Cerebrotendinous xanthomatosis: a defect in mitochondrial 26-hydroxylation required for normal biosynthesis of cholic acid.

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Research Article

Oxidation of side chain of 5 beta-cholestane-3 alpha,7 alpha,12 alpha-triol was studied in a patient with cerebrotendinous xanthomatosis (CTX) and in control subjects, using various subcellular fractions of liver homogenate and a method based on isotope dilution-mass spectrometry. In the control, 5 beta-cholestane-3 alpha,7 alpha,12 alpha-triol was converted into 5 beta-cholestane-3 alpha,7 alpha,12 alpha,26-tetrol and 3 alpha,7 alpha,12 alpha-trihydroxy-5 beta-cholestanoic acid by the mitochondrial fraction, and into 5 beta-cholestane-3 alpha,7 alpha,12 alpha,-25-tetrol by the microsomal fraction. In the CTX patient, liver mitochondria were completely devoid of 26-hydroxylase activity. The same mitochondrial fraction catalyzed 25-hydroxylation of vitamin D3. The microsomal fraction of liver of the subject with CTX contained more than 50-fold the normal amount of 5 beta-cholestane-3 alpha,7 alpha,12 alpha-triol. The basic metabolid defect in CTX appears to be a lack of the mitochondrial 26-hydroxylase. The excretion in the bile 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 observed in CTX patients may be secondary to the accumulation of the major substrate for the 26-hydroxylase, i. e., 5 beta-cholestane-3 alpha,7 alpha,12 alpha-triol, and exposure of this substrate to the normally less active microsomal 25-and 24-hydroxylases. It is concluded that the major pathway in the biosynthesis of cholic acid in human liver involves a mitochondrial C27-steroid 26-hydroxylation.

Cerebrotendinous Xanthomatosis

A DEFECT IN MITOCHONDRIAL 26-HYDROXYLATION
REQUIRED FOR NORMAL BIOSYNTHESIS OF CHOLIC ACID

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A B S T R A C T   Oxidation of the side chain of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol was studied in a patient with cerebrotendinous xanthomatosis (CTX) and in control subjects, using various subcellular fractions of liver homogenate and a method based on isotope dilution-mass spectrometry.

In the control, 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol was converted into 5β-cholestane-$3\alpha,7\alpha,12\alpha,26$-tetrol and $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanoic acid by the mitochondrial fraction, and into 5β-cholestane-$3\alpha,7\alpha,12\alpha,$-25-tetrol by the microsomal fraction.

In the CTX patient, liver mitochondria were completely devoid of 26-hydroxylase activity. The same mitochondrial fraction catalyzed 25-hydroxylation of vitamin D_3.

The microsomal fraction of liver of the subject with CTX contained more than 50-fold the normal amount of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol.

The basic metabolic defect in CTX appears to be a lack of the mitochondrial 26-hydroxylase. The excretion in the bile of 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol and 5β-cholestane-$3\alpha,7\alpha,12\alpha,24\alpha,25$-pentol observed in CTX patients may be secondary to the accumulation of the major substrate for the 26-hydroxylase, i.e., 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol, and exposure of this substrate to the normally less active microsomal 25- and 24-hydroxylases.

It is concluded that the major pathway in the biosynthesis of cholic acid in human liver involves a mitochondrial C_{27}-steroid 26-hydroxylation.

INTRODUCTION

From a great number of in vivo and in vitro experiments in humans as well as in rats, evidence has accumulated that in the biosynthesis of bile acids, the normal degradation of the cholesterol side chain starts with a 26-hydroxylation,[1] followed by oxidation of the hydroxyl group to yield $3\alpha,7\alpha$-dihydroxy-5β-cholestanoic acid and $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanoic acid (5–10; Fig. 1A). The mitochondrial fraction of both human and rat liver contains a 26-hydroxylase, which is active towards several 7α-hydroxylated C_{27}-steroids (11, 12). In rat liver (12, 13) the microsomal fraction is able to catalyze 26-hydroxylation of some C_{27}-steroids. In both rat (12) and human (11) liver, the microsomal fraction is able to catalyze the introduction of hydroxyl groups also in the 23-, 24-, and 25-position of the C_{27}-steroid side chain, but these

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[1] *Abbreviations and nomenclature used in this paper*: CTX, cerebrotendinous xanthomatosis; GC-MS, gas chromatography-mass spectrometry; HPLC, high-pressure liquid chromatography; Mops, 4-morpholino propane sulfonic acid; V_{max}, maximum velocity. According to Berséus (1) and to a recent work by Gustafsson (2) the mitochondrial fraction of rat liver hydroxylates the methyl group of cholesterol in position C-27 (the *pro-S*-methyl group [3]) and the microsomal fraction of rat liver hydroxylates the methyl group of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol in position C-26 (the 25-*pro-R*-methyl group [3]). Recently, however, Shefer et al. (4) found an opposite stereospecificity when studying side-chain hydroxylation of 5β-cholestane-$3\alpha,$-7α-diol in human liver mitochondria (4). Because the stereospecificity might be different in different species and with different substrates, we prefer here to denote the ω-hydroxylation of the side chain of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol as 26-hydroxylation.
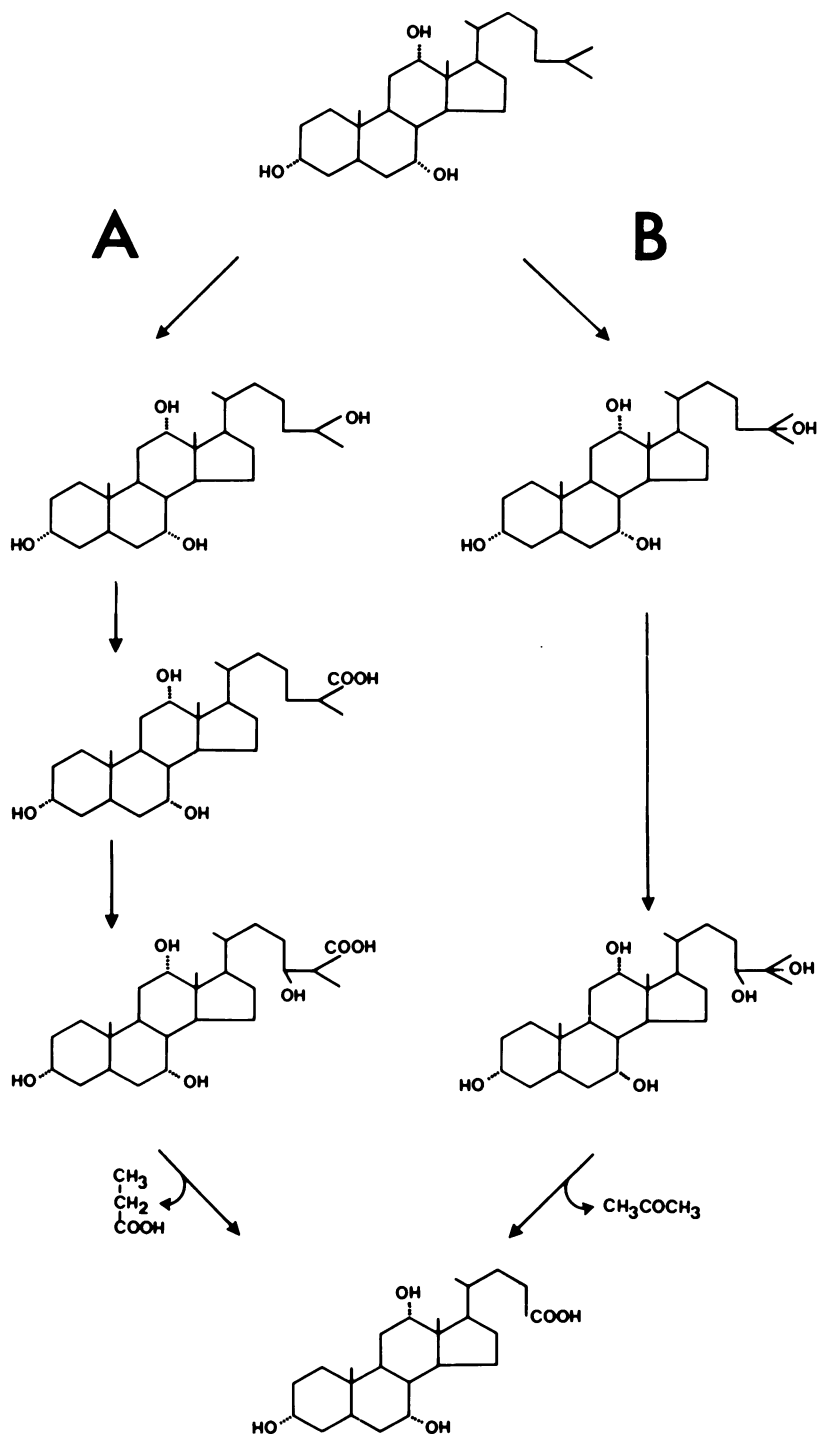
FIGURE 1 The 26-hydroxylase pathway (A) and the 25-hydroxylase pathway (B) for degradation of the C_{27}-steroid side chain in the biosynthesis of bile acids.

activities were originally believed to be of minor physiological significance (12).

In 1974, however, Setoguchi et al. (14) showed that ~10% of the total sterols excreted in feces of three patients with the rare lipid storage disease cerebrotendinous xanthomatosis (CTX), were 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,24,25-pentol. In later studies, this group of researchers

presented evidence that in the liver of healthy control subjects as well as in the liver of CTX patients, enzymes are present that are able to catalyze formation of cholic acid from 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol (15, 16; Fig. 1B). Both in vivo and in vitro experiments showed that patients with CTX had a lower capacity to metabolize 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol than control subjects. In particular, the rate of 24β-hydroxylation of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-tetrol was about four times higher in the microsomal fraction of liver of a control subject than in the corresponding fraction of a CTX patient (16, 17). It was concluded that the major metabolic defect in patients with CTX is a relative lack of 24β-hydroxylase activity. On the basis of these findings, it was further suggested that the pathway to cholic acid, involving a 25-hydroxylation, may be the major one in normal man (15, 16). However, the possibility that the pathway involving a 26-hydroxylation is the main route could not be excluded (15).

In the present work we have investigated the latter possibility and we show that the mitochondrial fraction from the liver of a patient with CTX was completely devoid of 26-hydroxylase activity. In addition, large amounts of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol were detected in the microsomal fraction. The exposure of this substrate to the action of the microsomal 23-, 24-, and 25-hydroxylases may explain the accumulation of 25-hydroxylated steroids in these patients. Based on our findings we suggest that the basic metabolic defect in CTX patients is the lack of a mitochondrial C_{27}-steroid 26-hydroxylase and that this enzyme is involved in the normal major pathway in cholic acid biosynthesis in man.

METHODS

Chemicals. Malic acid, glucose-6-phosphate, 4-morpholino propane sulfonic acid (Mops), ATP and NADP$^+$ were purchased from Sigma Chemical Co., St. Louis, Mo. Glucose-6-phosphate dehydrogenase was from Boehringer & Soehne, Mannheim, West Germany. N,N'-diphenyl-p-phenylene diamine (DPPD) was from Eastman Kodak Co., Rochester, N. Y. Other chemicals and solvents were standard commercial high purity materials.

Unlabeled steroids. Vitamin D$_3$ obtained from Sigma Chemical Co. was purified by high-pressure liquid chromatography (HPLC) on a silicic acid column (18) before use. 5β-Cholestane-$3\alpha,7\alpha,12\alpha$-triol and 5β-cholestane-$3\alpha,7\alpha,12\alpha,26$-tetrol were synthesized as described (13). $3\alpha,7\alpha,12\alpha$-Trihydroxy-5β-cholestanoic acid was obtained from alligator bile (13). This acid isolated from this species appears to be the 25R-isomer (19, 20). The alkaline conditions used in the isolation procedure might, however, to some extent change the stereospecificity at C-25. Most probably the $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanoic acid synthesized in human liver has the 25R-configuration (21). In any case, the 25R- and 25S-isomers are not separated under any of the chromatographic conditions used in the present work.

Tritium-labeled steroid. 5β-[7β-^{3}H]Cholestane-$3\alpha,7\alpha,12\alpha$-triol (7 Ci/mol) was synthesized as described (13).

Deuterium-labeled steroids. 25-Hydroxy[26^{2}H]vitamin D$_3$ was synthesized as described (22). 5β-[2,2,3,4,4-^{2}H$_5$]Cholestane-$3\alpha,7\alpha,12\alpha$-triol, 5β-[2,2,3,4,4-^{2}H$_5$]cholestane-$3\alpha,7\alpha,12\alpha$-25-tetrol, and 5β-[2,2,3,4,4-^{2}H$_5$]cholestane-$3\alpha,7\alpha,12\alpha,26$-tetrol were prepared as follows: $7\alpha,12\alpha$-Dihydroxy-5β-cholestane-3-one was prepared by Oppenhauer oxidation of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol (23). This compound, 20 mg, was refluxed in 5 ml of CH$_3$O^{2}H and 1 ml of ^{2}H$_2$O together with 12 mmol NaOH for 24 h. The deuterated solvents were obtained from Merck AG (Darmstadt, West Germany) and had a purity of >99% with respect to ^{2}H. The mixture was then diluted with ^{2}H$_2$O, acidified with ^{2}HCl (Merck AG, 99% pure with respect to ^{2}H) and extracted with diethyl ether. The ether phase was washed with ^{2}H$_2$O until neutral. After evaporation of the solvent in vacuo, the residue was treated with 5 ml of CH$_3$O^{2}H and 10 mg of NaB^{2}H$_4$ (Merck AG, 99% pure with respect to ^{2}H) for 1 h at room temperature. After dilution with ^{2}H$_2$O and acidification with ^{2}HCl, the mixture was extracted with ether as above. The reduced material in the ether phase was purified by preparative thin-layer chromatography, using ethyl acetate as solvent. Crystallization in methanol-water gave 5.7 mg of white needles. The material was pure as judged by thin-layer chromatography (ethyl acetate as solvent) and gas chromatography of the trimethylsilyl ether using a SE-30 column. A mass spectrum (LKB 9000, LKB Instruments, Inc., Stockholm, Sweden) of the trimethylsilyl ether of the compound confirmed the identity and showed that the material was a mixture of tetra- and pentadeuterium labeled 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol. Thus the peaks at the mass/charge ratio (m/e) 546, m/e 456, m/e 366, and m/e 253 in the mass spectrum of the trimethylsilyl ether of unlabeled 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol were shifted to m/e 551, m/e 461, m/e 371, and m/e 258 in the mass spectrum of the deuterium-labeled compound (Fig. 2).

^{2}H$_5$-Labeled 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol was then incubated with the microsomal fraction of a rat liver homogenate (11, 13). The products, ^{2}H$_5$-labeled 5β-cholestane-$3\alpha,7\alpha,12\alpha$,-25-tetrol and 5β-cholestane-$3\alpha,7\alpha,12\alpha,26$-tetrol were isolated by thin-layer chromatography (12, 13). According to gas chromatography of trimethylsilyl ether of the material (SE-30 column) the thin-layer chromatographic fraction contained a mixture of 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol (34%) and 5β-cholestane-$3\alpha,7\alpha,12\alpha,26$-tetrol (66%).

The mass spectrum of the trimethylsilyl ether of ^{2}H$_5$-labeled 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol was consistent with the proposed structure. Thus the peaks at m/e 544, m/e 454, and m/e 253 in the mass spectrum of trimethylsilyl ether of unlabeled 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol (14) were shifted to m/e 549, m/e 459, and m/e 258.

The mass spectrum of the trimethylsilyl ether of ^{2}H$_5$-labeled 5β-cholestane-$3\alpha,7\alpha,12\alpha,26$-tetrol was also consistent with the proposed structure. Thus the peaks at m/e 634, m/e 544, m/e 454, and m/e 253 in the mass spectrum of trimethylsilyl ether of unlabeled 5β-cholestane-$3\alpha,7\alpha,12\alpha,26$-tetrol were shifted to m/e 639, m/e 549, m/e 459, and m/e 258 in the mass spectrum of the trimethylsilyl ether of the ^{2}H$_5$-labeled compound (Fig. 3).

In each incubation assay, a fixed amount of the thin-layer chromatographic fraction containing ^{2}H$_5$-labeled 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol and 5β-cholestane-$3\alpha,7\alpha,12\alpha,26$-tetrol was used directly without further purification.

Clinical. Our patient was a women (I.J.) born in 1935 with typical clinical symptoms of CTX: Achilles tendon xanthomas, cataracts, mental reduction, and ataxia. Detailed descriptions of the patient and her sister who also suffers from CTX have been published (24). The diagnosis was established by the demonstration of a high level of 5α-cholestane-3β-ol (cholestanol) in serum (3.2 mg/dl, normal
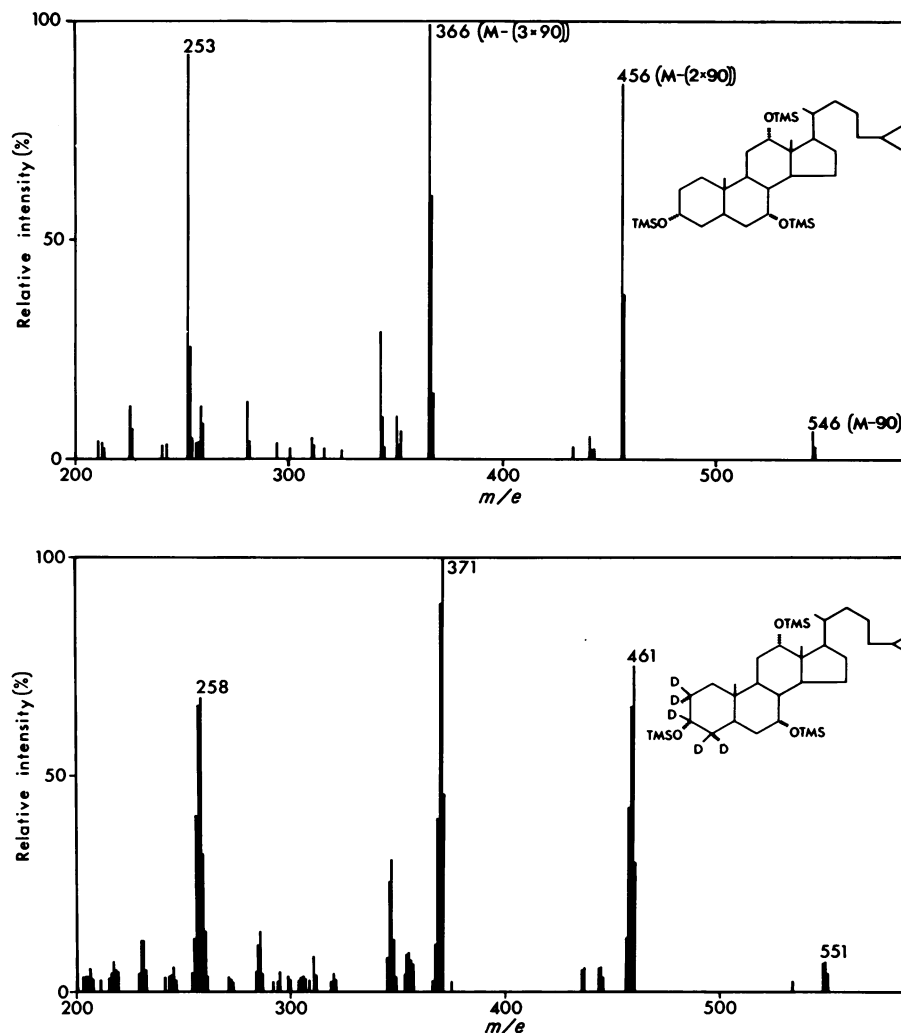
FIGURE 2   Partial mass spectrum of trimethylsilyl ether of unlabeled (upper spectrum) and $^2H_5$-labeled 5β-cholestane-3α,7α,12α-triol (lower spectrum).

reference value 0.9±0.2 [25]) and of excess cholestanol in a tendon xanthoma (17% of the sterol fraction). Cholestanol was separated from cholesterol by thin-layer chromatography of lipid extracts (26) of the xanthoma and serum on plates coated with 20% AgNO$_3$ (27). Quantitative analyses by gas liquid chromatography (Varian 2100 instrument, Varian Associates, Palo Alto, Calif.) were performed after treatment with trifluoroacetic anhydride, using 3% QF 1 columns at 220°C, and α-cholestane as internal standard. The identity of cholestanol was established by mass spectrometry (Varian CH7 instrument). The spectrum was the typical one (28) with peaks at 388, 373, 355, 234, 233, and 215.

A specimen of bile was analyzed in 1976 by Dr. Gerald Salen. The relative contents of bile acids and bile alcohols were: 5β-cholestane-3α,7α,12α,triol 1%, 5β-cholest-25-ene-3α,7α,12α-triol 4%, 5β-cholestane-3α,7α,12α,25-tetrol 32%, cholic acid 41%, deoxycholic acid 2%, chenodeoxycholic acid 1%, and unidentified bile alcohols 19%. As compared with normal bile (14, 25) the most striking findings were thus a deficiency in chenodeoxycholic acid (normal amounts

40–45%), and large amounts of bile alcohols (normally not detectable).

In 1978, the patient was admitted to the hospital (Haukeland sykehus, Bergen, Norway) for acute cholecystitis. Because of numerous gallstones it was decided to perform a cholecystectomy. Liver function tests were normal. With the informed consent of the patient and her family, a liver biopsy (1.4 g) was removed during the operation in June 1979.

Control subjects.   Normal human liver material was made available from a renal transplant donor, a 32-yr-old man, dead according to officially adopted Norwegian criteria. Needle biopsies (30–35 mg) taken routinely during abdominal surgery were obtained from six patients free of liver diseases.

Preparation of subcellular fractions of liver biopsies. Immediately after the biopsies were taken, they were put on ice-cold 0.25 M sucrose, 25 mM Mops buffer, pH 7.4, and homogenized in a Potter-Elvehjem homogenizer. Mitochondria were prepared without delay as described (29) with the exception that they were washed only twice. Microsomes were prepared from the postmitochondrial
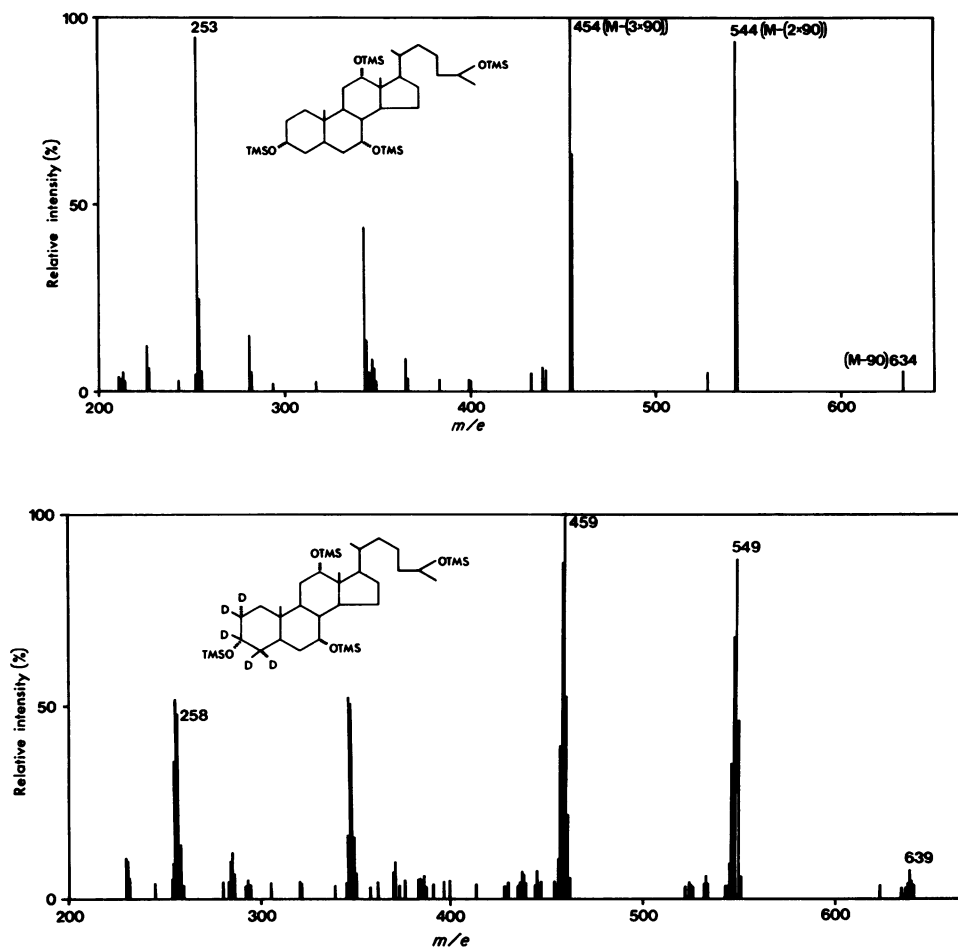
FIGURE 3 Partial mass spectrum of trimethylsilyl ether of unlabeled (upper spectrum) and $^2H_5$-labeled (lower spectrum) 5β-cholestane-3α,7α,12α,26-tetrol.

supernate by centrifugation at 105.000 g for 75 min. The resulting mitochondrial and microsomal pellets were resuspended in 0.25 M sucrose, 25 mM Mops, pH 7.4. All incubations were performed immediately after the cell fractionation processes were completed. The protein concentration was determined by the Lowry method (30) using bovine serum albumin as a standard.

Incubation, extraction, and chromatographic procedures. Each incubation mixture contained the following in 1.0 ml of 0.25 M sucrose, 27 mM Mops buffer, pH 7.4; 2.7 μmol ATP, 3 μmol potassium malate, 0.6 μmol glucose-6-phosphate, 0.5 unit glucose-6-phosphate-dehydrogenase, 0.7 μmol NADP$^+$ and 10 μmol MgCl$_2$. The incubations with vitamin D$_3$ also contained 2 μg DPPD. The amounts of protein from the different subcellular fractions were 0.3–1.8 mg and checked to be within the linearity range of the reaction.

The reaction was started by the addition of substrate (0.4–12 μg of tritium-labeled 5β-cholestane-3α,7α,12α-triol in 10 μl of ethanol) and allowed to proceed for 60 min at 37°C under oxygen. The reaction was terminated by the addition of 0.1 ml 1 N HCl. When $^2H_5$-labeled 5β-cholestane-3α,7α,12α-25 and 26-tetrols were used as internal standards, these were added together with the HCl.

After extraction with ethyl acetate (31) the solvent was evaporated under N$_2$ and the residue redissolved in 100–

300 μl of methanol. Aliquots of the samples were subjected to HPLC (SP 3500 B liquid chromatograph, Spectra-Physics, Inc., Santa Clara, Calif.) using a Spherisorb ODS column (4.6 × 250 mm, particle size 5 μm), and 13% H$_2$O in methanol as eluting solvent (1 ml/min) (32). The collected fractions (l/min) were counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) and the rate of conversion of 5β-cholestane-3α,7α,12α-triol was calculated from the distribution of the radioactivity. Any loss of highly polar material to the water phase (<1% of the total radioactivity was recovered in this phase) was not taken into account.

Assay of 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,26-tetrol, and 3α,7α,12α-trihydroxy-5β-cholestanoic acid by isotope dilution-mass spectrometry. (For review of this technique see ref. 33.) The content of 5β-cholestane-3α,7α,12α-triol in homogenates of liver was assayed using $^2H_5$-labeled 5β-cholestane-3α,7α,12α-triol as internal standard. The labeled steroid, 200 ng, was added to 100 μl of the liver homogenate. The mixture was extracted with ethyl acetate as above and was purified by HPLC, using 13% H$_2$O in methanol as solvent and a Spherisorb ODS column (4.6 × 250 mm). The fraction corresponding to 5β-cholestane-3α,7α,12α-triol was converted into trimethylsilyl ether and analyzed by gas

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chromatography-mass spectrometry (GC-MS), using a 1.5% SE-30 column at 280°C (LKB 9000 instrument, equipped with a multiple ion detector). The multiple ion detector was focused on the ion at m/e 456 and on the ion at m/e 461 (corresponding to M − 2 × 90 fragment) (Fig. 2). The ratio between the two tracings were strictly linear with increasing amounts of unlabeled 5β-cholestane-3α,7α,12α-triol in the presence of a fixed amount of $^2H_5$-labeled 5β-cholestane-3α,7α,12α-triol (Fig. 4A). The amount of unlabeled 5β-cholestane-3α,7α,12α-triol was calculated from the ratio between the two tracings and the standard curve.

The amounts of 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,26-tetrol obtained in the different incubations were assayed using the corresponding deuterium-labeled compounds as internal standards. A fixed amount of the mixture of $^2H_5$-labeled 5β-cholestane-3α,7α,12α,25-tetrol (120 ng) and $^2H_5$-labeled 5β-cholestane-3α,7α,12α,26-tetrol (230 ng) was added to each incubation.

The steroids were extracted as described above and, without further purification, converted into trimethylsilyl ether, and subjected to analysis by GC-MS. The multiple ion detector was focused on the ion at m/e 544 and at m/e 549 (Fig. 3). The amount of unlabeled 5β-cholestane-3α,7α,12α,26-tetrol was calculated from the ratio between the two peaks occurring in the two tracings, with a retention time corresponding to trimethylsilyl ether of 5β-cholestane-3α,7α,12α,26-tetrol, and the linear standard curve obtained (Fig.

4B). The amount of unlabeled 5β-cholestane-3α,7α,12α,25-tetrol was calculated from the ratio between the two peaks occurring in the two tracings (m/e 544 and m/e 549), with a retention time corresponding to trimethylsilyl ether of 5β-cholestane-3α,7α,12α,25-tetrol.

Because of a lack of synthetic material no standard curve could be produced in this case, but the reasonable assumption was made that the ratio between unlabeled 25-tetrol and $^2H_5$-tetrol was the same as the ratio between the two peaks occurring in the two tracings. A ratio of 1.0 between the height of the peak at m/e 544 and the height of the peak at m/e 549 was thus assumed to correspond to 120 ng of unlabeled 5β-cholestane-3α,7α,12α,25-tetrol.

In the assay of 3α,7α,12α-trihydroxy-5β-cholestanoic acid, the lipid extract containing the mixture of $^2H_5$-labeled 25-tetrol and 26-tetrol was methylated with diazomethane and converted into the trimethylsilyl ether before analysis by GC-MS. The detector was focused on the ion at m/e 544 (to follow the $^2H_5$-labeled 26-tetrol) and on the ion at m/e 500 (to follow unlabeled trimethylsilyl ether of methylated 3α,7α,12α-trihydroxy-5β-cholestanoic acid). It was shown that the ratio between the peak obtained at m/e 500 (with a retention time corresponding to derivative of 3α,7α,12α-trihydroxy-5β-cholestanoic acid) and the peak at m/e 549 (with a retention time corresponding to derivative of the 26-tetrol) was approximately linear with the amount of unlabeled derivative of 3α,7α,12α-trihydroxy-5β-cholestanoic
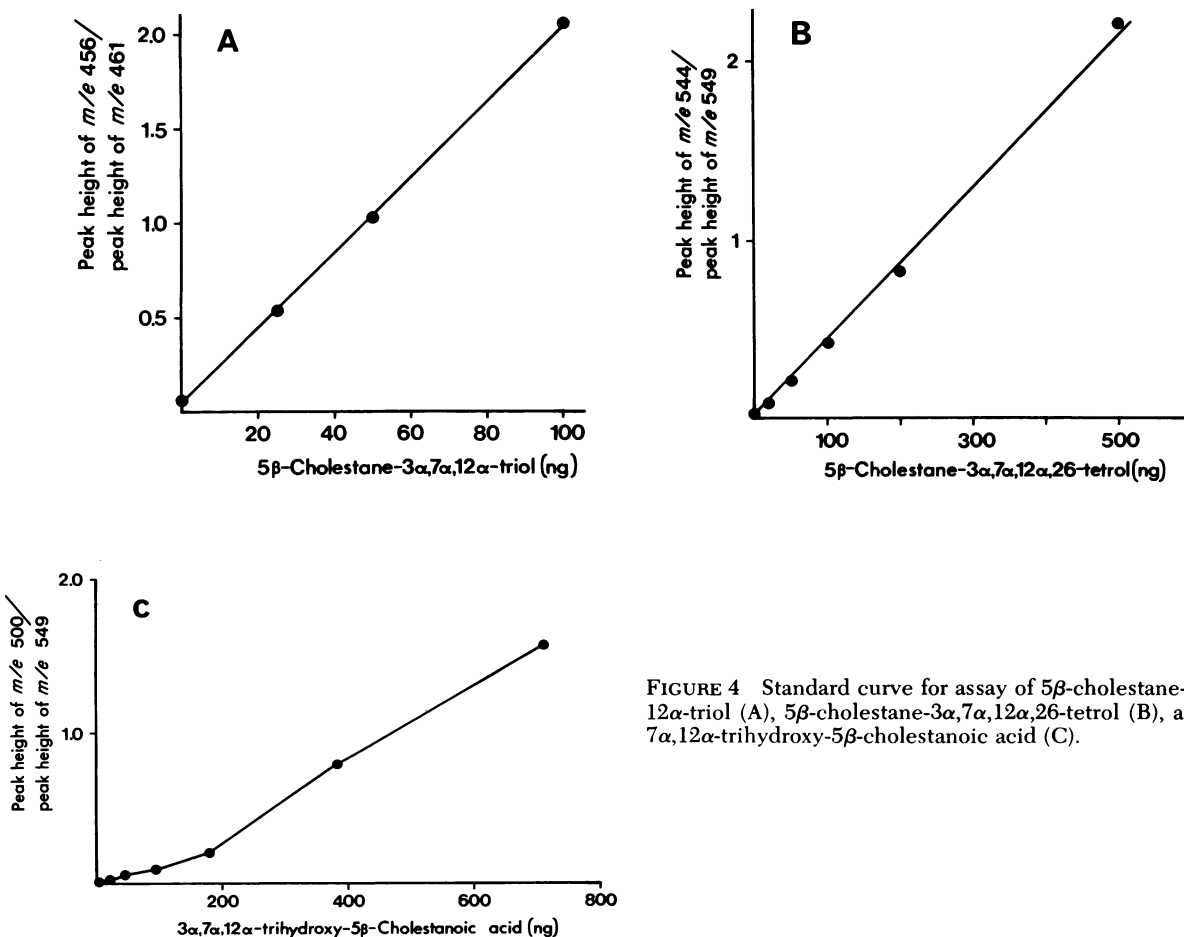

FIGURE 4 Standard curve for assay of 5β-cholestane-3α,7α,12α-triol (A), 5β-cholestane-3α,7α,12α,26-tetrol (B), and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (C).

acid in the range of 200 to 800 ng (Fig. 4C). When the amount of unlabeled 3α,7α,12α-trihydroxy-5β-cholestanoic acid was <200 ng, the ratio between the peak heights deviated from linearity, however, and the quantitation in this range must be considered as approximate.

In the assay of 25-hydroxylation of vitamin D_3 by the mitochondrial fraction of human liver, the assay conditions used were identical to those used for assay of 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (6.3 mg of mitochondrial protein, 10 μg of substrate). Before ethyl acetate extraction as above, 50 ng of $^2H_3$-labeled 25-hydroxyvitamin D_3 was added. The extract was purified by HPLC, converted into the trimethylsilyl/t-butyldimethylsilyl derivative, and subjected to GC-MS as described (34).

Kinetic analysis of experimental data. Apparent K_m and maximum velocity (V_{max}) values were determined by fitting experimental data to a Michaelis Menten hyperbola by using program HYPER (35). Computations were carried out with a POP 11/03 computer.

RESULTS

Conversion of tritium-labeled 5β-cholestane-3α,-7α,12α-triol into more polar products by different subcellular fractions of normal liver and liver from the CTX patient. Two more polar product peaks were

detected on the radiochromatograms by HPLC of the extracts of reaction mixtures after incubating different subcellular fractions with 5β-cholestane-3α,7α,12α-triol. The major peak had the same retention time as 5β-cholestane-3α,7α,12α,25- and 26-tetrols (and possibly other tetrols), and the minor one had the same retention time as 3α,7α,12α-trihydroxy-5β-cholestanoic acid.

The rates of formation of these more polar products were markedly higher with the subcellular fractions from the liver of the control subject than from the CTX patient (Fig. 5). In particular, the hydroxylation activity of the mitochondrial fraction from the CTX subject was reduced to near the detection limit.

From the substrate saturation curves (Fig. 5) the V_{max} and K_m values for the hydroxylase activities of the various fractions could be determined and the results are summarized in Table I. The most striking feature is the reduction in the V_{max} for the mitochondrial hydroxylation activity of the CTX subject to 0.5% of that of the control. The microsomal activity of the CTX patient is apparently lowered in that it amounted
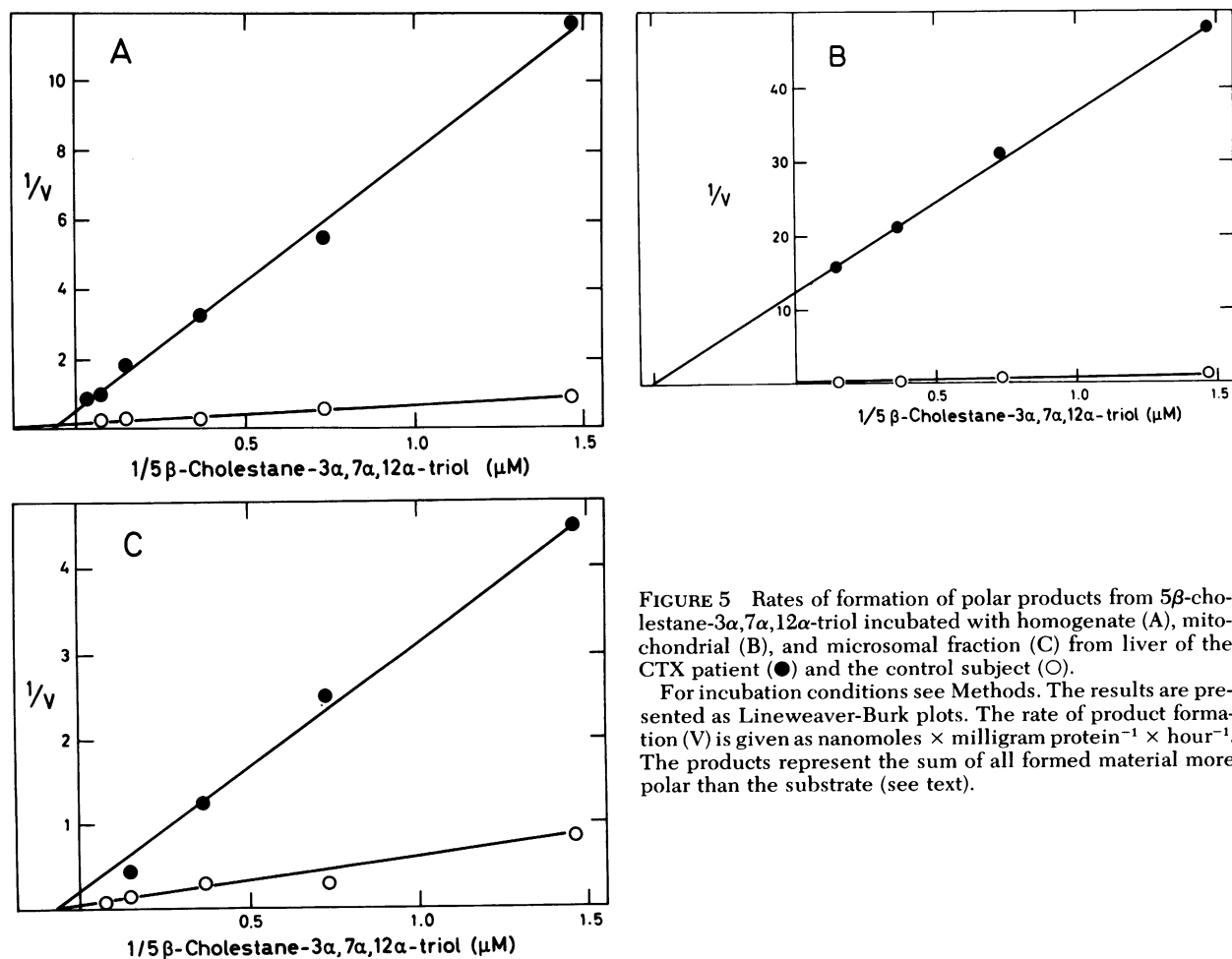

FIGURE 5 Rates of formation of polar products from 5β-cholestane-3α,7α,12α-triol incubated with homogenate (A), mitochondrial (B), and microsomal fraction (C) from liver of the CTX patient (●) and the control subject (○).

For incubation conditions see Methods. The results are presented as Lineweaver-Burk plots. The rate of product formation (V) is given as nanomoles × milligram protein^{-1} × hour^{-1}. The products represent the sum of all formed material more polar than the substrate (see text).

TABLE I

TABLE I

Conversion of Tritium-labeled 5β-Cholestane-3α,7α,12α-triol into More Polar Products* by Liver Subcellular Fractions from Control Subject and from CTX Patient

Subcellular fraction	V_{max}	K_m
	$nmol \times mg^{-1} \times h^{-1}$	μM
Whole homogenate		
Control subject	9.4	6.4
CTX patient	1.9	14.5
Mitochondria		
Control subject	14.4	7.1
CTX patient	0.08	2.0
Microsomes		
Control subject	22.8	15.2
CTX patient	6.2	14.7

* See legend to Fig. 5.

to only 27% of that of the control (see below). Identical K_m values were found for the homogenate and the mitochondria from the control subject, whereas the values were identical for the homogenate and the microsomes from the CTX patient (Table I). These

findings certainly reflect the importance of the different subcellular fractions to the overall hydroxylation activity of the homogenates, the mitochondrial fraction being more important in the control whereas the microsomal fraction is dominant in the CTX patient.

Conversion of 5β-cholestane-3α,7α,12α-triol into 5β-cholestane-3α,7α,12α-,25-tetrol, 5β-cholestane-3α,7α,12α,26-tetrol, and 3α,7α,12α-trihydroxy-5β-cholestanoic acid by subcellular fractions of normal liver and liver from the CTX patient. In Fig. 6A, a mass fragmentographic recording is shown of a derivative of the standard mixture consisting of deuterated 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,26-tetrol. The peak with the retention time at 5.7 min shown in the tracing at m/e 549 corresponds to the derivative of the deuterium-labeled 25-tetrol and the peak with the retention time of 6.3 min in the same tracing corresponds to the derivative of the 26-tetrol. No peaks were seen in the tracing at m/e 500 or m/e 544, corresponding to unlabeled 3α,7α,12α-trihydroxy-5β-cholestanoic acid and unlabeled 25- or 26-tetrols, respectively. A blank incubation with a crude homogenate of normal human liver gave the same chromatogram as that shown in Fig. 6A. Fig. 6B shows a typical chromatogram of derivative of extract from an
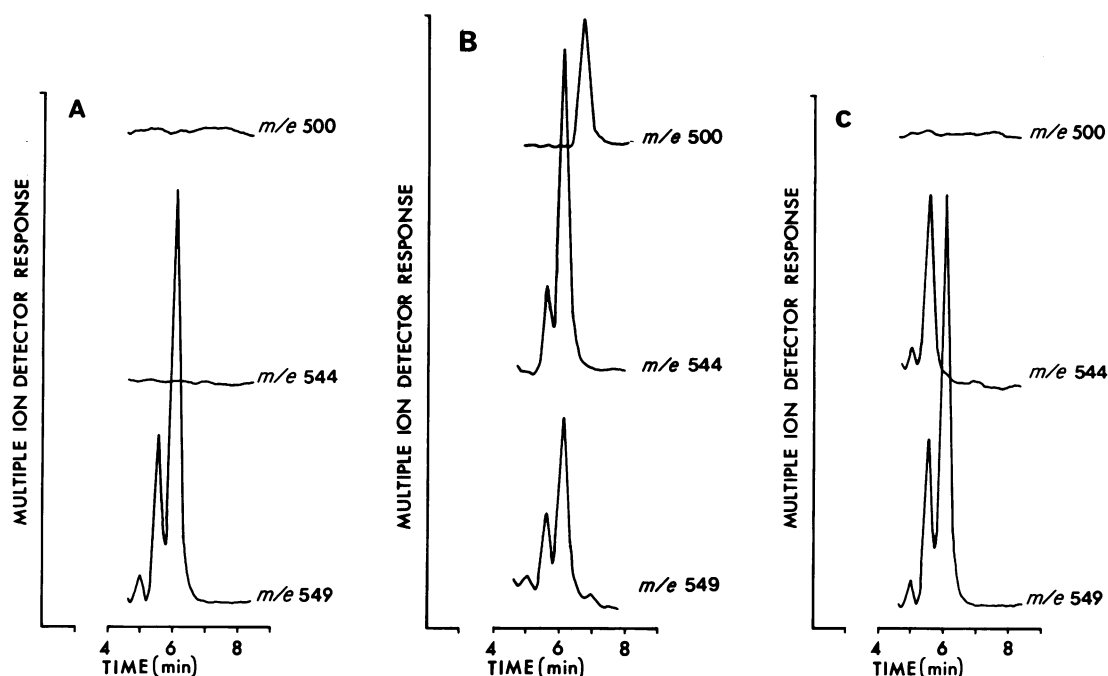

FIGURE 6 Mass fragmentographic recording of methylated and trimethylsilylated standard mixture of $^2H_5$-labeled 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,26-tetrol (A); methylated and trimethylsilylated extract of an incubation of whole homogenate of normal human liver with 5β-cholestane-3α,7α,12α-triol (B); methylated and trimethylsilylated extract of an incubation of whole homogenate of liver from the CTX patient with 5β-cholestane-3α,7α,12α-triol (C). The standard mixture of $^2H_5$-labeled 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,-12α,26-tetrol had been added to two incubations as described in Methods.

incubation of 5β-cholestane-3α,7α,12α-triol with whole homogenate of normal human liver. Peaks were obtained both in the tracing at m/e 544 (corresponding to unlabeled 25-tetrol and 26-tetrol) and in the tracing at m/e 500 (corresponding to unlabeled 3α,7α,12α-trihydroxy-5β-cholestanoic acid). The amount of unlabeled 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,26-tetrol, and 3α,7α,12α-trihydroxy-5β-cholestanoic acid could be calculated as described in Methods, using the standard curve shown in Figs. 4B and C. Fig. 6C shows a typical chromatogram of derivative of extract from an incubation of 5β-cholestane-3α,7α,12α-triol with whole homogenate of liver from the CTX patient. No significant peaks corresponding to unlabeled 26-tetrol or 3α,7α,12α-trihydroxy-5β-cholestanoic acid could be detected. However, there was a significant peak corresponding to unlabeled 5β-cholestane-3α,7α,12α,25-tetrol.

The results of the different incubations are summarized in Table II. In consonance with the previous work (11), the normal human liver microsomes catalyzed 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol, but not 26-hydroxylation. The microsomal fraction from the CTX patient had a somewhat lower capacity to 25-hydroxylate 5β-cholestane-3α,7α,12α-triol than the microsomal fraction from the control subject. The mitochondrial fraction from the control subject catalyzed conversion of 5β-cholestane-3α,7α,-12α-triol into both 5β-cholestane-3α,7α,12α,26-tetrol and 3α,7α,12α-trihydroxy-5β-cholestanoic acid at high rates. The mitochondrial fraction from the CTX patient, however, was completely devoid of such activity.

The mitochondrial and microsomal fractions were isolated and assayed for 25- and 26-hydroxylase activity in only one control subject. Whole homogenate from seven control subjects were assayed for these activities. In all cases 26-hydroxylation was detected, but the rate varied considerably (mean 0.81, and range 0.33–2.45 nmol × mg^{-1} × h^{-1}) between the different preparations.

The lack of 26-hydroxylase activity in the mitochondrial fraction of the CTX patient might theoretically be the result of inactivation of mitochondrial fraction during preparation. This possibility was excluded, however, by incubation of the mitochondrial fraction from the CTX patient with 10 μg of vitamin D$_3$ under essentially the same incubation conditions as those used for assay of 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol. A significant conversion into 25-hydroxyvitamin D$_3$ was observed (6 pmol × mg protein^{-1} × h^{-1}). With the microsomal fraction a conversion of 3 pmol × mg protein^{-1} × h^{-1} was found.

TABLE II

Conversion of 5β-Cholestane-3α,7α,12α-Triol into 5β-Cholestane-3α,7α,12α, 25-Tetrol, 5β-Cholestane-3α,7α,12α,26-Tetrol and 3α,7α,12α-Trihydroxy-5β-Cholestan-26-oic Acid by Liver Subcellular Fractions from Control Subject and from the CTX Patient

	V$_{max}$		
Subcellular fraction	25-Hydroxylation	26-Hydroxylation*	Formation of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid
	nmol × mg^{-1} × h^{-1}		
Whole homogenate			
Control subject	0.51‡	2.45	0.44
CTX patient	1.20	0	0
Mitochondria			
Control subject	0	8.72	0.69
CTX patient	0.15§	0	0
Microsomes			
Control subject	5.78	0	0
CTX patient	1.47	0	0

* Includes the formation of 5β-cholestane-3α,7α,12α,26-tetrol and 3α,7α,12α-trihydroxy-5β-cholestanoic acid.

‡ Represents the value obtained with 5 μg of substrate/incubation. The V$_{max}$ could not be obtained from the substrate saturation curve because a straight line was not obtained by reciprocal plotting of the data.

§ This value was obtained with all substrate concentrations tested and could not be corrected for the blank value because of accidental error.

The considerably lower specific activity with this fraction excludes the possibility that the mitochondrial activity was the result of microsomal contamination.

Accumulation of 5β-cholestane-3α,7α,12α-triol in the liver of the CTX patient. By using $^2H_5$-labeled 5β-cholestane-3α,7α,12α-triol as internal standard, the amount of unlabeled 5β-cholestane-3α,7α,12α-triol was measured in whole homogenate and microsomal fraction from liver of two control subjects and the CTX patient. In Fig. 7, the mass fragmentographic recordings are shown that were obtained in the analysis of trimethylsilyl ether of purified extract from microsomal fraction from liver of the control subject (A) and the CTX patient (B). The tracing at *m/e* 456, corresponding to unlabeled 5β-cholestane-3α,7α,12α-triol, gave a much higher peak in the analysis of the material from the CTX patient than in the analysis of the material from the control subject. The exact amount of unlabeled 5β-cholestane-3α,7α,-12α-triol in the different subcellular fractions were calculated, using the standard curve shown in Fig. 4A. The results are summarized in Table III. The amount of 5β-cholestane-3α,7α,12α-triol was ~50-fold higher in the microsomal fraction and in the whole homogenate from the liver of the CTX patient as compared with the two control subjects.

Included in Table III are also the amounts of 5β-cholestane-3α,7α,12α,25-tetrol determined in the blank incubations with the homogenate and the microsomal fractions. Again, much higher levels were detected in the fractions from the CTX patient than from the control subject. The lower amount of the tetrol relative to the triol in the microsomes from the CTX patient but not in the homogenate should be noted.

The lower conversion of tritium-labeled 5β-cholestane-3α,7α,12α-triol to more polar products by the microsomal fraction of the CTX patient than by that of the control subject (Table I) may be explained by the large amount of endogenous 5β-cholestane-3α,7α,12α-triol diluting the labeled substrate.

DISCUSSION

According to the results presented here, the most likely metabolic defect in CTX is a lack of mitochondrial C_{27}-steroid 26-hydroxylase activity. Thus the mitochondrial fraction as well as the whole homogenate of the liver of the CTX patient was completely devoid of such activity. In addition, the major substrate for the 26-hydroxylase, 5β-cholestane-3α,7α,12α-triol, was accumulated in the liver to a greater extent than 5β-cholestane-3α,7α,12α,25-tetrol.

The inability of the isolated mitochondrial fraction to 26-hydroxylate C_{27}-steroids is not per se sufficient
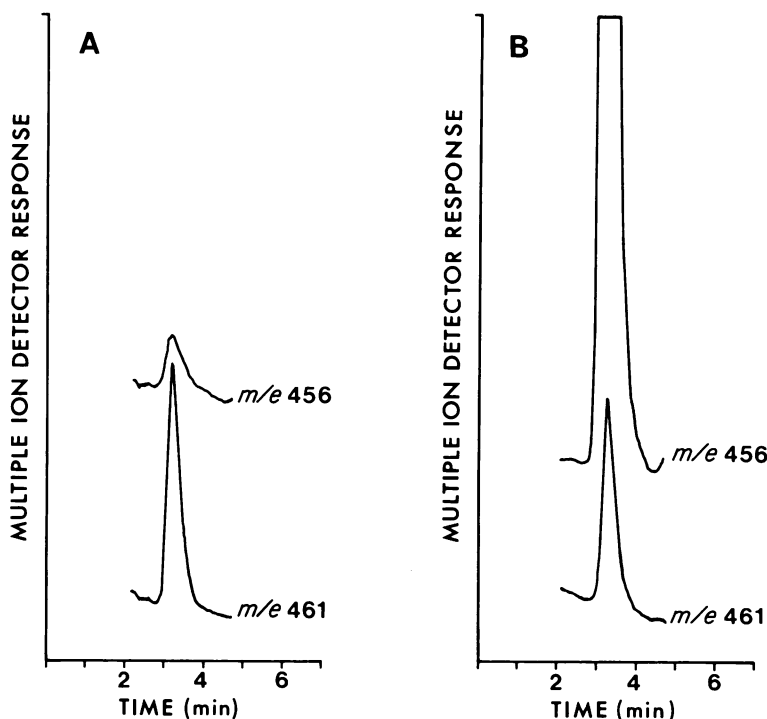

FIGURE 7 Mass fragmentographic recording of trimethylsilylated HPLC-purified ethyl acetate extract of liver microsomes from a control subject (A) and the CTX patient (B). $^2H_5$-labeled 5β-cholestane-3α,7α,12α-triol, 200 ng, had been added to the extract before purification.

TABLE III

Content of 5β-Cholestane-3α,7α,12α-Triol and 5β-Cholestane-3α,7α,12α,25-Tetrol
in Liver Fractions of the CTX Patient and Two Control Subjects

Subjects	5β-cholestane-3α,7α,12α-triol		5β-cholestane-3α,7α,12α,25-tetrol	
	Whole homogenate	Microsomal fraction	Whole homogenate	Microsomal fraction
	ng/mg protein			
CTX patient	105	480	107	33
Control subject 1	2	9	Not detectable	6
Control subject 2	3	—	Not detectable	—

evidence that the patient with CTX lacks 26-hydroxylase activity. The 26-hydroxylase activity varied also considerably in the different liver biopsies studied in the present work. When using fresh human liver homogenate, however, we have never previously failed to detect 26-hydroxylase activity unless the tissue had been frozen. Also, the ability of the mitochondrial fraction from the CTX patient to catalyze 25-hydroxylation of vitamin D_3 excludes the possibility of inactivation of this subcellular fraction. In a previous work, we have shown that the intramitochondrial localization is the same for the vitamin-D_3 25-hydroxylase and the C_{27}- steroid 26-hydroxylase (36). Furthermore, in both of these cytochrome P-450-dependent hydroxylations, liver ferredoxin and ferredoxin reductase seem to be involved as electron carriers (18, 32). In a recent work we have provided evidence that different species of cytochrome P-450 are involved in mitochondrial 25-hydroxylation of vitamin D_3 and 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol.[2] A selective inactivation of only one of these species in the mitochondrial fraction from the CTX patient seems highly unlikely.

The present observations make it less likely that the basic defect in CTX is a deficient microsomal 24β-hydroxylase active on 5β-cholestane-3α,7α,12α,25-tetrol as has been advocated by Salen et al. (17). Their conclusion was based on findings both of a delayed conversion of labeled 5β-cholestane-3α,7α,12α,25-tetrol into cholic acid and a relatively low 24β-hydroxylase activity (reduced by a factor of four) in patients with CTX as compared with control subjects (15–17). In principle such findings can be explained by an expanded endogenous pool of 5β-cholestane-3α,7α,12α,25-tetrol, which might dilute the administered exogenous steroid (Table III).

In a previous work, we observed that 5β-cholestane-3α,7α-diol was 25-hydroxylated by human liver microsomes to a considerably lower extent than was 5β-cholestane-3α,7α,12α-triol (11). Thus more cholic acid relative to chenodeoxycholic acid will probably be formed by the 25-hydroxylase pathway. If the 25-hydroxylase pathway is of importance under normal conditions, and the basic defect in CTX patients is a relative deficiency of the enzyme catalyzing 24-hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol, it would be expected that cholic acid biosynthesis should be more affected than the biosynthesis of chenodeoxycholic acid. In contrast, however, it has been reported that the production of chenodeoxycholic acid in patients with CTX is decreased more than the production of cholic acid (25) and a deficiency of chenodeoxycholic acid in the bile was found also in our patient. This is in accord with the contention that the basic metabolic defect in CTX is a lack of the 26-hydroxylase. It is believed that the major substrate for the mitochondrial 26-hydroxylase in the biosynthesis of chenodeoxycholic acid is 5β-cholestane-3α,7α-diol. If this compound accumulates in patients with CTX, it will be exposed to the action of the 12α-hydroxylase to a greater extent than in normal subjects (37, 38). The consequence will be an even higher accumulation of 5β-cholestane-3α,7α,12α-triol, which leads to a shift towards biosynthesis of cholic acid.

In addition to the results presented here, there are some other indications in the literature that the 26-hydroxylase pathway normally is more important than the 25-hydroxylase pathway. Thus Cronholm and Johansson (12) have reported that tritium-labeled 5β-cholestane-3α,7α,12α,26-tetrol was converted into cholic acid in a bile fistula rat much more efficiently than was tritium-labeled 5β-cholestane-3α,7α,12α,25-tetrol (12). Very recently the same experiment was repeated in human patients with a biliary fistula (39). Also in this case, labeled 5β-cholestane-3α,7α,-12α,26-tetrol was converted into cholic acid much

[2] Björkhem, I., I. Holmberg, H. Oftebro, and J. I. Pedersen. Properties of a reconstituted vitamin D_3 25-hydroxylase from rat liver. *J. Biol. Chem.* In press.

more efficiently than was labeled 5β-cholestane-3α,7α,12α,25-tetrol.

In conclusion, the presented results and the above considerations are all in agreement with the view that the 26-hydroxylase pathway is normally the major one in the biosynthesis of both cholic acid and chenodeoxycholic acid in man. In patients with CTX however, the 25-hydroxylase will partly compensate for the lack of the 26-hydroxylase and thus become of major importance in the biosynthesis of bile acids.

ACKNOWLEDGMENTS

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