**Bile Acids**

XIV. METABOLISM OF CHENODEOXYCHOLIC ACID-24-C\(\text{14}\) IN SURGICALLY JAUNDICED MICE*†


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Previous studies in this laboratory (1-5) have demonstrated that chenodeoxycholic acid-24-C\(\text{14}\) is metabolized in the rat to two hitherto unknown trihydroxycholanic acids formerly called Acid I (\(3\alpha,6\beta,7\beta\)-trihydroxycholanic acid) and Acid II (\(3\alpha,6\alpha,7\alpha\)-trihydroxycholanic acid). At the time the trivial names, \(\beta\)- and \(\alpha\)-muricholic acids, respectively, were suggested (6), it was realized that mur, muris includes both rat and mouse. Subsequently, it has been found that the mouse metabolizes chenodeoxycholic acid to \(\alpha\)- and \(\beta\)-muricholic acids. Danielsson and Kazuo (7) have identified these metabolites in the bile of mice by their chromatographic behavior and Ziboh and Hsia (8) have isolated them by isotopic dilution from the urine of surgically jaundiced mice. This report gives the details of identification of several metabolites of chenodeoxycholic acid-24-C\(\text{14}\), including \(\alpha\) and \(\beta\) muricholic acids, from the urine of surgically jaundiced mice.

**EXPERIMENTAL PROCEDURE**

**Preliminary Experiments**—Chenodeoxycholic acid-24-C\(\text{14}\) used in these studies was prepared from the norbromide through a nitrite synthesis by the method reported by Bergstrom et al. (9).

Eighteen mice (CF-1, Carworth Farms, Inc.) weighing between 27 g and 32 g were surgically jaundiced under anesthesia with sodium pentothal (50 mg/kg of body weight). Through a midline incision, a cholecystectomy was first performed, followed by double ligation of the common bile duct. Four days postoperatively, each of the five surviving animals received intragastrically approximately 0.1 mg (0.43 PC) of carboxyl-labeled chenodeoxycholic acid as the sodium salt in 0.5 ml of water. The mice were divided into three equal groups, housed in metabolism cages, and urine was collected at regular intervals. Alkaline digests of the carcasses contained approximately 30% of the administered C\(\text{14}\).

**Fractionation of Urinary Radioactivity**—Methods of fractionation of pooled urine from the animals were similar to those previously described (2). After alkaline hydrolysis of the conjugated bile acids, acidic fractions containing 4.82 \(\times\) 10\(^5\) d.p.m. were chromatographed\(^1\) in the system of 70% aqueous acetic acid and benzene-Skellysolve B (1). Of the chromatographed C\(\text{14}\), Fractions 20-1 and 20-2 contained 15%; Fractions 40-1 to 40-4, which usually contain dihydroxycholanic acids, contained 8%; Fractions 60-2 to 60-4, in which \(\beta\)-muricholic acid is usually eluted, contained 23%; and Fractions 80-3 to 100-1, in which \(\alpha\)-muricholic acid is usually eluted, contained 5%.

Since the survival of the mice and recovery of C\(\text{14}\) in the urine were not entirely satisfactory, improved operative and postoperative techniques were used in a subsequent experiment. Nevertheless, the preliminary studies showed extensive metabolism of chenodeoxycholic acid in the jaundiced mouse.

**Intraperitoneal Administration of Chenodeoxycholic Acid**—Twelve mice, weighing from 23 g to 35 g were surgically jaundiced in the preliminary experiment with more emphasis on combating shock and maintaining homeostasis. In addition, about 5 mg each of Aureomycin and sulfadiazine were sprinkled over the peritoneum before the incision was closed. These measures improved survival remarkably. Four days postoperatively each of the nine surviving animals received intraperitoneally approximately 0.4 mg (2.2 PC) of carboxyl-labeled chenodeoxycholic acid as the sodium salt in 0.5 ml of water. The mice were divided into three equal groups, housed in metabolism cages, and urine was collected at regular intervals. After 7 days, the administration of radioactive chenodeoxycholic acid was repeated. Aliquots of urine were radioassayed and the average daily excretion of C\(\text{14}\) is shown in Fig. 1.

The fractionation and extraction procedures were similar to those used in the preliminary work. Fig. 2 presents a chromatogram\(^1\) of the free bile acids. The data are similar to those obtained in the preliminary study. Of the chromatographed C\(\text{14}\), Fractions 20-1 and 20-2 contained 15.3%; Fractions 40-1 to 40-4 contained 9.6%; Fractions 60-2 to 60-4 contained 30%; Fractions 80-1 and 80-2 contained 4%; and Fractions 80-3 to 100-1 contained 7.5%. Approximately 10% of C\(\text{14}\) was detected in the final washing of the column with methanol.

**Identification of Trihydroxycholanic Acids**

Aliquots of the various radioactive fractions were diluted with appropriate nonradioactive authentic bile acids. These diluted materials were purified by chromatography of the acids and of their derivatives as well as by repeated crystallization. This according to the percentage of benzene in Skellysolve B. For example, 20-1 is the first fraction of the eluent containing 20% benzene in Skellysolve B.
Fig. 1. Average daily excretion of C\textsuperscript{14}. Each animal received approximately 0.4 mg of chenodeoxycholic acid-\textsuperscript{14}C intraperitoneally on the 1st day; the administration was repeated on the 8th day. The heights of the bars indicate the percentages of administered C\textsuperscript{14} recovered. The numbers of animals used are given in parentheses.

Fig. 2. Chromatographic separation of the bile acids obtained from the urine of surgically jaundiced mice. The composition of the eluent is given as per cent of benzene in Skellysolve B, and the volume is given in liters (L). Four fractions were collected for each eluent. The heights of the solid bars from the base line to the top indicate the percentages of the chromatographed C\textsuperscript{14}; the open bars indicate milligrams of eluate.

procedure has been demonstrated to yield more satisfactory evidence of the identity of the products of metabolism. The results of such studies are described in the following paragraphs.

\textit{β-Muricholic Acid—Fractions 60-2 to 60-4} in which β-muricholic acid should appear contained approximately 30\% of the chromatographed C\textsuperscript{14}. An aliquot of these fractions (3.72 \times 10\textsuperscript{6} d.p.m.) was added to 42.5 mg of authentic β-muricholic acid and the mixture acetylated and chromatographed as shown in Fig. 3. Most of the chromatographed C\textsuperscript{14} was eluted in Fractions 0-2 and 0-3 with the triacetate of β-muricholic acid. This material was hydrolyzed and the free β-muricholic acid was chromatographed and crystallized to constant specific activity from aqueous methanol and from a mixture of acetone and petroleum ether. The methyl ester was prepared and crystallized to constant specific activity from ether and petroleum ether. The data are presented in Table I; calculation from these data shows that 58.3\% of the C\textsuperscript{14} in Frations 60-2, 60-3, and 60-4 was present in β-muricholic acid.

\textit{α-Muricholic Acid—Fractions 80-3 to 100-1} in which α-muricholic acid should appear contained approximately 8\% of the chromatographed C\textsuperscript{14}. An aliquot of these fractions (4.68 \times 10\textsuperscript{6} d.p.m.) was added to 41.7 mg of authentic α-muricholic acid. The mixture was acetylated; a chromatogram of the product is shown in Fig. 4. The triacetate in Fractions 0-2 and 0-3 retained only a small proportion of the chromatographed C\textsuperscript{14} which nevertheless was sufficient to identify the metabolite. Most of the radioactivity was eluted in Fraction 20-3; the identity of this material has not been ascertained. The acetate in Fractions 0-2 and 0-3 was hydrolyzed, the free acid chromatographed and finally crystallized to constant specific activity as shown in Table II; 2.8\% of the C\textsuperscript{14} in the combined Fractions 80-3 to 100-1 was calculated to be present in α-muricholic acid.

\textit{Identification of Bile Acids with Two Nuclear Oxygen Functions}

Aliquots of the combined Fractions 40-1 through 40-4 were added to authentic unlabeled acids and the mixtures purified by chromatography of the acids and of their derivatives as well as by repeated crystallization.

\textit{Chenodeoxycholic Acid—}After chromatography of the isotopically diluted acid, most of the acid was obtained in a single fraction. This acid was treated with diazomethane, and the ester acetylated with a mixture of pyridine and acetic anhydride to form methyl 3α,7α-diacetoxycholanate. Chromatography of this material on silica gel resulted in the removal of a small
material was chromatographed on silica gel; about half of the acetate to form methyl 3α-acetoxy-7-ketocholanate. This was collected for each eluent. The heights of the solid bars from the base line to the top indicate the percentages of the chromatographed C14; the open bars indicate milligrams of eluate.

**TABLE II**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>No. of crystallizations</th>
<th>Amount (mg)</th>
<th>Specific activity (d.p.m. per mg x 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquous acetic acid†</td>
<td>3</td>
<td>17.2</td>
<td>3.24</td>
</tr>
<tr>
<td>Acetone-petroleum ether</td>
<td>2</td>
<td>12.1</td>
<td>3.20</td>
</tr>
<tr>
<td>Acetone-petroleum ether</td>
<td>2</td>
<td>6.9</td>
<td>3.21</td>
</tr>
<tr>
<td>Acetone-petroleum ether</td>
<td>2</td>
<td>2.8</td>
<td>3.18</td>
</tr>
</tbody>
</table>

† α-Muricholic acid is effectively purified by crystallization from aqueous acetic acid but variable amounts of acetic acid are retained; thus no specific activities were determined.

portion of C14 from the derivative. After crystallization to constant specific activity from aqueous methanol, the ester was hydrolyzed, the product purified by partition chromatography, and the free acid crystallized to constant specific activity from ethyl acetate and petroleum ether. The data are shown in Table III; 5.5% of the C14 in the original combined fractions (40-1 through 40-4) was calculated to be present in chenodeoxycholic acid.

**7-Ketolitholic Acid**—Another aliquot of Fractions 40-1 through 40-4 was added to authentic 7-ketolithocholic acid. After chromatography on the acetic acid partition column, the eluted acid in Fraction 40-1 was methylated and purified by chromatography in the same system. The principal fraction was acetylated to form methyl 3α-acetoxy-7-ketocholanate. This material was chromatographed on silica gel, about half of the radioactivity in the acetylated mixture was eluted with the derivative. After crystallization to constant specific activity from aqueous acetone, the ester was hydrolyzed. The product was purified by partition chromatography and crystallized to constant specific activity. The data are shown in Table IV;
7.3% of the C\textsuperscript{14} in the combined fractions (40-1 through 40-4) was calculated to be in 7-ketolithocholic acid.

**Ursodeoxycholic Acid**—Another aliquot was added to authentic ursodeoxycholic acid and purified by acetic acid partition chromatography. Fractions 40-2 and 40-3 were combined, methanolized, and chromatographed in the same system. The methyl ester recovered in Fractions 20-2 and 20-3 retained most of the chromatographed C\textsuperscript{14}. After crystallization to constant specific activity from ether and petroleum ether, the ester was hydrolyzed. The product was purified by partition chromatography and crystallized to constant specific activity from a mixture of acetone and petroleum ether. The data are shown in Table V; 3.9% of the C\textsuperscript{14} in the combined fractions (40-1 through 40-4) was calculated to be in ursodeoxycholic acid.

**Amount of Each Metabolite Positively Identified**—\( \beta \)-Muricholic acid was the major metabolite of chenodeoxycholic acid in the surgically jaundiced mouse. From the data presented, it can be calculated that about 15% of the chromatographed C\textsuperscript{14} was present in this acid, whereas less than 1% was present in each of the other metabolites, namely \( \alpha \)-muricholic acid (0.2%), 7-ketolithocholic acid (0.7%), and ursodeoxycholic acid (0.4%). Only 0.5% of the unmetabolized chenodeoxycholic acid was recovered. These are minimal values since small amounts of each acid may have been present in fractions adjacent to those used for identification.

**Examination for Other Metabolites**—In our preliminary experiment an aliquot from Fractions 40-1 to 40-4 was admixed with authentic 3\( \alpha \),6\( \beta \)-dihydroxycholic acid. After several crystallizations, the specific activity remained constant which suggested that this acid is a metabolite of chenodeoxycholic acid (8). However, continuation of these studies has not given convincing evidence that 3\( \alpha \),6\( \beta \)-dihydroxycholic acid occurs in the urine as a metabolite. These experiments must be redesigned to obtain conclusive evidence on this point.

In the search for other metabolites, exhaustive examination of Fractions 80-1 and 80-2 in which cholic acid is known to appear showed that less than 1% of the C\textsuperscript{14} in these fractions could be present in this acid. However, the jaundiced mice did excrete cholic acid as measured by the method of Reinhold and Wilson (12) during the period in which metabolism of the radioactive chenodeoxycholic acid occurred.

**Discussion**

Fractionation of the urinary C\textsuperscript{14} from these experiments indicated that extensive metabolism of the administered radiolabeled chenodeoxycholic acid had occurred. \( \beta \)-Muricholic acid was the main metabolite of chenodeoxycholic acid whereas \( \alpha \)-muricholic acid was a minor metabolite in surgically jaundiced mice. A small percentage of the C\textsuperscript{14} in the dihydroxycholic acid fractions was found to be present in chenodeoxycholic acid, ursodeoxycholic acid, and 7-ketolithocholic acid. These results resemble those obtained in the study of the metabolism of chenodeoxycholic acid in the rat (2, 3, 13, 14).

Mahowald et al. (13) and Samuelsson (14) have suggested that the major metabolic pathway of chenodeoxycholic acid in the rat consists of 6\( \beta \)-hydroxylation to form \( \alpha \)-muricholic acid, followed by inversion of the hydroxyl group at carbon 7 to form \( \beta \)-muricholic acid, probably through a 7-keto intermediate. Samuelsson has further suggested that a minor pathway involves oxidation of chenodeoxycholic acid to form 7-ketolithocholic acid, reduction of the latter acid to ursodeoxycholic acid, and subsequent 6\( \beta \)-hydroxylation to form \( \beta \)-muricholic acid. These postulates appear to be equally applicable to the mouse in view of the similarity of metabolites identified.

The familiar characteristics of the bile acids in forming complexes necessitate especial care in their identification. Chromatography and crystallization alone are often inadequate to differentiate trace amounts of radioactive bile acids, particularly those of similar chromatographic characteristics (15). The importance of the preparation of derivatives in the purification and identification of bile acids has been emphasized by these experiments.

**Summary**

Approximately 0.4 mg (2.2 \( \mu \)g) of carboxyl-labeled chenodeoxycholic acid was given intraperitoneally to each of a group of surgically jaundiced mice. Examination of the urine resulted in the identification of \( \alpha \)-muricholic, \( \beta \)-muricholic, ursodeoxycholic, and 7-ketolithocholic acids as metabolites of chenodeoxycholic acid.

**References**
