Stereospecific Side Chain Hydroxylations in the Biosynthesis of Chenodeoxycholic Acid*

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The present studies demonstrate that in rat, guinea pig, and rabbit, liver mitochondrial enzymes catalyze predominantly the formation of the 25R diastereomer of 5α-cholestane-3α,7α,26-triol, while the microsomal enzymes produce mainly 5β-cholestane-3α,7α,25-triol.

EXPERIMENTAL PROCEDURES

Materials

Animals—The rats used were males of the Wistar strain weighing 200 to 250 g. Male guinea pigs were obtained from the Otisville Laboratories of the New York City Health Department and weighed about 250 g. The rabbits used were male New Zealand Whites weighing about 1 kg. All animals were given food and water ad libitum prior to being killed.

Preparation of Unlabeled Compounds—5β-Cholestane-3α,7α-diol (m.p. 87–88°C) and 5β-cholestane-3α,7α,12α-triol (m.p. 184–186°C) were a gift from Dr. I. Björkhem, Karolinska Institutet, Stockholm, Sweden (7, 8). 5β-Cholestane-3α,7α,25-triol was synthesized according to Lettré et al. (9). 24R and 24S 5β-cholestane-3α,7α,24-triols (24) and 25R and 25S 5β-cholestane-3α,7α,26-triols were synthesized according to Dayal et al. (6), and the chirality at C-24 and C-25 was assigned tentatively.

Preparation of Labeled 5β-[14C]Cholesterol-3α,7α-diol—5β-[14C]-
Stereoactive Hydroxylations of 5β-Cholesterol-3α,7α-diol

Fig. 1. Conversion of 5β-cholesterol-3α,7α-diol to the corresponding 5β-cholenetriol by hepatic microsomal or mitochondrial fractions. A, 5β-cholesterol-3α,7α-diol; B, 5β-cholesterol-3α,7α,24-triol (24β); C, 5β-cholesterol-3α,7α,24,25-triol (24β,25β); D, 5β-cholesterol-3α,7α,26-triol; E, 5β-cholesterol-3α,7α,26,27-triol; F, 5β-cholesterol-3α,7α,26-triol (26α,27α); G, 5β-cholesterol-3α,7α,26,27-triol (26α,27α,25β).

Methods

Fractionation of Liver Homogenates—Rats and guinea pigs were killed by cervical dislocation and rabbits by air embolism, and their livers were removed rapidly and transferred to beakers immersed in crushed ice. All liver specimens were obtained at about 10:00 a.m. to minimize possible effects of diurnal variations. The tissue fractionation procedures and all subsequent operations were carried out at 0-4°C. Portions of the chilled livers were extruded through a tissue press (Harvard Apparatus Co., Millis, MA) and homogenized for about 40 s in ice-cold 0.25 M sucrose (8 ml per 2 g of fresh tissue) with a Potter-Elvehjem homogenizer loosely fitted with a Teflon pestle. The homogenate was centrifuged at 800 x g for 12 min to remove nuclei and cell debris. The mitochondrial fraction was obtained by centrifugation of the 800 x g supernatant at 8,500 x g for 1 h and washed twice as described by Wilgram and Kennedy (13). The final pellet was suspended in ice-cold 0.25 M sucrose in a volume of 0.5 ml per 1 g of liver. The mitochondrial fraction was obtained by centrifuging the 8,500 x g supernatant solution for 12 min at 20,000 x g. The precipitate was discarded, and the supernatant solution was centrifuged at 100,000 x g for 1 h (14). The microsomal pellet was washed by resuspension in 0.25 M sucrose and centrifugation at 1 h 100,000 x g. The final pellet was suspended in 0.25 M sucrose in a volume of 1 ml per 1 g of liver.

Protein was determined according to Lowry et al. (15). The protein content of the mitochondrial and microsomal fractions was approximately 10 mg/ml.

Standard Enzyme Assays and Analyses of Incubation Mixtures—In standard incubations with the mitochondrial fraction, 85 nm phosphate buffer, pH 7.4, 1.7 mM MgCl₂, 4 mM dioisocitrate, and 1 ml of the mitochondrial fraction containing 1 to 1.5 mg of protein in a final volume of 1.0 ml were preincubated for 5 min at 37°C (16). The reaction was initiated by the addition of 5β-[3H]-cholestanetriol (100 nM, specific activity 1.61 X 10⁶ dpm/nmol) in 15 μl of acetone. The aceton solution was injected below the surface of the aqueous incubation medium with a 25-μl Hamilton syringe. In some cases the isocitrate was replaced by 3 mM NADPH. 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.

Enzymes with either microsomal or mitochondrial systems were carried out with shaking for 15 min at 37°C and were terminated by the addition of 0.5 ml of 1 M HC1. At the end of the reaction the assays were carried out in duplicate, and zero time controls were run with each experiment. The unreacted 5β-cholesterol-3α,7α-diol and the reaction product were immediately extracted with 2 X 5.0 ml of ethyl acetate, shaking for 5 min each time. The combined ethyl acetate extracts were washed twice with water and evaporated to dryness under N₂.

For routine assay the 5β-cholenetriol was formed during the incubation was separated by TLC on 0.25-mm-thick alumina plates (Analtech, Inc., Newark, DE), solvent system chloroform:acetone (73 v/v) and on 0.25-mm thick Silica Gel H plates (Analtech, Inc., Newark, DE), solvent system benzene:ethyl acetate (73 v/v). The material was further purified by TLC on Neutral aluminum oxide grade III (Bio-Rad Laboratories, Richmond, CA) with increasing concentrations of ethyl acetate-benzene (11). The material was further purified by TLC on 0.25-mm thick Silica Gel G plates (Analtech, Inc., Newark, DE), solvent system chloroform:acetone:ethanol (70-50-1.5 v/v/v) and on 0.25-mm thick Silica Gel G plates (Analtech, Inc., Newark, DE), solvent system chloroform:acetone:ethanol (70:50-1.5 v/v/v) to constant specific radioactivity (2 X 10⁻¹⁶ dpm/mg, radioactivity 98.8%).

Cofactors and Inhibitors—NADPH and D,L-isocitrate were purchased from Calbiochem (La Jolla, CA). Aminyl, antmycin A, rotenone, and dicumarol were purchased from Sigma Chemical Co. (St. Louis, MO).

GLC—The 5β-cholenetriol was analyzed as the trimethylsilyl derivative on a column (180 cm x 1 mm) packed with 3% QP 1 on 80-100 mesh Gas-Chrom Q, column temperature 230°C, N₂ flow 40 ml/min (Hewlett-Packard, Palo Alto, CA, model 7610 gas chromatograph). The retention times relative to 5β-cholesterol (2.65 min) were: 5β-cholesterol-3α,7α-diol, 2.96; 5β-cholesterol-3α,7α,24-triol (24β), 3.29; 5β-cholesterol-3α,7α,24-triol (24β,25β), 3.33; 5β-cholesterol-3α,7α,26-triol (26α,27α), 3.35; 5β-cholesterol-3α,7α,26-triol (26α,27α,25β), 3.35, 5β-cholesterol-3α,7α,26,27-triol (26α,27α,25β), 3.03, and 5β-cholesterol-3α,7α,12α,24-triol, 1.64. The isomeric pairs, 5β-cholesterol-3α,7α,26,27-triol (26α,27α) and 5β-cholesterol-3α,7α,26,27-triol (26β,27β) cannot be separated by GLC under the conditions employed.

Mass Spectra—Mass spectra of the bile alcohols were obtained with a Varian MA 1111 gas chromatograph-mass spectrometer (Varian Associates, Palo Alto, CA) at an ion source pressure of 2 to 3 X 10⁻⁶ mm and an electron energy of 70 eV, as described previously (12).

Results

Properties of the Mitochondrial Assay System

A. Optimal Conditions—5β-[3H]-Cholesterol-3α,7α-diol was incubated under various conditions with liver mitochondrial fractions of rat, guinea pig, and rabbit. The corresponding labeled 5β-cholenetriols formed were separated by TLC, and their radioactivity was determined as described under "Methods.

The relationship between reaction rates and enzyme concentrations for each species is illustrated in Fig. 3, a, b, and c. In all cases, reaction rates were linear with respect to protein concentration up to 1.5 mg of protein during a 15 min with a substrate concentration of 100 nmol/ml.

B. Cofactors: NADPH, Isocitrate—Table I illustrates the effect of substituting NADPH for isocitrate (generator of intramitochondrial NADPH) upon the 26-hydroxylation of

1. The abbreviations used are: TLC, thin layer chromatography; GLC, gas-liquid chromatography.

2. V. Steric effects on the metabolic fate of cholesterol-3α,7α-dihydroxy-25R,26S,27R-triol (25R,26S,27R). The abbreviations used are: TLC, thin layer chromatography; GLC, gas-liquid chromatography.
Stereospecific Hydroxylations of 5β-Cholestane-3α,7α-diol

Fig. 2. Thin layer chromatogram of the 5β-cholestanetriols obtained from an incubation of 5β-[G-3H]cholestone-3α,7α-diol with mammalian subcellular liver fractions. Samples were applied with authentic markers on 0.25-mm-thick alumina G (Analtech) plates and developed twice, first with chloroform:acetone:methanol, 35:25:3.5 (v/v/v) and next with benzene:ethyl acetate:methanol, 90:20:7 (v/v/v). The bile alcohols have been designated by the same numerals as in Fig. 1. RF values of the reference compounds used were: Z, 5β-cholestone-3α,7α-diol, 0.86; ZZ, 5β-cholestone-3α,7α,24-triol (24S), 0.70; III and IV, 5β-cholestone-3α,7α,24-triol (24R) + 5α-cholestone-3α,7α,25-triol, 0.61; V, 5β-cholestone-3α,7α,26-triol (25S), 0.51; VI, 5β-cholestone-3α,7α,26-triol (25R), 0.44; VII, 5β-cholestone-3α,7α,12α-triol, 0.37. Compounds III and IV are not separated on alumina G plates and must be rechromatographed on Silica Gel G (0.25 mm thick) with the solvent system benzene:acetone:methanol, 70:50:1.5 (v/v/v). RF values of III, 0.58 and IV, 0.54 (see also “Methods”).

5β-cholestone-3α,7α-diol by mitochondrial fractions of rat or guinea pig liver. In the rat comparable rates of hydroxylation were obtained with NADPH and isocitrate under standard assay conditions as described under “Methods” (on the average 28.5 pmol/mg/min for the 25R diastereomer and 7.5 pmol/mg/min for the 25S diastereomer). In the guinea pig, on the other hand, the rate of formation of 5β-cholestone-3α,7α,26-triol (25R and 25S) with NADPH was less than 10% of that observed with isocitrate as cofactor. However, when the isocitrate was replaced by NADPH and the reaction was initiated by addition of the mitochondrial fraction (omitting preincubation of this fraction with NADPH prior to the addition of substrate, see “Methods”), the observed reaction rate was essentially identical with that obtained under standard assays conditions for the rat and guinea pig, respectively.

C. Effect of Respiratory Chain Inhibitors on 5β-Cholesterol-3α,7α-diol 26-Hydroxylase System—The effect of respiratory chain inhibitors on the 26-hydroxylase system was studied in guinea pig liver mitochondria, using NADPH as the electron donor. The results are summarized in Table II. As can be noted, all the inhibitors tested produced considerable stimulation of the mitochondrial 26-hydroxylation, resulting in increased formation of both diastereomers (25R and 25S) of 5β-cholestone-3α,7α,26-triol.

Properties of the Microsomal Assay System

Optimal Assay Conditions—5β-[G-3H]Cholesterol-3α,7α-diol was incubated under various conditions with liver micro-

TABLE I

Effect of reduced coenzymes on mitochondrial 5β-cholestane-3α,7α,26-hydroxylase activity

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Rat</th>
<th>Guinea pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>25R</td>
<td>25S</td>
<td>25R</td>
</tr>
<tr>
<td>pmol/mg protein/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocitrate</td>
<td>28.3</td>
<td>7.00</td>
</tr>
<tr>
<td>NADPH</td>
<td>28.5</td>
<td>7.13</td>
</tr>
<tr>
<td>NADPH'</td>
<td></td>
<td>8.03</td>
</tr>
</tbody>
</table>

a Standard assay system as described under “Methods.” Isocitrate was employed to generate intramitochondrial NADPH.

b Standard assay system as described under “Methods” with the exception that isocitrate is replaced by NADPH (3 mM).

c Standard assay system as described under “Methods” with the exceptions 1) isocitrate is replaced by NADPH (3 mM); and 2) the reaction is initiated by addition of the mitochondrial fraction without preincubation.

Fig. 3. Effect of enzyme concentration on hydroxylation of 5β-[G-3H]cholestone-3α,7α-diol by rat (a), guinea pig (b), and rabbit (c) liver mitochondria. Standard assay conditions were employed except for protein concentration. □—□, 5β-cholestone-3α,7α,12α-triol; ▲—▲, 5β-cholestone-3α,7α,24-triol (24R); △—△, 5β-cholestone-3α,7α,24-triol (24S); ×—×, 5β-cholestone-3α,7α,25-triol; ●—●, 5β-cholestone-3α,7α,26-triol (25R); and ○—○, 5β-cholestone-3α,7α,26-triol (25S).
somal fractions of rat, guinea pig, and rabbit. The corresponding labeled 5β-cholestanetriols formed were separated by TLC, and their radioactivity was determined as described under "Methods." Reaction rates were linear with time for 15 min, and substrate saturation was reached at a concentration of 100 μM. The relationship between reaction rates and enzyme concentrations for each species is illustrated in Fig. 4, a, b, and c. In all cases, reaction rates were linear with respect to protein concentration up to 1 to 1.5 mg.

Identification and Radioactive Purity of Reaction Products—To establish the identity and radioactive purity of the two diastereomers, 25R and 25S, of 5β-cholestan-3α,7α,26-triol and the other 5β-cholestanetriols formed from 5β-[G-3H]cholestan-3α,7α-diol, the assay system described under "Methods," using hepatic mitochondrial or microsomal fractions, was scaled up 10-fold. The labeled products formed during a 1-h incubation were extracted and subjected to thin layer chromatography on alumina as described above, without addition of carrier, and the bands corresponding to known triol reference compounds were eluted with methanol. The biosynthetic bile alcohols were analyzed by GLC mass spectrometry, as illustrated below for 5β-cholestan-3α,7α,26-triol (25R).

The mass spectrum was identical with that of the authentic 25R diastereomer of 5β-cholestan-3α,7α,26-triol with main peaks at m/z 546 [M – 90], 531 [M – (90 + 13)], 456 [M – (2 × 90)], 441 [M – (2 × 90 + 15)], 366 [M – (3 × 90)], 351 [M – (3 × 90 + 15)], and 255 [M – (2 × 90 + side chain)]. The other biosynthetic bile alcohols, particularly the 25S diastereomer of 5β-cholestan-3α,7α,26-triol, were identified in the same manner. Known alcohols from each band were then applied with 50 μg of carrier on alumina G plates and developed with chloroform:acetone:methanol, 35:25:3.5 (v/v/v). After elution with methanol two aliquots were taken from each sample; one was used for determining mass by GLC and the other for radioassay by liquid scintillation counting. The samples were chromatographed again on alumina G plates with a different solvent system, benzene:ethyl acetate:methanol, 90:30:7 (v/v/v). After elution, the bile alcohols were chromatographed a third time on Silica Gel G with chloroform:acetone:methanol, 70:50:5 (v/v/v). Each time the specific radioactivity of each band was determined by GLC and liquid scintillation counting. The data dealing with 5β-cholestan-3α,7α,26-triol (25R) and 5β-cholestan-3α,7α,26-triol (25S) isolated from incubation mixtures of guinea pig liver mitochondria or microsomes are summarized in Table III. In each case the specific radioactivity remained constant during repeated chromatography. The other bile alcohols formed during the incubation of 5β-[G-3H]cholestan-3α,7α-diol yielded similar results.

Conversion of 5β-[G-3H]cholestan-3α,7α-diol to 5β-Cholesterolatriens by Liver Mitochondria—Table IV illustrates the conversion of 5β-cholestan-3α,7α,26-triol into the 25R diastereomer of 5β-cholestan-3α,7α,26-triol and other 5β-cholestanetriols by hepatic mitochondrial fractions of rat, guinea pig, and rabbit. In all species studied, the major product (50 to 73% of total hydroxylations) was 5β-cholestan-3α,7α,26-triol (25R). The rates of formation of this 25R diastereomer of 5β-cholestan-3α,7α,26-triol were higher in rabbit than in rat and guinea pig liver. In the rat and guinea pig there was also considerable hydroxylation at C-24 (24S).

Conversion of 5β-[G-3H]cholestan-3α,7α-diol to 5β-Cholesterolatriens

**Table II**

<table>
<thead>
<tr>
<th>Additions</th>
<th>5β-Cholestan-3α,7α,26-triols formed</th>
<th>pmol/mg protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25R</td>
<td>3.53</td>
</tr>
<tr>
<td>KCN (1.0 mM)</td>
<td>25S</td>
<td>13.9</td>
</tr>
<tr>
<td>Antimycin A* (0.02 mM)</td>
<td>25S</td>
<td>3.49</td>
</tr>
<tr>
<td>Rotenone** (0.2 mM)</td>
<td>25S</td>
<td>43.9</td>
</tr>
<tr>
<td>Amytal*** (1.0 mM)</td>
<td>25S</td>
<td>11.7</td>
</tr>
<tr>
<td>Dicumarol*** (0.05 mM)</td>
<td>25S</td>
<td>10.7</td>
</tr>
</tbody>
</table>

*Antimycin A, rotenone, amytal, and dicumarol were dissolved in ethanol. The controls contained identical amounts of ethanol.

**Table III**

<table>
<thead>
<tr>
<th>Additions</th>
<th>TLC</th>
<th>Specific radioactivity after TLC in 5β-cholestan-3α,7α,26-triols</th>
<th>Specific radioactivity after TLC in 5β-cholestan-3α,7α,26-triols</th>
<th>Specific radioactivity after TLC in 5β-cholestan-3α,7α,26-triols</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25R</td>
<td>0.46</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>KCN (1.0 mM)</td>
<td>25S</td>
<td>514</td>
<td>255</td>
<td></td>
</tr>
<tr>
<td>Antimycin A* (0.02 mM)</td>
<td>25S</td>
<td>532</td>
<td>224</td>
<td></td>
</tr>
</tbody>
</table>

*The 25R isomer was isolated from an incubation with guinea pig mitochondria.
**The 25S isomer was isolated from an incubation with guinea pig microsomes.
***The samples were applied with 50 μg of carrier on 0.25-mm-thick alumina G plates and developed twice with chloroform:acetone:methanol, 35:25:3.5 (v/v/v).
****The samples were reapplied on 0.25-mm-thick silica G plates and developed twice with benzene:ethyl acetate:methanol, 90:30:7 (v/v/v).
*****The samples were reapplied on 0.25-mm-thick silica G plates and developed with chloroform:acetone:methanol, 70:50:5 (v/v/v).
Stereospecific Hydroxylations of 5β-Cholestan-3α,7α-diol

The activity of the hepatic mitochondrial and microsomal enzymes converting 5β-cholestane-3α,7α-diol to the isomeric 5β-cholestanetriols can be determined by an isotope incorporation procedure. The biosynthetic 5β-cholestanetriols hydroxylated at C-24 (24R and 24S) or C-12 by neutral alumina and Silica Gel G plates (Fig. 2). The sensitivity of the method is such that 10 pmol of any 5β-cholestanetriol formed can be detected (Tables IV and V), using 5β-[3H]cholestan-3α,7α-diol (specific activity 1.61 X 10^4 dpm/μmol) as substrate.

The identity of the reaction products was determined by GLC mass spectrometry of their trimethylsilyl derivatives. The mass spectra of the biosynthetic products were identical with those of the authentic compounds.

The radioactive purity of the biosynthetic polar sterols was established by multiple TLC using two types of plates (Silica Gel G and alumina G) and three different solvent systems (see Table III). The specific radioactivity of the biosynthetic 5β-cholestanetriols hydroxylated at C-24 (24R and 24S) differed considerably, depending on the species studied. However, considering side chain hydroxylations only, the microsomal 25-hydroxylation was higher in all species (40 to 78%) than the hydroxylations at C-12, C-24, and C-26. Furthermore, the 26-hydroxylation catalyzed by the microsomal fraction resulted in the formation of the two diastereomers of 5β-cholestanetriol in ratios of 1:2 (24R and 24S) for rat and guinea pig and about 2:1 for rabbit. In experiments measuring the formation of all six 5β-cholestanetriols, a reaction time of 15 min and a mitochondrial or microsomal protein concentration of 1.0 mg/ml were chosen to assure optimal assay conditions. Under these conditions we were able to compare the relative rates of hydroxylations at different carbon atoms in a given species.

It has been shown previously that the mitochondrial and microsomal enzymes converting 5β-cholestan-3α,7α-diol to the isomeric 5β-cholestanetriols can be determined by an isotope incorporation procedure. The biosynthetic 5β-cholestanetriols hydroxylated at C-24 (24R and 24S) or C-12 by neutral alumina and Silica Gel G plates (Fig. 2). The sensitivity of the method is such that 10 pmol of any 5β-cholestanetriol formed can be detected (Tables IV and V), using 5β-[3H]cholestan-3α,7α-diol (specific activity 1.61 X 10^4 dpm/μmol) as substrate.

The identity of the reaction products was determined by GLC mass spectrometry of their trimethylsilyl derivatives. The mass spectra of the biosynthetic products were identical with those of the authentic compounds.

TABLE IV
Conversion of 5β-[3H]cholestan-3α,7α-diol to 5β-cholestanetriols by hepatic mitochondria

Mitochondrial fractions were prepared and products were analyzed as described under "Methods." Standard assay conditions were employed.

<table>
<thead>
<tr>
<th>Species</th>
<th>Formation of 5β-cholestanetriol</th>
<th>pmol/mg protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (5)</td>
<td>3α,7α,24-Triol</td>
<td>25R  25S</td>
</tr>
<tr>
<td>Guinea pig (5)</td>
<td>3α,7α,25-Triol</td>
<td>24R  24S</td>
</tr>
<tr>
<td>Rabbit (4)</td>
<td>3α,7α,12α-Triol</td>
<td>24R  24S</td>
</tr>
</tbody>
</table>

" Number of animals in each group in parentheses. The data in the table represent the average for each group (S.E. = ±6%).

TABLE V
Conversion of 5β-[3H]cholestan-3α,7α-diol to 5β-cholestanetriols by hepatic microsomes

Microsomal fractions were prepared and products were analyzed as described under "Methods." Standard assay conditions were employed.

<table>
<thead>
<tr>
<th>Species</th>
<th>Formation of 5β-cholestanetriol</th>
<th>pmol/mg protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (5)</td>
<td>3α,7α,24-Triol</td>
<td>25R  25S</td>
</tr>
<tr>
<td>Guinea pig (5)</td>
<td>3α,7α,25-Triol</td>
<td>24R  24S</td>
</tr>
<tr>
<td>Rabbit (4)</td>
<td>3α,7α,12α-Triol</td>
<td>24R  24S</td>
</tr>
</tbody>
</table>

" Number of animals in each group in parentheses. The data in the table represent the average for each group (S.E. = ±6%).

The rates of hydroxylation of 5β-cholestan-3α,7α-diol during incubation with the microsomal fractions obtained from livers of rat, guinea pig, and rabbit. The relative rates of hepatic microsomal hydroxylations at C-12, C-24 (24R and 24S), C-25, and C-26 (25R and 25S) differed considerably, depending on the species studied. However, considering side chain hydroxylations only, the microsomal 25-hydroxylation was higher in all species (40 to 78%) 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 diastereomers of 5β-cholestan-3α,7α,26-triol in ratios of 1:2 (24R:24S) for rat and guinea pig and about 2:1 for rabbit. It should be noted here that in rat and rabbit the major product (48 and 74% of total triols) was 5β-cholestan-3α,7α,12α-triol, while in the guinea pig 12α-hydroxylation amounted only to 4.8% of total hydroxylations.

DISCUSSION

The results of the present paper demonstrate that 5β-cholestan-3α,7α-diol (Fig. 1, I) can be hydroxylated at C-12, C-24, C-25, and C-26 (II to VII) by mammalian hepatic mitochondrial and microsomal fractions. Hydroxylation at C-24 or C-26 of the steroid side chain is accompanied by the introduction of an additional asymmetric carbon atom at C-24 or C-25, respectively, leading to the formation of two distinct pairs of diastereomers. The biosynthesis of the 24R and 24S diastereomers of 5β-cholestan-3α,7α,24-triol has been demonstrated previously in subcellular fractions of guinea pig liver. Similarly, 5β-cholestan-3α,7α,12α-triol was hydroxylated by the microsomal fraction of rat liver homogenates in both the 24R and 24S positions (10). However, previous studies of the stereospecificity of the 26-hydroxylation were confined to cholesterol as the substrate (17).
utilized during the preincubation period, probably via NADPH:NADH transhydrogenation to a larger extent by the mitochondrial fractions of guinea pig liver than by those of rat liver (see discussion of respiratory chain inhibitors below). We, therefore, chose to use isocitrate as cofactor in the standard assay with the mitochondrial fractions of the different mammalian species tested. Furthermore, isocitrate is preferable as cofactor in experiments with the mitochondrial fractions, since isocitrate cannot be utilized by the different hydroxylation systems present in the microsomes that always contaminate the mitochondrial fraction (4).

As illustrated in Table II, inhibitors of respiratory electron transfer, such as antimycin A and rotenone, considerably enhanced the rate of 26-hydroxylation contaminating the mitochondrial fraction (4). Since isocitrate cannot be utilized by the different hydroxylation systems, the formation of both diastereomers to a smaller extent (3 to 4 times). The stimulation of NADH-dependent side chain hydroxylation by respiratory chain inhibitors can be explained if an NADPH:NADH transhydrogenase system were normally operational in hepatic mitochondria and inhibited by intramitochondrial NADH which accumulated in the presence of respiratory inhibitors. Indeed, Björkhem and Gustafsson (18) have shown that a transhydrogenation of NADH

\[
\text{NAD}^{+} \Leftrightarrow \text{NADH} \Leftrightarrow \text{NADP}^{+} \Leftrightarrow \text{NADPH}
\]

could in principle occur in rat liver mitochondria with subsequent utilization of NADPH for 26-hydroxylation, since ATP stimulated 26-hydroxylation of cholesterol in the presence of NADP and NADH. The stimulatory effect of respiratory chain inhibitors on the 26-hydroxylation of 5β-cholestane-3a,7a-diol by guinea pig mitochondria might be ascribed to the accumulation of NADH during the preincubation period. Consequently, increased amounts of this coenzyme would be available for transhydrogenation and prevent the oxidation of NADPH via the respiratory chain, allowing it to be used exclusively by the cytochrome mixed function oxidase system. A similar effect has been reported in the NADH- or isocitrate-dependent cleavage of the cholesterol side chain by ovarian mitochondria in the presence of rotenone (19).

In agreement with the work of Björkhem and Gustafsson with rat liver mitochondrial fractions (4, 18), and our experiments with guinea pig liver mitochondria, hepatic mitochondria of the three species studied catalyzed predominantly the hydroxylation of 5β-cholestane-3a,7a-diol at C-26 (Table IV). This reaction was stereospecific, and the major product was the 25R diastereomer of 5β-cholestane-3a,7a,26-triol amounting to 50, 54, and 73% of the total hydroxylations for rat, guinea pig, and rabbit, respectively. The ratio of 25R/25S diastereomers of 5β-cholestane-3a,7a,26-triol was about 5:1 in all species. In the rat and guinea pig there was also considerable hydroxylation at C-24 (24S), but the importance of 5β-cholestane-3a,7a,24-triol as an intermediate in chenodeoxycholic acid synthesis is not known. In addition, 5β-cholestane-3a,7a,25-triol was formed to a small extent (2 to 7%) in all species. 25-Hydroxylation activity on vitamin D has been demonstrated in rat liver mitochondria (20), but whether this is the same enzyme involved in the 25-hydroxylation of 5β-cholestane-3a,7a-diol remains to be established. Part of the observed hydroxylations in the mitochondrial fractions is the result of contamination with the microsomal fraction, but this is difficult to evaluate exactly. According to measurements of microsomal glucose-6-phosphatase activity, the microsomal fraction was contaminated to about 3% with the microsomal fraction. Using the microsomal 12α-hydroxylase as a marker enzyme it can be estimated that the microsomal contamination of the mitochondria ranged from 2 to 9% (Tables IV and V).

Table V illustrates the rates of hydroxylation at C-12, C-24, C-25, and C-26 of 5β-cholestane-3a,7a-diol by hepatic microsomal fractions of rat, guinea pig, and rabbit. Considering only side chain hydroxylations, 5β-cholestane-3a,7a,25-triol was the major product in all species and amounted to 40, 79, and 96% of the total side chain hydroxylations for rat, guinea pig, and rabbit, respectively. Hydroxylation at C-26 were lower and amounted to 13, 4, and 10% for the 25R diastereomer of 5β-cholestane-3a,7a,26-triol and 25, 9, and 6% for the 25S isomer. Unlike the findings with mitochondria there was a relative lack of stereospecificity, and both diastereomers were produced in ratios of 1:2 (25R:25S) in rat and guinea pig and 2:1 in rabbit. Applicable quantities of 5β-cholestane-3a,7a,24-triol (24R and 24S) were also formed (24R, 4 to 9% and 24S, 4 to 14%), but the participation of this compound in chenodeoxycholic acid synthesis is not known. As illustrated in Table V, 5β-cholestane-3a,7a-diol was also hydroxylated in the 12α position by microsomal fractions. 12α-Hydroxylation activity varied widely among the species tested. For example, in the guinea pig with less than 1% of cholic acid in its bile (21), 12α-hydroxylation activity amounted to less than 5% of all hydroxylations, while in the rabbit where cholic and deoxycholic acids amount to more than 95% (22) of its biliary bile acids, 12α-hydroxylation was as high as 74%.

In summary, the mitochondria catalyzed predominantly the formation of the 25R diastereomer of 5β-cholestane-3a,7a,26-triol, while the major product of the microsomal fraction was 5β-cholestane-3a,7a,25-triol in all three species tested. These findings suggest that if the first step in the side chain hydroxylation is mediated by the mitochondria, then the major pathway of chenodeoxycholic acid formation involves 26-hydroxylated intermediates (25R). On the other hand, if the microsomes are the site of the initial step of the side chain hydroxylation, the major pathway probably would involve C-25 hydroxylated intermediates, although the role of 5β-cholestane-3a,7a,26-triol (25S) requires further exploration. In addition, it was recently observed that 5β-cholestane-3a,7a,25-triol is a poor precursor of chenodeoxycholic acid in man in vivo (23). Obviously, the quantitative importance of the different pathways needs to be established.

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