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Conversion of Cholesterol to Trihydroxyprostanic Acid and Cholic Acid in Man*  

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Some lower vertebrates, such as certain fishes, amphibia, and reptiles, do not shorten the cholesterol side chain in the formation of bile acids but simply oxidize the terminal methyl groups to "primitive" 27-carbon bile alcohols or acids. Haslewood has proposed that some of these C-27 compounds may be intermediates in the biochemical pathway by which more recently evolved animals transform cholesterol to "modern" C-24 bile acids (1).

This proposal is supported by experimental evidence showing that a C-27 bile acid, trihydroxyprostanic acid,1 is derived from cholesterol in the alligator (2) and rapidly converted to cholic acid in the rat possessing a bile fistula (3). In man the administration of cholesterol-26-C14 gives rise to a radioactive fraction in bile that crystallizes to constant specific activity with added trihydroxyprostanic acid (4).

Crystallization of naturally occurring trihydroxyprostanic acid from human bile (5) identical with the chief bile acid of alligators and crocodiles (6) led to the present experiment to determine if this compound is a normal intermediate in the conversion of cholesterol to cholic acid in man.

Methods and Results

Cholesterol-4-C14 was homogeneous on silicic acid Super-Cel columns (7) and had a constant specific activity when recrystallized with unlabeled cholesterol of known purity. Approximately 27 μg of the labeled cholesterol, dissolved in 0.25 ml of ethanol, was injected intravenously into an 80-year-old male who had cholelithiasis, choledochocholithiasis, and cholecystitis treated by cholecystectomy and placement of a T-tube in the common duct 5 days before the experiment. Laboratory values the day before surgery were as follows: total serum bilirubin, 4.6 mg per 100 ml; zinc turbidity, 7 U; cephalin cholesterol flocculation, 2+; thymol turbidity, 5 U; serum albumin, 3.1 g per 100 ml; alkaline phosphatase, 29 King-Armstrong U; blood urea nitrogen, 14 mg per 100 ml; prothrombin time, 12.6 seconds, control 11.6 seconds. Two days after the operation the total serum bilirubin declined to 3.2 mg per 100 ml, and jaundice disappeared. The patient was discharged the day after the experiment and had an uneventful recovery.

Excretion of radioactivity in bile after cholesterol-4-C14 injection. All bile was collected in ethanol; each fraction was adjusted to contain 50% ethanol and stored at 5°C. Of the administered radioactivity, approximately 30% was excreted in the bile in the first 5 days. The cumulative percentage of the administered radioactivity excreted in the bile is plotted against time in Figure 1. The isotope content of the bile was determined by evaporating 0.1 ml of the 50% bile-ethanol mixture on stainless steel planchets in triplicate and using a gas flow counter.
Isolation of trihydroxycoprostanic acid-4-C\(^{14}\) from bile. All bile excreted for the first 120 hours (5 days) after injection of the labeled cholesterol-4-C\(^{14}\) totaled 1,958 ml. This quantity was extracted in approximately 200-ml batches; the labeled trihydroxycoprostanic acid was isolated and identified by the same procedure as previously described (5). Briefly this entails ethanol extraction, hydrolysis (4.5 N NaOH), chromatography on Celite-acetic acid columns, methylation, and further purification on alumina and Celite-ethanol columns. The crystalline methyl ester thus obtained (35.6 mg), melting point, 153 to 155° C, had an infrared spectrum in KBr identical with that of authentic methyl-3\(\alpha\), 7\(\alpha\), 12\(\alpha\)-trihydroxycoprostanate. About 20 \(\mu\)g of the free acid obtained from the methyl ester as described in the next paragraph was chromatographed on paper (8), stationary phase, 70% acetic acid in water (vol/vol), moving phase, 20% (vol/vol) isopropyl ether in petroleum ether (boiling point, 80 to 100° C), and had a mobility and color of fluorescence with the antimony trichloride reagent (9) the same as the control trihydroxycoprostanic acid. A 5-mg fraction was recrystallized to constant specific activity (Table 1). The radioactivity of the methyl ester was measured by dissolving the crystals in 10 ml of toluene containing 0.3% 2,5-diphenyloxazole and counting in a coincidence type liquid scintillation counter.

Conversion of trihydroxycoprostanic acid-4-C\(^{14}\) to cholic acid 4-C\(^{14}\). Methyl trihydroxycoprostanate, obtained as described above, was hydrolyzed with 0.1 ml of 5 N KOH in 0.5 ml of ethanol for 40 minutes on the steam bath. An equal volume of water and 2 drops of 12 N HCl caused a precipitate that was extracted from the aqueous phase with anhydrous ethyl ether. The residue from the evaporated ether phase was dissolved in 1 ml of 50% ethanol containing 0.05 mM NaOH. Approximately 0.49 mmole per L of the labeled sodium trihydroxycoprostanate containing 10,500 cpm was dissolved in 2 ml of 50% ethanol and injected through the tubing of an intravenous saline drip. The recipient was a 70-year-old woman who had had a T-tube placed in the common bile duct 5 days previously because of a leiomyosarcoma of the pancreas with metastasis to the liver. The patient was not jaundiced; laboratory values the day before surgery were as follows: alkaline

### TABLE 1

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Weight mg</th>
<th>SA mg</th>
<th>cpm</th>
<th>cpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone-petroleum ether</td>
<td>4.44</td>
<td>2,125</td>
<td>480</td>
<td></td>
</tr>
<tr>
<td>Acetone-petroleum ether</td>
<td>4.28</td>
<td>2,070</td>
<td>493</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>3.85</td>
<td>1,950</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td>Ether-petroleum ether</td>
<td>2.96</td>
<td>1,450</td>
<td>490</td>
<td></td>
</tr>
<tr>
<td>Ether</td>
<td>1.98</td>
<td>1,110</td>
<td>561</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>506</td>
<td></td>
</tr>
</tbody>
</table>
phosphatase, 7 King-Armstrong U; Bromsulphalein retention, 5%; serum amylase, 50 Somogyi U.

The total quantity of bile (65 ml) excreted from the T-tube for 5 hours after the injection was collected in ethanol; it contained 8,600 cpm or 82% of the injected radioactivity. This was diluted with 4 vol of ethanol, filtered, and evaporated under nitrogen. The residue was hydrolyzed in 200 ml of 4.5 N NaOH for 5 hours at 15 pounds per square inch. The hydrolysate was diluted with an equal volume of water, pH adjusted to less than 2 with 12 N HCL, and the solution extracted twice with 500 ml of chloroform. The residue from the chloroform extract was placed on a 3.6-× 30-cm Celite column with 70% acetic acid-water (vol/vol) as stationary phase and increasing concentrations of benzene in petroleum ether as moving phase (10). Of the radioactivity excreted in the bile, 86.5% appeared as a single peak in the cholic acid fraction (Figure 2). The center of this peak (first half of 80% benzene fraction) was evaporated, and a few milligrams was removed for paper chromatography. The remainder of the residue (95 mg contaminated with pigment) was methylated with diazomethane and chromatographed on 500 mg of dry Al2O3 to which 0.02 ml of water had been added. The column was prepared in benzene and eluted with benzene (45 ml), ethyl ether (20 ml), acetone (25 ml), and methanol (15 ml). Most of the solid material was eluted with acetone and contained traces of yellow pigment. Repeated crystallizations from ether-petroleum ether and acetone-petroleum ether yielded 52.0 mg of a white crystalline product that was then recrystallized 6 times at relatively constant specific activity (Table II).

A paper chromatogram, stationary phase, 70% acetic acid in water (vol/vol), moving phase, 60% isopropyl ether in petroleum ether (vol/vol), with 1 mg of the labeled free acid applied along the starting line and cholic acid controls on either side, was run descending for 2 hours so that the solvent front remained on the paper. A strip cut lengthwise from the paper was developed with the antimony trichloride reagent and revealed the free acid to have the same mobility and color of fluorescence as the cholic acid control. No other spots appeared on the chromatogram. The remainder of the paper was cut into 6 equal sections; section 1 included the source, and section 6, the front. The sections were eluted with ethanol that was evaporated and counted on planchets in a gas flow counter. All the radioactivity eluted was confined to the cholic acid section.

The infrared spectrum of the methyl ester (melting point, 153 to 154° C) was identical with authentic methyl cholate.

\[ \text{Excretion of unchanged trihydroxycoprostanic acid-4-C}^{14} \]  
To determine if any of the administered trihydroxycoprostanic acid were excreted in the bile unchanged (except for probable conjugation with taurine and glycine), the fractions containing small amounts of radioactivity preceding

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**TABLE II**

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Weight</th>
<th>C.P.M.</th>
<th>C.P.M./mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl ether-petroleum ether</td>
<td>5.7</td>
<td>450</td>
<td>79</td>
</tr>
<tr>
<td>Ethyl ether-petroleum ether</td>
<td>3.9</td>
<td>310</td>
<td>79</td>
</tr>
<tr>
<td>Acetone-petroleum ether</td>
<td>3.4</td>
<td>280</td>
<td>82</td>
</tr>
<tr>
<td>Ethyl ether-petroleum ether</td>
<td>3.4</td>
<td>273</td>
<td>80</td>
</tr>
<tr>
<td>Acetone-petroleum ether</td>
<td>3.2</td>
<td>270</td>
<td>83</td>
</tr>
<tr>
<td>Ethyl ether-petroleum ether</td>
<td>2.8</td>
<td>233</td>
<td>83</td>
</tr>
</tbody>
</table>

Average = 80
the cholic acid peak (Figure 2) were combined and rechromatographed on a small (1-× 30-cm) Celite column using the same solvent system employed for the column in Figure 2. The effluent was collected in approximately 5-ml fractions distributed over 120 tubes. Fractions in the 40% benzene effluent were assayed for trihydroxyco- prostanic acid by glass paper chromatography (11) with isooctane, 100 ml, and acetic acid, 1.5 ml (vol/vol), as the moving phase, ascending for 12 minutes. The fractions containing trihydroxyco- prostane (II) and trihydroxyco- prostanic acid (IV), both having the cholic acid nucleus but retaining the 8-carbon side chain, are rapidly converted to cholic acid in the rat (13, 3). A possible intermediate in this sequence, 3a, 7a, 12a, 26-tetrahydroxyco- prostane (III), is formed from II by rat and mouse liver homogenates and mitochondria, and is converted to IV by mouse liver homogenates (14). Oxidation of cholesterol to II has been achieved in supernatant fluids from rat liver.
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homogenates (15), and IV has been derived from cholesterol in the alligator (2) and in man (4). In rat liver mitochondrial preparations the 3 terminal carbon atoms of the side chain are split off as propionyl CoA (16).

In none of these animal experiments, however, has trihydroxyprostanic acid been isolated from mammalian bile without the addition of carrier compounds, nor has the stepwise conversion of cholesterol $\rightarrow$ IV $\rightarrow$ V been demonstrated in mammals alone. The crystallization of naturally occurring trihydroxyprostanic acid from human bile (5) provided a means to demonstrate the oxidation of cholesterol via trihydroxyprostanic acid to cholic acid in bile fistula patients.

These studies do not imply that the sequence shown in Figure 3 represents the only pathway for cholic acid synthesis in man. Quite probably, however, it is at least one of the major routes. The efficient conversion of bishomocholic acid (VII) to cholic acid in the rat (17) demonstrates that other pathways are possible. The very poor conversion of scy mol (VIII), the chief bile alcohol of sharks, to cholic acid (5%) by the rat (18) is further evidence of alternative pathways in mammals, but these pathways are of very low efficiency when compared to the rapid oxidation of trihydroxyprostanic acid to cholic acid.

The quantity of trihydroxyprostanic acid isolated from the bile fistula patient in this study and the three patients in the previous study (5) must be regarded as minimal because losses undoubtedly occurred in the process of purification. An estimate of the minimal quantities in fistula bile from these four patients would give values of 0.01 to 0.08 mg per ml. This amount of trihydroxyprostanic acid represents about 0.3 to 2.7% of the primary bile acids (chenodeoxycholic, 0.45 mg per ml; cholic, 2.50 mg per ml) found in the bile collections studied. The primary bile acids were quantitated by a previously described method (19). It is likely that smaller amounts of trihydroxyprostanic acid normally occur in bile when the enterohepatic circulation remains intact and that excretion of this compound is increased with a bile fistula, for external drainage of bile greatly accelerates the synthesis rates of both cholesterol (20) and bile acids (21).

Primary bile acids are those formed from cholesterol in the liver. Trihydroxyprostanic acid may therefore be regarded as a primary bile acid in man and a precursor for another primary bile acid, cholic acid. Secondary bile acids are formed from primary bile acids by microorganisms in the colon. When cholic acid reaches the colon, it is converted by bacteria to deoxycholic acid (VI), which is one of the final excretory products of cholesterol. Deoxycholic acid has been isolated from feces (22), and its origin from cholesterol (23, 24) via cholic acid (25, 26) has been described. A number of other secondary bile acids derived from cholic acid have also been described (27). It is thus possible to delineate a pathway in man for the synthesis of cholic acid from cholesterol with trihydroxyprostanic acid as an intermediate and deoxycholic acid as one of the final excretory metabolites.

The fact that trihydroxyprostanic acid is the major bile acid end product derived from cholesterol in certain primitive reptiles and amphibians but is an intermediate in the formation of cholic acid in man provides strong support for Haslem’s proposal (28) concerning the evolutionary significance of bile acids. An interesting implication is that in the formation of cholic acid, man performs a biochemical recapitulation of human evolutionary history.

Summary

After intravenous administration of cholesterol-4-C14 to a patient with a bile fistula, a cholesterol metabolite, trihydroxyprostanic acid-4-C14 was isolated from the bile, identified, and crystallized to constant specific activity. This product was given intravenously to a second bile fistula patient; and cholic acid-4-C14, the major radioactive compound excreted, was isolated from the bile, identified, and crystallized to constant specific activity.

This study has led to these conclusions: 1) Trihydroxyprostanic acid is a naturally occurring intermediate in the conversion of cholesterol to cholic acid in man. 2) Cholic acid is the major product of trihydroxyprostanic acid oxidation in man. 3) Trihydroxyprostanic acid may be regarded as another human primary bile acid. 4) Small amounts of trihydroxyprostanic acid may be excreted without further oxidation by the liver in bile fistula patients.
Acknowledgments

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References