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Metabolism of 3α,7β,12α-Trihydroxycholanic Acid in the Rat

Bile Acids and Steroids 95

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Tritium labelled 3α, 7β, 12α-trihydroxycholanic acid has been prepared. The metabolism of this acid after intraperitoneal or intracecal injection into bile fistula rats has been studied. It is not attacked by the liver enzymes but is extensively transformed by the intestinal microorganisms. Deoxycholic- and 12-ketolithocholic acids were identified in the feces and cecum and cholic, deoxycholic and 12-ketolithocholic acids in the bile.

In a recent investigation on the transformation of cholic acid-24-^{14}C in the rat during the enterohepatic circulation a metabolite was isolated with properties identical with 3α, 7β, 12α-trihydroxycholanic acid. Material with the same chromatographic behaviour and sulfuric acid spectrum was also isolated from human bile samples [2]. 3α, 7β, 12α-Trihydroxycholanic acid is probably only formed by reduction of a bacterial metabolite of cholic acid, 7-ketodeoxycholic acid (3α, 12α-dihydroxy-7-ketocholanic acid) in the liver [3].

The metabolism of randomly tritium labelled 3α, 7β, 12α-trihydroxycholanic acid in the rat liver and intestine and the liver metabolism of the intestinal metabolites has been studied in the present investigation.

EXPERIMENTAL

Bile acid samples

Tritium labelled 3α, 7β, 12α-trihydroxycholanic acid. The inactive acid [4] (5 mg) was exposed to tritium gas [5] (2 Curie, 99 % pure, 200 mm Hg. Purchased from Isotope Division, A. E. R. E. Harwell, England) at room temperature for 4 days. The tritium labelled product was purified by chromatography with solvent system C3 after dilution with 30 mg of inactive 3α, 7β, 12α-trihydroxycholanic acid. It was crystallized by acidification of an aqueous solution of the sodium salt, yielding 19 mg, m. p. 126—128°. Specific activity: 20 μC/mg. The radioactivity of this sample was further checked by recrystallizations of the free acid with inactive material and after conversion of the methylester to the tri-*p*-nitrobenzoate [4].

7-Ketodeoxycholic acid was prepared according to the method of Hoehn and Linsk [6], m. p. 197–198°.

12-Ketolithocholic acid (3α-hydroxy-12-ketocholanic acid) was prepared according to the method of Bergström and Haslewood [7], m. p. 160–161°.

Animal experiments

Bile duct cannulated rats (Sprague-Dawley strain, 200–250 g, male) were prepared as described earlier [8]. The sodium salt of the labelled bile acid in 0.9 % aqueous NaCl was injected intraperitoneally 12 h after the bile duct cannulation. The bile was collected in ethanol. The excreted radioactive products represent the pure liver metabolites of the administered acid. In another series of experiments the labelled bile acid was administered by intracecal injection during the operation [1]. The rats were killed after 36 h. Labelled material present in feces and the large intestine of these animals represents transformation products of the injected acid by the intestinal microorganisms. The radioactive compounds excreted in the bile during this period consist of the intestinal metabolites, absorbed from the large intestine as well as the products formed by the action of liver enzymes on these metabolites.

Isolation procedures and chromatographic methods

The bile was filtered, evaporated and hydrolyzed with 2 N NaOH for 8 h at 120° in a closed steel tube. The solution was acidified with hydrochloric acid and extracted with ether.

Feces and intestinal contents were refluxed twice with 80 % aqueous ethanol. After evaporation the residue was hydrolyzed, acidified and extracted with ether. The residue from the ether extracts was distributed between equal volumes of 70 % ethanol and light petroleum by a 3-stage counter-current extraction. The residue from the combined aqueous phases was chromatographed.

Separation of the free bile acids was made by reversed phase partition chromatography as described by Bergström and Sjövall [9] and Norman and Sjövall [1].

Solvent system	Moving phase	(ml)	Stationary phase	(ml)
F (Ref.[9])	Mathanol-water	165: 135	Chloroform-heptane	45 : 5
C3 (Ref.[1])	Methanol-water	140 : 160	Chloroform-isooctanol	15 : 15

4 ml of the stationary phase was supported on 4.5 g of hydrophobic Super Cel. Temperature 23°.

The radioactivity of the fractions collected from the column was determined by counting of an aliquot in an infinitely thin layer on aluminium planchets. A gas flow counter Frieseke Hoepfner FH 51, was used.

RESULTS

Intraperitoneal injection of tritium labelled 3α, 7β, 12α-trihydroxycholanic acid to bile duct cannulated rats. Bile fistulas were made on two rats and tritium labelled 3α, 7β, 12α-trihydroxycholanic acid (0.2 mg) injected intraperitoneally 12 h after the operation. Of the administered ^3H 78–92 % was excreted in the bile within the first 24 h. The hydrolyzed bile was chromatographed with solvent system C3. The only radioactive material, eluted, consisted of unchanged 3α, 7β, 12α-trihydroxycholanic acid as judged by rechromatography together with the inactive acid and by isotope-dilution. The stationary phase retained less than 1 % of the chromatographed ^3H.

Intracecal injection of tritium labelled 3α, 7β, 12α-trihydroxycholanic acid into bile duct cannulated rats. Tritium labelled 3α, 7β, 12α-trihydroxycholanic

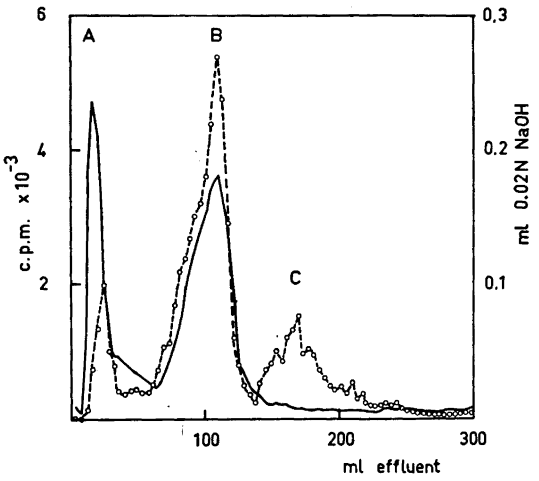

Fig. 1. Chromatographic separation of labelled products in the large intestine and feces after injection of 0.5 mg of ^{3}H labelled 3α, 7β, 12α-trihydroxycholanic acid into the cecum of a bile fistula rat. Inactive deoxycholic acid (15 mg) was added. Column: 9 g of hydrophobic Super Cel. Solvent system: F. ———— Titration values; - - - - - - - Radioactivity.

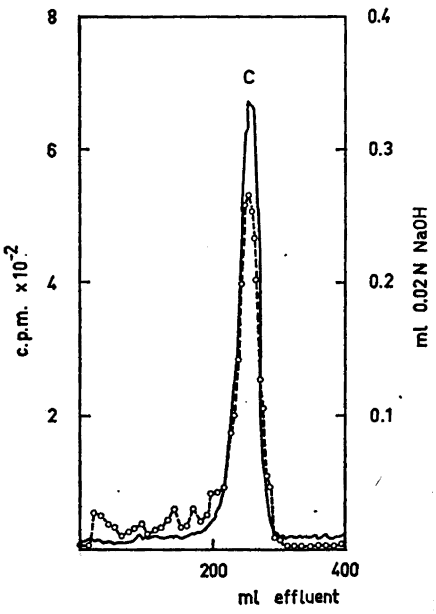

Fig. 2. Chromatographic separation of peak C of the chromatogram shown in Fig. 1 Inactive 12-ketolithocholic acid (15 mg) was added. Column: 9 g of hydrophobic Super Cel. Solvent system: F. ———— Titration values; - - - - - - - Radioactivity.

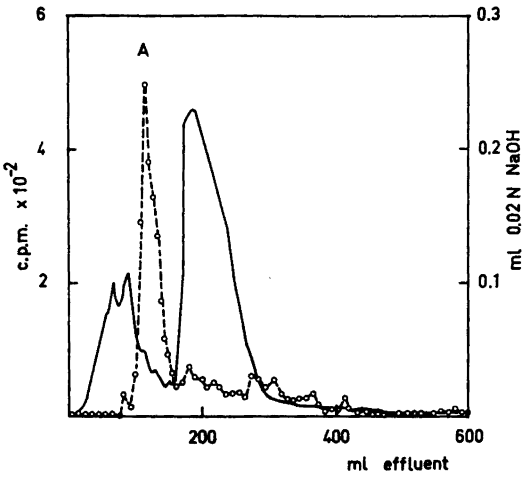

Fig. 3. Chromatographic separation of peak A of the chromatogram shown in Fig. 1 Inactive 7-ketodeoxycholic acid (15 mg) was added. Column: 9 g of hydrophobic Super Cel. Solvent system: C 3. ———— Titration values; - - - - - - - Radioactivity.

acid (0.5 mg to each of three rats) was injected into the cecum immediately after cannulation of the bile duct. The rats (RI, RII, RIII) were killed after 36 h.

Metabolites in the large intestine and feces. The labelled material (15 % (RI), 32 % (RII) and 36 % (RIII) of the injected ^3H) present in the large

Table 1. Recrystallizations of radioactive bands isolated by chromatography from the large intestine and feces or from the bile after intracecal administration of tritium labelled 3α, 7β, 12α-trihydroxycholanic acid to bile fistula rats.

Sample	Inactive bile acid added	Crystallizing solvent	Weight mg	*c. p. m.* mg
Dihydroxycholanic acid band from the large intestine and feces (Fig. 1)	Deoxycholic acid		100	7 300
		Aqueous acetic acid	87	7 650
		Ethyl acetate	70	7 600
		Aqueous ethanol	47	7 900
		Aqueous acetic acid	34	7 700
12-Ketolithocholic acid band from the large intestine and feces (Fig. 2)	12-Ketolithocholic acid		100	1 230
		Ethyl acetate	83	1 320
		Aqueous ethanol	61	1 160
		Aqueous acetone	44	1 060
		Ethylacetate	32	1 280
Cholic acid band from the bile (Fig. 4)	Cholic acid		100	4 700
		Aqueous acetic acid	80	4 920
		Ethyl acetate	68	4 840
		Ethyl acetate	53	4 720
		Aqueous ethanol	37	5 020

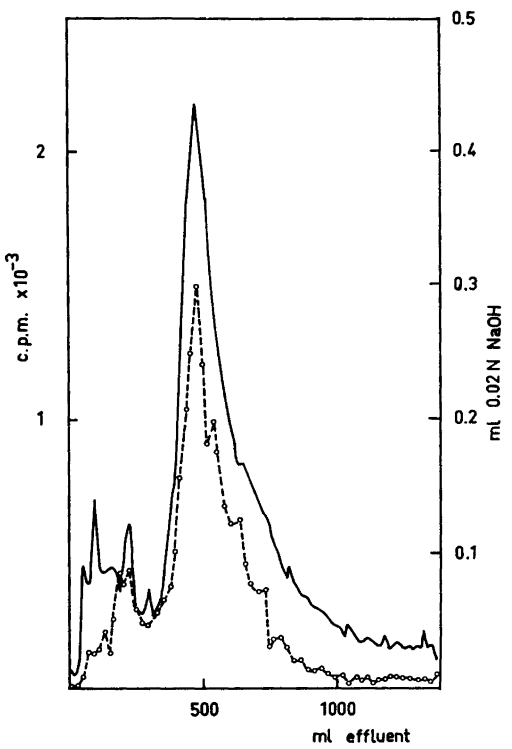

Fig. 4. Chromatographic separation of acids from hydrolyzed bile, excreted during 36 h following intracecal administration of 0.5 mg of ^{3}H-labelled 3α, 7β, 12α-trihydroxycholanic acid. Column: 13.5 g of hydrophobic Super Cel. Solvent system: C 3. ——— Titration values; - - - - - - - Radioactivity.

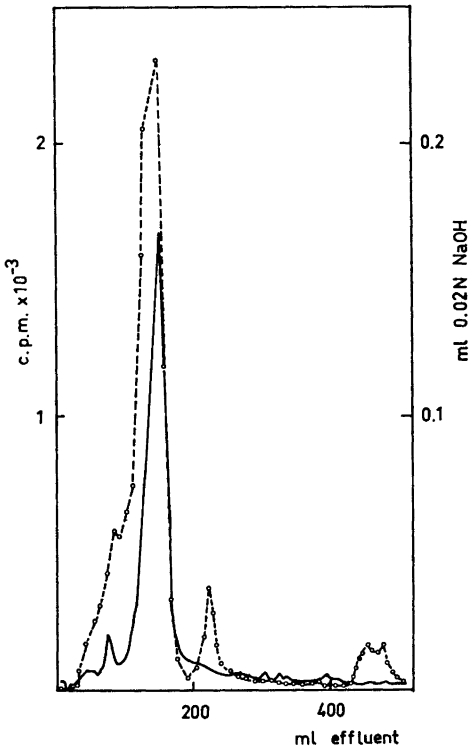

Fig. 5. Chromatographic separation of acids remaining in the stationary phase of the chromatogram shown in Fig. 4. Inactive deoxycholic acid (19 mg) was added. Column: 9 g of hydrophobic Super Cel. Solvent system: F. ——— Titration values; - - - - - - - Radioactivity.

intestine and feces was first chromatographed together with inactive deoxycholic acid with solvent system F. A chromatogram from one of the rats is shown in Fig. 1. (1.5 % of the chromatographed radioactivity remained in the stationary phase). The main peak of radioactivity (B) coincides with the titration peak caused by added inactive deoxycholic acid. The identity was established by recrystallizations with unlabelled deoxycholic acid in different solvents (Table 1). The third radioactive peak (C) was rerun together with unlabelled 12-ketolithocholic acid (Fig. 2). The identity was prooved by isotope dilution (Table 1). The radioactivity being eluted almost with the front (A in Fig. 1) was rechromatographed with the more polar solvent system C3 together with unlabelled 7-ketodeoxycholic acid (Fig. 3). The main part of the radioactivity appeared at the position of 3α, 7β, 12α-trihydroxycholanic

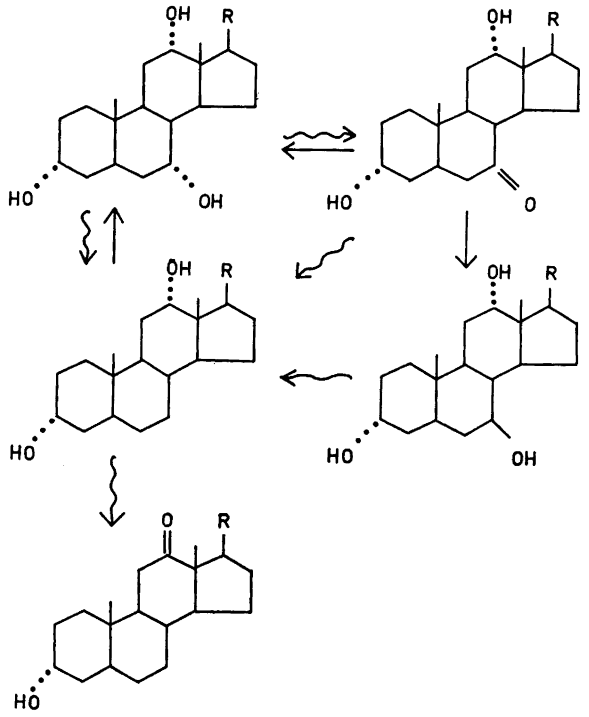

Fig. 6. ——→ Reaction caused by liver enzymes. ~~→ Reaction caused by intestinal microorganisms.

acid as shown by rechromatography with the unlabelled compound. No significant amount of tritium was eluted at the position of 7-ketodeoxycholic acid (160—280 ml effluent) or cholic acid (300—450 ml effluent).

Metabolites excreted in the bile. The hydrolyzed bile (containing 53 % (RI), 44 % (RII) and 38 % (RIII) of the injected ^3H) was chromatographed with solvent system C3 (Fig. 4). The main part of the radioactivity coincides with the titration peak (350—750 ml effluent) of the inactive cholic acid present in the bile. The identity was established by isotope dilution (Table 1). Traces of radioactivity appear at the position of 3α, 7β, 12α-trihydroxycholanic acid. The material remaining in the stationary phase was chromatographed with solvent system F together with unlabelled deoxycholic acid (Fig. 5). The main peak of radioactivity is eluted at the same position as the added inactive deoxycholic acid. At the beginning of this peak, however, contaminating labelled material of unknown nature is eluted. This could be removed by rechromatography with the same solvent system. The identity of labelled deoxycholic acid was established by isotope dilution. The following radioactive band (200—240 ml effluent) was identified as 12-ketolithocholic acid. The structure of the least hydrophilic compound (440—500 ml effluent) has not been determined.

DISCUSSION

When tritium labelled 3α, 7β, 12α-trihydroxycholanic acid was administered intraperitoneally to bile fistula rats it was excreted unchanged in the bile. In the large intestine, however, when administered by intracecal injection it was extensively transformed. The identified metabolites consisted of deoxycholic acid and 12-ketolithocholic acid. The labelled products excreted in the bile in these experiments, were identified as cholic, deoxycholic and 12-ketolithocholic acids. The labelled cholic acid found in the bile is most probably only formed by 7α-hydroxylation of deoxycholic acid absorbed from the intestine.

The numerous possibilities of converting cholic, deoxycholic, 7-ketodeoxycholic and 3α, 7β, 12α-trihydroxycholanic acids into each other are summarized in Fig. 6. Thus, different oxygen functions at C7 (ketone, α- and β-hydroxyl groups) in bile acids with the parent structure of deoxycholic acid are eliminated by microorganisms in the rat. Results available at present are not sufficient to establish whether cholic acid is the common denominator in the microbiological formation of deoxycholic acid by the following reactions:

$$7\alpha\text{-ol} \rightleftharpoons \text{7-ketone} \rightleftharpoons 7\beta\text{-ol}$$

or if the different functions are eliminated *per se*.

From experiments [10] with cholic acid-7-^{3}H-24-^{14}C, it is known that deoxycholic acid is synthesized *in vivo* with a negligibly intermediate formation of 7-ketodeoxycholic acid and that the dehydrogenation of cholic acid to 7-ketodeoxycholic acid is reversible in suspensions of *E. coli* [11].

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