

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

Bile Acids and Steroids 89

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The mechanism of the formation of hyodeoxycholic acid has been studied in the pig with chenodeoxycholic acid- $7\beta$ - $^3\text{H}$ ,  $24$ - $^{14}\text{C}$ . 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 ( $3\alpha,6\alpha,7\alpha$ -trihydroxycholanic acid), which is then dehydroxylated to hyodeoxycholic acid ( $3\alpha,6\alpha$ -dihydroxycholanic acid) by the microorganisms in the intestine. Determination of the  $^3\text{H}$  and  $^{14}\text{C}$  content of chenodeoxycholic, hyocholic and hyodeoxycholic acids, isolated from the bile, showed that the  $^3\text{H}$ -label is retained during the hyodeoxycholic acid formation. The mechanism of the elimination of the  $7\alpha$ -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 <sup>1</sup>. The metabolism of the above mentioned bile acids in the pig has recently been investigated in our laboratories <sup>2</sup>. It was found that chenodeoxycholic acid ( $3\alpha,7\alpha$ -dihydroxycholanic acid) is  $6\alpha$ -hydroxylated to hyocholic acid ( $3\alpha,6\alpha,7\alpha$ -trihydroxycholanic acid) in the liver, and the latter acid is then dehydroxylated to hyodeoxycholic acid ( $3\alpha,6\alpha$ -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 <sup>2</sup>. Furthermore, after administration of cholesterol- $4$ - $^{14}\text{C}$  to a bile fistula pig the only labelled acidic products excreted in the bile are chenodeoxycholic and hyocholic acids <sup>3</sup>. All these experiments demonstrate

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- $7\beta$ - $^3\text{H}$ ,  $24$ - $^{14}\text{C}$ .

#### MATERIAL AND METHODS

*Labelled acids:* Chenodeoxycholic acid  $7\beta$ - $^3\text{H}$  was prepared by reducing 7-ketolithocholic acid with tritium-labelled sodium borohydride as described previously <sup>4</sup>. Specific activity:  $0.5 \mu\text{C}/\text{mg}$ .

Chenodeoxycholic acid- $24$ - $^{14}\text{C}$  was prepared according to the method of Bergström *et al.*<sup>5</sup> Specific activity:  $10 \mu\text{C}/\text{mg}$ .

A stock solution of chenodeoxycholic acid- $7\beta$ - $^3\text{H}$ ,  $24$ - $^{14}\text{C}$  was prepared by dissolving 21.0 mg of chenodeoxycholic acid- $7\beta$ - $^3\text{H}$  and 0.5 mg of chenodeoxycholic acid- $24$ - $^{14}\text{C}$  in acetone. The ratio between tritium and  $^{14}\text{C}$  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 <sup>2</sup>, 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  $120^\circ$  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 <sup>6,7</sup> was used for separation of the free bile acids. The following solvent systems were used:

System	Moving phase	ml	Stationary phase	ml
C <sup>6</sup>	Methanol:water	150:150	Chloroform:isooctanol	15:15
F <sup>7</sup>	Methanol:water	165:135	Chloroform:heptane	45:5

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

*Isotope analyses.* The administered and isolated bile acids were combusted to  $\text{CO}_2$  and water. The water was converted to butane with butyl magnesium bromide and the  $^{14}\text{C}$  and  $^3\text{H}$  of the  $\text{CO}_2$  and butane was determined by gas phase counting as described by Glascock <sup>6</sup>. Two determinations were made on each bile acid sample.

#### RESULTS

Chenodeoxycholic acid  $7\beta$ - $^3\text{H}$ ,  $24$ - $^{14}\text{C}$  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  $6\alpha$ -position to hyocholic acid ( $3\alpha,6\alpha,7\alpha$ -trihydroxycholanic acid) in the liver. The latter acid is dehydroxylated to hyodeoxycholic acid ( $3\alpha,6\alpha$ -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.

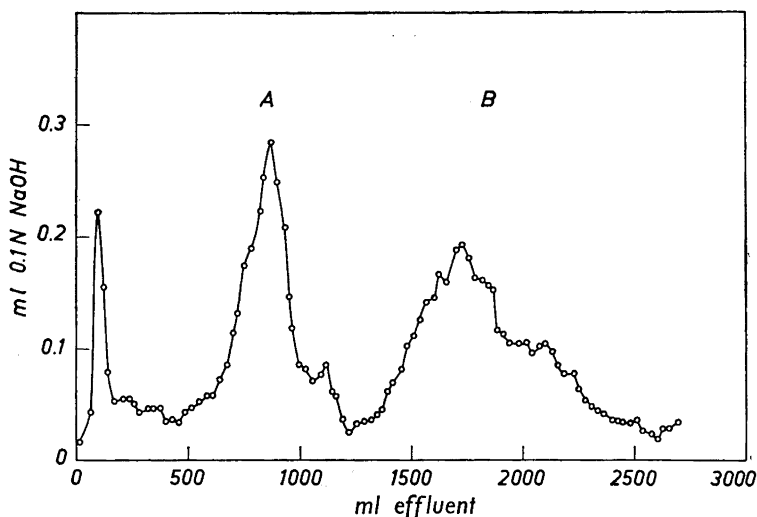


Fig. 1. Chromatographic separation of acids from hydrolyzed pig bile, excreted during 5h following the preparation of the bile fistula. Chenodeoxycholic acid- $7\beta$ - $^3\text{H}$ , $24$ - $^{14}\text{C}$  was administered 3 days before the operation. Column: 45 g Hostalene. Solvent system: Type C.

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 (Fig 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.<sup>10</sup> 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 dihydroxycholic 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.<sup>10</sup> m. p. 197–198°) and 95.8 mg of chenodeoxycholic acid, m. p. 140–141° (Lit.<sup>11</sup> m. p. 140–142°).

The  $^3\text{H}$  and  $^{14}\text{C}$  content of the administered chenodeoxycholic acid  $7\beta$ - $^3\text{H}$ ,  $24$ - $^{14}\text{C}$  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  $^3\text{H}$ -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  $3\alpha$ -hydroxy-6-ketocholic acid (m.p. 156–160°)<sup>12</sup> which had the same  $^3\text{H}$  content as the starting material (Table 1).

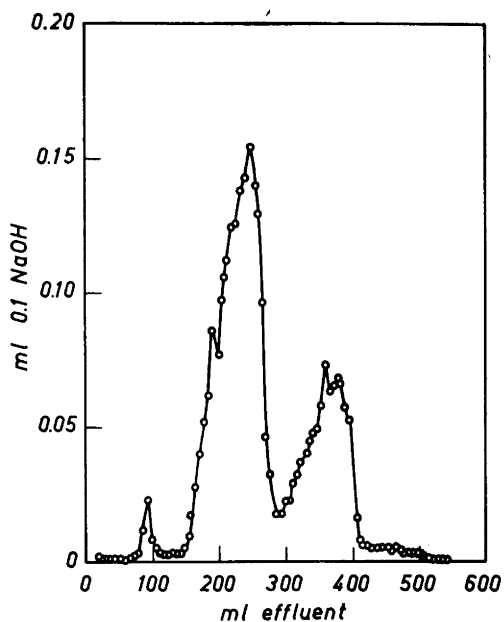


Fig. 2. Chromatographic separation of peak B of the chromatogram, shown in Fig. 1. Column: 22.5 g Hostalene. Solvent system: Type F.

Table 1.

Compound	$^3\text{H}$ , c.p. m/mg	$^3\text{H}$ , c.p. m/mg mean	$^{14}\text{C}$ , c.p. m/mg	$^{14}\text{C}$ , c.p. m. mean	$^3\text{H}^{14}\text{C}$	Per cent $^3\text{H}$ retained
Administered chenodeoxy- cholic acid-7 $\alpha$ - $^3\text{H}$ , 24- $^{14}\text{C}$	220		192			
Chenodeoxycholic acid, Pig I	215	218	194	193	1.13	
» » Pig I	29	27	25	25	1.08	96
» » Pig II	19		17	17	1.18	104
» » Pig II	20	20	17	17		
Hyocholic acid, Pig I	75		76			
» » Pig I	83	79	73	75	1.05	93
» » Pig II	93		78			
» » Pig II	87	90	79	79	1.14	101
Hyodeoxycholic acid, Pig I	58		53			
» » Pig I	56	57	58	56	1.02	90
» » Pig II	74		73			
» » Pig II	76	75	74	74	1.01	89
3 $\alpha$ -Hydroxy-6-keto cholic acid	72		69			
» »	71	72	70	70	1.03	91
3 $\alpha$ -Hydroxy-6-keto allo- cholic acid	5		70			
» »	2	4	73	72	0.06	5

The keto acid (200 mg) was dissolved in 15 ml 2 N KOH and refluxed for 24 h. The product was crystallized from ethylacetate yielding 112 mg of 3 $\alpha$ -hydroxy-6-keto allocholic acid (m. p. 187–188°)<sup>12</sup>. This acid contained only 5 % of the original <sup>3</sup>H labelling.

#### DISCUSSION

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

This type of elimination reaction was originally demonstrated in the rabbit<sup>13</sup>, 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<sup>14</sup> and in the rat<sup>15</sup>. 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 7 $\alpha$ -hydroxyl group has been studied in the rabbit with the aid of 7 $\beta$ -tritio-cholic acid<sup>16</sup>. The fact that the tritium label was retained in the deoxycholic acid excluded that an intermediate ketone formation occurs, although 7-ketodeoxycholic 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 7 $\alpha$ -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 7 $\alpha$ -position. In the present investigation the formation of hyodeoxycholic acid through an analogous reaction was studied with chenodeoxycholic acid-7 $\beta$ -<sup>3</sup>H, 24-<sup>14</sup>C. Determination of <sup>3</sup>H and <sup>14</sup>C in the administered and isolated chenodeoxycholic acid and isolated hyocholic and hyodeoxycholic acids showed that the <sup>3</sup>H-label is retained in the molecule during these transformations, *i. e.* hydroxylation at C6 and dehydroxylation at C7. The <sup>3</sup>H-label was still present in 3 $\alpha$ -hydroxy-6-ketocholic 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 <sup>3</sup>H-label was retained at C7 in hyodeoxycholic acid. The available data do not indicate if the label was in the 7 $\alpha$ - or 7 $\beta$ -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  $\Delta^6$  or  $\Delta^7$ -double bond.  
b) Reduction of this double bond.
- 3) a) Dehydration to the  $\Delta^6$ -unsaturated derivative.  
b) Enol-ketone-rearrangement to 3 $\alpha$ -hydroxy-6-ketocholic acid.  
c) Reduction of the 6-ketoderivative to hyodeoxycholic acid.

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

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.

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