# Cholic Acid Biosynthesis

## THE ENZYMATIC DEFECT IN CEREBROTENDINOUS XANTHOMATOSIS

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A B S T R A C T   Cholic acid biosynthesis is defective in individuals with cerebrotendinous xanthomatosis (CTX) and is associated with the excretion of 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$,25-tetrol, an intermediate in the 25-hydroxylation pathway of cholic acid in CTX. To define the enzymatic defect in CTX, two suspected precursors of cholic acid, namely 5$\beta$-[7$\beta$-$^3$H]cholestane-3$\alpha$,7$\alpha$,12$\alpha$-triol and 5$\beta$-[24-$^{14}$C]cholestane-3$\alpha$,7$\alpha$,12$\alpha$,24S,25-pentol were examined by both in vivo and in vitro experiments. A third precursor, 5$\beta$-[7$\beta$-$^3$H]-cholestane-3$\alpha$,7$\alpha$,12$\alpha$,25-tetrol, was compared with them in vitro.

In the in vivo experiments, each one of the labeled precursors was administered intravenously to two CTX and two control subjects. In the controls, 5$\beta$-[7$\beta$-$^3$H]cholestane-3$\alpha$,7$\alpha$,12$\alpha$-triol as well as 5$\beta$-[24-$^{14}$C]-cholestane-3$\alpha$,7$\alpha$,12$\alpha$,24S,25-pentol were rapidly converted to labeled cholic acid. Maximum specific activity values were reached within 1 d after pulse labeling, followed by exponential decay of the cholic acid specific activity curves. In contrast, these two precursors differed widely when administered to two CTX patients. While 5$\beta$-[24-$^{14}$C]cholestane-3$\alpha$,7$\alpha$,12$\alpha$,24S,25-pentol was rapidly converted to [24-$^{14}$C]cholic acid and yielded identical decay curves with those obtained in the control subjects, maximum specific activity values in [7$\beta$-$^3$H]cholic acid were much lower and peaked only on the second day after the injection of 5$\beta$-[7$\beta$-$^3$H]cholestane-3$\alpha$,7$\alpha$,12$\alpha$-triol. Furthermore, an appreciable amount of $^3$H label was present in the 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$,25-tetrol isolated from the bile of the subjects with CTX.

In the in vitro experiments, three enzymes on the 25-hydroxylation pathway of cholic acid were examined in both control and CTX subjects. The rate of the 25-hydroxylation of 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$-triol in CTX

patients was comparable to that in the controls. Similarly, the transformation of 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$,24S,25-pentol to cholic acid, catalyzed by soluble enzymes, proceeded at approximately equal rates in CTX and in control individuals. On the other hand, the rate of 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$,24S,25-pentol formation was about four times greater in the control subjects than in the CTX patients.

The results of the in vivo as well as the in vitro experiments suggest that the site of the enzymatic defect in CTX is at the 24S-hydroxylation of 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$,25-tetrol. The relative deficiency of this hydroxylase in CTX patients, accompanied by the accumulation of its substrate in bile and feces, probably accounts for the subnormal production of bile acids in CTX patients.

## INTRODUCTION

Cholesterol is the obligatory precursor of bile acids in all vertebrates (1). In the last few years, considerable information has been accumulated concerning the biosynthetic mechanisms whereby the weakly polar molecule of cholesterol is transformed into the strongly polar bile acid molecule. The liver appears to be the sole organ capable of transforming cholesterol into bile acids. In man, the primary bile acids, i.e., those formed directly from cholesterol in the liver, are chenodeoxycholic acid and cholic acid.[1] The most commonly accepted mechanism whereby cholesterol is converted to cholic acid involves 26-hydroxylated intermediates, and 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$,26-tetrol and 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholestanoic acid are postulated as key intermediates in this biosynthetic pathway (2). Loss of the isopropyl group of the side chain follows the forma-

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[1] *Nomenclature:* The following systematic names are given to bile acids referred to by trivial names: chenodeoxycholic acid, 3$\alpha$,7$\alpha$-dihydroxy-5$\beta$-cholanoic acid; cholic acid, 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholanoic acid.

tion of a 24-hydroxylated intermediate, $3\alpha,7\alpha,12\alpha$, $24\xi$-tetrahydroxy-$5\beta$-cholestanoic acid, which results in the formation of cholic acid and propionic acid (3).

During the last few years, we have studied the bile acid synthetic defect in the rare, inherited lipid storage disease, cerebrotendinous xanthomatosis (CTX).[2] This condition is characterized by the extensive accumulation of cholesterol and cholestanol throughout the body with particularly large sterol deposits in the brain, the lung, and the Achilles tendons (4). Symptoms develop secondarily to the location of the xanthomatous deposits. However, defective regulation of hepatic cholesterol and bile acid metabolism has been implicated as the major pathogenic mechanism in this condition (5). Specifically, CTX patients oversynthesize cholesterol and cholestanol, but are unable to produce sufficient quantities of bile acids ($\cong 50\%$ of normal). In addition, CTX subjects secrete substantial quantities of bile alcohols in bile and feces, in particular, $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol (6). This $5\beta$-cholestanetetrol has been demonstrated to be a key intermediate in the alternate pathway of cholic acid biosynthesis in CTX and normolipidemic subjects (7). To further investigate the defect in cholic acid synthesis in CTX patients, two putative precursors of cholic acid, namely $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha$-triol and $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha,24S$, 25-pentol, were examined by both in vivo and in vitro experiments. The results of these studies clearly show that the site of the enzymatic defect in cholic acid synthesis in CTX is associated with a deficiency of a microsomal enzyme-system catalyzing the conversion of $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol to $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha,24S,25$-pentol.

## METHODS

*Clinical.* Studies were conducted in six subjects: two CTX patients, one normolipidemic subject, and three hyper-

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[2] *Abbreviations used in this paper*: CTX, cerebrotendinous xanthomatosis; TLC, thin-layer chromatography.

lipidemic individuals (types IIb and IV). The latter four served as controls. Brief clinical summaries are given in Table I, while detailed descriptions of the CTX subjects have been published previously (4). The patients were hospitalized at the East Orange N. J. Veterans Administration Hospital and were fed regular hospital diets. Liver chemistries and blood coagulation tests were normal. Liver biopsies were obtained from J.C. and E.D.S. (CTX) for diagnostic light and electron microscopic examinations (8) and hepatic enzyme analysis. Liver specimens from O.W. and H.J. (controls) were obtained during surgery for chronic peptic ulcer disease (subtotal gastrectomies and vagotomies). The experimental protocol was approved by the Human Study Committee of the East Orange Veterans Administration Hospital, and the College of Medicine and Dentistry of New Jersey, New Jersey Medical School. Informed consent was obtained before surgery. After the induction of anesthesia with Enflurane (Ohio Medical Corp., Dayton, Ohio), the abdomen was opened and liver specimens which weighed 2–4 g were obtained.

*Preparation of unlabeled compounds.* $5\beta$-Cholestane-$3\alpha,7\alpha,12\alpha$-triol was prepared by electrolytic coupling according to Bergström and Krabisch (9), $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha$, 25-tetrol was prepared from cholic acid and purified as described by Dayal et al. (10). $5\beta$-Cholestane-$3\alpha,7\alpha,12\alpha,24S$, 25-pentol (melting point: 203–205°C) was synthesized from $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol as described previously (11).

*Preparation of labeled compounds.* $5\beta$-[$7\beta$-³H]cholestane-$3\alpha,7\alpha,12\alpha$-triol was prepared according to Björkhem and Gustafsson (12). Similarly, $5\beta$-[$7\beta$-³H]cholestane-$3\alpha$, $7\alpha,12\alpha,25$-tetrol was prepared by N-bromosuccinimide oxidation of $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol and reduction of the resulting 7-keto compound (purified by preparative thin-layer chromatography [TLC] on silica gel G plates with chloroform:acetone:methanol, 70:50:8 [vol/vol/vol]) with [³H]sodium borohydride. $5\beta$-[24-¹⁴C]cholestane-$3\alpha,7\alpha,12\alpha$, 24S,25-pentol was prepared according to Salen et al. (13). The final compounds were purified by crystallization from acetone. The following specific activities were obtained: $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha$-triol, $1.61 \times 10^7$ dpm/$\mu$mol (radiopurity 97.8%); $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol, $1.60 \times 10^4$ dpm/ $\mu$mol (radiopurity 98.4%); and $5\beta$-cholestane-$3\alpha,7\alpha,12\alpha,24S$, 25-pentol, $1.36 \times 10^4$ dpm/$\mu$mol (radiopurity 93.7%).

*Cofactors.* NAD, NADP, NADPH, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Calbiochem, San Diego, Calif.

*Gas-liquid chromatography.* The bile acids and bile alcohols were analyzed as the trimethylsilyl ether derivatives on 180 × 4-mm columns packed with 3% methylfluoroalkyl silicone on 80–100 mesh GAS Chrom Q (Ap-

TABLE I

*Data on Patients*

| Patient | Sex | Age | Height | Weight | % of ideal weight | TC* | TG‡ | Diagnosis |
|---------|-----|-----|--------|--------|-------------------|-----|-----|-----------|
| | | | *cm* | *kg* | | *mg/100 ml* | | |
| J.C. | M | 39 | 165 | 55 | 95 | 144 | 84 | CTX |
| E.D.S. | F | 53 | 161 | 47 | 94 | 132 | 62 | CTX |
| A.S. | M | 55 | 175 | 88 | 135 | 195 | 205 | Hyperlipidemia (type IIb), cholelithiasis |
| J.S. | M | 57 | 168 | 96 | 154 | 309 | 1,008 | Hyperlipidemia (type IV) |
| O.W. | M | 52 | 166 | 66 | 124 | 236 | 253 | Hyperlipidemia (type IIb), chronic peptic ulcer disease |
| H.J. | M | 59 | 196 | 78 | 134 | 211 | 74 | Chronic peptic ulcer disease, (normolipidemic) |

\* TC, total cholesterol.
‡ TG, triglycerides.

plied Science Labs. Inc., State College, Pa.) column temperature 230°C (gas chromatograph, model 7610, Hewlett-Packard Co., Palo Alto, Calif.) (10).

*Mass spectra.* Mass spectra of the bile alcohols were obtained with a Varian MAT-III gas chromatograph-mass spectrometer, Varian Associates, Palo Alto, Calif., as described previously (11).

*Experimental design in vivo.* To study the formation of cholic acid, each one of the labeled precursors, 5β-[7β-³H]cholestane-3α,7α,12α-triol and 5β-[24-¹⁴C]cholestane-3α,7α,12α,24S,25-pentol, was administered intravenously in separate experiments to two CTX and two control subjects. Labeled cholic acid was isolated from daily specimens of bile, its mass and radioactivity were determined, and the specific radioactivity decay curves were plotted vs. time (14). In addition, 5β-[7β-³H]cholestane-3α,7α,12α,25-tetrol was isolated from the bile specimens of the two CTX patients and the specific radioactivity decay curves were plotted vs. time as described above.

*Experimental design in vitro.* Liver homogenates of CTX patients and control subjects were fractionated by differential ultracentrifugation and the microsomal and soluble fractions were prepared and incubated separately with the following putative precursors: 5β-[7β-³H]cholestane-3α,7α,12α-triol, 5β-[7β-³H]cholestane-3α,7α,12α,25-tetrol, and 5β-[24-¹⁴C]cholestane-3α,7α,12α,24S,25-pentol. After 15 min of incubation the reaction was stopped and the activities of the different enzymes were determined by a combination of TLC-liquid scintillation counting of the pertinent products.

*Isolation of cholic acid and 5β-cholestane-3α,7α,12α,25-tetrol from specimens of bile.* After the injection of a tracer dose of a labeled putative bile acid precursor, labeled cholic acid was isolated from specimens of bile from CTX as well as control subjects; labeled 5β-cholestane-3α,7α,12α,25-tetrol was isolated only from CTX patients as described previously by Salen et al. (14). In a typical experiment, a specimen of bile was made alkaline (pH 9.5) and extracted exhaustively with ethyl acetate. 5β-Cholestane-3α,7α,12α,25-tetrol was isolated from the ethyl acetate extract by TLC on silica gel G plates (0.25-mm thick, Analtech, Inc., Newark, Del.) with a solvent system of chloroform:acetone:methanol (70:50:10 [vol/vol/vol]). The aqueous layer was made strongly alkaline (2.5 N NaOH) and autoclaved at 115°C for 3 h. After acidification (pH 2) the bile acids were extracted with ether. Cholic acid was isolated from the ether extract by TLC on silica gel G plates (Analtech, Inc.) with benzene:dioxane:acetic acid (15:5:2 [vol/vol/vol]). Labeled 5β-cholestane-3α,7α,12α,25-tetrol and cholic acid thus obtained were quantitated by GLC and their radioactivity was determined by liquid scintillation counting (14).

*Fractionation of liver homogenates.* All specimens of human liver were obtained at about 10:00 a.m. to minimize the diurnal variation of bile acid synthesis. The liver tissue was placed immediately in ice-cold 0.1 M Tris-Cl buffer, pH 7.4, which contained 2.5 mM EDTA, and was transported to the laboratory in an ice bath within 1 h. All subsequent operations were carried out at 4°C. The liver was extruded through a tissue press (Harvard Apparatus Co., Inc., Millis, Mass.). A 1-g aliquot was homogenized in a loose-fitting Potter-Elvehjem homogenizer with 4 ml of 0.1 M Tris-Cl buffer, pH 7.4, which contained 2.5 mM EDTA. The microsomal fraction was obtained by centrifuging the homogenate for 12 min at 20,000 g, followed by centrifugation at 100,000 g for 1 h (15). The microsomal pellet was washed by resuspension in 0.1 M Tris-Cl buffer, pH 7.4, with EDTA, followed by centrifugation for 1 h at 100,000 g. The final pellet was suspended in 0.1 M Tris-Cl buffer, pH 7.4, with EDTA in a

volume corresponding to the original 20,000 g supernatant solution from which it had been prepared.

Protein was determined according to Lowry et al. (16). The protein content of the microsomal fraction was about 5 mg/ml and, of the 100,000 g supernatant fraction, about 12 mg/ml.

## Enzyme Assays

*Conversion of 5β-cholestane-3α,7α,12α-triol to 5β-cholestane-3α,7α,12α,25-tetrol.* The complete system contained in a volume of 1.0 ml: 85 mM phosphate buffer, pH 7.4, 1.7 mM MgCl₂, 3 mM NADPH, and 0.1 ml of the microsomal fraction that contained 1–1.5 mg protein. The reaction was initiated by the addition of 5β-[7β-³H]cholestane-3α,7α, 12α-triol (100 nmol, sp act, 1.61 × 10⁷ dpm/μmol) in 15 μl acetone. The incubation mixture was shaken in air at 37°C for 15 min and the reaction was terminated by the addition of 0.1 ml 1 N HCl. All enzyme assays were carried out in duplicate and zero time controls were run with each experiment. The unreacted 5β-cholestane-3α,7α,12α-triol and the reaction products were immediately extracted with 2 × 5.0 ml ethyl acetate, shaking for 5 min each time. The combined ethyl acetate extracts were washed twice with water and evaporated to dryness under N₂. The 5β-[7β-³H]cholestane-3α,7α,12α, 25-tetrol formed during the incubation was isolated by TLC as follows: 5β-cholestane-3α,7α,12α,25-tetrol was separated from the other 5β-cholestanetetrols by TLC on 0.25-mm thick silica gel G plates (Brinkmann Instruments Inc., Westbury, N. Y.) with chloroform:acetone:methanol (70:50:7 [vol/vol/ vol], developed three times). Unlabeled cholestanetetrols (5 μg each) were applied with the extracts as markers. The pertinent spots were made visible with a spray reagent which consisted of 3.5% phosphomolybdic acid in isopropanol. Retardation factor values were as follows: 5β-cholestane-3α,7α,12α,24R-tetrol, 0.35; 5β-cholestane-3α,7α,12α,25-tetrol, 0.41; 5β-cholestane-3α,7α,12α,26-tetrol (25R), 0.44; and 5β-cholestane-3α,7α,12α,24S-tetrol + 5β-cholestane-3α,7α,12α, 26-tetrol (25S), 0.47. Enzyme activity was calculated after removing the spot that corresponded to 5β-cholestane-3α, 7α,12α,25-tetrol from the plate and measuring its radioactivity in a liquid scintillation counter. Because the specific radioactivity of the substrate was known, the radioactivity data could be expressed in terms of pmol of product formed.

To confirm the identity and the radioactive purity of the biosynthetic 5β-[7β-³H]cholestane-3α,7α,12α,25-tetrol, a large-scale incubation was carried out and the pertinent band from the preparative silica gel G plate (Brinkmann Instruments Inc.) was eluted with methanol. The methanol extract was divided into three aliquots: one aliquot was used for mass and structure determination by GLC-mass spectrometry, a second aliquot was used for radioactivity determination in a liquid scintillation counter (Beckman LS-200B, Beckman Instruments, Inc., Fullerton, Calif.), and a third aliquot was reapplied on alumina G plate (0.25-mm thick, Analtech, Inc.) and developed with benzene:ethyl acetate:methanol (90:20:14 [vol/vol/vol]). After visualizing the spot with iodine, it was removed from the plate, eluted with methanol, and its mass and radioactivity were determined by GLC and liquid scintillation counting.

*Conversion of 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestane-3α,7α,12α,24S,25-pentol.* 5β-[7β-³H]cholestane-3α,7α,12α,25-tetrol (200 nmol; sp act 1.60 × 10⁴ dpm/ μmol) in methanol was mixed with 0.15 mg of Tween-80 (Fisher Scientific Co., Springfield, N. J.). The organic solvent was evaporated to dryness and the residue was solubilized by vigorous mixing (Vortex Scientific Industries, Inc., Bohemia, N. Y.) in 0.1 ml of 0.1 M Tris-Cl buffer, pH 7.4. In a

final volume of 1.0 ml, the incubation mixture contained: 0.2 mM labeled 5β-cholestane-3α,7α,12α,25-tetrol; 85 mM Tris-Cl buffer, pH 7.4; 1.0 mM EDTA; 1.0 mM NADP; 3.6 mM glucose-6-phosphate; 7 enzyme U of glucose-6-phosphate dehydrogenase; and ≅0.7 mg of microsomal protein. The incubation of the entire system was carried out with shaking for 10 min at 37°C in air and was terminated by the addition of 1.0 ml of 10% KOH in methanol:H$_2$O (90:10 [vol/vol]). The 5β-[7β-$^3$H]cholestane-3α,7α,12α,24S,25-pentol was isolated from the incubation mixture and its radioactivity determined as described by Cheng et al. (17).

*Conversion of 5β-cholestane-3α,7α,12α,24S,25-pentol to cholic acid.* In standard incubation procedures 200 nmol of 5β-[24-$^{14}$C]cholestane-3α,7α,12α,24S,25-pentol was dissolved in 30 μl of acetone which was quickly dispersed into 0.2 ml 0.1 M Tris Cl-buffer, pH 7.4. In a volume of 0.72 ml, the incubation medium contained: 83 mM Tris-Cl buffer, pH 7.4; 4.2 mM NAD$^+$; and 100,000 g supernatant solution which contained ≅0.5 mg of protein. The incubation was carried out by shaking in air at 37°C for 10 min. The reaction was terminated by the addition of 2% NaOH in 95% ethanol. The [24-$^{14}$C]cholic acid formed was isolated and its radioactivity was determined as described previously (7).

## RESULTS

*In vivo conversion of 5β-[7β-$^3$H]cholestane-3α, 7α,12α-triol to cholic acid.* 5β-[7β-$^3$H]cholestane-3α,7α,12α-triol (2 μCi) was injected intravenously to two control and two CTX subjects. The $^3$H-labeled cholic acid formed was isolated from daily specimens of bile collected over a period of 1 wk and its specific activity decay-curves were plotted as shown in Fig. 1. In all subjects, radioactive cholic acid was detected within 1 d of pulse labeling. In the controls, maximum specific activity values were reached within 24 h, followed by a linear decay of the cholic acid specific activity curves. In contrast, the maximum specific activity of cholic acid in the CTX patients was reached only 2 d after pulse labeling and then declined linearly.

More detailed studies of the products formed from the labeled 5β-cholestane-3α,7α,12α-triol showed the presence of $^3$H-labeled 5β-cholestane-3α,7α,12α,25-tetrol in the bile specimens of the CTX patients; this labeled tetrol was absent in the bile of the control subjects. Fig. 2 illustrates the specific activity decay-curves of 5β-[7β-$^3$H]cholestane-3α,7α,12α,25-tetrol and that of $^3$H-labeled cholic acid in one of the CTX patients (E.D.S.). The specific activity decay-curve of the tetrol intersected with the rising specific activity curve of cholic acid, thus showing a precursor-product relationship.

*In vivo conversion of 5β-cholestane-3α,7α,12α, 24S,25-pentol to cholic acid.* After the intravenous injection of 5β-[24-$^{14}$C]cholestane-3α,7α,12α,24S,25-pentol (2 μCi) to two control and two CTX subjects, $^{14}$C-labeled cholic acid was isolated from daily specimens of bile. The results of this experiment are shown in Fig. 3. Radioactivity was present in the cholic acid
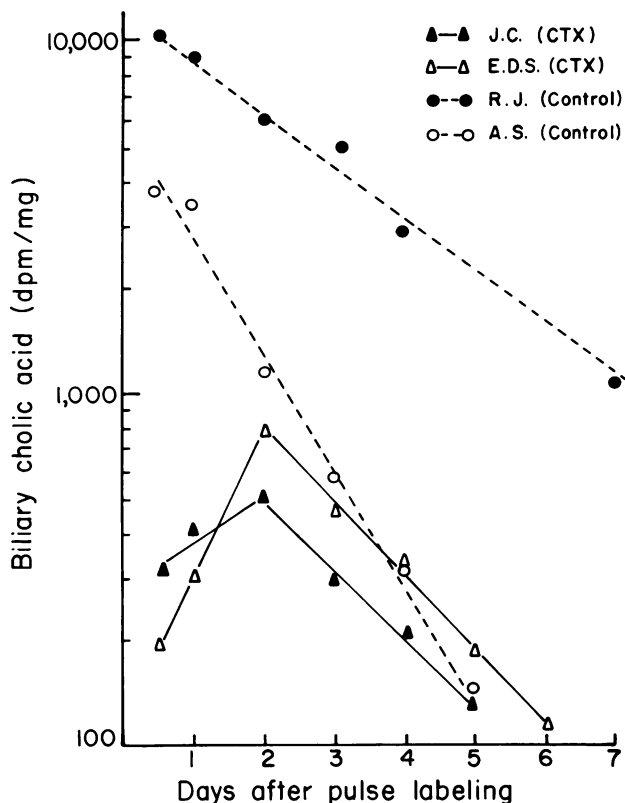


FIGURE 1 Specific activity vs. time decay curves of cholic acid from bile of two normolipidemic and two CTX subjects after intravenous pulse labeling with 5β[7β-$^3$H]3α,7α,12α-triol.

from all subjects (controls, as well as CTX patients) and the specific activity vs. time curves decayed exponentially, reaching a maximum within 24 h.

*In vitro conversion of 5β-cholestane-3α,7α,12α-triol to cholic acid.* To examine the individual steps on the pathway from 5β-cholestane-3α,7α,12α-triol to cholic acid (Fig. 4), the following reactions were carried out with labeled substrates: (a) conversion of 5β-cholestane-3α,7α,12α-triol to 5β-cholestane-3α,7α, 12α,25-tetrol by hepatic microsomes, (b) conversion of 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestane-3α, 7α,12α,24S,25-pentol by hepatic microsomes, and (c) transformation of 5β-cholestane-3α,7α,12α,24S,25-pentol to cholic acid by hepatic soluble enzyme preparations. Comparisons were made between subcellular hepatic preparations of control and CTX subjects. The results are illustrated in Table II and show that the enzyme activity catalyzing the 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol to yield 5β-cholestane-3α,7α,12α,25-tetrol was active in both control and CTX subjects. In contrast, the transformation of 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestane-
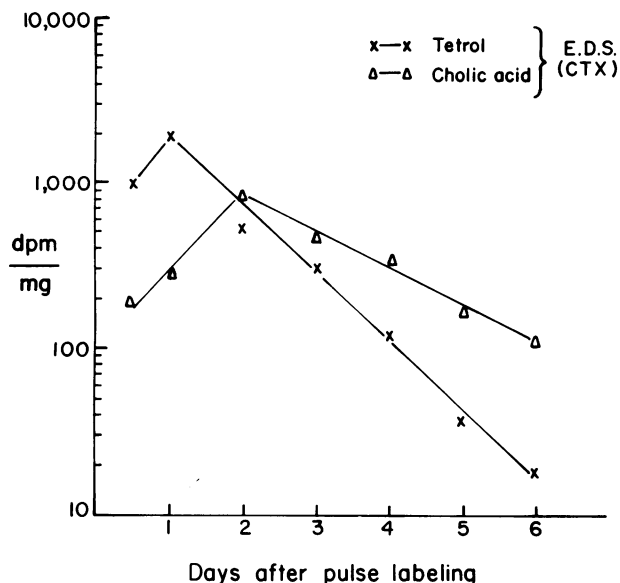
FIGURE 2 Specific activity vs. time decay curves of 5β-cholestane-3α,7α,12α,25-tetrol and cholic acid isolated from bile of a CTX patient after intravenous pulse labeling with 5β[7β-³H]3α,7α,12α-triol.

3α,7α,12α,24S,25-pentol by hepatic microsomes was four times greater in the control subjects than in the CTX patients. Finally, the rate of the side-chain cleavage of 5β-cholestane-3α,7α,12α,24S,25-pentol by hepatic soluble enzymes in CTX patients was comparable to that in the controls.

## DISCUSSION

The results of the present investigation show that 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,25-tetrol, and 5β-cholestane-3α,7α,12α,24S,25-pentol were converted into cholic acid in both control and CTX subjects. In the control subjects, 5β-cholestane-3α,7α,12α-triol was rapidly transformed to cholic acid so that peak cholic acid specific activity was detected within the first 24 h after pulse labeling. In contrast, the specific activity of biliary cholic acid in the CTX subjects rose slowly over the first 24 h and did not reach a maximum value until 48 h after pulse labeling (Fig. 1). When this transformation was examined more closely in one of the CTX subjects, a characteristic precursor-product relationship between biliary cholic acid and 5β-cholestane-3α,7α,12α,25-tetrol was observed (Fig. 2), which implies that most of the cholic acid originated from this tetrol (18). Furthermore, no 5β-cholestane-3α,7α,12α-triol was detected in the bile of this CTX patient which suggests a rapid and complete conversion of this triol to cholic acid via 5β-cholestane-3α,7α,12α,25-tetrol. After 5β-(24-¹⁴C)cholestane-3α,7α,12α,24S,25-pentol was administered, peak biliary
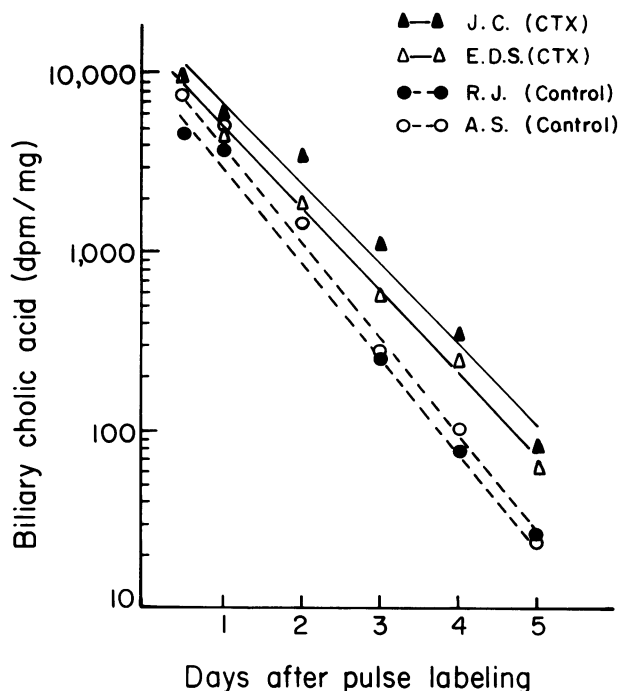


FIGURE 3 Specific activity vs. time decay curves of cholic acid from bile of two normolipidemic and two CTX subjects after intravenous pulse labeling with 5β[24-¹⁴C]3α,7α,12α, 24S,25-pentol.

cholic acid specific activities were reached within 24 h in both the CTX and control subjects; followed by similar exponential decay curves of radioactive cholic acid.

The results of the in vivo experiments (vide supra) indicate that in both CTX and control subjects there was no block in the formation of cholic acid from 5β-cholestane-3α,7α,12α,24S,25-pentol, whereas the transformation of 5β-cholestane-3α,7α,12α-triol to
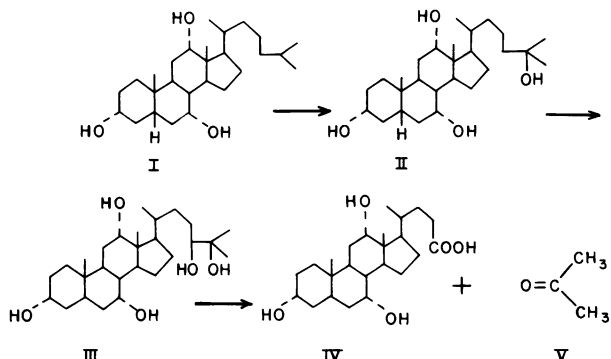


FIGURE 4 Pathway of cholic acid biosynthesis showing side chain degradation via 25-hydroxylated intermediates. I, 5β-cholestane-3α,7α,12α-triol; II, 5β-cholestane-3α,7α,12α,25-tetrol; III, 5β-cholestane-3α,7α,12α,24S,25-pentol; IV, cholic acid; V, acetone.

## TABLE II
*The Activity of Enzymes on the 25-Hydroxylation Pathway in the Conversion of 5β-Cholestane-3α,7α,12α-triol to Cholic Acid**

| Enzyme | Rate of product formation | |
| --- | --- | --- |
| | Control | CTX |
| | *pmol/mg protein/10 min* | |
| 25-hydroxylase‡ | 5,480 (5,631; 5,329)¶ | 7,110 (7,320; 6,900) |
| 24S-hydroxylase§ | 636 (670; 602) | 151 (143; 159) |
| Cleavage enzyme‖ | 9,160 (9,008; 9,312) | 7,310 (7,560; 7,058) |

* Microsomal and soluble fractions were prepared and products were analyzed as described in Methods.
‡ Enzyme catalyzing the conversion of 5β-cholestane-3α, 7α,12α-triol (I, Fig. 4) to 5β-cholestane-3α,7α,12α,25-tetrol (II).
§ Enzyme catalyzing the conversion of 5β-cholestane-3α, 7α,12α,25-tetrol (II) to 5β-cholestane-3α,7α,12α,24S,25-pentol (III).
‖ Enzymes catalyzing the conversion of 5β-cholestane-3α,7α,12α,24S,25-pentol (III) to cholic acid (IV).
¶ Average values with individual determinations in parenthesis.

cholic acid was hindered in the CTX patients and was associated with the accumulation of 5β-cholestane-3α,7α,12α,25-tetrol. Thus, defective 24S-hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol appears to be the enzymatic abnormality which causes the deficiency of cholic acid in CTX. This possibility was supported by the results of the in vitro incubation experiments with hepatic microsomal and soluble enzyme preparations (Table II). When 5β-cholestane-3α,7α,12α-triol was incubated with hepatic microsomes prepared from control as well as CTX liver specimens, active 25-hydroxylation was observed in both cases with the formation of 5β-cholestane-3α,7α,12α,25-tetrol. Similarly, the cleavage of 5β-cholestane-3α,7α,12α, 24S,25-pentol to yield cholic acid by the soluble enzyme fractions was comparable in both cases. In contrast, the rate of 24S-hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol by hepatic microsomes from CTX patients was only 0.25 of that from the controls. Thus, a complete 25-hydroxylation pathway of cholic acid from 5β-cholestane-3α,7α,12α-triol is demonstrated in CTX as well as control subjects (Fig. 4). However, 24S-hydroxylase activity is depressed in CTX and apparently results in the accumulation of 5β-cholestane-3α,7α,12α,15-tetrol. Although small amounts of 5β-cholestane-3α,7α,12α,24R,25-pentol and 5β-cholestane-3α,7α,12α,23ξ,25-pentol were formed by hepatic microsomes (17) and were found in the feces and bile of CTX subjects (11), neither bile alcohol is converted

to cholic acid (7). We postulate that both compounds accumulate in CTX subjects because deficient 24S-hydroxylation prolongs the exposure of 5β-cholestane-3α,7α,12α,25-tetrol to the microsomal 24R- and 23-hydroxylation systems.

The block in bile acid synthesis may be related to the development of the clinical picture in CTX. Brain, tendon, and lung xanthomas and coronary atherosclerosis are the major clinical manifestations that result from the excessive deposition of cholesterol and cholestanol in these vital tissues. Recently, we have presented evidence that the increased, neutral sterol deposits result from the overproduction of both cholesterol and cholestanol (5). However, because CTX is inherited as an autosomal recessive disease, the genetic defect should result from a single enzyme defect which must account directly or indirectly for all the clinical and biochemical aspects of this hereditary metabolic disease (19). The demonstration that bile acid synthesis is abnormal with a specific defect in 24S-hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol fits well into the definition of a recessively inherited genetic disease. As a result of diminished bile acid pool, cholesterol production increases; but, because of the block in side-chain cleavage, bile acid precursors are excreted and the augmented cholesterol production is only partially converted to bile acids. The remainder is available for deposition in the tissues and for cholestanol formation.

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