On the Mechanism of the Formation of Hyodeoxycholic Acid in the Pig

Bile Acids and Steroids 89

SUNE BERGSTROM, AKE GORANSSON and BENGT SAMUELSSON

Department of Chemistry, Karolinska Institutet, Stockholm, and Djursjukhuset, Hälsingborg, Sweden

The mechanism of the formation of hyodeoxycholic acid has been studied in the pig with chenodeoxycholic acid-7β-3H, 24-14C. This acid was injected intraperitoneally into pigs and a bile fistula was prepared three days later. By this procedure the administered acid is hydroxylated in the liver to hyocholic acid (3α,6α,7α-trihydroxycholanic acid), which is then dehydroxylated to hyodeoxycholic acid (3α,6α-dihydroxycholanic acid) by the microorganisms in the intestine. Determination of the 3H and 14C content of chenodeoxycholic, hyocholic and hyodeoxycholic acids, isolated from the bile, showed that the 3H-label is retained during the hyodeoxycholic acid formation. The mechanism of the elimination of the 7α-hydroxyl group is discussed.

The main bile acids in the normal pig bile are chenodeoxycholic, hyocholic and hyodeoxycholic acids, which occur conjugated mainly with glycine but also to a small extent with taurine^1. The metabolism of the above mentioned bile acids in the pig has recently been investigated in our laboratories^2. It was found that chenodeoxycholic acid (3α,7α-dihydroxycholanic acid) is 6α-hydroxylated to hyocholic acid (3α,6α,7α-trihydroxycholanic acid) in the liver, and the latter acid is then dehydroxylated to hyodeoxycholic acid (3α,6α-dihydroxycholanic acid) during the enterohepatic circulation. Hyodeoxycholic acid is not rehydroxylated in the liver. Immediately after the preparation of a bile fistula all three acids are present in the bile. However, hyodeoxycholic acid gradually vanishes and after two days only chenodeoxycholic and hyocholic acids are present^2. Furthermore, after administration of cholesterol-4,14C to a bile fistula pig the only labelled acidic products excreted in the bile are chenodeoxycholic and hyocholic acids^3. All these experiments demonstrate

Acta Chem. Scand. 13 (1959) No. 9
that hyodeoxycholic acid is formed secondarily from hyocholic acid in the intestine and exclude that it may be synthesized primarily in the liver.

The aim of the present investigation was to study the mechanism of hyodeoxycholic acid formation with the aid of chenodeoxycholic acid-7β-3H, 24-14C.

MATERIAL AND METHODS

*Labelled acids:* Chenodeoxycholic acid 7β-3H was prepared by reducing 7-ketolitchocholic acid with tritium-labelled sodium borohydride as described previously *1. Specific activity: 0.5 µC/mg.*

Chenodeoxycholic acid-24-14C was prepared according to the method of Bergström *et al.* * Specific activity: 10 µC/mg.*

A stock solution of chenodeoxycholic acid-7-β-3H, 24-14C was prepared by dissolving 21.0 mg of chenodeoxycholic acid-7-β-3H and 0.5 mg of chenodeoxycholic acid-24-14C in acetone. The ratio between tritium and 14C of this acid was 1.13, when determined by the gas phase counting procedure.

*Animal experiments.* 10 mg of doubly labelled chenodeoxycholic acid was injected intraperitoneally as the sodium salt in 0.9 % aqueous sodium chloride into each of two young female pigs weighing about 20 kg. The bile fistulas were prepared three days after the injection of the labelled bile acid, according to the technique described earlier *1, and bile collection in ethanol immediately started.

*Fractionation of the bile.* The bile from 5 h collection periods was filtered, evaporated to dryness and hydrolyzed with 150 ml of 2 N KOH at 120° for 6 h in a sealed flask. The free bile acids were extracted from the acidified solution with ether.

*Chromatographic separations.* Reversed phase partition chromatography as described by Bergström, Norman and Sjövall *6,7* was used for separation of the free bile acids. The following solvent systems were used:

<table>
<thead>
<tr>
<th>System</th>
<th>Moving phase</th>
<th>ml</th>
<th>Stationary phase</th>
<th>ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>C*</td>
<td>Methanol:water</td>
<td>150:150</td>
<td>Chloroform:isoctanol</td>
<td>15:15</td>
</tr>
<tr>
<td>F*</td>
<td>Methanol:water</td>
<td>165:135</td>
<td>Chloroform:heptane</td>
<td>45:5</td>
</tr>
</tbody>
</table>

3 ml of the stationary phase was supported on 4.5 g of Hostalene (Farbwerke Hoechst G.m.b.h., West Germany) *8. Hostalene was purified before use by extraction with 95 % ethanol for 48 h in a Soxhlet extractor and then dried at 75°. All the chromatograms were run at a constant temperature of +23°.*

*Isotope analyses.* The administered and isolated bile acids were combusted to CO2 and water. The water was converted to butane with butyl magnesium bromide and the 14C and 3H of the CO2 and butane was determined by gas phase counting as described by Glasecock *4. Two determinations were made on each bile acid sample.

RESULTS

Chenodeoxycholic acid 7β-3H, 24-14C was injected intraperitoneally into two pigs and bile fistulas were prepared three days after the injection. It has previously been demonstrated that chenodeoxycholic acid is hydroxylated at the 6α-position to hyocholic acid (3α,6α,7α-trihydroxycholanic acid) in the liver. The latter acid is dehydroxylated to hyodeoxycholic acid (3α,6α-dihydroxycholanic acid) by the intestinal microorganisms during the enterohepatic circulation. By the above-mentioned procedure it was thus expected that the injected doubly labelled chenodeoxycholic acid would be converted into both hyocholic and hyodeoxycholic acids.

Fig. 1. Chromatographic separation of acids from hydrolyzed pig bile, excreted during 5 h following the preparation of the bile fistula. Chenodeoxycholic acid-7β-3H, 24-14C was administered 3 days before the operation. Column: 45 g Hostalene. Solvent system: Type C.

The hydrolyzed bile was chromatographed with solvent system C. In Fig. 1 is shown a chromatogram of the bile acids excreted during 5 h immediately following the operation (Pig 1).

The material in the fractions of the peak (A. 650—1 000 ml effluent) which has the elution volume characteristic of hyocholic acid, was crystallized from aqueous acetic acid and aqueous acetone. Yield: 141.6 mg of hyocholic acid, m. p. 184—185°. (Lit.10 m. p. 188—189°.) The second peak of the chromatogram shown in Fig. 1 (B. 1 350—2 400 ml effluent) was rechromatographed with solvent system F (Fig. 2), which is suitable for separation of dihydroxycholanic acids. Two major peaks appear, of which the first one (150—290 ml effluent) is eluted with the elution volume of hyodeoxycholic acid and the second one (300—410 ml effluent) with that of chenodeoxycholic acid. The material in these peaks was crystallized from ethyl acetate and ethylacetate/light petroleum, respectively, yielding 135.7 mg of hyodeoxycholic acid m. p. 198—199°, (Lit.10 m. p. 197—198°) and 95.8 mg of chenodeoxycholic acid, m. p. 140—141° (Lit.11 m. p. 140—142°).

The 3H and 14C content of the administered chenodeoxycholic acid 7β-3H, 24-14C and the isolated chenodeoxycholic, hyocholic and hyodeoxycholic acids from two pigs was determined. From the results of these determinations (Table 1) it is obvious that the original 3H-labelling in chenodeoxycholic acid is retained both in hyocholic acid and in hyodeoxycholic acid.

Hyodeoxycholic acid, isolated from the bile, was oxidized with chromic acid in aqueous acetic acid at 2—3° to 3α-hydroxy-6-ketocholanic acid (m. p. 156—160°)12 which had the same 3H content as the starting material (Table 1).

_Acta Chem. Scand._ 13 (1959) No. 9
Fig. 2. Chromatographic separation of peak B of the chromatogram, shown in Fig. 1. Column: 22.5 g Hostalene. Solvent system: Type F.

Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^3$H, c.p. m/mg</th>
<th>$^3$H, c.p. m/mg</th>
<th>$^{14}$C, c.p. m/mg</th>
<th>$^{14}$C, c.p. m. mean</th>
<th>$^{3}$H/$^{14}$C</th>
<th>Per cent $^3$H retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Administered chenodeoxycholeic acid-7a-$^3$H, 24-$^{14}$C</td>
<td>220</td>
<td>215</td>
<td>192</td>
<td>194</td>
<td>193</td>
<td>1.13</td>
</tr>
<tr>
<td>Chenodeoxycholic acid, Pig I</td>
<td>29</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyocholic acid, Pig I</td>
<td>75</td>
<td>76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyodeoxycholic acid, Pig I</td>
<td>58</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a-Hydroxy-6-keto cholanic acid</td>
<td>72</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a-Hydroxy-6-keto allo-cholanic acid</td>
<td>5</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The keto acid (200 mg) was dissolved in 15 ml 2 N KOH and refluxed for 24 h. The product was crystallized from ethyl acetate yielding 112 mg of 3a-hydroxy-6-keto allocholanic acid (m. p. 187–188°) 12. This acid contained only 5% of the original 3H labelling.

DISCUSSION

The formation of hyodeoxycholic acid in the pig involves elimination of the 7α-hydroxyl group from hyocholic acid, which is formed in the liver through 6α-hydroxylation of the primarily synthesized chenodeoxycholic acid.

This type of elimination reaction was originally demonstrated in the rabbit 13, in which the main bile acid, deoxycholic acid, is formed by dehydroxylation of cholic acid during the enterohepatic circulation. The same reaction has also been found in man 14 and in the rat 15. The elimination of the hydroxyl group in hyocholic acid as well as in cholic acid is carried out by the microorganisms in the intestine. The mechanism of the elimination of the 7α-hydroxyl group has been studied in the rabbit with the aid of 7β-tritio-cholic acid 16. The fact that the tritium label was retained in the deoxycholic acid excluded that an intermediate ketone formation occurs, although 7-keto-deoxycholic acid was rapidly converted into deoxycholic acid. This makes it probable that the dehydroxylation consists of a dehydration-reduction reaction sequence or a direct elimination. As 7α-hydroxylation of the formed deoxycholic acid in the rat liver resulted in complete elimination of the tritium label it appears probable that it had been moved into the 7α-position.

In the present investigation the formation of hyodeoxycholic acid through an analogous reaction was studied with chenodeoxycholic acid-7β-3H, 2414C. Determination of 3H and 14C in the administered and isolated chenodeoxycholic acid and isolated hyocholic and hyodeoxycholic acids showed that the 3H-label is retained in the molecule during these transformations, i.e. hydroxylation at C6 and dehydroxylation at C7. The 3H-label was still present in 3α-hydroxy-6-ketocholanic acid, formed by chromic acid oxidation of hyodeoxycholic acid, but could be eliminated by a base-catalyzed exchange of the keto-derivative, indicating that the 3H-label was retained at C7 in hyodeoxycholic acid. The available data do not indicate if the label was in the 7α- or 7β-position (cf. above).

The most probable mechanisms for the elimination reaction, which are consistent with the isotopic experiments, are summarized below.

1) Direct elimination of the hydroxyl group at C7.
2) a) Dehydration with the formation of a Δ6 or Δ7-double bond.
   b) Reduction of this double bond.
3) a) Dehydration to the Δ6-unsaturated derivative.
   b) Enol-ketone-rearrangement to 3α-hydroxy-6-ketocholanic acid.
   c) Reduction of the 6-ketoderivative to hyodeoxycholic acid.

The first two mechanisms have been discussed previously for the elimination of the 7α-hydroxyl group from cholic acid. Nor is it possible to differentiate between these two possibilities by the experiments reported in this paper.

Acta Chem. Scand. 13 (1959) No. 9
In the hyodeoxycholic acid formation, however, a third mechanism cannot be excluded. Dehydration of hyocholic acid to the $\Delta^6$-unsaturated derivative entails the formation of a compound with an enol configuration that might very easily rearrange to the ketone. Further work is in progress to clarify the above-mentioned questions.

Acknowledgements. We are very grateful to Dr. Fritz Sevelius, Djurafjukhuset, Hälsingborg, for the facilities and help placed at our disposal. The technical assistance of Miss B. Turedotter, Miss B. Holmberg and Mr. S. Jonsson is gratefully acknowledged. This work is part of investigations supported by Knut och Alice Wallenbergs Stiftelse, Sweden, and the National Heart Institute, National Institutes of Health, Bethesda, Maryland, USA (H 2842).

REFERENCES


Received July 8, 1959.