Cerebrotendinous xanthomatosis: defective liver mitochondrial hydroxylation of chenodeoxycholic acid precursors

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Abstract Oxidation of the side chain of 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one, and 5-cholestene-3β,7α-diol has been studied in subcellular fractions of liver from a patient with cerebrotendinous xanthomatosis (CTX) and a control subject. All intermediates were efficiently 26-hydroxylated and further converted to the corresponding 26-carboxylated derivatives by the mitochondrial fraction of normal human liver. No such conversion was observed with the mitochondria from the liver of the CTX patient. All intermediates were poor substrates for the microsomal 25-hydroxylase both in the liver of the CTX patient and the control subject. 12α-Hydroxylation of the substrates was very efficient with the microsomal fractions from both subjects. Based on these and previous findings (Oftebro, H., I. Björkhem, S. Skrede, A. Schreiner, and J. I. Pedersen. 1980. *J. Clin. Invest.* **65:** 1418–1430), it is concluded that the metabolic defect in CTX is a complete lack of mitochondrial C_{27}-steroid 26-hydroxylase. In CTX the precursors of chenodeoxycholic acid are first attacked by the microsomal 12α-hydroxylase and subsequently by the microsomal 25-hydroxylase as an alternate route to cholic acid formation. This explains the increased ratio of cholic acid to chenodeoxycholic acid observed in the bile of these patients. In the normal liver the formation of both cholic acid and chenodeoxycholic acid involves a mitochondrial 26-hydroxylation.—**Oftebro, H., I. Björkhem, F. C. Størmer, and J. I. Pedersen.** Cerebrotendinous xanthomatosis: defective liver mitochondrial hydroxylation of chenodeoxycholic acid precursors. *J. Lipid Res.* 1981. **22:** 632–640.

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Cerebrotendinous xanthomatosis (CTX) is a rare inherited disease characterized by deposits of cholesterol and cholestanol in the brain, tendons, and other tissues in conjunction with progressive neurologic and mental dysfunction. The production of the primary bile acids is considerably reduced. In particular, the synthesis of chenodeoxycholic acid is almost completely abolished, but the formation of cholic acid is also seriously affected (6, 7). Bile acid intermediates, 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,24,25-pentol, and 5β-cholestane-3α,7α,12α,23,25-pentol are excreted in large quantities in the bile and feces of the patients (7–9), while no 26-hydroxylated intermediates have been detected (9).

In a recent work we reported that the mitochondria isolated from the liver of a CTX patient were completely devoid of 26-hydroxylase activity towards 5β-cholestane-3α,7α,12α-triol (10). Increased amounts of this intermediate were also detected in the liver, especially in the microsomal fraction. We concluded that the metabolic defect in CTX is a complete lack of a mitochondrial hydroxylation of 5β-cholestane-3α,7α,12α-triol and that this hydroxylation is required for the normal production of cholic acid. In CTX the absence of the mitochondrial 26-hydroxylase can to some extent be compensated by a microsomal 25-hydroxylase. Thus, there is a pathway to cholic acid involving 5β-cholestane-3α,7α,12α,25-tetrol as intermediate (11, 12).

Abbreviations: CTX, cerebrotendinous xanthomatosis; DHCA, 3α,7α-dihydroxy-5β-cholestan-26-oic acid; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; HPLC, high pressure liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; R_t, retention time; Mops, morpholinopropane sulfonic acid. According to Berséus (1) and to Gustafsson and Sjöstedt (2), the mitochondrial fraction of rat liver hydroxylates the methyl group of the cholesterol side chain in position C-27 (the 25-*pro-S*-methyl group, derived from C-3′ carbon of mevalonic acid (3)). The microsomal fraction of rat liver hydroxylates the methyl group of 5β-cholestane-3α,7α,12α-triol in position C-26 (2) (the 25-*pro-R*-methyl group, derived from C-2 of mevalonate). Recent studies indicate that in human liver the mitochondrial oxidation of both 5β-cholestane-3α,7α-diol (4) and 5β-cholestane-3α,7α,12α-triol starts with hydroxylation at C-27 (5). Because the stereochemistry might be different with different substrates and since no general agreement has been adopted as to which nomenclature should be used, we prefer here to denote the ω-hydroxylation of the C_{27}-steroid side chains as "26"-hydroxylation.

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In CTX the biosynthesis of chenodeoxycholic acid is even more reduced than that of cholic acid. In this study we have investigated the in vitro hydroxylations of three bile acid intermediates, 5-cholestene-3β,7α-diol, 7α-hydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α-diol. According to current concepts these compounds are considered to be the main precursors of chenodeoxycholic acid. The results show that in the liver of the CTX patient none of these substrates is hydroxylated in the 26-position. The almost complete lack of chenodeoxycholic acid production in CTX is explained by the finding that 5β-cholestane-3α,7α-diol and 7α-hydroxy-4-cholesten-3-one are very poor substrates for the microsomal 25-hydroxylase in human liver. Only after 12α-hydroxylation will this hydroxylase attack the side chain as an alternate route to cholic acid formation.

MATERIALS AND METHODS

Tritium-labeled and unlabeled steroids

[7β-^{3}H]5-Cholestene-3β,7α-diol (6 Ci/mol), [6β-^{3}H]-7α-hydroxy-4-cholesten-3-one (7 Ci/mol), [7β-^{3}H]5β-cholestane-3α,7α-diol (7 Ci/mol), [7β-^{3}H]5β-cholestane-3α,7α,12α-triol (7 Ci/mol), [6β-^{3}H]7α,12α-dihydroxy-4-cholesten-3-one (7 Ci/mol), [7β-^{3}H]5β-cholestane-3α,7α,26-triol (7 Ci/mol), [7β-^{3}H]5-cholestene-3β,7α,26-triol (6 Ci/mol), [7β-^{3}H]3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) (200 Ci/mol), and [3β,7β-^{3}H]3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) (12 Ci/mol), and unlabeled steroids were synthesized as described (13–15).

Other chemicals and solvents were standard commercial high purity materials.

Clinical

A liver biopsy (1.4 g) was removed from a 44-year-old woman with cerebrotendinous xanthomatosis during cholecystectomy in June 1979. Informed consent was obtained from the patient and her family. Details concerning the clinical findings of this patient are given in a previous work (10). Normal human liver material was from a 32-year-old renal transplant donor, dead according to officially adopted criteria. Research protocol was approved by this institution.

Preparation of subcellular fractions of liver biopsies

The liver biopsies were put on ice-cold 0.25 M sucrose, 25 mM Mops buffer, pH 7.4, and homogenized as quickly as possible. The mitochondrial and microsomal fractions were prepared by standard procedures previously described (10). The subcellular fractions were incubated immediately after preparation. Protein was determined by the method of Lowry et al. (16).

Incubation procedure and analysis of the reaction products

The incubation mixture contained the following in 1 ml of 0.25 M sucrose, 27 mM Mops buffer, pH 7.4, 2.7 μmol ATP, 3 μmol potassium malate, 0.7 μmol $NADP^+$, 0.6 μmol glucose-6-phosphate, 0.5 unit glucose-6-phosphate dehydrogenase, 10 μmol $MgCl_2$, and the subcellular fraction corresponding to 0.4–1.9 mg of protein. The reaction was started by the addition of 10 μg of unlabeled substrate together with 100,000 cpm of labeled substrate. The reaction was allowed to proceed under oxygen for 60 min at 37°C, and was terminated by the addition of 0.1 ml of 1 N HCl. The incubation mixtures were extracted with ethyl acetate (17), evaporated under nitrogen, and the residues were 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, CA) using a Zorbax ODS column (4.6 × 250 mm). The eluting solvent was 7.5% water in methanol when 5β-cholestane-3α,7α-diol was the substrate, and 10% water in methanol was used when 7α-hydroxy-4-cholesten-3-one and 5-cholestene-3β,7α-diol were the substrates. The flow rate was 1 ml/min and 1-ml fractions were collected. After addition of 4 ml of counting solution (Insta-Gel II, Packard Instruments, Inc.), the fractions were counted in a Packard Tri-Carb liquid scintillation spectrometer. Recovery of the injected radioactivity was essentially complete. The rates of conversion into the different products (see below) were calculated from the percentage distribution of radioactivity in the eluates. The retention times of tritium-labeled authentic standards were determined by injecting aliquots containing about 1,500 cpm of each standard separately. The eluting solvent was 7.5% water in methanol for [7β-^{3}H]5β-cholestane-3α,7α,26-triol (R_t 11 min), [7β-^{3}H]5β-cholestane-3α-7α,12α-triol (R_t 19 min), [3β,7β-^{3}H]3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) (R_t 8 min), and [7β-^{3}H]3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) (R_t 6 min). The eluting solvent was 10% water in methanol for [7β-^{3}H]5-cholestene-3β,7α,26-triol (R_t 9 min), [6β-^{3}H]7α,26-dihydroxy-4-cholesten-3-one (R_t 10 min, R_t as detected by a UV detector, 9.4 min), [6β-^{3}H]7α,12α-dihydroxy-4-cholesten-3-one (R_t 16 min, R_t as detected by a UV detector, 15.6 min), and [7β-^{3}H]3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) (R_t 6 min).

Other aliquots of the samples were subjected to HPLC as above. The radioactive peaks that eluted

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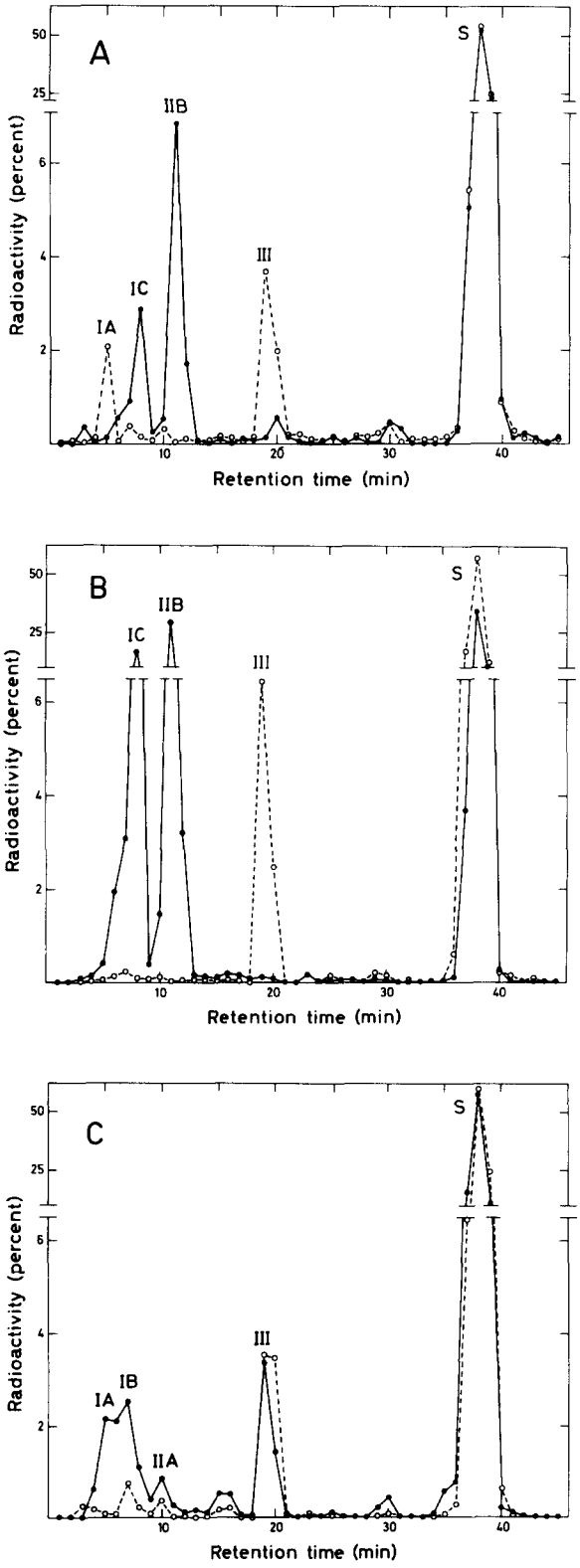

Fig. 1. High pressure liquid chromatography of the extracts of incubations with 5β-cholestane,3α,7α-diol and the whole homogenate fraction (A), the mitochondrial fraction (B), and the microsomal fraction (C) of liver from control subject (● —— ●) and from the CTX patient (○ - - - ○). The incubation, extraction, and HPLC procedures are described in Materials and Methods. Peaks 1A and 1B were not identified, peak 1C corresponds to 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA), peak IIA to 5β-cholestane-3α,7α,25 triol, peak IIB to 5β-cholestane-3α,7α,26-triol and peak III to 5β-cholestane-3α,7α,12α-triol. S denotes the substrate.

from the column were collected, the material was converted into the trimethylsilyl ether derivatives and analyzed by combined gas–liquid chromatography–mass spectrometry using a 1.5% SE column at 280°C and the LKB 9000 instrument (LKB Instruments Inc., Stockholm, Sweden), equipped with a multiple ion detector. Further details of the selected ion monitoring (mass fragmentography) used to identify the products are given in the section below.

RESULTS

Incubations with 5β-cholestane-3α,7α-diol

As shown in **Fig. 1A**, 5β-cholestane-3α,7α-diol was converted into at least three more polar products by the homogenate fraction of normal liver. The most polar metabolite, IC, had a retention time on HPLC identical with that of authentic DHCA. This material was methylated and converted into the trimethylsilyl ether and analyzed by GLC–MS. The multiple ion detector was focused at m/e 502 (corresponding to the M-90 ion in the mass spectrum of trimethylsilyl derivative of methylated DHCA) and at m/e 412 (corresponding to the M-2 × 90 ion). A peak was obtained in both tracings with a retention time identical with that of the derivative of authentic DHCA.

Metabolite IIB had a retention time on HPLC identical with that of 5β-cholestane-3α,7α,26-triol. This material was converted into the trimethylsilyl ether and analyzed by GLC–MS. The multiple ion detector was focused at m/e 546 (corresponding to the M-90 ion in the mass spectrum of trimethylsilyl ether of 5β-cholestane-3α,7α, 26-triol (13), at m/e 531 (corresponding to the M-90-15 ion), and at m/e 456 (corresponding to the M-2 × 90 ion). A peak was obtained in all these tracings with a retention time identical with that of authentic 5β-cholestane-3α,7α,-26-triol.

Metabolite III had a retention time on HPLC identical with that of 5β-cholestane-3α,7α,12α-triol. Also, this material was converted into the trimethylsilyl ether and analyzed by GLC–MS. Using the selective ion monitoring technique, it was shown that in similarity with derivative of authentic 5β-cholestane-3α,7α,12α-triol, the material contained ions at m/e 456 (M-2 × 90) and at m/e 366 (M-3 × 90).

With homogenate isolated from the CTX patient, one additional metabolite appeared (peak IA). Due to the small amounts available, it was not possible to identify this material. No conversion into 5β-choles-

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tane-3α,7α,26-triol (peak IIB) and DHCA (peak IC) occurred with the homogenate from the CTX patient (Fig. 1A and **Table 1**). The formation of metabolite III by the homogenate from the CTX patient was higher than normal. Using the same technique as above, it was confirmed that this material was identical with 5β-cholestane-3α,7α,12α-triol.

The mitochondrial fraction of normal liver catalyzed conversion of 5β-cholestane-3α,7α-diol into metabolites IC and IIB (Fig. 1B and Table 1). The identity of these products as DHCA and 5β-cholestane-3α,7α,26-triol was confirmed using the same technique as above. None of these metabolites was produced with mitochondria from the CTX patient.

A peak corresponding to 5β-cholestane-3α,7α,12α-triol (peak III) was observed in the chromatogram shown in Fig. 1B. The identity of this material was confirmed by the same technique as above. It is difficult to explain the formation of 5β-cholestane-3α,7α,12α-triol by the mitochondrial fraction of the CTX patient as entirely due to microsomal contamination (see discussion).

The microsomal fraction of normal liver catalyzed the conversion of 5β-cholestane-3α,7α-diol into a product with retention time as 5β-cholestane-3α,7α,-12α-triol (peak III, Fig. 1C, Table 1). The identity of this material was confirmed as described above.

A rather small peak(IIA), slightly more polar than 5β-cholestane-3α,7α,26-triol, was observed in the chromatogram. This material was converted into the trimethylsilyl derivative and analyzed by GLC–MS. The multiple ion detector was focused at m/e 456 (corresponding to M-2 × 90 ion in the mass spectrum of trimethylsilyl ether of 5β-cholestane-3α,7α,25-triol (13)), and at m/e 131 (characteristic for a trimethylsilyloxo function at C-25 in a C_{27}-steroid side chain (18, 19)). A peak was obtained in both tracings with a retention time identical with that of derivative of authentic 5β-cholestane-3α,7α,25-triol.

The most polar peaks in the chromatogram (IA and IB) had retention times similar to that of THCA and a tetrahydroxylated C_{27}-steroid, respectively. The possibility that the material in peak IA was identical with THCA was excluded, however, using the selective ion monitoring technique (cf.ref. 10). No further attempts were made to establish the structure of this material and the material corresponding to peak IB.

With microsomes from the CTX patient, 5β-cholestane-3α,7α,12α-triol (peak III) was formed at a relatively high rate (Table 1). The identity of this material was confirmed with the same technique as above. The formation of the more polar products was apparently decreased (see discussion). The material corresponding to peak IIA was identical with 5β-cholestane -3α,7α,25-triol as shown with the same technique as above.

The rate of formation of the different metabolites by the different subcellular fractions is summarized in Table 1. It is seen that 5β-cholestane-3α,7α-diol is efficiently 26-hydroxylated by normal liver mitochondria, but no such hydroxylation occurs with the mitochondria from the CTX patient. The rate of 12α-hydroxylation is high with the microsomal fractions from both subjects, while the rate of 25-hydroxylation is low with both microsomal fractions.

Incubations with 7α-hydroxy-4-cholesten-3-one

7α-Hydroxy-4-cholesten-3-one was converted into three more polar products by the whole homogenate fraction of normal liver (**Fig. 2A**). A major product (peak III) had the same retention time as 7α,12α-dihydroxy-4-cholesten-3-one. Using the selective ion

TABLE 1. Conversion of ^{3}H-labeled 5β-cholestane-3α,7α-diol into more polar products by liver from control subject and from the CTX patient

Subcellular fraction	Peak IA Unidentified	Peak IB Unidentified	Peak IC DHCA	Peak IIA 5β-cholestane-3α,7α,25-triol	Peak IIB 5β-cholestane-3α,7α,26-triol	Peak III 5β-cholestane-3α,7α,12α-triol
	nmol × mg protein^{-1} × hr^{-1}					
Whole homogenate						
Control subject			3.28		6.33	0.60
CTX patient	0.28			0.08	not detectable	0.80
Mitochondrial fraction						
Control subject			5.17		7.81	
CTX patient	0.11				not detectable	2.12
Microsomal fraction						
Control subject	1.61	2.05[a]		0.57		2.02
CTX patient		0.25[a]		0.12		2.66

[a] This compound most probably represents 5β-cholestane-3α,7α,12α,25-tetrol (c.f. text).

The incubation, extraction, and HPLC procedures are given in Materials and Methods. For identification of the different peaks, see Fig. 1A–C.

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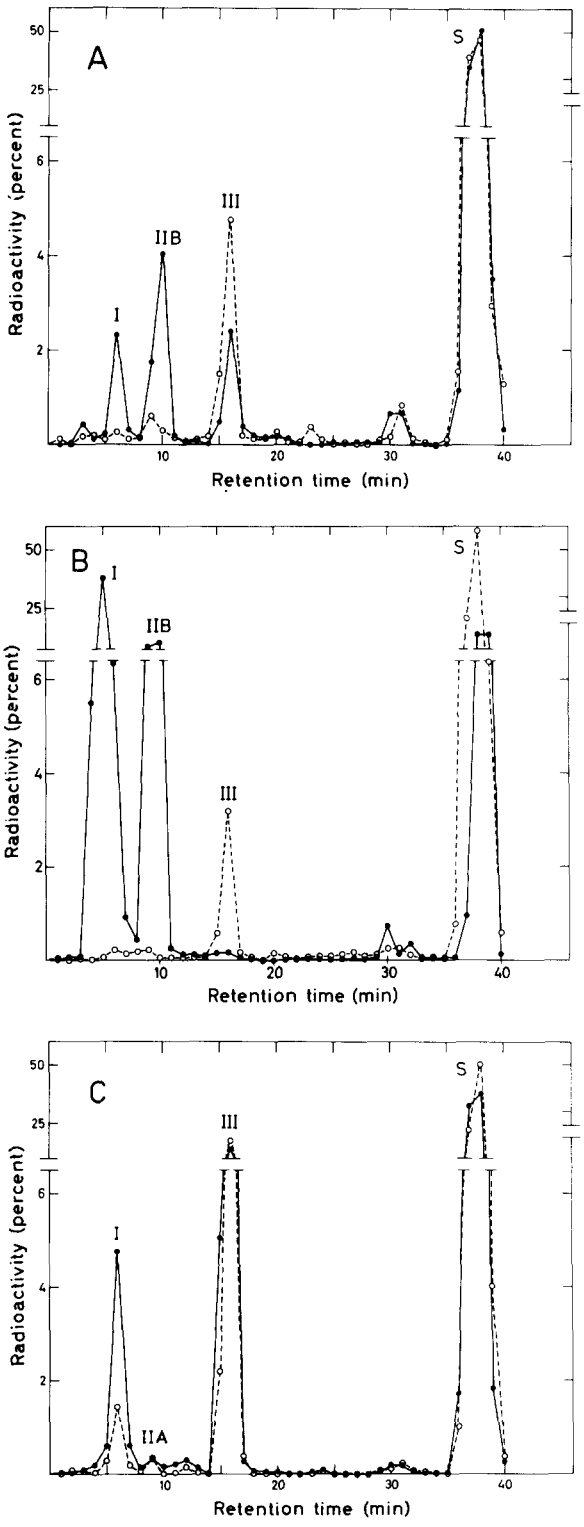

Fig. 2. High pressure liquid chromatography of the extracts of incubations with 7α-hydroxy-4-cholesten-3-one and the whole homogenate fraction (A), the mitochondrial fraction (B), and the microsomal fraction (C) of liver from control subject (● —— ●) and from the CTX patient (○ – – – ○). The incubation, extraction, and HPLC procedures are given in Materials and Methods. Peak I is assumed to represent 7α-hydroxy-4-cholesten-3-one-26-oic acid in (A) and (B). Peak IIA most probably represents 7α,25-dihydroxy-4-cholesten-3-one. Peak IIB has the retention time corresponding to 7α,26-dihydroxy-4-cholesten-3-one. Peak III was identified as 7α,12α-dihydroxy-4-cholesten-3-one (c. f. text). S denotes the substrate.

monitoring technique, it was shown that the trimethylsilyl ether of this material had a retention time identical with that of the derivative of authentic 7α,12α-dihydroxy-4-cholesten-3-one. Furthermore, the characteristic ions at m/e 380 (M-2 × 90) and at m/e 268 (20) were present in its mass spectrum.

Peak IIB had the retention time on HPLC corresponding to 7α,26-dihydroxy-4-cholesten-3-one (cf.ref. 21). Due to the known difficulties (21) of subjecting this compound to gas–liquid chromatography, no attempts were made to identify this compound with GLC–MS.

Peak I had a retention time similar to that of THCA and to that expected for the 26-carboxy derivative of 7α-hydroxy-4-cholesten-3-one. Using the selective ion monitoring technique, the possibility was excluded that the compound was identical with THCA (10). In view of the fact that no authentic carboxy derivative of 7α-hydroxy-4-cholesten-3-one was available, no further attempts were made to identify the compound.

The whole homogenate from the liver of the CTX patient efficiently catalyzed conversion of 7α-hydroxy-4-cholesten-3-one into metabolite III (Fig. 2A and **Table 2**). Using the same technique as above, it was shown that this material was identical with 7α,12α-dihydroxy-4-cholesten-3-one. There was no significant conversion into more polar products.

7α-Hydroxy-4-cholesten-3-one was efficiently converted into the polar metabolites I and IIB by the mitochondrial fraction of the normal liver (Fig. 2B and Table 2). In this case also, it was shown that the material corresponding to peak I was not identical with THCA. It should be mentioned that it has previously been shown by indirect means that the mitochondrial fraction of human liver efficiently catalyzes conversion of 7α-hydroxy-4-cholesten-3-one into 7α,-26-dihydroxy-4-cholesten-3-one (21) (peak IIB in the chromatogram shown in Fig. 2A and 2B). It seems likely that metabolite I is identical with the 26-carboxylated derivative of 7α-hydroxy-4-cholesten-3-one.

The mitochondrial fraction from the liver of the CTX patient did not catalyze formation of the polar metabolites I and IIB from 7α-hydroxy-4-cholesten-3-one (Fig. 2B and Table 2). There was a significant formation of metabolite III. Using the same technique as above, it was shown that this material was

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TABLE 2. Conversion of ^{3}H-labeled 7α-hydroxy-4-cholesten-3-one into more polar products by liver subcellular fractions from control subject and from the CTX patient

Subcellular fraction	Peak I	Peak IIA[c]	Peak IIB (7α,26-dihydroxy-4-cholesten-3-one)	Peak III (7α,12α-dihydroxy-4-cholesten-3-one)
		nmol × mg protein^{-1} × hr^{-1}		
Whole homogenate				
Control subject	2.61[a]		4.54	2.43
CTX patient	0.12	0.10	not detectable	0.86
Mitochondrial fraction				
Control subject	11.83[a]		3.95	0.08
CTX patient	not detectable		not detectable	0.94
Microsomal fraction				
Control subject	2.50[b]	0.15		8.37
CTX patient	0.78[b]	0.13		7.55

[a] Assumed to represent 7α-hydroxy-4-cholesten-3-one-26-oic acid.
[b] Not identified.
[c] Assumed to represent 7α,25-dihydroxy-4-cholesten-3-one.
The incubation, extraction, and HPLC procedures are given in Materials and Methods. For identification of the different peaks, see Fig. 2A–C and text.

identical with 7α,12α-dihydroxy-4-cholesten-3-one (see discussion).

7α-Hydroxy-4-cholesten-3-one was efficiently converted into metabolite III by the microsomal fraction from both the normal liver and the liver of the CTX patient (Fig. 2C and Table 2). In both cases, the identity of metabolite III as 7α,12α-dihydroxy-4-cholesten-3-one was established by GLC–MS as above.

In addition to 7α,12α-dihydroxy-4-cholesten-3-one, a polar metabolite (peak I) was formed both in the microsomal fraction from the normal liver and in that from the liver of the CTX patient. This polar metabolite was not identical with THCA. The small peak IIA (Fig. 2C), slightly more polar than 7α,26-dihydroxy-4-cholesten-3-one, probably represents 7α,25-dihydroxy-4-cholesten-3-one. In view of the small amounts available, no further attempts were made to identify these products.

The rates of formation of the different metabolites from 7α-hydroxy-4-cholesten-3-one by the different subcellular fractions are summarized in Table 2. It is seen that the substrate is efficiently 26-hydroxylated by the normal liver mitochondria, but no 26-hydroxylation is observed with the mitochondria from the liver of the CTX patient. The rate of 12α-hydroxylation is high with the microsomal fractions from both subject, while these same fractions have very low ability to catalyze 25-hydroxylation of 7α-hydroxy-4-cholesten-3-one.

Incubations with 5-cholestene-3β,7α-diol

Incubations of 5-cholestene-3β,7α-diol with the different subcellular fractions of normal liver resulted in a very complex pattern of products. Part of the explanation may be that 5-cholestene-3β,7α-diol is efficiently converted into 7α-hydroxy-4-cholesten-3-one by the microsomal fraction. Thus a mixture of hydroxylated metabolites of both 5-cholestene-3β,7α-diol and 7α-hydroxy-4-cholesten-3-one are to be expected under the conditions used in the present work. The detailed results of the incubations with 5-cholestene-3β,7α-diol are therefore not presented. The most important finding was, however, that the mitochondrial fraction of normal liver catalyzed formation of a product with retention time corresponding to 5-cholestene-3β,7α,26-triol at a rate of 5.2 nmol × mg protein^{-1} × hr^{-1}. It has previously been shown that the mitochondrial fraction of normal human liver efficiently catalyzes formation of this product from 5-cholestene-3β,7α-diol (21). No formation of 5-cholestene-3β,7α-26-triol occurred, however, with the mitochondrial fraction from the CTX patient.

DISCUSSION

In a previous work on hydroxylation of the C_{27}-steroid side chain by the microsomal and the mitochondrial fraction of human liver homogenate (21), optimal assay conditions were used with a saturating concentration of substrate. In order to obtain a higher percentage conversion, not only to the primary products but also to products occurring later in the sequence of reactions leading to bile acids, a lower concentration of substrate with higher specific radio-

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activity and a longer incubation time was used in the present study. HPLC was found to be a very suitable technique for separation and isolation of the different products. In view of the small amounts of metabolites obtained with the present conditions, it was not possible to identify the different metabolites with a full mass spectrum. Thus it was necessary to use the more sensitive technique of selected ion monitoring (mass fragmentography). The criteria used for identification should, however, be sufficient. Thus, a metabolite was regarded as identical with a specific compound if the two materials had identical retention time on HPLC and GLC and, in addition, contained two or three common characteristic ions in their mass spectra.

In a recent work we showed that liver mitochondria from a patient with CTX were unable to catalyze hydroxylation of 5β-cholestane-3α,7α,12α-triol into 5β-cholestane-3α,7α,12α,26-tetrol (10). We suggested that the major metabolic defect in CTX is the lack of the mitochondrial 26-hydroxylase. Furthermore, very high levels of 5β-cholestane-3α,7α,-12α-triol were detected in subcellular fractions from the liver of the CTX patient (10, 22). In the liver of the CTX patient the accumulated 5β-cholestane-3α,-7α,12α-triol is converted into 5β-cholestane-3α,7α,12α,25-tetrol by the microsomal 25-hydroxylase with subsequent formation of cholic acid (11, 12).

The alternative 25-hydroxylase pathway for cholic acid biosynthesis (11, 12) is evidently considerably less efficient than the pathway involving the mitochondrial 26-hydroxylase, thus explaining the reduced secretion of cholic acid in the bile of the CTX patients (7).

The secretion of chenodeoxycholic acid is even more reduced than that of cholic acid in these patients (7), and in our patient chenodeoxycholic acid amounted to only about 1% of the total bile acid constituents (10). The main precursors in the biosynthesis of chenodeoxycholic acid are considered to be 5-cholestene-3β,7α-diol, 5β-cholestane-3α,7α-diol, and 7α-hydroxy-4-cholesten-3-one (23, 24). The present results confirm earlier findings that these substrates are 26-hydroxylated by normal human liver mitochondria (21). No such hydroxylation was detected in the mitochondria isolated from the liver of the CTX patient.

This lack of 26-hydroxylase activity could in principle be due to inactivation of the mitochondrial fraction during the isolation procedure. Using the assay conditions described above, however, we have never failed to detect 26-hydroxylase activity with human liver biopsies (10). Only after freezing the tissue or the isolated mitochondrial fractions is the activity lost, presumably because of loss of an iron sulfur protein (ferredoxin) essential for hydroxylase activity (25). This may explain the variability and even absence of 26-hydroxylase activity previously observed with subcellular fractions of normal human liver (21). Also, the mitochondrial fraction of the CTX patient had the ability to 25-hydroxylate vitamin D_3 (10). In a previous work we have shown that both the vitamin D_3 25-hydroxylase and the C_{27}-steroid 26-hydroxylase are cytochrome P-450 enzymes with similar intramitochondrial localization and requiring liver ferredoxin and ferredoxin reductase for activity (26). A selective inactivation of only one of these species in the mitochondrial fraction from the CTX patient seems highly unlikely.

The unusual high 12α-hydroxylase activity detected in the mitochondrial fraction of the CTX patient raises the question of a possible microsomal contamination of this fraction. Identical standard procedures (10) were used for preparation of mitochondria and microsomes from the liver of both the normal individual and the CTX patient. C_{27}-steroid 12α- and 25-hydroxylase activities are generally considered to be localized to the endoplasmic reticulum (23). No such activities were detected in the mitochondrial fraction of the normal liver (Tables 1 and 2), indicating minimal microsomal contamination of this fraction.

In the mitochondrial fraction of the CTX patient, however, the specific 12α-hydroxylase activity appeared to amount to 80% of that in the microsomal fraction when 5β-cholestane-3α,7α-diol was the substrate (Table 1) and to 12% when 7α-hydroxy-4-cholesten-3-one was the substrate (Table 2). Megamitochondria have been observed in some of the liver cells of a patient with CTX, but only minor morphological changes of the endoplasmatic reticulum were observed (27). It therefore seems unlikely that a microsomal contamination can explain the very high 12α-hydroxylase activity towards 5β-cholestane-3α,7α-diol in the mitochondrial fraction. The absence of 25-hydroxylase activity in the mitochondrial fraction also supports this conclusion. We therefore cannot exclude the possibility that 12α-hydroxylase activity, in particular towards 5β-cholestane-3α,7α-diol, which normally is not detectable in liver mitochondria may become manifest in the mitochondria of CTX patients. If so, this higher 5β-cholestane-3α,7α-diol 12α-hydroxylase in CTX may be a contributing factor in the low production of chenodeoxycholic acid in this disease.

As demonstrated before (21), both 5β-cholestane-

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3α,7α-diol and 7α-hydroxy-4-cholesten-3-one served as very poor substrates for the microsomal 25-hydroxylase both in normal liver and in that from the CTX patient (Tables 1 and 2). Evidently there is no alternative 25-hydroxylase pathway for chenodeoxycholic acid biosynthesis in man. Our results indicate that in the liver of CTX patients both 5β-cholestane-3α,7α-diol and 7α-hydroxy-4-cholesten-3-one are primarily attacked by the microsomal 12α-hydroxylase and subsequently 25-hydroxylated, thereby entering the alternative pathway (11, 12) for cholic acid biosynthesis. This interpretation is strongly supported by our findings of very high levels of both 5β-cholestane-3α,7α,12α-triol (10, 22) and 7α,12α-dihydroxy-4-cholesten-3-one (22) in the liver of the CTX patient.

Intravenously administered 5β-cholestane-3α,7α,-26-triol is more efficiently converted into chenodeoxycholic acid than is 5β-cholestane-3α,7α,25-triol in normal human subjects (28). In a study on the in vivo conversion of intravenously administered radioactive 5β-cholestane-3α,7α,25-triol and 5β-cholestane-3α,-7α,26-triol into bile acids in a CTX patient, it was concluded that the conversion of both of these intermediates was impaired (29). However, the data given do not appear to support such a conclusion. In fact, the specific activity of chenodeoxycholic acid derived from 5β-cholestane-3α,7α,26-triol reached a level several times higher in the CTX patient than in the control subjects (Tables 5 and 6, ref. 29). In light of our own results, we are inclined to interpret these data to indicate that the liver of the CTX patient has the capacity to efficiently convert administered 5β-cholestane-3α,7α,26-triol into chenodeoxycholic acid. The lack of formation of this intermediate will accordingly result in an impaired biosynthesis of chenodeoxycholic acid.

In conclusion, the presented results, in addition to those published elsewhere (10, 22), indicate that the mitochondrial C_{27}-steroid 26-hydroxylase is required not only for the normal biosynthesis of cholic acid (10, 30), but also for that of chenodeoxycholic acid. In CTX the 26-hydroxylase is absent and bile acids are formed by an alternative pathway (11, 12) involving both the 12α, and the 25-hydroxylases. The low capacity of the microsomal 25-hydroxylase for chenodeoxycholic acid precursors explains the reduced formation of chenodeoxycholic acid in CTX and also explains why there is no accumulation of 25-hydroxylated precursors of this acid in the liver of the patients. We believe that the above explanation is more plausible than the previous suggestion (11, 12) that the 25-hydroxylase pathway is of major importance in the normal biosynthesis of bile acids and that the metabolic defect in CTX is a deficient 24-hydroxylase active on 5β-cholestane-3α,7α,12α,25-tetrol (31).

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