Biosynthesis of Chenodeoxycholic Acid in Man

STEREOSPECIFIC SIDE-CHAIN HYDROXYLATIONS OF 5β-CHOLESTANE-3α,7α-DIOL

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ABSTRACT Stereospecific side-chain hydroxylations of 5β-cholestane-3α,7α-diol were studied in mitochondrial and microsomal fractions of human liver. Incubation of 5β-cholestane-3α,7α-diol resulted in hydroxylations at C-12, C-24, C-25, and C-26. Hydroxylations at C-24 and C-26 were accompanied by the introduction of additional asymmetric carbon atoms at C-24 and C-25 respectively, that led to the formation of two distinct pairs of diastereoisomers, namely 5β-cholestane-3α,7α,24-triols (24R and 24S) and 5β-cholestane-3α,7α,26-triols (25R and 25S). A sensitive and reproducible radioactive assay to measure the formation of the different biosynthetic 5β-cholestanetriols was developed. Optimal assay conditions for human mitochondrial and microsomal systems were tentatively established.

The mitochondrial fraction was found to predominantly catalyze the 26-hydroxylation of 5β-cholestane-3α,7α-diol with the formation of the 25R-diastereoisomer of 5β-cholestane-3α,7α,26-triol as the major product. In the microsomal fraction, on the other hand, 25-hydroxylation was more efficient than 26-hydroxylation and accounted for 6.4% of the total hydroxylations. The microsomes catalyzed the formation of both diastereoisomers of 5β-cholestane-3α,7α,26-triol (25R and 25S, 4.2 and 1.6% respectively).

These experiments suggest that the initial step in the degradation of the steroid side chain during the biosynthesis of chenodeoxycholic acid in man is mediated by the mitochondria, and involves the formation of the 25R-diastereoisomer of 5β-cholestane-3α,7α,26-triol. The role of the microsomal 25- and 26-hydroxylated intermediates requires further exploration.

INTRODUCTION

5β-Cholestane-3α,7α-diol has been postulated as a key intermediate in the biosynthesis of chenodeoxycholic acid (1).[1] The sequence of reactions that leads to the formation of 5β-cholestane-3α,7α-diol in man has been studied (2), but the mechanism of degradation of the side chain has not been completely established. There are indications that one mechanism of side-chain degradation in man involves 26-hydroxylation, because 3α,7α-dihydroxy-5β-cholestanoic acid has been isolated from human bile and has been shown to be formed from cholesterol and metabolized into chenodeoxycholic acid (3). Recently, a 25-hydroxylation pathway of cholic acid[1] synthesis in man has been demonstrated (4), which suggests that side-chain degradation might start with a 25-hydroxylation step. Studies, in vitro, with preparations from human liver (5) show that the 26-hydroxylation of 5β-cholestane-3α,7α-diol is catalyzed very efficiently by the mitochondria, whereas only very small 26-hydroxylase activity could be detected in the microsomal fraction. The microsomal 25-hydroxylase system was very active with 5β-cholestane-3α,7α,12α-triol as a substrate but exhibited much lower activity with the corresponding -3α,7α,-diol. Although hydroxylation of 5β-cholestane-3α,7α-diol at C-26 yields an additional asymmetric

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

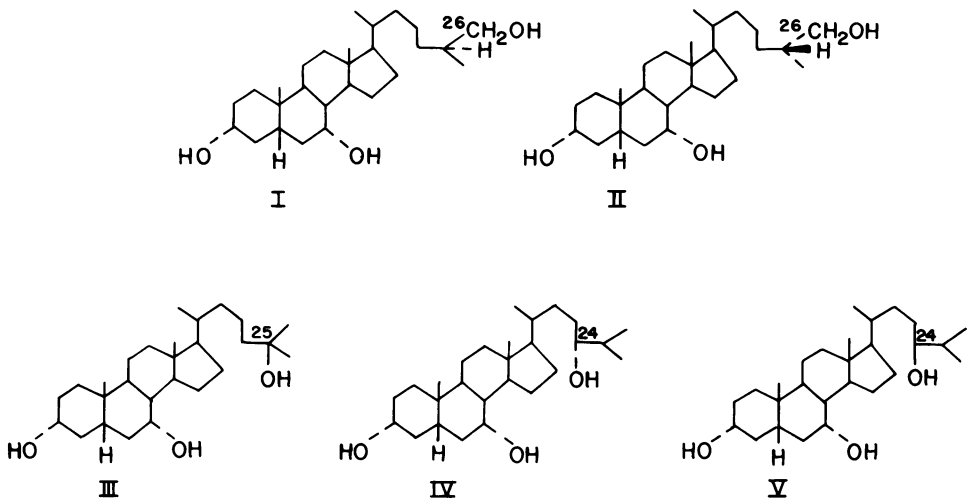

FIGURE 1 Structures of 5β-cholestanetriols: I, 5β-cholestane-3α,7α,26-triol (25R); II, 5β-cholestane-3α,7α,26-triol (25S); III, 5β-cholestane-3α,7α,25-triol; IV, 5β-cholestane-3α,7α,24-triol (24R); and V, 5β-cholestane-3α,7α,24-triol (24S).

carbon atom at C-25, that forms two stereochemically distinct isomers, 25R and 25S, of 5β-cholestane-3α,7α, 26-triol (I, II, Fig. 1), none of the studies mentioned above attempted to determine the stereospecificity of this hydroxylation. Previous studies on the stereospecificity of the 26-hydroxylation were carried out with cholesterol (6) and 5β-cholestane-3α,7α,12α,26-tetrol (7) as substrates.

It is the purpose of this paper to study side-chain hydroxylations of 5β-cholestane-3α,7α-diol in man, in vitro, with special emphasis on the stereospecificity of the mitochondrial and microsomal 26-hydroxylation.

METHODS

Materials

Clinical procedures. Liver tissue was obtained from nine male normolipidemic subjects (38—65 yr of age) with chronic peptic ulcer disease. The patients were hospitalized at the East Orange, N. J. Veterans Administration Hospital, and were fed regular hospital diets. Specimens of liver were obtained during surgery (8), and immediately immersed in an ice-cold 0.25-M sucrose solution. Liver chemistries and blood coagulation tests were normal. The experimental protocol was approved by the Human Study Committee of the East Orange Veterans Administration Hospital and the College of Medicine and Dentistry of New Jersey, New Jersey Medical School. Informed consent was obtained before surgery.

Preparation of unlabeled compounds. 5β-Cholestane-3α, 7α-diol (melting point: 87–88°C) and 5β-cholestane-3α,7α, 12α-triol (melting point: 184–186°C) were a gift from Dr. I. Björkhem.

25R- and 25S-5β-cholestane-3α,7α,26-triols (I, II, Fig. 1) and 24R- and 24S-5β-cholestane-3α,7α,24-triols (IV, V) were synthesized according to Dayal et al., and the chirality at C-24 and C-25 was assigned tentatively (9). 5β-Cholestane-3α,7α,25-triol (III) was synthesized according to Lettré et al. (10).

Preparation of labeled 5β-[G-^{3}H]cholestane-3α,7α-diol. 5β-[G-^{3}H]Cholestane-3α,7α-diol was prepared from the unlabeled diol by the Wilzbach technique (New England Nuclear; Boston, Mass.) (11). The labeled substrate was purified by column and thin-layer chromatography (TLC)[2] as previously described (12, 13), to constant specific radioactivity (1.61×10^7 dpm/μmol; radiopurity 98.8%).

Cofactors. NADPH and DL-isocitrate were purchased from Calbiochem (San Diego, Calif.).

Gas-liquid chromatography (GLC)-mass spectrometry. The 5β-Cholestanetriols, as the trimethylsilyl derivatives, were analyzed by GLC-mass spectrometry as previously described (9) (Hewlett-Packard Co., Palo Alto, Calif., model no. 7610 gas chromatograph; and Varian MAT-III gas chromatograph-mass spectrometer, Varian Associates, Palo Alto, Calif.).

Methods

Fractionation of liver homogenates. Liver specimens were obtained at about 10 a.m. to minimize possible effects of diurnal variations. All of the manipulations described in this section were performed at 4°C. The tissue was minced with a razor blade and homogenized for 40 s in 0.25 M sucrose (4 ml/1 g of tissue) with a Potter-Elvehjem homogenizer, loosely fitted with a Teflon (E. I. Du Pont deNemours & Co., Inc., Wilmington, Del.) pestle. The homogenate was centrifuged at 800 *g* for 12 min to remove nuclei and cell debris. The mitochondrial fraction was obtained by centrifuging the 800-g supernate for 12 min at 8,500 *g* and by washing as described by Wilgram and Kennedy (14). The final pellet was suspended in ice-cold 0.25 M sucrose in a volume of 0.5 ml/1 g liver. The purity of the mitochondrial pellet was determined with the microsomal glucose-6-phosphatase (15) as a marker enzyme, and it was found to be contaminated to about 3% with microsomes. The microsomal fraction was obtained by centrifuging the

[2] *Abbreviations used in this paper*: GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

8,500 *g* supernatant solution for 12 min at 20,000 *g*. The precipitate was discarded and the supernatant solution was centrifuged at 100,000 *g* for 1 h (16). The microsomal pellet was washed and resuspended in 0.25 M sucrose in a vol of 1 ml/1 g liver.

Protein was determined according to Lowry et al. (17). The protein content of the mitochondrial and microsomal fractions was approximately 10 mg/ml.

Standard enzyme assays and analysis of incubation mixtures. In standard incubations with the mitochondrial fraction, 85 mM-phosphate buffer (pH 7.4), 1.7 mM $MgCl_2$, 4 mM DL-isocitrate, and 0.1 ml of the mitochondrial fraction that contained 1–1.5 mg protein in a final volume of 1.0 ml were preincubated for 5 min at 37°C (18). The reaction was initiated by the addition of 5β-[G-^{3}H]cholestane-3α,7α-diol (100 nmol, sp act 1.61×10^7 dpm/μmol) in 15 μl acetone. In standard incubations with the microsomal fractions the same procedure was followed with the exception that NADPH (3 mM) was used as the sole coenzyme.

Incubations with either microsomal or mitochondrial systems were carried out with shaking for 15 min at 37°C in air, and were terminated by the addition of 0.1 ml 1 N HCl. All enzyme assays were carried out in duplicate, and zero time controls were run with each experiment. The unreacted-5β-cholestane-3α,7α-diol and the reaction products were immediately extracted with 2×5.0 ml ethyl acetate, shaking it for 5 min each time. The combined ethyl acetate extracts were washed twice with water, and evaporated to dryness under N_2. For routine assay, the 5β-cholestanetriols formed during the incubation were identified and quantitated by a combination of TLC and liquid-scintillation counting as follows: The bile alcohols were separated by TLC on 0.25 mm-thick alumina plates (Analtech, Inc., Newark, Del.) and developed twice: first with chloroform:acetone:ethanol, 35:25:3.5 (vol/vol/vol), and next with benzene:ethyl acetate: methanol, 90:20:7 (vol/vol/vol). The second development was necessary only when the retardation factor values obtained were too low to assure adequate separations. Activation of the TLC plate at 105°C for 15 min was required, and samples were applied when the plate had cooled to 40°C on a constant temperature hot plate. Unlabeled 5β-cholestane-3α, 7α-diol and 5β-cholestanetriols (10 μg each) were applied with the extracts and to each side of the plate as markers. The markers along the sides of the plate were made visible with spray reagent that consisted of 3.5% phosphomolybdic acid in isopropanol. Retardation factor values of the reference compounds used were: 5β-cholestane-3α,7α,12α-triol, 0.37; 5β-cholestane-3α,7α,26-triol (25R), 0.44, 5β-cholestane-3α,7α, 26-triol (25S), 0.51; 5β-cholestane-3α,7α,25-triol + 5β-cholestane-3α,7α,24R-triol, 0.61; 5β-cholestane-3α,7α,24S-triol, 0.70; and 5β-cholestane-3α,7α-diol, 0.86. To separate 5β-cholestane-3α,7α,24R-triol from 5β-cholestane-3α,7α,25-triol, the mixture that contained these two compounds was reapplied to a 0.25-mm thick silica gel G plate (Brinkmann Instruments Inc., Westbury, N. Y.), by use of a finely-drawn micropipette to keep spot size small, and developed twice with benzene:acetone:methanol, 70:50:1.5 (vol/vol/vol). Retardation factor values of 5β-cholestane-3α,7α,24R-triol and 5β-cholestane-3α,7α,25-triol were 0.58 and 0.54, respectively.

Enzyme activities were calculated by removing individual spots from the TLC plates and measuring their radioactivity in a liquid scintillation counter (Beckman LS-200B, Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Identification of the 5β-cholestanetriols formed from 5β-[G-^{3}H]-cholestane-3α,7α-diol. The assay system was scaled up 10-fold, and the labeled products formed during a 1-h incubation period were extracted and subjected to TLC on alumina without addition of carriers (see Methods). The bands that corresponded to known triol reference compounds were eluted with methanol and analyzed by GLC-mass-spectrometry. Fig. 2A illustrates the mass spectrum of the biosynthetic 5β-cholestane-3α,7α,26-triol (25R) obtained from a large scale incubation of human hepatic mitochondria which was identical with that of the authentic compound (Fig. 2B) (9). The other biosynthetic bile alcohols were identified in the same manner. (The two pairs of diastereoisomeric bile alcohols, namely 5β-cholestane-3α,7α,24-triol [24R and 24S] and 5β-cholestane-3α,7α,26-triol [25R and 25S], which were clearly resolved on TLC, had identical mass spectra (9).)

Radioactive purity of reaction products. To establish the radioactive purity of the individual biosynthetic 5β-cholestanetriols, a known aliquot from the pertinent band (see above) was applied with 50 μg of carrier on alumina G plate and developed with chloroform:acetone:methanol, 35:25:3.5 (vol/vol/vol). After elution with methanol and analysis of an aliquot by GLC and liquid-scintillation counting, the sample was chromatographed again on alumina G plate with a different solvent system, benzene:ethyl acetate:methanol, 90:20:7 (vol/vol/vol). After elution, the triol was chromatographed a third time on silica-gel G with chloroform:acetone:methanol, 70:50:5 (vol/vol/vol). Each time the specific radioactivity was determined by GLC and liquid-scintillation counting. Table I summarizes the data that deals with 5β-cholestane-3α, 7α,26-triol (25R) isolated from incubations of human hepatic mitochondria. The specific radioactivity remained constant within the precision of measurement (±7%) during repeated chromatography. The other bile alcohols, formed during the incubation of 5β-[G-^{3}H]cholestane-3α,7α-diol by the mitochondrial and microsomal fractions, yielded similar results.

Optimal conditions of hepatic mitochondrial and microsomal hydroxylations of 5β-cholestane-3α,7α-diol. 5β-[G-^{3}H]Cholestane-3α,7α-diol was incubated under various conditions with liver mitochondrial and microsomal fractions. The corresponding labeled 5β-cholestanetriols formed were separated by TLC and their radioactivity was determined as described above. In both cases, the enzyme systems appeared to be saturated when the concentration of the substrate was 100 nmol/ml. The rate of formation of the different 5β-cholestanetriols formed was proportional to incubation time during 15 min for the 24-hydroxylation and 30 min for the 12-, 25- and 26-hydroxylations. The relationship between reaction rates and enzyme concentrations for the mitochondrial and microsomal fractions was linear with respect to protein concentration up to 1.5 mg protein (Figs. 3A

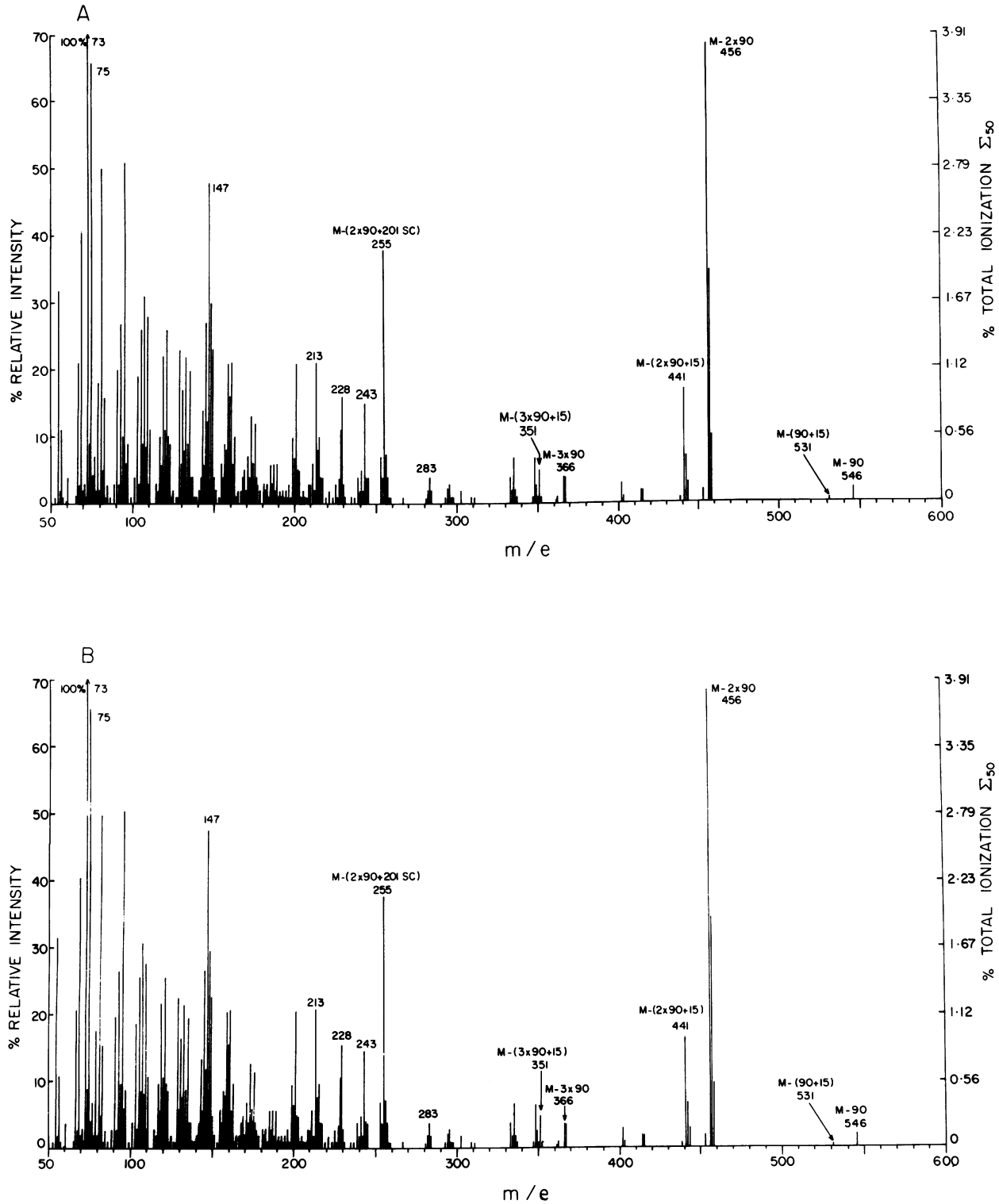

FIGURE 2 Mass spectrum of the biosynthetic 5β-cholestane-3α,7α,26-triol (25R) obtained from a large scale incubation experiment with human liver mitochondria (A) compared with the authentic compound (B).

TABLE I

*Radioactive Purity of Biosynthetic 5β-[G-^{3}H]Cholestane-3α,7α,26-triol (25R) Obtained from Incubation of 5β-[G-^{3}H]Cholestane-3α,7α-diol with Human Hepatic Mitochondria**

TLC	Specific radioactivity in 5β-cholestane-3α,7α,26-triol (25R) after TLC
	dpm/μg
Alumina G‡	387
Alumina G§	362
Silica gel G‖	376

* Determined as described in Results.

‡ The sample was applied with 50 μg of carrier on 0.25 mm-thick alumina G (Analtech, Inc.) plates and developed twice with chloroform:acetone:methanol, 35:25:3.5 (vol/vol/vol).

§ The sample was reapplied on 0.25 mm-thick alumina G plates and developed twice with benzene:ethyl acetate:methanol, 90:20:7 (vol/vol/vol).

‖ The sample was reapplied on 0.25 mm-thick silica gel G plates and developed with chloroform:acetone:methanol, 70:50:5 (vol/vol/vol).

and B). In experiments which measured the formation of all 6 5β-cholestanetriols, a reaction time of 15 min and a mitochondrial or microsomal protein concentration of 1.0 mg/ml was chosen to assure optimal assay conditions. With these conditions we were able to compare the relative rates of hydroxylations of different carbon atoms in human liver preparations.

Incubation of 5β-[G-^{3}H]cholestane-3α,7α-diol with hepatic mitochondrial and microsomal fractions. Table II illustrates the conversion of 5β-[G-^{3}H] cholestane-3α,7α-diol into 5β-cholestane-3α,7α,26-triol, (25R and 25S), 5β-cholestane-3α,7α,25-triol, and other 5β-cholestanetriols by human hepatic mitochondria and microsomes. In the mitrochondria, the major product was the 25R diastereoisomer of 5β-cholestane-3α,7α-26-triol (71% of total triols), while in the microsomes 25-hydroxylation was higher (6.4%) than the hydroxylations at C-24 and C-26. Furthermore, the 26-hydroxylation catalyzed by the microsomal fraction resulted in the formation of the two diastereoisomers of 5β-cholestane-3α,7α,26-triol and amounted to 4.2 and 1.6% of the total hydroxylations for the 25R- and 25S-diastereoisomers, respectively. It should be noted that the major product formed by the microsomal fraction was 5β-cholestane-3α,7α,12α-triol (85% of total triols).

DISCUSSION

The results of this paper demonstrate that, in man, hepatic mitochondria catalyze predominantly the 26-hydroxylation of 5β-cholestane-3α,7α-diol, producing the 25R diastereoisomer of 5β-cholestane-3α,7α,26-triol (I, Fig. 1). In contrast, the main side-chain hydroxylation product formed by hepatic microsomes

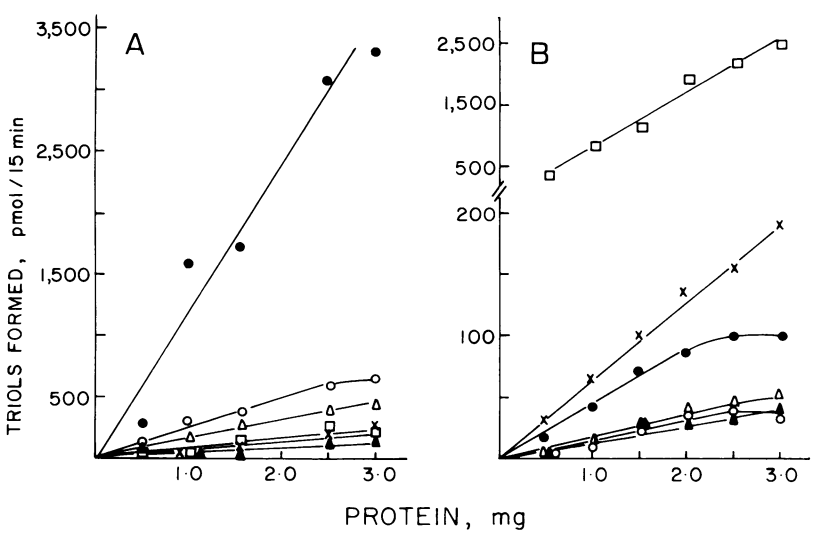

FIGURE 3 Effect of enzyme concentration on hydroxylation of 5β-[G-^{3}H]cholestane-3α,7α-diol by human liver mitochondria (A) and microsomes (B). Standard assay conditions were employed, except for protein concentration. □ —— □, 5β-cholestane-3α,7α,12α-triol; ▲ —— ▲, 5β-cholestane-3α,7α,24-triol (24R); △ —— △, 5β-cholestane-3α,7α,24-triol (24S); × —— ×, 5β-cholestane-3α,7α,25-triol; ● —— ●, 5β-cholestane-3α,7α,26-triol (25R), and ○ —— ○, 5β-cholestane-3α,7α,26-triol (25S).

TABLE II

Conversion of 5β-[G-^{3}H]Cholestane-3α,7α-diol to 5β-Cholestanetriols by Hepatic Mitochondria and Microsomes‡*

5β-Cholestanetriols formed	Rate of 5β-cholestanetriol formation	
	Mitochondria	Microsomes
	pmol/mg protein/15 min	
3α,7α,12α-triol	80.0±2.49§	829.5±12.85§
3α,7α,24-triol (24R)	37.9±1.43	12.6±0.66
3α,7α,24-triol (24S)	169.5±4.82	15.02±0.71
3α,7α,25-triol	70.9±3.33	63.0±2.07
3α,7α,26-triol (25R)	1609.5±20.8	41.3±1.69
3α,7α,26-triol (25S)	286.6±7.67	16.05±0.60

* The convesion of the randomly labeled 5β-[G-^{3}H]cholestane-3α,7α-diol was compared to that of the specifically-labeled 5β-[7β-^{3}H]cholestane-3α,7α-diol (13) in rat liver mitochondria. Little tritium was lost during the side-chain hydroxylations of the randomly labeled substrate (<5%) except in the case of the 26-hydroxylation (≅30%).

‡ Mitochondrial and microsomal fractions were prepared and products were analyzed as described in Methods. Standard assay conditions were employed.

§ Each value represents the average of five experiments with human hepatic subcellular fractions±SEM.

is 5β-cholestane-3α,7α,25-triol (III) and, to a smaller extent, the 25R and 25S diastereoisomers of 5β-cholestane-3α,7α,26-triol. Presumably, in the synthesis of chenodeoxycholic acid in biological systems, hydroxylation of 5β-cholestane-3α,7α-diol leads to the formation of only one diastereoisomer of 5β-cholestane-3α,7α,26-triol, 25R or 25S, which is further oxidized, yielding a C_{27}-acid, 3α,7α-dihydroxy-5β-cholestanoic acid which has the identical configuration at C-25. This assumption is based on a number of analyses of bile from Alligator mississippiensis (19) and from man (20), in which only a single diastereoisomer (25R) of 3α,7α,12α-trihydroxy-5β-cholestanoic acid, the analogous C_{27}-acid in the cholic acid pathway, was isolated. However, the configuration of 3α,7α-dihydroxy-5β-cholestanoic acid isolated either from human bile (3) or from the bile of Alligator mississippiensis (21) has not been established so far, although both isomeric forms of this bile acid have been synthesized and characterized (22).

Recently, an alternate pathway of cholic acid synthesis in man and in the rat has been described which involves 25-hydroxylated intermediates and is catalyzed by microsomal and soluble enzymes (4). By analogy, a 25-hydroxylation pathway can be postulated in the chenodeoxycholic acid biosynthesis.

The availability of methods for the preparation and identification of the various 5β-cholestanetriols (9) has now enabled us to study in greater detail side-chain hydroxylations in subcellular fractions of man, in vitro, using an isotope incorporation procedure. The sensitivity of the method is such that 10 pmol of any 5β-cholestanetriol formed can be detected (Table II).

The mitochondrial 26-hydroxylation was stereospecific in that the major product was the 25R-diastereoisomer of 5β-cholestane-3α,7α,26-triol that amounted to 71% of the total hydroxylations. The ratio of 25R:25S diastereoisomers of 5β-cholestane-3α,7α,26-triol was about 6:1. There was also some hydroxylation at C-24 (24S, 7.5%), but the importance of 5β-cholestane-3α,7α,24-triol as an intermediate in chenodeoxycholic acid synthesis is not known. In addition, 5β-cholestane-3α,7α,25-triol was formed to a small extent (3%). Although the mitochondrial fraction was contaminated with microsomes to about 3%, the microsomal hydroxylations were minimal under the conditions employed because the intramitochondrial NADPH generated via NADP-dependent isocitrate dehydrogenase (23) cannot be utilized by the different hydroxylating systems present in the microsomes.

In the microsomes, hydroxylation at C-26 was lower than at C-25 and the two diastereoisomers (25R and 25S) of 5β-cholestane-3α,7α,26-triol were formed in a ratio of 2.5:1. Small quantities of 5β-cholestane-3α,7α,24-triol (24R, 1.3% and 24S, 1.5%) were also detected.

In all the studies described above, a randomly tritiated substrate was used, although specifically tritiated materials are usually preferred (24). However, 5β-[G-^{3}H]cholestane-3α,7α-diol could be prepared with a higher specific radioactivity than the 7β-^{3}H-labeled diol and was therefore used when enzyme activities were relatively low and maximum sensitivity was required for the assay system. In the present case, with rat liver mitochondria (see footnote to Table II), it was found that the amounts of 5β-cholestanetriols calculated from the specific activities of G-^{3}H- and 7β-^{3}H-labeled substrates were similar except in the case of 5β-cholestane-3α,7α,26-triol, where [^{3}H] was lost from a terminal carbon. This observation agrees with previous findings (8). By taking the above findings into consideration, the mitochondrial 5β-cholestane-3α,7α,26-triol would amount to 74% for the 25R-isomer and 13% for the 25S-isomer, while the microsomal 5β-cholestane-3α,7α,26-triol (25R and 25S) would be 5.3 and 2.1% of the total hydroxylations, respectively.

Thus, the high rate and stereospecificity of the 26-hydroxylation of 5β-cholestane-3α,7α-diol by human liver mitochondria, suggest that the initial step in the degradation of the steroid side-chain during the biosynthesis of chenodeoxycholic acid in man, is the formation of the 25R-diastereoisomer of 5β-cholestane-

3α,7α,26-triol, catalyzed by the mitochondria. The role of the microsomal 5β-cholestane-3α,7α,25-triol and 5β-cholestane-3α,7α,26-triols (25R and 25S) appears to be of little importance.

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