A 25-Hydroxylation Pathway of Cholic Acid Biosynthesis in Man and Rat

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Abstract This paper describes a pathway of cholic acid synthesis, in man and in the rat, which involves 25-hydroxylated intermediates and is catalyzed by microsomal and soluble enzymes. The subcellular localization, stereospecificity, and other properties of the enzymes involved were studied with liver fractions of normolipidemic subjects, cerebrotendinous xanthomatosis patients, and rats.

5β-Cholestane-3α,7α,12α,25-tetrol was converted to 5β-cholestane-3α,7α,12α,24β,25-pentol by the microsomal fraction in the presence of NADPH and O_2. 5β-Cholestane-3α,7α,12α,24α,25-pentol, 5β-cholestane-3α,7α,12α,-23ξ,25-pentol, and 5β-cholestane-3α,7α,12α,25,26-pentol were also formed. In the presence of NAD, 5β-cholestane-3α,7α,12α,24β,25-pentol, but not the other 5β-cholestanepentols formed, was converted to cholic acid by soluble enzymes in good yield.

These experiments demonstrate the existence of a pathway for side-chain degradation in cholic acid synthesis which does not involve hydroxylation at C-26 or the participation of mitochondria.

INTRODUCTION

In the formation of cholic acid from cholesterol in mammalian liver, the ring system is probably hydroxylated first, forming 5β-cholestane-3α,7α,12α-triol (1). As far as is known at present, the initial reaction in the side-chain oxidation is a 26-hydroxylation of 5β-cholestane-3α,7α,-12α-triol (I, Fig. 1) (1–4). The further oxidation of 5β-cholestane-3α,7α,12α,26-tetrol (II) leads to formation of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (III) (5) which in turn is converted into cholic acid (V) via varanic acid, 3α,7α,12α,24ξ-tetrahydroxy-5β-cholestan-26-oic acid (IV) (6, 7).

Recent work in this and other laboratories has indicated that the degradation of the cholesterol side chain to cholic acid may involve intermediates hydroxylated at C-25. Of particular importance in this respect are the findings by Cronholm and Johansson in the rat (2) and by Björkhem et al. in man (8) that the major product formed during the incubation of 5β-cholestane-3α,7α,12α-triol with liver microsomes was 5β-cholestane-3α,7α,12α,-25-tetrol and not 5β-cholestane-3α,7α,12α,26-tetrol. Our own studies have shown that patients with cerebrotendinous xanthomatosis (CTX)[1] accumulate 25-hydroxylated bile alcohols in bile and feces (9), and that 5β-cholestane-3α,7α,12α,25-tetrol is transformed into cholic acid both in CTX patients and in normolipidemic subjects (10), a result previously reported by Yamada in the rat (11). While it was known, therefore, that 5β-cholestane-3α,7α,12α-triol can be converted to 5β-cholestane-3α,7α,-12α,25-tetrol in vitro and that the latter could be metabolized to cholic acid in vivo, the individual steps of this pathway remained to be demonstrated.

In the present study it was shown that in man and in the rat, liver microsomal enzymes catalyze the 24β-hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol (VI, Fig. 2) and that the resultant 5β-cholestane-3α,7α,12α,24β,25-pentol (VII) is rapidly transformed into cholic acid (V) by soluble enzymes. Since 5β-cholestane-3α,7α,12α-triol (I) is converted to 5β-cholestane-3α,7α,12α,25-tetrol by a microsomal system (2), the pathway of cholic acid

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[1] *Abbreviations used in this paper:* CTX, cerebrotendinous xanthomatosis; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; TMSi, trimethylsilyl.

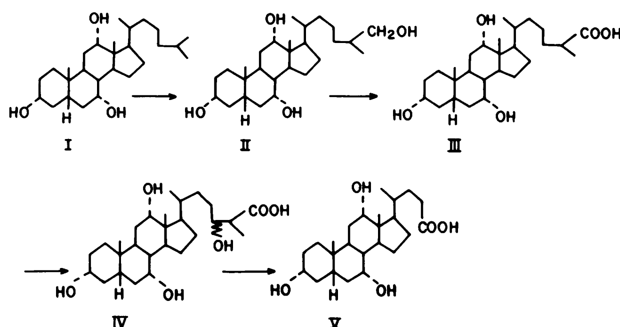

FIGURE 1 Pathway of cholic acid biosynthesis showing side-chain degradation via 26-hydroxylated intermediates. I, 5β-cholestane-3α,7α,12α-triol; II, 5β-cholestane-3α,7α,12α,26-tetrol; III, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; IV, 3α,7α,12α,24ξ-tetrahydroxy-5β-cholestan-26-oic acid; V, cholic acid.

synthesis involving 25-hydroxylated intermediates apparently does not involve the participation of mitochondrial enzymes.

EXPERIMENTAL PROCEDURES

Materials

Clinical. Studies were conducted in two normolipidemic subjects with chronic peptic ulcer disease and in two CTX subjects (J. C. and E. S.). Complete clinical descriptions of the CTX subjects have been published previously (12). The patients were hospitalized at the East Orange Veterans Administration Hospital and were fed regular hospital diets. Liver chemistries and blood coagulation tests were normal. Specimens of liver were obtained during surgery: subtotal gastrectomies with vagotomies were performed in the subjects with peptic ulcers. In the CTX subjects, liver biopsies were obtained for diagnostic light and electron microscopic examination and hepatic enzyme analysis. The experimental protocol was approved by the Human Study Committee of the East Orange Veterans Hospital and the College of Medicine and Dentistry of New Jersey, New Jersey Medical School. Informed consent was obtained before surgery. After the induction of anesthesia with Enflurane (Ohio Medical Corp., Dayton, Ohio), the abdomen was opened and liver specimens weighing 2–4 g were obtained.

Animals. Male rats of the Wistar strain weighing 200–250 g were used. They were fed Purina rat chow before the experiments.

Preparation of unlabeled compounds. 5β-Cholestane-3α,7α,12α,25-tetrol (mp 189–191°C) was prepared from cholic acid and purified as described by Dayal et al. (13). 5β-Cholestane-3α,7α,12α,24α,25-pentol (IX, Fig. 3) (mp 212–214°C) and 5β-cholestane-3α,7α,12α,24β,25-pentol (VII) (mp 203–205°C) were synthesized from 5β-cholestane-3α,7α,12α,25-tetrol as described previously (13, 14). 5β-Cholestane-3α,7α,12α,25,26-pentol (X) (mp 182–184°C; lit. mp 178.5°C [15]), was obtained during the synthesis of 5β-cholestane-3α,7α,12α,24ξ,25-pentol (13) by eluting the alumina column with 15–17% methanol in ethyl acetate. The 5β-cholestane-3α,7α,12α,25,26-pentol was crystallized from methanol/ethyl acetate or from ethyl acetate. The identity of this pentol was further confirmed by mass spectrometry of the trimethylsilyl (TMSi) derivative of the crystalline material. There was no molecular ion M; the first detectable peak was m/e 797 ($M-15$). There were three major series of peaks, the most prominent of which occurred at m/e 709 ($M-103$), 619 ($M-103-90$), 529 ($M-103-2\times 90$), 439 ($M-103-3\times 90$), and 349 ($M-103-4\times 90$). This series arises from α scission of the C-25-26 bond induced by the trimethylsiloxyl group at carbon 25, together with the loss of trimethylsilanol (90 amu) (9, 14). The other two series were at m/e 722 ($M-90$), 632 ($M-2\times 90$), 542 ($M-3\times 90$), 452 ($M-4\times 90$), and m/e 707 ($M-90-15$), 617 ($M-2\times 90-15$), 527 ($M-3\times 90-15$), 437 ($M-4\times 90-15$), 347 ($M-5\times 90-15$), which are due to successive losses of trimethylsilanol (90 amu) and, in the case of the last series, the additional loss of a methyl group (15 amu). The effect of the vicinal C-25, C-26-trimethylsiloxyl substituents was reflected in the rather prominent peaks at m/e 633, 543, 453, and 363 which are caused by the loss of the trimethylsiloxyl group alone (89 amu) (16). One of the largest peaks in the spectrum was at m/e 219 and corresponds to scission of the bond between carbons 24 and 25 induced by the trimethylsiloxyl substituent at carbon 25 (14).

5β-Cholestane-3α,7α,12α,23ξ,25-pentol (VIII, Fig. 3) (mp 210–211°C) was isolated from the feces of CTX patients, purified, and crystallized as previously described (14).

Preparation of labeled compounds. [G-^{3}H]5β-Cholestane-3α,7α,12α,24α,25-pentol, [G-^{3}H]5β-cholestane-3α,7α,12α,24β,25-pentol, and [G-^{3}H]5β-cholestane-3α,7α,12α,23ξ,25-pentol (5 mg of each) were tritiated by the Wilzbach procedure at New England Nuclear, Boston, Mass. (17), and purified by column and thin-layer chromatography (TLC), (13, 14) to constant specific radioactivity. The following specific activities were obtained: 24α-epimer, 1.78×10^4 dpm/nmol (radiopurity, 94.8%); 24β-epimer, 1.36×10^4 dpm/nmol (93.7% pure); and 5β-cholestane-3α,7α,12α,23ξ,25-pentol, 1.86×10^4 dpm/nmol (radiopurity, 92.4%).

[G-^{3}H]5β-Cholestane-3α,7α,12α,25,26-pentol was prepared enzymatically by incubating [G-^{3}H]5β-cholestane-3α,7α,12α,25-tetrol with rat liver microsomes as described in the section entitled "Enzyme Assays" (see below). 5β-Cholestane-3α,7α,12α,24α,25-pentol and 5β-cholestane-3α,7α,12α,25,26-pentol, which have identical R_f values on silica gel G, were separated by TLC on Alumina G (0.25 mm thick, Analtech, Inc., Newark, Del.) with benzene: ethyl acetate: methanol (75:20:25 [vol/vol/vol]) as a solvent system. The R_f values of 5β-cholestane-3α,7α,12α,24α,25-pentol and 5β-cholestane-3α,7α,12α,25,26-pentol were 0.51 and 0.45, re-

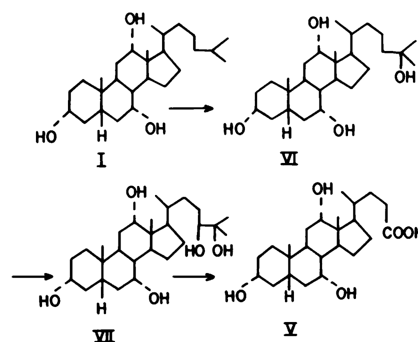

FIGURE 2 Pathway of cholic acid biosynthesis showing side-chain degradation via 25-hydroxylated intermediates. I, 5β-cholestane-3α,7α,12α-triol; VI, 5β-cholestane-3α,7α,12α,25-tetrol; VII, 5β-cholestane-3α,7α,12α,24β,25-pentol; V, cholic acid.

spectively. The latter was identified by gas-liquid chromatography (GLC) (14) and mass spectrometry. Its mass spectrum was identical with that of the synthetic 5β-cholestane-3α,7α,12α,25,26-pentol, described above. The specific radioactivity was 0.8×10^4 dpm/nmol and the radiopurity was 96.1%.

Cofactors. NAD and NADPH were purchased from Calbiochem, La Jolla, Calif.

GLC. The 5β-cholestanepentol fraction was analyzed as the TMSi derivatives on 180 × 0.4-cm columns packed with 3% QF-1 on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.), column temperature 230°C (model 7610 gas chromatograph, Hewlett-Packard Co., Palo Alto, Calif.). The retention times relative to 5β-cholestane were described previously (14).

Mass spectra. Mass spectra of the bile alcohols were obtained with a Varian MAT-111 gas chromatograph-mass spectrometer (Varian Associates, Palo Alto, Calif.) as described previously (14).

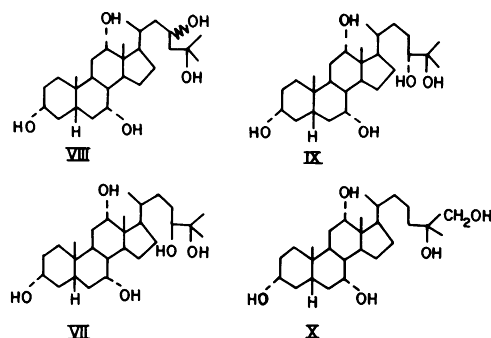

FIGURE 3 Structures of 5β-cholestanepentols. VIII, 5β-cholestane-3α,7α,12α,23ξ,25-pentol; IX, 5β-cholestane-3α,7α,12α,24α,25-pentol; VII, 5β-cholestane-3α,7α,12α,24β,25-pentol; X, 5β-cholestane-3α,7α,12α,25,26-pentol.

Methods

FRACTIONATION OF LIVER HOMOGENATES

All specimens of human liver were obtained at about 10:00 a.m. to minimize the diurnal variation in bile acid synthesis. The liver tissue was placed immediately in ice-cold 0.1 M Tris-Cl buffer, pH 7.4, containing 2.5 mM EDTA and was transported to the laboratory in an ice bath within 1 h.

In animal experiments, rats were killed by cervical dislocation and the liver was removed immediately and chilled on ice. All subsequent operations with human or rat liver were carried out at 0–4°C. The liver was extruded through a tissue press (Harvard Apparatus Co., Millis, Mass.). A 1-g aliquot was homogenized in a loose-fitting Potter-Elvehjem homogenizer with 4 ml of 0.1 M-Tris-Cl buffer, pH 7.4, containing 2.5 mM EDTA. The homogenate was centrifuged for 12 min at 800 *g*, and nuclei and cell debris were discarded. The microsomal fraction was obtained by centrifuging the 800-*g* supernatant solution for 12 min at 20,000 *g* followed by centrifugation at 100,000 *g* for 1 h (18). The microsomal pellet was washed by resuspension in 0.1 M Tris-Cl buffer, pH 7.4, with EDTA, and centrifugation for 1 h at 100,000 *g*. The final pellet was suspended in 0.1 M Tris-Cl buffer, pH 7.4, with EDTA in a volume corresponding to the original 20,000-*g* supernatant solution from which it had been prepared. The mitochondrial fraction was obtained by resuspension of the 20,000 *g* pellet in 0.25 M sucrose and centrifugation at 8,500 *g* for 12 min (19). A more highly purified mitochondrial fraction was obtained by following the procedure of Wilgram and Kennedy (20). This method results in a fraction of intact mitochondria with minimal microsomal contamination. The final mitochondrial pellet was suspended in half the volume of the original 20,000-*g* supernatant solution with 0.1 M Tris-Cl buffer, pH 7.4, with EDTA. When it was desired to carry out incubations with ruptured mitochondria, the method of Taniguchi et al. (21) was employed.

Protein was determined according to Lowry et al. (22). The protein content of the microsomal fraction was about 5 mg/ml; of the mitochondrial fraction, about 3 mg/ml; and of the 100,000-*g* supernatant fraction, about 12 mg/ml.

ENZYME ASSAYS

Conversion of 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestanepentols. 5β-Cholestane-3α,7α,12α,25-tetrol (2 μmol) in methanol was mixed with 1.5 mg of Tween-80 (Fisher Scientific Co., Springfield, N. J.) in acetone. The organic solvents were evaporated to dryness. The residue was solubilized by vigorous mixing (Vortex, Scientific Industries, Inc., Bohemia, N. Y.) in 1.0 ml of 0.1 M Tris-Cl buffer, pH 7.4. The incubation mixture contained 0.2 mM 5β-cholestane-3α,7α,12α,25-tetrol; 85 mM Tris-Cl buffer, pH 7.4; 1.0 mM EDTA; 1.0 mM NADPH; and approximately 10 mg of microsomal protein. Final volume was 10.0 ml. Zero-time controls were run with each experiment. The incubation of the entire system was carried out with shaking for 10 min at 37°C in air and was terminated by the addition of 10 ml of 10% KOH in methanol:H_2O (90:10 [vol/vol]).

The bile alcohols were extracted twice with 20 ml of ethyl acetate, washed twice with water, and evaporated to dryness. The 5β-cholestanepentols formed during the incubation were identified and quantitated by a combination of TLC and GLC (14) as follows: The bile alcohols were separated by preparative TLC on 0.25-mm-thick silica gel G plates (Analtech, Inc.) with chloroform:acetone:methanol (35:25:7.5 [vol/vol/vol]) (14). Two major bands were obtained ($R_f = 0.30$ and R_f 0.32–0.34), which were made visible with iodine vapor. The bands at R_f 0.30 (5β-cholestane-3α,7α,12α,24α,25-pentol + 5β-cholestane-3α,7α,12α,25,26-pentol) and at R_f 0.32–0.34 (5β-cholestane-3α,7α,12α,24β,25-pentol + 5β-cholestane-3α,7α,12α,23ξ,25-pentol) were removed separately from the plate and eluted with methanol. The solvent was evaporated under N_2, and the composition of each band was determined by GLC and confirmed by GLC-mass spectrometry (14). Retention time of TMSi ethers relative to 5α-cholestane were: 5β-cholestane-3α,7α,12α,23ξ,25-pentol, 3.94; 5β-cholestane-3α,7α,12α,24α,25-pentol, 4.23; 5β-cholestane-3α,7α,12α,24β,25-pentol, 4.35; and 5β-cholestane-3α,7α,12α,25,26-pentol, 4.63 (retention time of 5β-cholestane, 2.95 min).

Conversion of 5β-cholestane-3α,7α,12α,24β,25-pentol to cholic acid. In standard incubation procedures 200 nmol of the tritiated substrate were dissolved in 30 μl of acetone which was quickly dispersed into 0.2 ml of 0.1 M Tris-Cl buffer, pH 7.4. The incubation medium contained in a volume of 0.72 ml: 83 mM Tris-Cl buffer, pH 7.4; 4.2 mM NAD^+; 100,000-*g* supernatant solution containing approximately 0.5 mg of protein. The incubation was carried out by shaking in air at 37°C for 10 min. The reaction was terminated by addition of 1 ml of 2% NaOH in 95% ethanol. After addition of 1 ml water the unreacted substrate was extracted three times with 4 ml ethyl acetate. The remaining water layer was cooled on ice, acidified (pH 2) with 6

TABLE I

*Composition of Pentol Fraction after Incubation of 5β-Cholestane-3α,7α,12α,25-tetrol with Hepatic Microsomes**

Species	% of total pentol fraction in 5β-cholestane-			
	3α,7α,12α,23ξ,25-pentol	3α,7α,12α,24α,25-pentol	3α,7α,12α,24β,25-pentol	3α,7α,12α 25,26-pentol‡
Man				
Control	23.4§	10.5	40.7	25.4
CTX	18.2§	10.2	44.5	27.3
Rat	17.0‖	35.6	36.4	11.0

* Microsomal fractions were prepared and products were analyzed as described in the Methods section.
‡ 5β-Cholestane-3α,7α,12α,25,26-pentol was most probably of microsomal origin, since the microsomal fraction was minimally contaminated with mitochondria (19).
§ The numbers represent average values obtained from studies of two male normolipidemic subjects and one male and one female patient with CTX.
‖ The numbers represent average values from five animals.

N HCl, and the cholic acid formed was extracted three times with 5 ml peroxide-free ether. The combined ether layers were washed and evaporated to dryness. The residue was dissolved in 50 μl methanol and applied together with 100 μg unlabeled cholic acid on silica gel G plates, 0.25 mm thick (Analtech, Inc.). The TLC plates were developed with benzene: dioxane: glacial acetic acid, 15:5:2 (vol/vol/vol) (23), and the cholic acid spots were visualized by iodine vapor or phosphomolybdic acid (3.5% in isopropanol). The pertinent spots were removed from the plate by suction and transferred into liquid scintillation vials containing 1 ml methanol and 12 ml of dioxane/naphthalene scintillation solution. The radioactivity was determined in a liquid scintillation counter (LS-200B, Beckman Instruments, Inc., Fullerton, Calif), with suitable corrections for background and quenching. Since the specific radioactivity of the substrate was known, the radioactivity data could be expressed in terms of picomoles of cholic acid formed. All enzyme assays were carried out in duplicate. Zero-time controls were carried out with each experiment.

RESULTS

Conversion of 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestanepentols. Table I illustrates the composition of the 5β-cholestanepentol fractions obtained from incubation of 5β-cholestane-3α,7α,12α,25-tetrol with hepatic microsomal fractions of normolipidemic subjects, CTX patients, and rats. In man the major product (approximately 40%) was 5β-cholestane-3α,7α,12α,24β,25-pentol with lesser amounts of 5β-cholestane-3α,7α,12α,24α,25-pentol, 5β-cholestane-3α,7α,12α,23ξ,25-pentol, and 5β-cholestane-3α,7α,12α,25,26-pentol (Fig. 3). In the rat relatively larger amounts of the 24α-epimer were produced than in man.

The subcellular distribution of the hydroxylation system which catalyzes the conversion of 5β-cholestane-3α,-7α,12α,25-tetrol to cholestanepentols is shown in Table

TABLE II

*Subcellular Distribution of Side-Chain Hydroxylation System Acting upon 5β-Cholestane-3α,7α,12α,25-tetrol**

Fraction	Rate of production of 5β-cholestanepentols		
	Human liver		Rat liver
	Control	CTX	
		pmol/mg protein per 10 min	
Whole homogenate	317	91	2,690
Intact mitochondria‡	10	4	73
Microsomes	1,458	345	11,680
100,000-*g* supernate	436	105	70
Mitochondria + 100,000-*g* supernate	—§	—§	65
Microsomes + 100,000-*g* supernate	—§	—§	3,630

* Subcellular fractionation was performed as described in Methods.
‡ When the 5β-cholestane-3α,7α,12α,25-tetrol was incubated with partially broken rat liver mitochondria the rate of hydroxylation increased to 950 pmol/mg protein per 10 min, which was less than 10% of the rate observed with the microsomal fraction.
§ Not analyzed.

II. It was found that the hydroxylation was catalyzed predominantly by the microsomal fraction.

The side-chain hydroxylation of 5β-cholestane-3α,7α,-12α,25-tetrol required NADPH and O_2. The activity was greatly reduced when NADH was the electron donor or when oxygen was absent as illustrated in Table III.

Conversion of 5β-cholestane-3α,7α,12α,24β,25-pentol to cholic acid. Table IV illustrates the subcellular distribution of the enzyme system which transforms 5β-choles-

TABLE III

*Cofactor Requirement of the Microsomal Side-Chain Hydroxylation System Acting on 5β-Cholestane-3α,7α,12α,25-tetrol**

	Rate of formation of 5β-cholestanepentols
	pmol/mg protein per 10 min
Complete system‡	13,500
Minus NADPH	446
Minus NADPH plus NADH	2,250
Under N_2	1,275
Boiled microsomal fraction	<20

* Rat liver microsomes.
‡ Standard assay system, see Methods.

TABLE IV

*Subcellular Distribution of Hepatic Enzyme Activity Transforming 5β-Cholestane-3α,7α,12α,24,25-pentols to Cholic Acid**

Fraction	Rate of cholic acid formation: Human, Normal, 24α-Pentol‡	Human, Normal, 24β-Pentol§	Human, CTX, 24α-Pentol‡	Human, CTX, 24β-Pentol§	Rat, 24α-Pentol‡	Rat, 24β-Pentol§
			pmol/mg protein per min			
Whole homogenate	13.3	430	9.1	303	15.0	450
Mitochondria‖	1.9	73.1	1.3	42.0	2.2	72.6
Microsomes	0.8	20.7	0.5	11.9	1.2	20.6
100,000-*g* supernate	25.1	916	17.2	731	27.2	1,084
Mitochondria + 100,000-*g* supernate	5.6	203	4.3	182.7	5.4	216
Microsomes + 100,000-*g* supernate	10.5	382	6.8	282	11.0	452

* Subcellular fractionation was performed as described in Methods.
‡ 5β-Cholestane-3α,7α,12α,24α,25-pentol.
§ 5β-Cholestane-3α,7α,12α,24β,25-pentol.
‖ Partially broken mitochondria (21).

tane-3α,7α,12α,24,25-pentol to cholic acid. The two pentols epimeric at C-24 were used as substrates. The results demonstrate that the enzyme system is located predominantly in the soluble fraction and that the 24β-epimer is the preferred substrate for cholic acid formation. It was found in these experiments that only free, i.e. unconjugated, cholic acid was formed, since alkaline hydrolysis (120°C, 3 h, 10% NaOH [wt/vol]) liberated no additional amounts of cholic acid.

Additional data on the substrate specificity of the enzyme system are shown in Table V. 5β-Cholestane-3α,7α,-12α,24β,25-pentol was the only substrate tested which was transformed into cholic acid at a rapid rate. The rate of production of acidic material from the other isomeric pentols amounted to 5% or less of that observed with 5β-cholestane-3α,7α,12α,24β,25-pentol.

The requirement of the cholic acid-forming enzyme system for NAD is shown in Table VI. The enzyme was inactive in the absence of NAD^+ but 10–15% of the total activity was restored in the presence of excess $NADP^+$.

To identify and establish the radioactive purity of the biosynthetic cholic acid the assay system described in the Methods section was scaled up about 10-fold and the labeled cholic acid formed was extracted and separated by TLC. The cholic acid band was visualized with iodine vapor and eluted with methanol. The identity and radioactive purity of the biosynthetic cholic acid was determined by reverse isotope dilution of the free acid and two derivatives, as shown in Table VII. The specific

TABLE V

*Substrate Specificity of Soluble Enzyme System Transforming 5β-Cholestanepentols into Cholic Acid**

Substrate	Rate of cholic acid production: Human liver, Control	Human liver, CTX	Rat liver
		pmol/mg protein per min	
5β-Cholestane-3α,7α,12α,23ξ,25-pentol‡	33.8	40.3	37.5
5β-Cholestane-3α,7α,12α,24α,25-pentol‡	25.1	17.2	22.8
5β-Cholestane-3α,7α,12α,24β,25-pentol	916	731	983
5β-Cholestane-3α,7α,12α,25,26-pentol‡	1.9	1.5	2.6

* Standard assay system, see Methods.
‡ The small amounts of radioactivity found in the cholic acid area of the TLC plate after incubation with 5β-cholestane-3α,7α,12α,23ξ,25-pentol, 5β-cholestane-3α,7α,12α,24α,25-pentol, and 5β-cholestane-3α,7α,12α,25,26-pentol were not positively identified as cholic acid.

TABLE VI

Cofactor Requirement of Soluble Enzyme Activity Transforming 5β-Cholestane-3α,7α,12α,24β,25-pentol into Cholic Acid

Cofactors added	Rate of cholic acid formation: Human liver, Control	Human liver, CTX	Rat liver
		pmol/mg protein per min	
Complete system*	1,082	811	995
Minus NAD^+	30.7	21.3	24.1
Minus NAD^+ plus $NADP^+$ (3 μmol)	165.9	121.8	110.6
Boiled 100,000-*g* supernate	21.6	14.7	20.0

* Standard assay system, see Methods.

TABLE VII
Identification of Biosynthetic Cholic Acid Obtained from Incubation of [G-^{3}H]5β-Cholestane-3α,7α,12α,-24β,25-pentol with 100,000-g Supernatant Solution

	Specific radioactivity of cholic acid formed by liver of:	
	CTX patient	Rat
	dpm/μmol	
[G-^{3}H]Cholic acid from preparative TLC*	1,568,000	5,812,000
[G-^{3}H]Cholic acid after addition of carrier	1,612‡	4,760§
1st crystallization (ethanol/water)	1,520	4,870
2nd crystallization (ethanol/water)	1,609	4,910
Methyl cholate (methanol/water)	1,575	4,810
Methyl cholate triacetate‖	1,581	4,880

* The biosynthetic [G-^{3}H]cholic acid obtained from large scale incubation experiments of pentol with 100,000-*g* supernatant solution was separated from the incubation mixture by extraction followed by TLC (0.5-mm plates) as described in the Results section.
‡ 0.025 μmol of [G-^{3}H]cholic acid was diluted with 24.4 μmol unlabeled cholic acid.
§ 0.04 μmol of [G-^{3}H]cholic acid was diluted with 48.8 μmol unlabeled cholic acid.
‖ Specific radioactivity was determined by GLC and scintillation counting of separate aliquots.

radioactivity of the biosynthetic cholic acid remained constant within the precision of measurement (±7%).

DISCUSSION

The results of the present paper demonstrate that 5β-cholestane-3α,7α,12α-triol can be converted to cholic acid in human and rat liver in vitro, via a 25-hydroxylation pathway (Fig. 2), without the participation of 26-oxygenated derivatives. Incubation of 5β-cholestane-3α,7α,-12α,25-tetrol with the microsomal fraction under the conditions employed resulted in the formation of a mixture of 5β-cholestanepentols. When microsomes prepared from human liver, either from normolipidemic controls or CTX patients, were used, 5β-cholestane-3α,7α,12α,24β,25-pentol was the predominant component, while with rat liver microsomes 5β-cholestane-3α,7α,12α,24α,25-pentol and 5β-cholestane-3α,7α,12α,24β,25-pentol were formed in equal amounts and constituted the major products formed (Table I).

The 25-hydroxylation pathway of cholic acid biosynthesis, just like the one proceeding via 26-hydroxylated intermediates, involves the introduction of a hydroxyl group at C-24. It seems, however, that the enzymes which carry out the 24-hydroxylation are not similar for the two pathways, suggesting that different mechanisms are involved. In the 25-hydroxylation pathway, the reaction is catalyzed solely by a microsomal enzyme which was not stimulated by the addition of 100,000-*g* supernatant solution. Mitochondria and 100,000-*g* supernatant solution, either alone or in combination did not catalyze the formation of 24-hydroxylated 5β-cholestanepentols (Table II). In contrast, Masui and Staple and, more recently, Gustafsson found that the introduction of the C-24 hydroxyl group into 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (III, Fig. 1) was catalyzed either by the mitochondrial fraction (6, 24) or by the microsomal fraction fortified with 100,000-*g* supernatant solution. In the case of the microsomal fraction large amounts of ATP were required (24). Moreover, the 24-hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol (II, Fig. 2) requires O_2 and NADPH (Table III) and is probably catalyzed by a mixed function oxidase, although the participation of cytochrome P-450 was not investigated by us. Recent studies by Gustafsson (24) postulated that in the pathway involving 26-oxygenated intermediates the 24-hydroxylation step was not catalyzed by a mixed function oxidase but was analogous to fatty acid β-oxidation and involved an acyl dehydrogenase and enoyl hydrase as had also been suggested by Masui and Staple (6).

In the present studies, the rate of 5β-cholestanepentol formation was considerably higher in rat than in human liver microsomes. Hydroxylation was about four times greater in the human control subjects than in the CTX patients (Table II). The reduced rate of 24β-hydroxylation is presumably due to a relative deficiency of this hydroxylase in CTX patients as compared to the controls and may explain the accumulation of 5β-cholestane-3α,7α,12α,25-tetrol in bile and feces of the former (9). It seems unlikely that the observed difference was due to differences in tetrol concentration in the microsomal preparations since the amount of tetrol present in the microsomes was found to be negligible in comparison to the amount of substrate added to saturate the enzyme. It is therefore conceivable that the relative lack of this 24β-hydroxylase represents a major metabolic defect in CTX.

The enzyme catalyzing the transformation of 5β-cholestane-3α,7α,12α,24,25-pentol to cholic acid was located predominantly in the 100,000-*g* supernatant fraction (Table IV). This transformation proceeded at approximately equal rates in man and in the rat. In both species the reaction appeared to be quite stereospecific in that only the 24β-epimer was transformed to cholic acid at an appreciable rate (Table V). This stereospecificity and the rapid metabolism of the 24β-epimer to cholic acid might explain the fact that only 5β-cholestane-3α,7α,12α,23ξ,25-pentol and 5β-cholestane-3α,7α,12α,24α,25-pentol could be detected in bile and feces of CTX patients. We were unable to find 5β-cholestane-3α,7α,12α,24β,25-pentol, presumably because it was rapidly transformed into cholic acid (14). The soluble enzyme system required NAD^+

for the formation of cholic acid (Table VI). The reaction might involve a 24-ketone intermediate, which was however not observed in the present study. Until further work has been done it may be speculated that the desmolase which catalyzes cleavage of the side chain between carbons 24 and 25 is a dehydrogenase/hydroxylase as has been postulated for the mechanism of adrenal hormone synthesis (25).

Thus, the results of the present study suggest that there exists an alternate pathway of cholic acid biosynthesis in man and in the rat involving the 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol (I, Fig. 2). This pathway proceeds via 5β-cholestane-3α,7α,12α,25-tetrol (VI) and 5β-cholestane-3α,7α,12α,24β,25-pentol (VII) and does not involve 5β-cholestanoic acids as intermediates. While it is known that the 25-hydroxylation pathway is important in the formation of cholic acid in patients with CTX, the quantitative significance of 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol for cholic acid synthesis in normal man and in the rat remains to be evaluated.

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