BILE ACIDS

VIII. METABOLISM OF 7-KETOLITHOCHOLIC ACID-24-\textsuperscript{14}C IN THE RAT*

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The metabolism of C\textsuperscript{14}-labeled chenodeoxycholic acid in the rat results in the formation of two new trihydroxycholanic acids, Acid I and Acid II (1–4), which have also been isolated from the bile of normal rats (1) and from the urine of surgically jaundiced rats. Acid I is the major bile acid excreted by the latter type of experimental animals (5). Chemical studies on these new bile acids (3–6) have suggested that Acid I contains a 7\(\alpha\)-hydroxyl group. The conversion of chenodeoxycholic acid to Acid I then would involve inversion of the 7\(\alpha\)-hydroxyl group to the 7\(\beta\) orientation which was discussed in a previous publication (3). Therefore, as an aid in identifying the course of metabolism of chenodeoxycholic acid, as well as to observe the metabolism of a ketonic bile acid, we have extended our studies to the metabolism of C\textsuperscript{14}-labeled 7-ketolithocholic acid.\textsuperscript{1}

EXPERIMENTAL\textsuperscript{2}

\textit{Synthesis of 7-Ketolithocholic Acid-24-C\textsuperscript{14}}—A mixture of 28 mg. of chenodeoxycholic acid, previously labeled in the carboxyl position (2), and 56

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\textsuperscript{*} A preliminary report of the studies contained in this paper was presented at the meeting of the Federation of American Societies for Experimental Biology at Chicago, Illinois, April, 1957.

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\textsuperscript{1} We have learned in a private communication from Dr. Bergström of a metabolic study of this acid in his laboratories. These investigators observed that ursodeoxycholic acid and Acid I are metabolites of 7-ketolithocholic acid in the rat. In addition, their study of the metabolism of 7\(\beta\)-tritiochenodeoxycholic acid in the rat led to the observation that during conversion to Acid I the label is lost, whereas the Acid II formed retains tritium.

\textsuperscript{2} All melting point determinations were taken on the Fisher-Johns apparatus and are reported as read. Specific rotations were taken in a 1 dm. tube. For a complete explanation of the method of chromatography used in this study, see Matschiner \textit{et al.} (1). We have abbreviated the designation of the fractions from this column.

581
mg. of unlabeled chenodeoxycholic acid was oxidized by 15.7 mg. of chromic oxide in aqueous acetic acid at 5°. The product was purified by partition chromatography. The labeled acid eluted in Fraction 40-1 was crystallized from a mixture of acetone and petroleum ether to yield 55 mg., m.p. 204-206°, which were not depressed on admixture with authentic 7-ketolithocholic acid (3). Radioassay of the crystals showed them to contain $3.09 \times 10^6$ d.p.m. per mg.$^3$

Collection of Bile—Three adult male rats from the St. Louis University Colony were prepared for collection of bile by cannulation of the bile duct. Under light ether anesthesia, each animal received by stomach tube approximately 1 mg. of the labeled acid as the sodium salt in 1 ml. of water. The urine, feces, and bile were collected at regular intervals,

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recovery of C$^{14}$ in Bile, Urine, and Feces</strong></td>
</tr>
<tr>
<td><strong>Time</strong></td>
</tr>
<tr>
<td>hrs.</td>
</tr>
<tr>
<td>0-24</td>
</tr>
<tr>
<td>24-48</td>
</tr>
<tr>
<td>48-72</td>
</tr>
<tr>
<td>0-72</td>
</tr>
<tr>
<td>0-72</td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

and aliquots were taken for radioassay. Table I shows the average daily recovery of C$^{14}$ in the bile and the average total recovery in the urine and feces. Nearly all of the radioactivity was recovered in the bile with very little in the urine and feces.

Fractionation of Bile—Methods for the fractionation of pooled bile from individual animals were the same as those previously described (2). Use of the aqueous methanol and octanol-chloroform reversed phase partition column of Bergström and Norman (7) permitted the elution of nearly all of the biliary C$^{14}$ in the taurine-conjugated fractions with an average of 5.3 per cent appearing during subsequent elution.

After alkaline hydrolysis of the conjugated bile acids, the ether-soluble according to the per cent of benzene in Skellysolve B. Each solvent mixture was collected in four portions. For example, 60-3 represents the third fraction of the eluate containing a mixture of 60 per cent benzene and 40 per cent Skellysolve B.

$^3$ A dilution experiment with authentic unlabeled chenodeoxycholic acid and the radioactive 7-ketolithocholic acid indicated that less than 1.0 per cent of the administered radioactivity could have been in chenodeoxycholic acid.
acidic fractions were chromatographed on the 70 per cent aqueous acetic acid partition column previously described (1). The data obtained from fractionation of the bile of one animal are presented in Fig. 1; 2.42 \times 10^6 d.p.m. were chromatographed. The principal radioactive zone (Fractions 40-1, 40-2, 40-3, and 40-4) contained 82.7 per cent of the chromatographed C\textsuperscript{14}. Smaller amounts of radioactivity appeared in subsequent fractions. The zone of elution of Acid I (Fractions 60-3, 60-4, 80-1, and 80-2) contained 11.1 per cent and that of Acid II (Fractions 80-4 and 100-1) 3.1 per cent of the chromatographed radioactivity.

![Fig. 1. Chromatographic analysis of the free bile acids obtained from the bile of an animal given radioactive 7-ketolithocholic acid. The heights of the open bars indicate the percentages of the chromatographed C\textsuperscript{14}. The heights of the diagonally lined bars from the base line to the top indicate the mg. of cholic acid as determined by colorimetric assay (8).](image_url)

Identification of Metabolites of 7-Ketolithocholic Acid—Separate aliquots of the combined residues from Fractions 40-1, 40-2, 40-3, and 40-4 were diluted with either authentic 7-ketolithocholic acid, chenodeoxycholic acid, or ursodeoxycholic acid, and the mixtures were purified to constant specific activity. The results of this study with the bile of a single animal are given in detail.

An aliquot, 7.65 \times 10^4 d.p.m., amounting to 0.04 of the combined residues was added to 49.847 mg. of authentic 7-ketolithocholic acid, and the mixture was chromatographed on the acetic acid partition column used above. The diluted acid, which was eluted sharply in Fraction 40-1, was purified as follows: (1) The sample was crystallized three times from aqueous acetic acid to yield 38 mg. with a specific activity of 1.44 \times 10^6 d.p.m. per mg. Crystallization from the above solvent was repeated
three more times to yield 22 mg. with a specific activity of $8.90 \times 10^2$ d.p.m. per mg. The sample was then crystallized from a mixture of acetone and petroleum ether to yield 10 mg. with a specific activity of $3.73 \times 10^2$ d.p.m. per mg. (2) The acid was methylated and acetylated, and the product was crystallized six times from aqueous acetic acid to yield 3 mg. of methyl 3α-acetoxy 7-ketochohanolactone with a specific activity of $3.90 \times 10^2$ d.p.m. per mg. 4 (3) The ester was chromatographed on silica gel and subsequently crystallized three times from aqueous acetone to yield crystals with a specific activity of $3.76 \times 10^2$ d.p.m. per mg. From the final specific activity it was calculated that 7-ketolithocholic acid contained 19.4 per cent of the chromatographed C14.

An aliquot, 0.24 of the combined residues containing $4.41 \times 10^5$ d.p.m., was added to 74.992 mg. of chenodeoxycholic acid, and the mixture was chromatographed on an aqueous acetic acid partition column 4 times as large as the basic size previously described (1). The diluted acid eluted in Fraction 40-1 was crystallized four times from a mixture of ethyl acetate and petroleum ether, and the product was found to have a specific activity of $1.77 \times 10^2$ d.p.m. per mg. Three additional crystallizations from the same solvents gave 48 mg. of crystals with a specific activity of $1.29 \times 10^3$ d.p.m. per mg. Methyl 3α,7α-diacetoxychohanolate was prepared as a derivative and crystallized three times from aqueous methanol, yielding 27 mg. with a specific activity of $8.83 \times 10^2$ d.p.m. per mg. This material was chromatographed on silica gel and crystallized three times from aqueous methanol to yield crystals with a specific activity of $8.70 \times 10^2$ d.p.m. per mg. With this specific activity, chenodeoxycholic acid was calculated to contain 11.3 per cent of the chromatographed C14.

In addition, an aliquot, 0.16 of the same combined residues containing $3.06 \times 10^5$ d.p.m., was added to 22.488 mg. of ursodeoxycholic acid. 6 Chromatography of the mixture gave the results shown in Table II. Fractions 40-2, 40-3, and 40-4 were combined and crystallized three times from aqueous methanol, yielding 13 mg. of ursodeoxycholic acid (m.p. 202-203°) with a specific activity of $8.13 \times 10^3$ d.p.m. per mg. The sample was further crystallized two times from a mixture of acetone and petroleum.

4 Throughout this paper the specific activities of derivatives are expressed as disintegrations per minute (d.p.m.) per mg. of parent acid.

6 Ursodeoxycholic acid was prepared by reduction of 7-ketolithocholic acid with sodium in propanol according to the method of Kanazawa et al. (9). The product was purified by partition chromatography and finally crystallized from aqueous methanol; $[\alpha]_p^{27} +55° \pm 2°$ (c, 0.504, methanol), m.p. 202-203°. The methyl ester was prepared as a derivative; $[\alpha]_p^{27} +57° \pm 2°$ (c, 0.530, methanol), m.p. 154-155°. Reported values for the acid are m.p. 197-198°; $[\alpha]_p^{20} +53.2°$ (alcohol) (9); m.p. 203°, $[\alpha]_p^{20} +57°$ (alcohol) (10). Reported values for the methyl ester are m.p. 152°; $[\alpha]_p^{20} +58.6°$ (alcohol) (9).
ether, yielding 11 mg. The acid was methylated with methanolic hydrogen chloride (3 per cent), and the product was purified by crystallization from a mixture of ether and petroleum ether and finally from aqueous methanol. The crystalline ester had a specific activity of $8.02 \times 10^3$ d.p.m. per mg.; therefore, ursodeoxycholic acid contained 46.6 per cent of the chromatographed $^{14}C$.

**Table II**

Chromatography of Isotopically Diluted Ursodeoxycholic Acid

<table>
<thead>
<tr>
<th>Eluting solvent</th>
<th>Volume</th>
<th>$C^{14}$</th>
<th>Residue</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>per cent*</td>
<td>ml.</td>
<td>d.p.m.</td>
<td>mg.</td>
<td>d.p.m. per mg.</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>$1.14 \times 10^3$</td>
<td>6</td>
<td>$1.90 \times 10^3$</td>
</tr>
<tr>
<td>40-1</td>
<td>25</td>
<td>$2.52 \times 10^4$</td>
<td>7</td>
<td>$7.53 \times 10^3$</td>
</tr>
<tr>
<td>40-2</td>
<td>25</td>
<td>$1.05 \times 10^5$</td>
<td>14</td>
<td>$7.50 \times 10^3$</td>
</tr>
<tr>
<td>40-3</td>
<td>25</td>
<td>$1.35 \times 10^4$</td>
<td>2</td>
<td>$6.75 \times 10^3$</td>
</tr>
<tr>
<td>60-1</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Per cent of benzene in Skellysolve B.

**Table III**

Percentages of Chromatographed $^{14}C$ Found in 7-Ketolithocholic, Chenodeoxycholic, and Ursodeoxycholic Acids

<table>
<thead>
<tr>
<th>Animal</th>
<th>Amount eluted in 40 per cent benzene fractions</th>
<th>Chromatographed $^{14}C$ found in each acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount eluted in 40 per cent benzene fractions</td>
<td>7-Ketolithocholic</td>
</tr>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>BF$_1$</td>
<td>82.7</td>
<td>19.4</td>
</tr>
<tr>
<td>BF$_2$</td>
<td>75.1</td>
<td>21.9</td>
</tr>
<tr>
<td>BF$_3$</td>
<td>81.5</td>
<td>25.2</td>
</tr>
<tr>
<td>Average</td>
<td>79.8</td>
<td>22.2</td>
</tr>
</tbody>
</table>

* Three rats with bile fistulas, see the text.

The two zones of lesser elution of $^{14}C$ shown in Fig. 1 (Fractions 60-3, 60-4, 80-1, and 80-2 and Fractions 80-4 and 100-1) were similar to the zones of elution of Acid I and Acid II, respectively. The metabolites in these fractions were subjected to dilution experiments with the appropriate authentic acid as previously described (1). The specific activities of the diluted acids remained constant during the experiment, indicating that the radioactivity in these fractions was present in Acid I and Acid II, respec-
tively. The results of these studies of the metabolism of 7-ketolithocholic acid are summarized in Table IV. The results previously obtained for the metabolism of chenodeoxycholic acid (2) are included for comparison.

**Table IV**

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>7-Ketolithocholic acid</th>
<th>Chenodeoxycholic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chenodeoxycholic (3α,7α-diol)</td>
<td>9.9</td>
<td>55</td>
</tr>
<tr>
<td>Ursodeoxycholic (3α,7β-diol)</td>
<td>47.1</td>
<td>2.5*</td>
</tr>
<tr>
<td>7-Ketolithocholic (3α,ol,7-keto)</td>
<td>23.3</td>
<td></td>
</tr>
<tr>
<td>Acid I† (3α,6β,7β-triol)</td>
<td>14.1</td>
<td>15</td>
</tr>
<tr>
<td>II † (3α,6β,7α-triol)</td>
<td>3.6</td>
<td>20</td>
</tr>
</tbody>
</table>

* Subsequent to the observed conversion of 7-ketolithocholic acid to ursodeoxycholic acid, the appropriate chromatographic fractions, obtained after the administration of chenodeoxycholic acid-24-C\(^14\), were reexamined for the presence of radioactive ursodeoxycholic acid. Approximately 2.5 per cent of the administered C\(^14\) was found in ursodeoxycholic acid in the bile of one animal. In another experiment, we have isolated 8 mg. of ursodeoxycholic acid from approximately 2.5 liters of rat bile (127 rat days). The acid was identified by melting point and mixed melting point of the acid and its methyl ester.

† Structure assigned on the basis of the evidence presented by Hsia et al. (3, 4, 6).

**DISCUSSION**

Table IV shows that 9.9 per cent of the C\(^14\) of the administered 7-ketolithocholic acid is present in chenodeoxycholic acid. The ratio of the amounts of C\(^14\) appearing in chenodeoxycholic acid and Acid II is the same in these experiments (Table IV, 9.9:3.6) as in the experiments in which labeled chenodeoxycholic acid is studied (55:20). On this basis, the labeled Acid II derived from 7-ketolithocholic acid-24-C\(^14\) may have been formed secondarily as a metabolite of chenodeoxycholic acid. The amount of Acid I that could have been formed in the same manner secondarily from chenodeoxycholic acid approximates one-fifth of the total radioactive Acid I obtained from 7-ketolithocholic acid. The remainder of the labeled Acid I found must originate from 7-ketolithocholic acid by some other pathway.

According to the experimental observations (1) that only small amounts of ursodeoxycholic acid are obtained from the metabolism of chenodeoxycholic acid, (2) that ursodeoxycholic acid is the principal metabolite of 7-ketolithocholic acid, and (3) that chenodeoxycholic acid is converted to Acid I, it appears that the course of the metabolism of chenodeoxycholic
acid to Acid I is not directly through 7-ketolithocholic acid. However, the conversion may take place by oxidation and subsequent reduction at carbon 7 after β-hydroxylation at position 6. Thus Acid II (3α,6β,7α-trihydroxycholanic acid (5)) may be an intermediate in the formation of Acid I from chenodeoxycholic acid according to Scheme 1:

\[
\begin{align*}
7α-\text{ol} & \xrightarrow{\text{OH}} 6β,7α-\text{diol} \xrightarrow{\text{H}_2} 6β-\text{ol,7-keto} \xrightarrow{\text{H}_2} 6β,7β-\text{diol}
\end{align*}
\]

Scheme 1. Formation of Acid I from chenodeoxycholic acid

This would be consistent with the observation that Acid II is excreted in only trace amounts during obstructive jaundice in the rat, while Acid I becomes the principal bile acid (4, 11). Under such conditions bile acid levels in the tissues are elevated, and the acids remain in the animal for a longer period (2).

The presence of both ursodeoxycholic acid and chenodeoxycholic acid in the bile of normal rats suggests that interconversion of hydroxyl groups at carbon 7 in the bile acid series may occur in this animal. The recovery of both epimers after the administration of 7-ketolithocholic acid is consistent with this view; however, the disproportion of epimers suggests that oxidation of the 7α-ol is insignificant in the normal rat.

SUMMARY

7-Ketolithocholic acid-24-C\textsuperscript{14} was prepared from chenodeoxycholic acid-24-C\textsuperscript{14} and administered intragastrically to rats with cannulated bile ducts. 94.7 per cent of the C\textsuperscript{14} appeared in the fraction containing taurine conjugates.

After hydrolysis, 14 per cent of the C\textsuperscript{14} was found in Acid I, 3.6 per cent in Acid II, 47 per cent in ursodeoxycholic acid, 10 per cent in chenodeoxycholic acid, and 23 per cent in 7-ketolithocholic acid.

BIBLIOGRAPHY


\footnote{The bile of another species has already been observed to contain all three members of such a metabolic sequence. Ursodeoxycholic acid, chenodeoxycholic acid, and 7-ketolithocholic acid have been observed in the bile of the coypu (12, 13).}
BILE ACIDS: VIII. METABOLISM OF 7-KETOLITHOCHOLIC ACID-24-C14 IN THE RAT


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