On the Formation of Hyodeoxycholic Acid in the Rat

BILE ACIDS AND STEROIDS 154*

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SUMMARY

1. Lithocholic acid-24-14C was administered to rats and labeled hyodeoxycholic acid was isolated from feces and bile collected after 3 days of intact enterohepatic circulation. Labeled 3α-hydroxy-6-keto-5β-cholanoic acid was isolated from feces. No labeled hyodeoxycholic acid was formed when lithocholic acid-24-14C was administered to bile fistula rats. One of the major metabolites in these experiments was 3α,6β-dihydroxy-5β-cholanoic acid.

2. After administration of 3α,6β-dihydroxy-5β-cholanoic acid-24-14C to intact rats, labeled hyodeoxycholic acid was isolated from feces and bile. Labeled 3α-hydroxy-6-keto-5β-cholanoic acid was isolated from feces. In the bile fistula rat, 3α,6β-dihydroxy-5β-cholanoic acid-24-14C was metabolized mainly into β-muricholic acid and no labeled hyodeoxycholic acid was formed.

3. In the intact rat, 3α-hydroxy-6-keto-5β-cholanoic acid-24-11C was transformed mainly into hyodeoxycholic acid, whereas in the bile fistula rat 3α,6β-dihydroxy-5β-cholanoic acid was the main metabolite.

4. It was suggested that hyodeoxycholic acid is formed from lithocholic acid through the following reaction:

Lithocholic acid ←→ 3α,6β-dihydroxy-5β-cholanoic acid


Matschiner et al. (4). More recently, Lin, Rubinstein, and Holmes (5) described the presence of two additional dihydroxycholanoic acids in bile fistula bile. These acids had the same gas chromatographic properties as hyodeoxycholic acid and deoxycholic acid, respectively, and both acids were absent from fistula bile collected later than 24 hours after the fistula operation. Makita and Wells (6) have reported that an acid with gas chromatographic properties similar to those of hyodeoxycholic acid is a major constituent of the bile acid fraction of rat feces. The formation and metabolism of bile acids in the rat as well as in other species have been studied extensively (7), but no information is available concerning the mechanism of formation of hyodeoxycholic acid in the rat. Hyodeoxycholic acid and hyocholic acid have been considered to be species-specific bile acids, being present only in the bile of Sus (8). The metabolism of these bile acids has been examined in the pig and it has been shown that hyodeoxycholic acid is formed from hyocholic acid by the action of intestinal microorganisms during the enterohepatic circulation of bile (9).

The present communication reports studies on the formation of hyodeoxycholic acid in the rat and presents evidence that this acid is formed from chenodeoxycholic acid during the enterohepatic circulation of bile.

EXPERIMENTAL PROCEDURE

Materials—Cholesterol-4-14C (specific activity, 60 μC per mg) was obtained from the Radiochemical Centre, Amersham, England. Lithocholic acid-24-14C (specific activity, 8 μC per mg) was prepared according to Bergström, Rottenberg, and Voltz (10) and was a generous gift of Dr. A. Normann. 3α,6β-Dihydroxy-5β-cholanoic acid was prepared as described by Hohm, Linsk, and Moffett (11) and Ratliff et al. (12) and had a melting point of 206–208° (recorded (11), 209–210°). 3α-Hydroxy-6-keto-5β-cholanoic acid was obtained by chromic acid oxidation of hyodeoxycholic acid (13) and had a melting point of 150° (recorded (14), 164°). 3α,6β-Dihydroxy-5β-cholanoic acid-24-14C was prepared biosynthetically from lithocholic acid 24-14C (see under “Results”), and 3α-hydroxy-6-keto-5β-cholanoic acid-24-14C was obtained by chromic acid oxidation of 3α,6β-dihydroxy-5β-cholanoic acid-24-14C (14).

Animal Experiments—White male rats of the Sprague-Dawley strain weighing 200 to 300 g were used. Bile fistulas were prepared in the usual manner. Bile and feces were collected in ethanol. The labeled compounds were administered intraperitoneally, cholesterol-4-14C and 3α-hydroxy-6-keto-5β-cholanoic acid-24-14C as emulsions stabilized with serum albumin and the other bile acids as sodium salts dissolved in NaCl.

The major bile acids in rat bile are cholic and chenodeoxycholic acids (2). The presence of small amounts of other bile acids has been reported. Ursodeoxycholic acid and α- and β-muricholic acids were isolated by Mahowald et al. (3) and...
Analysis of Bile and Feces—Bile acids were extracted from feces by refluxing three times for 2 hours with 70% aqueous ethanol. The combined ethanol extracts were concentrated, diluted with water, acidified with hydrochloric acid, and extracted with ether. The ether extract was washed with water until neutral and the solvent was evaporated. Bile was hydrolyzed with 3 KOH in 50% aqueous ethanol for 12 hours at 110°. The saponification mixture was diluted with water, acidified, and extracted with ether as above. The residues of the ether extracts were subjected to reversed phase partition chromatography (15). The solvent systems used are listed in Table I. Of the stationary phase, 4 ml/4.5 g of hydrophobic Hyflo Super Cel were used. Thin layer chromatography was done with solvent systems described by Eneroth (16). Gas chromatography was performed as described by Sjövall (17).

RESULTS

Hyodeoxycholic Acid in Bile—Bile was collected from a bile fistula rat in two fractions, one between 0 and 72 hours after operation, the other between 72 and 168 hours. Cholesterol-4,14C, 0.1 mC, was administered to the rat 72 hours after the operation. The hydrolyzed bile fractions were chromatographed with Phase System F 1; Fig. 1A shows this chromatogram of the first bile fraction. For further analysis, the effluent fractions were combined to give three main fractions: Fraction I contained the material eluted between 15 and 27 ml of effluent; Fraction II contained that eluted between 30 and 50 ml of effluent; and Fraction III contained the material in 50 to 75 ml of effluent. Fraction I, containing the trihydroxycholanoic acids, was chromatographed with Phase System C 1. Gas chromatographic and thin layer chromatographic analysis of a number of the fractions collected showed the presence of cholic acid and α- and β-muricholic acids. No evidence was obtained for the presence of hyocholic acid. In this connection, it is worth mentioning that no hyocholic acid could be detected in two large samples of bile fistula bile (750 and 150 ml, respectively). Fraction II was chromatographed with Phase System F 1 (Fig. 1B). The material eluted with its peak at 34 ml of effluent had the same elution volume as ursodeoxycholic and hyodeoxycholic acids, which were not separated with this solvent system. The fractions between 30 and 50 ml of effluent were combined, methylated with diazomethane, and analyzed by gas chromatography (Fig. 2). Two main peaks were observed in the gas chromatogram with the same retention times as those of authentic methyl ursodeoxycholate and methyl hyodeoxycholate, respectively. Fraction III was analyzed by gas chromatography and was found to consist of chenodeoxycholic acid together with small amounts of deoxycholic acid. The second fraction of bile was analyzed in the same manner. Hyodeoxycholic acid was present only in trace amounts. Table II summarizes the results obtained. The radioactivity in the bile acids isolated from the second fraction of bile was found to be distributed in proportion to the weight of the bile acids.

Metabolism of Lithocholic Acid—24-14C—Lithocholic acid-24-14C, 5 μC, was administered to an intact rat. After 3 days, a bile fistula operation was performed and bile was collected for 1 day. Feces were collected daily during the whole experimental period. Fig. 3A shows the chromatogram of the fecal bile acids excreted

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Table I

<table>
<thead>
<tr>
<th>Phase system</th>
<th>Moving phase</th>
<th>Stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1</td>
<td>Methyl alcohol-water</td>
<td>Chloroform-isooctyl alcohol</td>
</tr>
<tr>
<td></td>
<td>(150:150)</td>
<td>(15:15)</td>
</tr>
<tr>
<td>F 1</td>
<td>Methyl alcohol-water</td>
<td>Chloroform-heptane</td>
</tr>
<tr>
<td></td>
<td>(165:135)</td>
<td>(45:5)</td>
</tr>
<tr>
<td>F 2</td>
<td>Methyl alcohol water</td>
<td>Chloroform heptane</td>
</tr>
<tr>
<td></td>
<td>(180:120)</td>
<td>(45:5)</td>
</tr>
</tbody>
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* Ratios are volume for volume (milliliters).
during the first day after administration of isotope. The radioactivity eluted from the column was distributed in four main peaks. The third peak (between 30 and 45 ml of effluent) was identified as 3α-hydroxy-6-keto-5β-cholanoic acid by its thin layer chromatographic behavior (see Fig. 4B) and by crystallization to constant specific activity with the unlabeled acid. The fourth peak (between 70 and 90 ml of effluent) consisted mainly of unchanged lithocholic acid. The fractions eluted between 18 and 25 ml of effluent were rechromatographed together with unlabeled hyodeoxycholic acid (Fig. 3B), and the identity of the labeled material with hyodeoxycholic acid was established by thin layer chromatography (Fig. 4A) and by crystallization to constant specific activity with carrier hyodeoxycholic acid. The analyses are summarized in Table III.

Lithocholic acid-24-14C in doses of 5 μC was administered to bile fistula rats. The trihydroxycholanoic acids were separated with Phase System C 1 from the less polar bile acids, which are retained in the stationary phase in this phase system. These acids were eluted from the column with ethanol and were chromatographed with Phase System F 1. Fig. 3C shows the portion of this chromatogram where dihydroxycholanoic acids are eluted. The peak with a maximum at 23 ml of effluent was shown to consist of 3α,6β-dihydroxy-5β-cholanoic acid by thin layer chromatography (Fig. 4C) and crystallization to constant specific activity with unlabeled material. No hyodeoxycholic acid was detected in this fraction. Table III summarizes the composition of lithocholic acid metabolites in experiments with bile fistula rats.

**Table II

<table>
<thead>
<tr>
<th>Bile acids</th>
<th>Fraction A</th>
<th>Fraction B</th>
</tr>
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<tbody>
<tr>
<td>Trihydroxycholanoic</td>
<td>80.6</td>
<td>60.4</td>
</tr>
<tr>
<td>Chenodeoxycholic</td>
<td>12.7</td>
<td>39.5</td>
</tr>
<tr>
<td>Ursodeoxycholic</td>
<td>2.7</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Hyodeoxycholic</td>
<td>3.9</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Deoxycholic</td>
<td>0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

**Fig. 3. Curve A**, chromatogram of bile acids in feces collected during the first day after intraperitoneal administration of lithocholic acid-24-14C to an intact rat. Unlabeled lithocholic acid was added as reference compound. Column, 13.5 g of hydrophobic Super-Cel; Phase System F 2. ---, titration values; ––, radioactivity. **Curve B**, rechromatography of the second radioactive peak shown in Curve A together with unlabeled hyodeoxycholic acid. Column, 4.5 g of hydrophobic Hyflo Super-Cel; Phase System F 1. **Curve C**, chromatogram of the dihydroxycholanoic acid fraction of hydrolyzed bile from bile fistula rat which received an intraperitoneal injection of lithocholic acid-24-14C. Column, 9 g of hydrophobic Hyflo Super-Cel; Phase System F 1. Symbols as in Fig. 3A.
3α,6β-Dihydroxy-5β-cholanoic acid-24-14C was administered to intact rats and the experimental procedure followed was that described above for lithocholic acid-24-14C. Fig. 5C shows the chromatogram of the extract of the feces collected during the first day. The second radioactive peak (between 20 and 35 ml of effluent) was shown to consist predominantly of hyodeoxycholic acid by means of thin layer chromatography (Fig. 6B) and crystallization to constant specific activity. In the same manner, the material eluted between 60 and 100 ml of effluent (Fig. 5C) was identified as 3α-hydroxy-6-keto-5β-cholanoic acid (Fig. 6C).

Metabolism of 3α-Hydroxy-6-keto-5β-cholanoic Acid-24-14C—After administration of this acid to a bile fistula rat, 50% of the radioactivity was excreted in the first 24-hour portion of bile.

TABLE III
Composition of metabolites of lithocholic acid-24-14C in feces and bile

<table>
<thead>
<tr>
<th></th>
<th>Trihydroxy-&lt;br&gt;cholanoic acids</th>
<th>Cholesterol-&lt;br&gt;oxyacidic acid</th>
<th>Hydroxy-&lt;br&gt;cholanoic acid</th>
<th>3α,6β-Dihydroxy-&lt;br&gt;5β-cholanoic acid</th>
<th>3α-Hydroxy-6-keto-&lt;br&gt;5β-cholanoic acid</th>
<th>Lithocholic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithocholic acid</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>24-14C administered to intact rat*</td>
<td>17.3</td>
<td>13.3</td>
<td>25.5</td>
<td>2.7</td>
<td>12.2</td>
<td>29.0</td>
</tr>
<tr>
<td>Feces, 1st day</td>
<td>17.3</td>
<td>13.3</td>
<td>25.5</td>
<td>2.7</td>
<td>12.2</td>
<td>29.0</td>
</tr>
<tr>
<td>Feces, 2nd day</td>
<td>29.0</td>
<td>13.7</td>
<td>33.7</td>
<td>3.5</td>
<td>14.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Feces, 3rd day</td>
<td>50.5</td>
<td>8.3</td>
<td>25.4</td>
<td>2.7</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>Bile, 4th day</td>
<td>73.7</td>
<td>6.5</td>
<td>18.1</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>24-14C administered to bile fistula rat†</td>
<td>49.2</td>
<td>14.6</td>
<td>15.7</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>Bile, 1 day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The sum of lithocholic acid and its metabolites has been put at 100%. The total recovery of administered radioactivity was 94%.
† The figures given represent the average of three experiments.

The first 24-hour portion of bile contained 95% of administered radioactivity.

After hydrolysis, the bile acids were chromatographed with Phase System F 2 (Fig. 7A). The first peak was rechromatographed on Phase System F 1 and was found to consist of a mixture of 3α,6β-dihydroxy-5β-cholanoic acid and an acid with chromatographic properties similar to those of β-muricholic acid. The 3α,6β-dihydroxy-5β-cholanoic acid, which constituted about 30% of the mixture, was identified by thin layer chromatography (Fig. 8A) and crystallization to constant specific activity. The material eluted between 25 and 40 ml of effluent (Fig. 7A) was found to consist mainly of a compound with chromatographic properties similar to those of 3α-hydroxy-6-keto-5α-cholanoic acid, an artifact produced from 3α-hydroxy-6-keto-5β-cholanoic acid during alkaline hydrolysis of bile.

3α-Hydroxy-6-keto-5β-cholanoic acid-24-14C was also administered to an intact rat and the experiment was conducted in the same manner as described above. Chromatography of the bile acids excreted in feces during the first day showed the presence...
Hyodeoxycholic Acid in Rat

50 (x2)

ml effluent

5 ml 0.01N KOH

1.2

5 c.p.m. x 10^4

Fig. 7. Curve A, chromatogram of hydrolyzed bile from bile fistula rat which received an intraperitoneal injection of 3α-hydroxy-6-keto-5β-cholanoic acid-24-14C. Column, 9 g of hydrophobic Hyflo Super-Cel; Phase System F 2. Curve B, chromatogram of bile acids in feces collected during the first day after intraperitoneal administration of 3α-hydroxy-6-keto-5β-cholanoic acid-24-14C to an intact rat. Column, 18 g of hydrophobic Hyflo Super-Cel; Phase System F 2.

FIG. 8. Thin layer chromatogram of the descending part of the first peak in the chromatogram shown in Fig. 7A (Diagram A), of the ascending part of the first peak, and of the descending part of the second peak in the chromatogram shown in Fig. 7B (Diagrams B and C, respectively). Phase System S 11. Reference compounds as in Fig. 4.

of two peaks (Fig. 7B). The ascending part of the first peak and the descending part of the second peak were analyzed by thin layer chromatography (Fig. 8, B and C). The material of the first peak could not be identified, whereas the isotopic material of the second peak was identified as hyodeoxycholic acid. No unchanged 3α-hydroxy 6-keto 5β cholanoic acid was present in the fecal extract. After 3 days, a bile fistula was made. Hyodeoxycholic acid was identified as one of the major labeled products in bile.

Discussion

In confirmation of the results of Lin, Rubinstein, and Holmes (5), the present investigation shows that hyodeoxycholic acid is practically absent from fistula bile collected from rats after the preformed bile acids have been excreted. This result indicates that an intact enterohepatic circulation of bile is required for the formation of hyodeoxycholic acid. The immediate precursor of hyodeoxycholic acid could be either a primary bile acid (an acid formed in the liver from cholesterol) or a microbially formed metabolite of a primary bile acid. In the former case, a primary bile acid could be a precursor. In the pig, hyodeoxycholic acid is formed by the action of intestinal microorganisms during the enterohepatic circulation of bile (9). However, no hyocholic acid could be detected in the bile samples examined. Instead, the results obtained indicate that hyodeoxycholic acid is formed from lithocholic acid through several steps catalyzed by enzymes in the liver as well as in the intestinal microorganisms. Lithocholic acid itself is a product of microbial action on chenodeoxycholic acid, which is a primary bile acid (18). According to the results of this investigation, the most probable pathway for the conversion of lithocholic acid into hyodeoxycholic acid entails hydroxylation in the liver to 3α, 6β-dihydroxy-5β-cholanoic acid, a reaction described earlier by Thomas et al. (19). The 3α, 6β-dihydroxy-5β-cholanoic acid formed is oxidized to 3α-hydroxy-6-keto-5β-cholanoic acid by intestinal microorganisms, which also transform this acid into hyodeoxycholic acid. Interestingly, 3α-hydroxy-6-keto-5β-cholanoic acid was found to be reduced to 3α, 6β-dihydroxy-5β-cholanoic acid in the liver. In agreement with the results obtained by Thomas et al. (20), the main metabolite formed from 3α, 6β-dihydroxy-5β-cholanoic acid in the liver was found to be β-muricholic acid.

At present, it cannot be excluded that, in addition to the pathway of hyodeoxycholic acid formation mentioned above, a direct hydroxylation of lithocholic acid by microbial enzymes to yield hyodeoxycholic acid could occur. It is, however, difficult to test this possibility by experiments in vivo.

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