantities of the bile acid precursor, THCA. Both of these patients had cholestasis and a paucity of the intrahepatic bile ducts, a finding similar to that present in the two patients reported by Eyssen et al. (1). However, Eyssen's patients had other congenital abnormalities (frontal bossing, epicanthal folds, simian creases) not seen in our patients. Both patients in our family developed rickets that failed to respond to vitamin D and calcium supplements, suggesting an abnormality in the metabolism of vitamin D. However, in our second patient (J. H.), these changes may be secondary to the effect of phenobarbital (14) and cholestyramine on the metabolism and absorption of vitamin D.

The histopathological syndrome represented by these patients and variously referred to as "intrahepatic biliary atresia" (15, 16), "intrahepatic bile duct hypoplasia" (17), and "paucity of the intrahepatic bile ducts" (13) is part of the differential diagnosis of cholestasis in infants. Children with this morphological syndrome have a patent extrahepatic biliary system and decreased numbers of bile ducts, as seen in liver biopsy specimens. In addition to cholestasis, these patients often present with severe pruritus (13, 15), hyperlipidemia (18), and in some instances pulmonary arterial stenosis (19), features not present in our patients. The overall survival of patients with cholestasis and paucity of the intrahepatic bile ducts is longer than for extrahepatic biliary atresia, and may extend into adulthood (13). The two patients described by Eyssen et al. (1) and the two patients in this report died by 2 yr of age, suggesting that this syndrome of neonatal cholestasis is distinct from other forms with this same histopathological entity.

Previous studies have shown that some patients with cholestasis and paucity of the intrahepatic bile ducts improve in liver function and grow when treated with cholestyramine (13). When cholestyramine was fed to the second patient in this report, J. H., the serum bilirubin concentration returned to normal, but no increase in weight occurred. However, at the same time that improvement in liver function tests was observed, cirrhosis developed.

The presence of excessive amounts of THCA in this condition could be explained either by an abnormally

increased production of this compound or by a failure to metabolize THCA into cholic acid. To determine which of these possibilities was correct, the metabolism of THCA was studied in J. H. As shown in Fig. 4, the specific activity of [<sup>3</sup>H]THCA, plotted as the natural logarithm vs. time, decayed in a linear fashion. Accurate interpretation of this study requires one to assume that the injected isotope was thoroughly mixed in the THCA pool before significant excretion took place. Evidence supporting this assumption is shown in Fig. 4. Urine was collected continuously, and the specific activities determined in the urine collected during the first two 8-h periods closely fit the decay curve plotted by using the specific activity measured in subsequent urine collections. If significant excretion of the injected [<sup>3</sup>H]-THCA occurred before mixing, the specific activity of THCA in the earlier collections would have been higher than expected, based on the later specific activity measurements. Therefore, with this specific activity decay curve, the pool size and synthesis rate of THCA were calculated as 95 mg/m<sup>2</sup> and 55.7 mg/m<sup>2</sup> per day, respectively. Previous studies, in normal infants, have shown that the pool size and daily synthesis rate of cholic acid are about 290 mg/m<sup>2</sup> and 110 mg/m<sup>2</sup> per day, respectively (20). If THCA is an obligatory intermediate in cholic acid synthesis, the daily synthesis rate of THCA in normal infants must also be at least 110/mg/m<sup>2</sup> per day. Thus, in J. H., total THCA synthesis was less than in normal infants, possibly as the result of the underlying liver disease or other undefined metabolic defects.

Therefore, the increased quantities of THCA in this condition must be due to a defect in the metabolism of this compound into cholic acid. As shown in Table I, cholic acid was present, albeit in reduced quantities, in patient J. H., suggesting that the metabolic block is incomplete or that cholic acid was being formed by an alternate route that did not involve THCA. A possible alternate route for cholic acid synthesis was recently proposed by Setoguchi, Salen, Fint, and Mosbach (21). These authors found large amounts of 5 $\beta$ -cholestane-3 $\alpha$ -, 7 $\alpha$ ,12 $\alpha$ ,25-tetrol and 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ ,24 $\alpha$ ,25-pentol in the bile and stool of patients with cerebrotendinous xanthomatosis (CTX), a condition associated with decreased cholic acid production. These studies suggest that in patients with CTX, 26-hydroxylation of 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol (leading to THCA) is defective and cholic acid synthesis may involve 25- and subsequent 24-hydroxylation of this compound, followed by side-chain cleavage. However, in the present study, the finding of [<sup>3</sup>H]cholic acid in patient J. H. after the administration of [<sup>3</sup>H]THCA (see Fig. 5) indicates that the metabolic block in the conversion of THCA into cholic acid is incomplete, and the small amount of cholic acid present was probably formed via THCA.

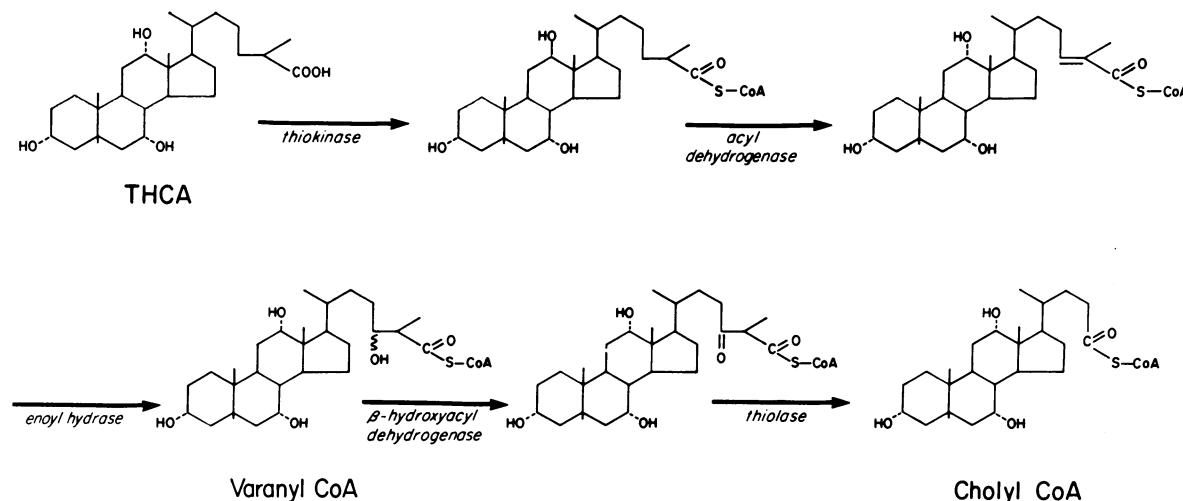


FIGURE 6 Pathway of  $\beta$ -oxidation of THCA to cholyl CoA through varanyl-CoA.

Carbon 25 of THCA is asymmetric and the bile of *Alligator mississippiensis* has been shown to contain 25-L-THCA (22). The natural isomer of THCA in man is not known. However, Bridgwater and Lindstedt (23) demonstrated that the rat is capable of metabolizing both the D and L isomers of THCA into cholic acid. Similarly, the human liver will metabolize a racemic mixture of D,L-DHCA into chenodeoxycholic acid (24). Therefore it is unlikely that the failure of our patient to metabolize [ $^3\text{H}$ ]THCA into cholic acid was due to the administration of the possible unnatural isomer of this compound.

The conversion of THCA into cholic acid is thought to take place in the mitochondria (12, 25, 26), and a generalized mitochondrial defect could explain the failure of conversion of THCA into cholic acid. However, as shown in Fig. 4, mitochondria examined by electron microscopy of a liver biopsy of J. H. appeared normal. More compelling evidence against this hypothesis is that no detectable amounts of the other C<sub>27</sub> bile acid, DHCA, could be identified in either of these two patients. Since this compound is metabolized to chenodeoxycholic acid (24), presumably by mitochondrial enzymes (27), the metabolic defect in this condition appears to be specific for the enzymes(s) involved in the conversion of THCA into cholic acid. It has been commonly assumed that the same enzyme systems are involved in the conversion of THCA to cholic acid and DHCA to chenodeoxycholic acid (27). Therefore, the "experiment of nature" represented by the patients in this study demonstrate that the conversion of DHCA into chenodeoxycholic acid must be carried out by a separate series of enzymes.

Previous studies (12, 25, 26) have suggested that the conversion of THCA into cholic acid takes place via

$\beta$ -oxidation through the series of intermediates shown in Fig. 6. The first step in this reaction sequence involves the activation of THCA with coenzyme A by a thiokinase. This reaction is also required for bile acid conjugation (28). As shown in Table II, conjugated THCA was found in the urine of J. H., indicating that activation of this compound with coenzyme A was occurring.

Varanic acid, 3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ ,24 $\alpha$ -tetrahydroxy-5 $\beta$ -cholestane-26-oic acid, occurring as its CoA derivative, is a well-defined intermediate formed during the metabolism of THCA (12) (see Fig. 6). Assuming varanyl-CoA, once formed, is either converted into cholyl-CoA or conjugated and excreted, we reasoned that if the enzymatic defect in our patients occurred after the formation of varanyl-CoA, both THCA and varanic acid should have been detected in these patients. However, if the enzymatic defect was located before the formation of varanyl CoA, this compound should not be present in excessive amounts. Since varanic acid was not detected in the bile, serum, or urine of J. H., nor in the serum or stool of T. H., it seems likely that the metabolic defect must be due to a deficiency of a C<sub>24</sub>-hydroxylating enzyme system involved in the conversion of THCA-CoA into varanyl-CoA.

As shown in Fig. 6, the 24-hydroxylation of THCA probably involves a C<sub>27</sub> bile acid side-chain acyl dehydrogenase and enoyl hydrase. Previous studies of fatty acid  $\beta$ -oxidation have demonstrated that several acyl dehydrogenase enzymes exist, with relative specificities depending upon the chain length of the fatty acid substrates (29). However, only a single enoyl hydrase has been isolated that has broad specificity for many different fatty acids (30). If, similarly, only a single enoyl hydrase is involved with bile acid side-chain cleavage, it

seems unlikely that this enzyme was deficient in our patients, because DHCA conversion to chenodeoxycholic acid would also have been defective. Thus, as a working hypothesis, we suggest that the deficient enzyme in this condition is an acyl dehydrogenase specific for THCA-CoA.

The syndrome present in our patients appears to be transmitted in a genetic, rather than in an acquired fashion, since the two affected children were separated by a normal sibling. An autosomal recessive mode of inheritance in which the parents are obligate heterozygotes seems most likely, since the affected children were of opposite sex. Failure to demonstrate increased amounts of THCA in either parent is not inconsistent with an autosomal recessive pattern of inheritance, because there are a variety of documented recessive conditions in which the substrates of the deficient enzymes are not increased in the heterozygous state. For example, the levels of homocystine and oxalate are normal in carriers of homocystinuria (31) and primary hyperoxaluria (32), respectively. If the parents are heterozygotes for this condition, their hepatocytes presumably contain about 50% of the enzyme, which was nearly totally deficient in their two children. Therefore, it seemed possible that under conditions of increased cholic acid synthesis, a partial enzymatic deficiency might become manifest. To test this possibility, the parents were fed cholestyramine; however, no increase in the percentage of THCA was observed in their bile. Thus, to document clearly the recessive genetic nature of the condition in this family, it will be necessary to demonstrate that the parents have a reduced activity of the affected enzyme in liver biopsy specimens.

It remains to be determined if the presence of increased amounts of THCA and the cholestasis in these patients are independent manifestations of an underlying condition or are somehow related in a cause-and-effect fashion. Our failure to demonstrate increased amounts of THCA in specimens obtained from 11 other patients with neonatal forms of intra- and extrahepatic bile duct anomalies indicates that cholestasis itself is not responsible for the metabolic defect in the metabolism of THCA. Certain bile acids, e.g., the monohydroxy bile acid, 3 $\alpha$ -hydroxy-5 $\beta$ -cholan-24-oic acid (lithocholic acid) has been shown to produce cholestasis (33, 34) and cirrhosis (35) in experimental animals. The toxicity of THCA has not been thoroughly investigated, although Lee and Whitehouse (36) demonstrated that THCA is a potent uncoupler of oxidative phosphorylation. Thus, one could speculate that excessive quantities of THCA might be the cause of the intrahepatic bile duct anomalies in these patients.

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