Biosynthesis of bile acids in man. Hydroxylation of the C27-steroid side chain.

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Research Article

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Biosynthesis of Bile Acids in Man

HYDROXYLATION OF THE C_{27}-STEROID SIDE CHAIN

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A B S T R A C T The first step in the degradation of the steroid side chain during biosynthesis of bile acids from cholesterol in man was studied in microsomal and mitochondrial fraction of homogenate of livers from 14 patients.

The microsomal fraction was found to catalyze an efficient 25-hydroxylation of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol. A small extent of 23-, 24-, and 26-hydroxylation of the same substrate was observed. 5β-Cholestane-$3\alpha,7\alpha$-diol was hydroxylated in the 25-position only to a very small extent.

The mitochondrial fraction was found to catalyze 26-hydroxylation of cholesterol, 5-cholestene-$3\beta,7\alpha$-diol, 5β-cholestane-$3\alpha,7\alpha$-diol, 7α-hydroxy-4-cholesten-3-one, and 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol. Addition of Mg^{++} stimulated the 26-hydroxylation of cholesterol but had no effect or an inhibitory effect on 26-hydroxylation of the other substrates, indicating a heterogeneity of the mitochondrial 26-hydroxylating system. The level of 26-hydroxylase activity towards different substrates varied considerably with different mitochondrial preparations.

The roles of the microsomal and mitochondrial 26-hydroxylations as well as the microsomal 25-hydroxylation in biosynthesis of bile acids in man are discussed. The results indicate that microsomal 26-hydroxylation is less important than mitochondrial 26-hydroxylation under normal conditions. The possibility that microsomal 25-hydroxylation is important cannot be ruled out.

INTRODUCTION

According to current concepts of the biosynthesis of bile acids from cholesterol in mammalian liver, the changes in the steroid nucleus precede the degradation of the steroid side chain (1), and 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol and 5β-cholestane-$3\alpha,7\alpha$-diol have been postulated as intermediates in the biosynthesis of cholic acid[1] and chenodeoxycholic acid,[1] respectively. The sequence of reactions leading to the formation of 5β-cholestane-$3\alpha,7\alpha$-diol in rat liver (1), guinea pig liver (2), as well as human liver (3) appears to be the following: cholesterol $\rightarrow$ 5-cholestene-$3\beta,7\alpha$-diol $\rightarrow$ 7α-hydroxy-4-cholesten-3-one $\rightarrow$ 7α-hydroxy-5β-cholestan-3-one $\rightarrow$ 5β-cholestane-$3\alpha,7\alpha$-diol. In the sequence of reactions leading to 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol, a 12α-hydroxyl group is introduced, probably at the stage of 7α-hydroxy-4-cholesten-3-one (1). The mechanisms of degradation of the side chains in 5β-cholestane-$3\alpha,7\alpha$-diol and 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol have not been completely established. Studies in vitro with preparations from rat liver show that in the major pathway for the degradation of the side chain, microsomal and/or mitochondrial 26-hydroxylation is the initial reaction (1, 4–7). The possibility that side chain degradation starts with a 25-hydroxylation has also been discussed, but there is little experimental evidence as yet to support this pathway.

No studies in vitro on the degradation of the steroid side chain have been performed with human liver. There are indications that one mechanism of side chain degradation in man involves 26-hydroxylation. $3\alpha,7\alpha$-Dihydroxy-5β-cholestanoic acid and $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanoic acid have been isolated from human bile and have been shown to be formed from cholesterol and metabolized into chenodeoxycholic acid

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

TABLE I
Data on Patients

Patient	Sex	Age	Diagnosis	Remarks
		yr		
1	F	67	Cholelithiasis	Bilirubin, 1.6 mg/100 ml; alkaline phosphatase, 130 U/liter; GOT, 20 U/liter; GPT, 30 U/liter.
2	M	78	Carcinoma of the pancreas	Bilirubin, 18.3 mg/100 ml; alkaline phosphatase, 91 U/liter; GOT, 33 U/liter; GPT, 36 U/liter; histologic examination, cholestasis.
3	M	71	Carcinoma of the stomach	
4	M	78	Carcinoma of the stomach	
5	F	51	Chronic myelocytic leukemia	Alkaline phosphatase, 316 U/liter; GOT, 27 U/liter; GPT, 42 U/liter.
6	M	41	Chronic myelocytic leukemia	Alkaline phosphatase, 281 U/liter; histologic examination, myeloid infiltration.
7	M	37	Hodgkin's disease	
8	M	36	Agranulocytosis	
9	M	36	Hereditary spherocytosis	Bilirubin, 2.2 mg/100 ml (mainly unconjugated).
10	M	29	Hodgkin's disease	Alkaline phosphatase, 86 U/liter; GOT, 21 U/liter; GPT, 33 U/liter.
11	M	40	Pheochromocytoma	Histologic examination, moderate fatty infiltration.
12	F	44	Lymphosarcoma	
13	F	29	Intoxication with tricyclic antidepressants	GOT, 65 U/liter; GPT, 123 U/liter.
14	F	10	Head injury	Serum analysis and histologic examination not performed.

GOT, glutamic-oxalacetic transaminase; GPT, glutamic-pyruvic transaminase.

and cholic acid, respectively (8–11). Experiments in vivo with 5-[4-^{14}C]cholestene-3β,7α-diol and tritium-labeled 5-cholestene-3β,26-diol suggest that if a 26-hydroxylation occurs, it might to some extent prevent subsequent 12α-hydroxylation (12). Setoguchi, Salen, Tint, and Mosbach (13) reported recently that patients with cerebrotendinous xanthomatosis excrete considerable amounts of 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,24,25-pentol in feces, suggesting that side chain degradation might start with a 25-hydroxylation.

In the present report, the mechanisms of side chain degradation in the biosynthesis of bile acids in man have been studied by analyzing hydroxylations of the side chain of different C$_{27}$-steroids catalyzed by the mitochondrial and microsomal fractions of liver homogenate.

METHODS

Subjects. The patients in this investigation are listed in Table I. Liver biopsies from subjects 1–12 were taken within 1 h after laparotomy. Before the operation, the patients were given 0.4 mg of scopolamine and 1 mg of oxikon, obtained from Ampin AB (Stockholm, Sweden). The general anesthesia included administration of sodium hexobarbital, nitrous oxide, succinyl choline, and halothane. Subjects 13 and 14 were donors for renal transplantation and the liver specimens were obtained shortly after the donor operation, about 30 and 45 min, respectively, after

death. Unless otherwise stated, preoperative laboratory tests were within the normal ranges (total bilirubin 0.2–0.8 mg/100 ml; alkaline phosphatase, 10–40 U/liter; glutamic-oxalacetic transaminase, 4–20 U/liter; glutamic-pyruvic transaminase, 2–17 U/liter) and liver biopsies were histologically normal.

Labeled compounds. [4-^{14}C]Cholesterol (sp act 145 μCi/mg) was obtained from the Radiochemical Centre (Amersham, Bucks, England). Before use, the material was purified by chromatography on aluminum oxide, grade III (Woelm, Eschwege, W. Germany) (7). 5-[7β-^{3}H]Cholestene-3β,7α-diol (sp act 13 μCi/mg), 7α-[6β-^{3}H]hydroxy-4-cholesten-3-one (sp act 16.7 μCi/mg), 5β-[7β-^{3}H]cholestane-3α,7α-diol (sp act 16.7 μCi/mg), and 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (sp act 16.7 μCi/mg) were prepared as described previously (5).

Unlabeled compounds. (25R)-5-Cholestene-3β,26-diol, 5-cholestene-3β,7α,26-triol, 5β-cholestane-3α,7α,26-triol, 5β-cholestane-3α,7α,12α,26-tetrol, and 5β-cholestane-3α,7α,12α,24-tetrol were prepared as described previously (4, 5). 5β-Cholestane-3α,7α,12α,24-tetrol was a mixture of the 24α- and 24β-epimers (4).

Enzymes and cofactors. NADP, isocitric acid, and isocitric acid dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Fractionation of liver homogenate. The samples of liver tissues were immediately transferred to chilled homogenizing medium consisting of 0.1 M Tris-Cl buffer, pH 7.4. The homogenization was performed 5–30 min after excision of the sample with a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle. The microsomal frac-

tion was obtained from the homogenate (20%, wt/vol) by centrifugation at 800 g, 20,000 g, and 100,000 g (5). The microsomal pellet was resuspended in the homogenizing medium and homogenized before incubation. In a few experiments the final resuspension was made in a modified Bucher medium (2). The mitochondrial fraction was obtained by resuspension in 0.25 M sucrose of the pellet obtained after centrifugation at 20,000 g and recentrifugation at 8,500 g for 12 min. The precipitate obtained was resuspended in 0.1 M Tris-Cl buffer, pH 7.4. The microsomal and the mitochondrial fractions were resuspended in the final step to a volume corresponding to that of the original 800 g supernate from which they had been prepared. The protein content of the microsomal fraction and the mitochondrial fraction was between 2.7 and 4.4 mg/ml and between 1.1 and 3.3 mg/ml, respectively, when determined according to the Lowry, Rosebrough, Farr, and Randall procedure (14). The cholesterol content of the mitochondrial fraction was about 1.0 mg/100 ml as determined by gas chromatography of the trimethylsilyl ether.

Incubation procedures and analysis of incubation mixtures. In standard incubations with the microsomal fractions, 250 μg of the steroid, dissolved in 50 μl of acetone, was incubated with 1.5 ml of the microsomal fraction in a total volume of 3 ml of the homogenizing medium (5). An isocitrate-dependent NADPH-generating system was used, containing 3 μmol of NADP (5). In standard incubations with the mitochondrial fraction, 100 μg of the steroid dissolved in 50 μl of acetone was incubated with 1.0 ml of the mitochondrial fraction in a total volume of 3 ml of the homogenizing medium (5). In these incubations, only isocitrate, 4.6 μmol, was used as cofactor. In some cases (Table IV), 30 μmol of $MgCl_2$ was added. All incubations were performed at 37°C for 20 min and were terminated by the addition of 20 vol of chloroform-methanol (2:1, vol/vol). After addition of 0.2 vol of 0.9% (wt/vol) sodium chloride solution, the chloroform extract was evaporated

and subjected to thin-layer chromatography as described previously (5). Ethyl acetate was the solvent in the incubations with 5β-cholestane-3α,7α-diol and 5-cholestene-3β,7α-diol; benzene-ethyl acetate (1:4, vol/vol) in the incubations with 7α-hydroxy-4-cholesten-3-one; benzene-ethyl acetate (1:1, vol/vol) in the incubations with cholesterol; system S7 (15) in the case of incubations with 5β-cholestane-3α,7α, 12α-triol. In most cases, the corresponding unlabeled 26-hydroxylated derivatives were added as external standards. Radioactivity in the different chromatographic zones was assayed with a thin-layer scanner (Berthold, Karlsruhe, W. Germany) and it was ascertained that this method of assay gave the same results as the method used earlier to assay extent of conversion (*cf.* refs. 2–7), *i.e.* elution of appropriate zones from the thin-layer chromatograms and determination of radioactivity in the methanol extracts by liquid scintillation counting. The chromatographic zones corresponding to the 26-hydroxylated products were extracted with methanol, converted into trimethylsilyl ethers (16) and subjected to radio-gas chromatography with a Barber-Colman 5000 instrument equipped with a 3% QF-1 column (Barber-Colman Company, Rockford, Ill.). When present, 23-, 24-, 25-, and 12α-hydroxylated products were found in the thin-layer chromatographic zone corresponding to the 26-hydroxylated products, and these products separated on gas chromatography (4, 5). The conversion into the different products was calculated from the amount of radioactivity found in the thin-layer chromatographic zone and the amount of radioactivity found with the appropriate retention time in the radio-gas chromatogram. As shown previously (5), there was a linear relationship between the amount of radioactivity injected into the radio-gas chromatograph and the peak area of the radioactivity tracing. When incubations with only buffer were performed, in some cases a small amount of radioactivity ($<0.2\%$) appeared in the zone corresponding to the 25- and 26-hydroxylated product. Radio-gas chromatography showed

TABLE II

Microsomal Hydroxylations

Substrate	Conversion into 26-hydroxylated product	Conversion into 25-hydroxylated product	Conversion into 12α-hydroxylated product
	nmol/mg protein/20 min		
Cholesterol ($n = 5$)	<0.1 ($n = 5$)	<0.1 ($n = 5$)	<0.1 ($n = 5$)
5-Cholestene-3β, 7α-diol ($n = 8$)	<0.2 ($n = 8$)	<0.1 ($n = 7$) 0.4 ($n = 1$)	2.3 ± 1.3 ($n = 5$) <0.2 ($n = 3$)
7α-Hydroxy-4-cholesten-3-one ($n = 8$)	<0.2 ($n = 8$)	<0.2 ($n = 8$)	5.9 ± 0.6 ($n = 8$)
5β-Cholestane-3α, 7α-diol ($n = 9$)	<0.1 ($n = 9$)	0.3 ± 0.0 ($n = 5$) <0.1 ($n = 4$)	1.8 ± 0.3 ($n = 9$)
5β-Cholestane-3α, 7α, 12α-triol ($n = 10$)	<0.1 ($n = 10$)	8.4 ± 2.0 ($n = 10$)	—

Values listed are means $\pm$SEM.
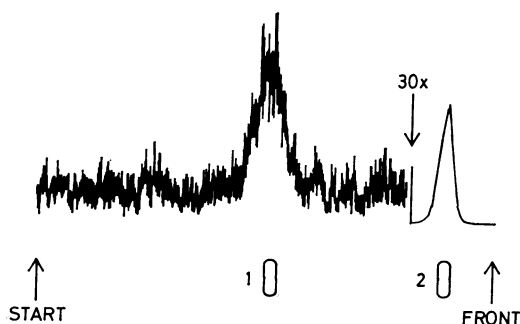
FIGURE 1 Radio scanning of thin-layer chromatogram of extract of incubation of 5β-[7β-³H]cholestane-3α,7α,12α-triol with the microsomal fraction of patient 14 (standard incubation conditions). Reference compounds were: 1, 5β-cholestane-3α,7α,12α,26-tetrol; 2, 5β-cholestane-3α,7α,12α-triol. Solvent, S7.

that these compounds were not identical with the side chain hydroxylated derivatives.

In all cases, conversion of cholesterol into the 26-hydroxylated product was calculated on the assumption that the added cholesterol was equilibrated with endogenous cholesterol (≈ 10 μg/ml of mitochondrial fraction). Even if there were no equilibration between the added cholesterol and the endogenous cholesterol, 26-hydroxylase activity would be overestimated by less than 10% under standard conditions (cf. ref. 7).

In some cases, aliquots of the trimethylsilyl ethers were analyzed by combined gas chromatography-mass spectrometry with the LKB 9000 instrument equipped with a 1.5% SE-30 column (LKB-Produkter AB, Stockholm,Sweden). In the analysis of the products obtained from incubations of 5β-cholestane-3α,7α,12α-triol with the microsomal fraction, the trimethylsilyl ether of the extract from the appropriate chromatographic zone was subjected to mass fragmentography. The mass fragmentography was performed with the above instrument equipped with a multiple ion detector. The four channels were focused on the ions m/e 131, m/e 145, m/e 159, and m/e 253, corresponding to a prominent peak or the base peak in the mass spectrum of the trimethylsilyl ether of the 25-, 24-, 23-, and 26-hydroxylated product, respectively (4).

RESULTS

Incubations with the microsomal fraction. Table II summarizes the results of incubations of the different substrates with the microsomal fraction under the standard incubation procedure with Tris-Cl as buffer. In accordance with previous work (3), 7α-hydroxy-4-cholesten-3-one and 5-cholestene-3β,7α-diol were hydroxylated efficiently in the 12α-position. Also 5β-cholestane-3α,7α-diol was converted efficiently into the corresponding 12α-hydroxylated product, as shown by thin-layer chromatography and radio-gas chromatography of the trimethylsilyl ether. 5β-Cholestane-3α,7α,12α-triol was converted efficiently into a compound with the thin-layer chromatographic and gas-chromatographic

properties of 5β-cholestane-3α,7α,12α,25-tetrol (Figs. 1 and 2). Combined gas chromatography-mass spectrometry of the trimethylsilyl ether established the identity of the product. The mass spectrum was identical with that of the authentic compound and with previously published mass spectra (4, 13) of trimethylsilyl ether of 5β-cholestane-3α,7α,12α,25-tetrol. The most prominent feature in the mass spectrum of this compound is the base peak at m/e 131, corresponding to cleavage between C₂₄ and C₂₅. In most incubations with 5β-cholestane-3α,-7α,12α-triol, small amounts of products with the thin-layer chromatographic and gas-chromatographic properties of 5β-cholestane-3α,7α,12α,24-tetrol and 5β-cholestane-3α,7α,12α,23-tetrol were formed. The trimethylsilyl ethers of these compounds had mass spectra identical to those of the authentic compounds (4). The most characteristic feature of the mass spectrum of the trimethylsilyl ether of 5β-cholestane-3α,7α,12α,24-tetrol is the prominent peak at m/e 145, corresponding to cleavage between C₂₃ and C₂₄ (4). The most prominent feature of the mass spectrum of the trimethylsilyl ether of 5β-cholestane-3α,7α,12α,23-tetrol is the base peak at m/e 159,
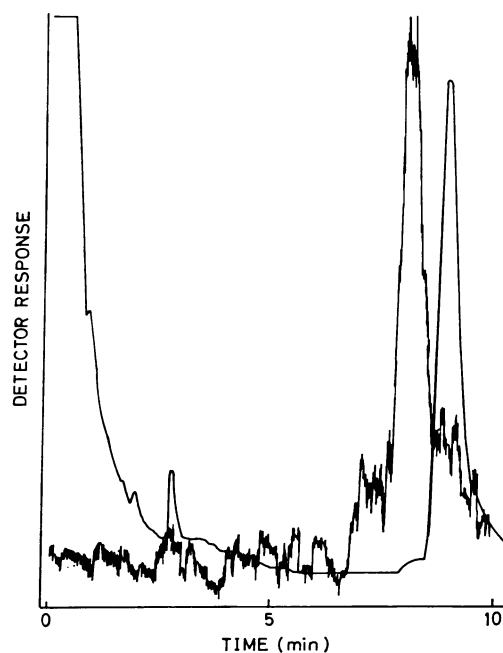

FIGURE 2 Radio-gas chromatogram of trimethylsilyl ether of radioactive product obtained from the thin-layer chromatogram shown in Fig. 1. Unlabeled trimethylsilyl ether of 5β-cholestane-3α,7α,12α,26-tetrol was added to the extract and had a retention time of 8.8 min. The radioactive peak occurring after 7.0 min corresponds to 5β-cholestane-3α,7α,12α,24-tetrol and the radioactive peak occurring after 8.0 min corresponds to 5β-cholestane-3α,7α,12α,25-tetrol. Column, 1% QF-1. Smooth tracing, mass; oscillating tracing, radioactivity.

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corresponding to cleavage between C$_{22}$ and C$_{23}$ (*cf.* ref. 4).

No attempts were made to separate 5β-cholestane-3α,-7α,12α,24α-tetrol from 5β-cholestane-3α,7α,12α,24β-tetrol (4). 26-Hydroxylation of 5β-cholestane-3α,7α,12α-triol could not be detected with either the radio-gas chromatographic technique (Fig. 2) or mass fragmentography (Fig. 3). In the mass fragmentography, the specific ions at *m/e* 253, *m/e* 131, *m/e* 145, *m/e* 159 were followed through the gas chromatogram. The ion at *m/e* 253 is the base peak in the mass spectrum of the trimethylsilyl ether of 5β-cholestane-3α,7α,12α,26-tetrol (4) and is prominent in mass spectra of trimethylsilyl ethers of all C$_{27}$-steroids with three hydroxyl groups in the steroid nucleus (4, 17). No significant peak with the retention time characteristic of 5β-cholestane-3α,7α,12α,26-tetrol could be detected in the recording of the ions at *m/e* 253, whereas peaks could be detected in this recording with retention times characteristic of trimethylsilyl ethers of 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,-12α,24-tetrol, and 5β-cholestane-3α,7α,12α,23-tetrol (Fig. 3). As expected there were peaks in the recording at *m/e* 131, *m/e* 145, and *m/e* 159 at retention times corresponding to trimethylsilyl ether of 5β-cholestane-3α,7α,-12α,25-tetrol, 5β-cholestane-3α,7α,12α,24-tetrol, and 5β-cholestane-3α,7α,12α,23-tetrol, respectively (Fig. 3). In addition to the gas-chromatographic peaks corresponding to the trimethylsilyl ethers of 23-, 24-, and 25-hydroxylated 5β-cholestane-3α,7α,12α-triols, several sharp peaks appeared in the chromatogram shown in Fig. 3. These peaks did not correspond to compounds of steroid nature as judged from their mass spectra.

In some of the incubations with 5β-cholestane-3α,7α-diol with the microsomal fraction, small amounts of a
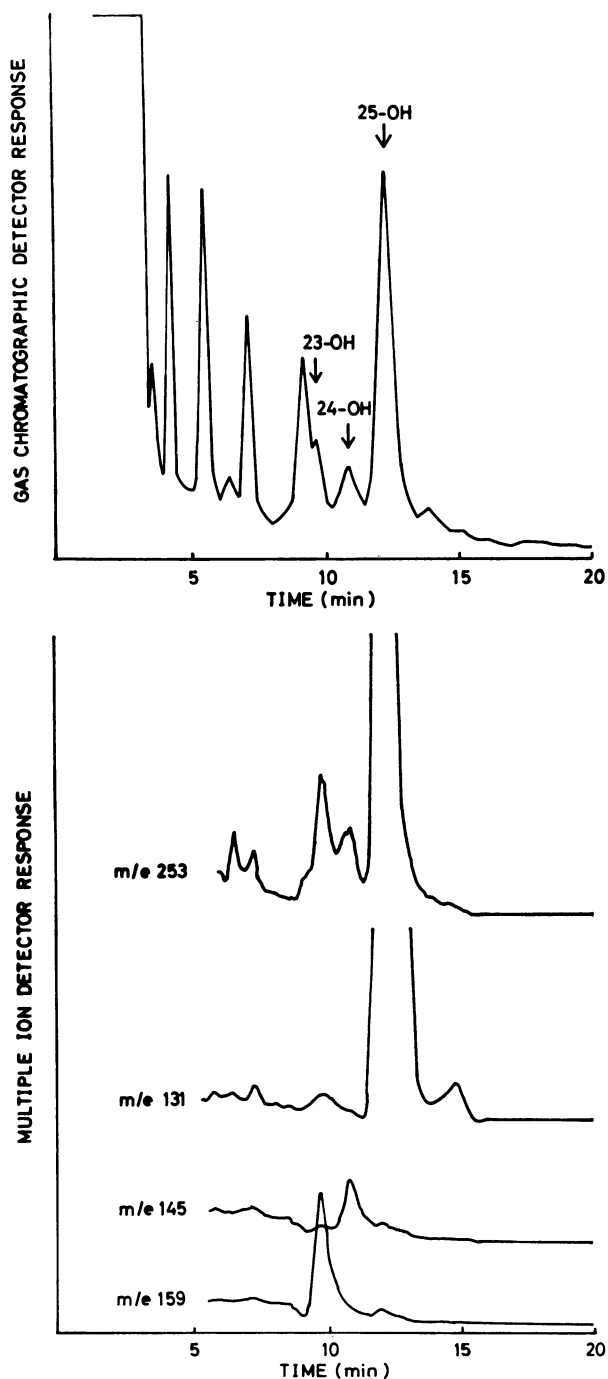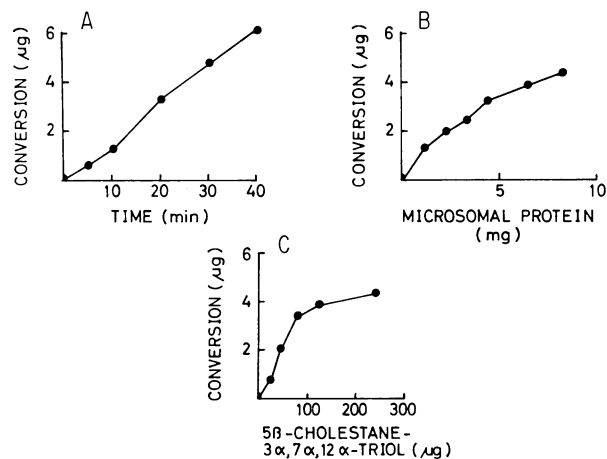
FIGURE 3 Mass fragmentogram of trimethylsilyl ether of radioactive product obtained from thin-layer chromatogram of extract of incubation of 5β-cholestane-3α,7α,12α-triol with the microsomal fraction. No reference compounds were added to the extract. The four channels of the multiple ion detector unit, focused on *m/e* 253, 131, 145, and 159, respectively, were all amplified to the same degree (300 times). Column, 1.5% SE-30. Under the chromatographic conditions employed, trimethylsilyl ether of 5β-cholestane-3α,7α,12α,26-tetrol has a retention time of about 13.8 min.

FIGURE 4 Effect of time (A), enzyme concentration (B) and substrate concentration (C) on microsomal 25-hydroxylation of 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol. Standard incubation conditions were used with the exception that 100 μg substrate was used in the experiments shown in A and B.

TABLE III

Mitochondrial 26-Hydroxylation

Substrate	Conversion into 26-hydroxylated product
	nmol/mg protein/20 min
Cholesterol ($n = 10$)	0.4 ± 0.1 ($n = 10$)
5-Cholestene-3β, 7α-diol ($n = 9$)	1.7 ± 0.9 ($n = 4$) <0.2 ($n = 5$)
7α-Hydroxy-4-cholesten-3-one ($n = 8$)	3.7 ± 1.2 ($n = 4$) <0.2 ($n = 4$)
5β-Cholestane-3α, 7α-diol ($n = 7$)	1.8 ± 0.3 ($n = 7$)
5β-Cholestane-3α, 7α, 12α-triol ($n = 10$)	2.0 ± 1.0 ($n = 4$) <0.1 ($n = 6$)

Values listed are means $\pm$SEM.

compound with the thin-layer chromatographic and gas chromatographic properties expected for 5β-cholestane-3α,7α,25-triol were formed. The mass spectrum of the trimethylsilyl ether of the compound had a base peak at m/e 131 (cf. above) and prominent peaks at m/e 546 (M-90), and m/e 456 (M-(2 × 90)). With one exception (Table II) 5β-cholestane-3α,7α,25-triol never exceeded 10% of total products.

In one incubation with 5-cholestene-3β,7α-diol with the microsomal fraction (Table II) small amounts of a product were formed with a retention time on gas chromatography expected of 5-cholestene-3β,7α,25-triol or 5-cholestene-3β,7α,26-triol. The small amounts of material available prevented further analysis of the compound. Significant 25-hydroxylation could not be detected with any of the other substrates tested. No significant 26-hydroxylation of any of the different substrates was detected (Table II).

The 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol was studied in more detail with microsomal fraction obtained from one of the liver samples (patient 14). The rate of 25-hydroxylation was found to be linear with microsomal protein up to 8 mg and with time up to about 40 min (Fig. 4). The enzyme appeared to be saturated with 250 μg of substrate.

In the above series of experiments, Tris-Cl was used as incubation medium since with rat liver microsomes 26-hydroxylation is more efficient with this buffer than with potassium phosphate buffer (5). In a few experiments with microsomal fraction from patients 13 and 14, a modified Bucher medium was used (2, 3). With this medium 12α-hydroxylation and 25-hydroxylation decreased but a significant conversion of 5β-cholestane-3α,-7α-diol and 5β-cholestane-3α,7α,12α-triol into products with the gas-chromatographic properties characteristic of the corresponding 26-hydroxylated compounds occurred. In two experiments with 5β-cholestane-3α,7α-diol and one with 5β-cholestane-3α,7α,12α-triol, the conversion into the assumed 26-hydroxylated product was

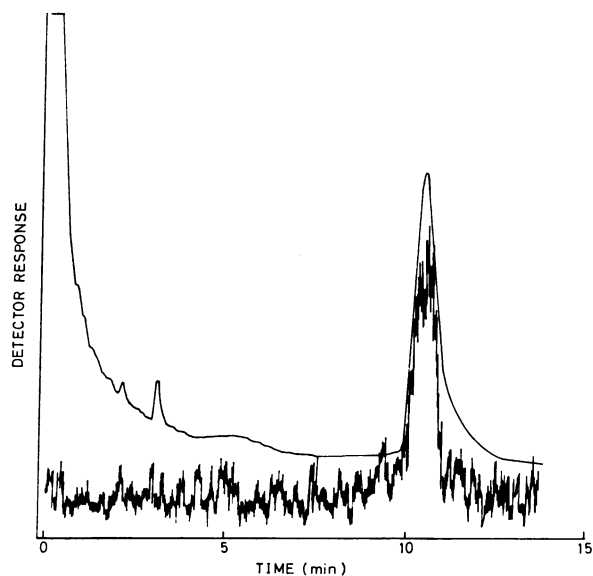

FIGURE 5 Radio-gas chromatogram of trimethylsilyl ether of radioactive product obtained from thin-layer chromatogram of extract of incubation of 5β-[7β-^{3}H]cholestane-3α, 7α,12α-triol with the mitochondrial fraction of liver from patient 14 (standard incubation conditions). Unlabeled trimethylsilyl ether of 5β-cholestane-3α,7α,12α,26-tetrol was added to the extract before injection. Chromatographic conditions as in Fig. 2.

about 0.1 nmol/mg protein/20 min or less (cf. Table II). In one experiment with 5β-cholestane-3α,7α,12α-triol, the conversion was about 0.5 nmol/mg protein/20 min. The small amounts of product obtained in the experiments with modified Bucher medium prevented final identification by combined gas chromatography-mass spectrometry.

Incubations with the mitochondrial fraction. Table III summarizes the results of incubations of the different substrates with the mitochondrial fraction. 26-Hy-

TABLE IV

Effect of Mg^{2+} on Mitochondrial 26-Hydroxylation

Substrate	Formation of 26-hydroxylated product	
	Without Mg^{2+}	With Mg^{2+}
	nmol/mg protein/20 min	
Cholesterol	0.1	0.2
5-Cholestene-3β, 7α-diol	0.6	0.5
7α-Hydroxy-4-cholesten-3-one	5.9	4.5
5β-Cholestane-3α, 7α-diol	2.4	2.0
5β-Cholestane-3α, 7α, 12α-triol	4.9	4.0

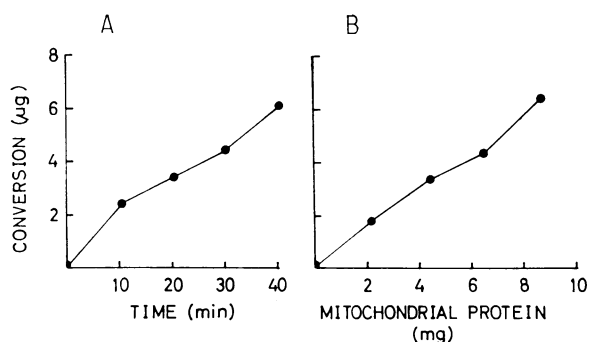The concentration of Mg^{2+} was 10 mM. The liver sample was obtained from patient 14.

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FIGURE 6 Effect of time (A), and enzyme concentration (B) on mitochondrial 26-hydroxylation of 5β-[7β-^{3}H]cholestane-$3\alpha,7\alpha,12\alpha$-triol. Standard incubation conditions were used.

droxylation could be demonstrated with every substrate tested, and no other hydroxylated product was formed in any case (Fig. 5). Cholesterol and 5β-cholestane-$3\alpha,7\alpha$-diol were 26-hydroxylated by every mitochondrial fraction tested, whereas 5-cholestene-$3\beta,7\alpha$-diol, 7α-hydroxy-4-cholesten-3-one, and 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol were 26-hydroxylated by about half of the different mitochondrial fractions tested. The mitochondrial fraction from one of the samples (patient 14) was incubated with all the different substrates (Table IV) and significant 26-hydroxylation was obtained in all cases. Cholesterol was 26-hydroxylated less efficiently than other substrates. With cholesterol as substrate, addition of Mg^{++} stimulated the reaction. With the other substrates, addition of Mg^{++} had no effect or a slight inhibitory effect.

In the case of incubations with cholesterol, 5-cholestene-$3\beta,7\alpha$-diol, 5β-cholestane-$3\alpha,7\alpha$-diol, and 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol, the final identification was performed directly with the trimethylsilyl ether of the product. The mass spectra obtained were identical to those of the authentic compounds. The characteristic features of these mass spectra have been published previously (5). In the case of incubations of 7α-hydroxy-4-cholesten-3-one, direct identification of the product by combined gas chromatography-mass spectrometry was difficult due to the long retention time on the column used. The material with the thin-layer chromatographic properties expected of $7\alpha,26$-dihydroxy-4-cholesten-3-one was therefore reduced with sodium borohydride, and the trimethylsilyl ether of the reduced product was identified by combined gas chromatography-mass spectrometry as 4-cholestene-$3\beta,7\alpha,26$-triol. The mass spectrum was identical to that reported previously (5). The mass spectrum had a prominent base peak at m/e 544 (M-90) and small peaks at m/e 529 [M — (90 + 15)], 454 [M — (2 × 90)], and 196(5). The peak at m/e 196 was found in mass spectra of trimethylsilyl ethers of a series of different 3-hydroxylated C_{27}-steroids with a Δ^4-double bond

and a 7α-hydroxyl group (Björkhem, I., J.-Å. Gustafsson, and J. Sjövall, unpublished observations).

The 26-hydroxylation of 5β-cholestane-$3\alpha,7\alpha$-diol by the mitochondrial fraction from patient 14 was studied in some detail (Fig. 6). The rate of 26-hydroxylation was linear with mitochondrial protein up to 8 mg and with time up to about 40 min.

DISCUSSION

Microsomal hydroxylation. Only very small 26-hydroxylase activity could be detected in the microsomal fraction of the human liver preparation under the experimental conditions used in the present study. It is possible that under other assay conditions the extent of 26-hydroxylation may be higher. In accordance with previous work (3) there was an efficient 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one, 5-cholestene-$3\beta,7\alpha$-diol, and 5β-cholestane-$3\alpha,7\alpha$-diol. In addition, the side chain of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol was hydroxylated efficiently in the 25-position and to a small extent also in the 23- and 24-positions. The efficient 25-hydroxylation of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol is of interest, and it is possible that this hydroxylation might be of importance in the biosynthesis of bile acids (*cf.* below). The same reaction has been shown to occur also with rat liver microsomes (4). The 25-hydroxylase system seems to have a rather high degree of substrate specificity. Of the other substrates tested, only 5β-cholestane-$3\alpha,7\alpha$-diol was 25-hydroxylated to a small extent in some of the experiments.

It seems unlikely that the small amount of 23- and 24-hydroxylase activity demonstrated with 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol as substrate is related to bile acid biosynthesis in man. 23-Hydroxylated bile acids have not been described in human bile, but are known to be present in bile of seals and reptiles (18). 5-Cholestene-$3\beta,23$-diol has been tentatively identified in human meconium (19). Introduction of a 24-hydroxyl group is probably an obligatory step in the biosynthesis of bile acids, but information from experiments with rat liver preparations indicates strongly that $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholestanoic acid and not 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol is the major substrate for the 24-hydroxylase system involved in cholic acid biosynthesis (1).

It is likely that cytochrome P-450-containing systems are responsible for the different side chain hydroxylations with the microsomal fraction. Preliminary work has shown that a partially purified cytochrome P-450 fraction from human liver is able to catalyze 25-hydroxylation of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol when combined with NADPH-cytochrome P-450 reductase from rat liver and a phospholipid (Björkhem, I., L. Kager, and K. Wikvall, unpublished observation).

Mitochondrial 26-hydroxylation. The mitochondrial

fraction was found to catalyze 26-hydroxylation of all the different C_{27}-steroids. Cholesterol and 5β-cholestane-$3\alpha,7\alpha$-diol were 26-hydroxylated by every mitochondrial fraction tested, whereas the other substrates were 26-hydroxylated efficiently by some preparations but not at all by others. This might have been due to partial inactivation of the system. It was not possible to correlate the absence of 26-hydroxylase activity with some specific diagnosis or treatment. The difficulties in working with mitochondrial 26-hydroxylase, due to the lability of the system, have been pointed out previously (20). In one case, the amount of liver obtained was sufficient to test every substrate with the same preparation. 26-Hydroxylation of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol, 5β-cholestane-$3\alpha,7\alpha$-diol, and 7α-hydroxy-4-cholesten-3-one was more efficient than that of cholesterol and 5-cholestene-$3\beta,7\alpha$-diol. It appears that the mitochondrial system in human liver has the same broad substrate specificity as that in rat liver (5). Isocitrate was used as cofactor in all incubations with the mitochondrial fraction. It was shown previously that isocitrate was suitable as cofactor for 26-hydroxylation of cholesterol as well as 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol by rat liver mitochondria (7, 20). The proposed role of isocitrate is to generate intramitochondrial NADPH via NADP-dependent isocitrate dehydrogenase (7). Isocitrate is also suitable as cofactor in experiments with the mitochondrial fraction, since isocitrate cannot be utilized by the different hydroxylating systems present in the microsomes that always contaminate the mitochondrial fraction (5). It was shown previously that addition of Mg^{++} stimulates mitochondrial 26-hydroxylation of cholesterol in rat liver (7) but inhibits or has no effect on 26-hydroxylation of the other C_{27}-steroids in bile acid biosynthesis (Gustafsson, J., unpublished observation). Similar but less marked effects of Mg^{++} were found with the mitochondrial 26-hydroxylating system in human liver. It remains to be established whether the different effects of Mg^{++} are due to presence of different 26-hydroxylating systems with different properties or whether there are different mechanisms for transfer of the steroids through the mitochondrial membranes.

Hydroxylation of the steroid side chain during biosynthesis of bile acids in man. Three different mechanisms for side chain degradation during biosynthesis of bile acids in mammals have been discussed (1, 4, 5, 13, 21). (*a*) Side chain degradation starts with 26-hydroxylation in the mitochondrial fraction. This step is followed by oxidation of the C_{26}-hydroxyl group to yield the corresponding carboxylic acid which is hydroxylated in the 24-position. The hydroxyl group in the 24-position is oxidized to the corresponding 24-oxo compound, which is further oxidized to the corresponding C_{24}-bile acid. (*b*) Side chain degradation starts with 26-hydroxy-

lation in the microsomal fraction. This step is then followed by the same sequence of reactions as above. (*c*) Side chain degradation starts with 25-hydroxylation, probably in the microsomal fraction. This step might be followed by a 24-hydroxylation. Oxidation of the 24-hydroxyl group and subsequent cleavage yields acetone and a C_{24}-bile acid.

There is evidence that all three pathways exist in rat liver. According to current concepts, a pathway involving acetone formation is of little importance, quantitatively (1, 22). 5β-Cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol is formed in rat liver microsomes in vitro (4), but is converted into cholic acid in vivo much less efficiently than 5β-cholestane-$3\alpha,7\alpha,12\alpha,26$-tetrol and 5β-cholestane-$3\alpha,-7\alpha,12\alpha$-triol (4, 23). The relative importance of microsomal and mitochondrial 26-hydroxylases is not clear. There is evidence to indicate that microsomal 26-hydroxylation plays a role in the regulation of the ratio of cholic acid to chenodeoxycholic acid formed from cholesterol. In the hyperthyroid rat, in which the normal cholic acid/chenodeoxycholic acid ratio is reversed, the microsomal 26-hydroxylase is increased and the 12α-hydroxylase decreased (6). Since the presence of a 26-hydroxyl group practically prevents a subsequent 12α-hydroxylation, the observed changes in 26- and 12α-hydroxylase activities in the hyperthyroid state may explain the reversal of the ratio of cholic acid to chenodeoxycholic acid (6).

Of the three pathways discussed, microsomal 26-hydroxylation appears to be of little importance in man, as judged from the present results. It is interesting in this connection that in contrast to the rat, the cholic acid/chenodeoxycholic acid ratio in man is affected only to a small extent by the thyroid state (24).

The relative importance of the pathways involving mitochondrial 26-hydroxylation or microsomal 25-hydroxylation in man is difficult to assess at present. The efficient formation of 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol in the microsomal fraction does not necessarily indicate that a pathway involving 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol is important in vivo. The excretion in bile and feces of 5β-cholestane-$3\alpha,7\alpha,12\alpha,25$-tetrol and 5β-cholestane-$3\alpha,7\alpha,12\alpha,24,25$-pentol by patients with cerebrotendinous xanthomatosis might have two explanations. There might be a subnormal 26-hydroxylation leading to accumulation and excretion of side products that are not intermediates in the normal pathways to bile acids. The other explanation is that the two sterols are important intermediates in the normal biosynthesis of cholic acid in man and that patients with cerebrotendinous xanthomatosis have subnormal capacity to convert these compounds into cholic acid. It was recently reported in preliminary form that patients with cerebrotendinous xanthomatosis as well as normal subjects

are able to convert 5β-cholestane-3α,7α,12α,25-tetrol into cholic acid (25). It was concluded that the basic genetic defect in these patients was a lack of 26-hydroxylase activity and that the major part of the bile acids formed in these patients is formed through the alternate pathway involving 5β-cholestane-3α,7α,12α,25-tetrol. As shown in the present work, the microsomal 25-hydroxylase appears to be specific for 5β-cholestane-3α,7α,12α-triol. This finding can be considered as support for the conclusion by Mosbach and Salen (25) that patients with cerebrotendinous xanthomatosis utilize pathways to cholic acid involving 25-hydroxylation, and explains why these patients have an abnormally high cholic acid/chenodeoxycholic acid ratio in bile (13).

ACKNOWLEDGMENTS

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