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Synthesis and Metabolism of 3α, 12α-Dihydroxy-Δ^6-cholenic Acid-24-^{14}C

Bile Acids and Steroids 117

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3α,12α-dihydroxy-Δ^6-cholenic acid-24-^{14}C has been synthesized from cholic acid-24-^{14}C. The metabolism of the former acid has been studied after intraperitoneal or intracecal injection to bile duct cannulated rats. The unsaturated acid is reduced in the intestine to deoxycholic acid, which is further oxidized to 12-ketolithocholic acid. Labelled deoxycholic and cholic acids were present in the bile. No transformation of 3α,12α-dihydroxy-Δ^6-cholenic acid occurs in the liver.

Studies concerning the transformation of bile acids during the enterohepatic circulation in different species have demonstrated that deoxycholic acid is formed from cholic acid by the action of intestinal microorganisms on cholic acid. This transformation entails dehydroxylation at C-7, a reaction that has been repeatedly shown to occur for different bile acid species containing either a 7α- or a 7β-hydroxyl group [1,2].

The mechanism of the dehydroxylation has been most thoroughly investigated regarding the transformation of cholic acid into deoxycholic acid whereby the hydroxyl group in 7α-position is eliminated [3,4]. Studies with cholic acid containing tritium in specific positions revealed that the hydrogen atom in 7β-position is retained whereas of the hydrogen atoms bound to carbon atoms adjacent to C-7 (6α,6β, and 8β) one is eliminated in the dehydroxylation, *e.g.* that in 6β position.

The result was interpreted to mean that cholic acid is initially dehydrated by a diaxial trans elimination (6β-H, 7α-OH) in which reaction 3α,12α-dihydroxy-Δ^6-cholenic acid is formed. The reduction of this postulated intermediate to deoxycholic acid was also studied from a stereochemical point of view by observing the change in configuration of the tritium labels retained in deoxy-

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cholic acid. By this approach it was evident that if the unsaturated acid were an intermediate reduction must have involved diequatorial transaddition of hydrogen into 6α- and 7β-positions.

In order to get further support for the above mentioned sequence of reactions as well as to make accessible a means of studying this reaction *in vitro* in separate steps we have synthesized ^{14}C labelled 3α,12α-dihydroxy-Δ^6-cholenic acid and studied its metabolism in the rat.

EXPERIMENTAL

Cholic acid-24-^{14}C was synthesized according to the method of Bergström *et al.*[5] Specific activity: 0.6 μC/mg.

Methyl-3α,12α-diacetoxy-7-ketocholanate-24-^{14}C (*I*). The methyl ester from 108 mg of cholic acid-24-^{14}C was dissolved in 5 ml of acetone and 2 ml of water and oxidized with 0.13 g of N-bromosuccinimide during 6 h at room temperature. The solution was diluted with water and extracted with ether. The ether solution was washed with sodium bicarbonate (8 %) and water. The dried ether solution was evaporated and the residue acetylated by refluxing with 2 ml of pyridine and 2 ml of acetic anhydride for 6 h. The reaction mixture was diluted with water and extracted with ether. The ether solution was washed with 2 N hydrochloric acid and water, dried over sodium sulfate and evaporated. Crystallization from aqueous methanol yielded 83 mg of I, m.p. 118–119°[6].

Methyl-3α,12α-diacetoxy-6α-bromo-7-keto cholanate-24-^{14}C (*II*). To a solution of 83 mg of I in 1 ml of acetic acid was added 30.6 mg of bromine and 0.033 ml of 30 % hydrobromic acid in acetic acid. Water was added dropwise to turbidity after 24 h at room temperature. The crystalline product was filtered, washed and dried yielding 84 mg of II, m.p. 164–165°[6].

3α,12α-Dihydroxy-Δ^6-cholenic acid-24-^{14}C (III). An ice-cooled solution of II (80 mg) in 4 ml of methanol was reduced with 80 mg of sodium borohydride for 1 h. Dilution with water, acidification and ether extraction yielded after evaporation 75 mg of residue. This compound was treated without further purification with 0.5 g of Zn in 10 ml of acetic acid and refluxed for 16 h. The solution was diluted with water and extracted with ether. The residue obtained after evaporation was refluxed with 6 ml of aqueous 1 N sodium hydroxide for 4 h. Ether extraction and evaporation gave a residue (43 mg), which was purified by chromatography with solvent system F. Crystallization from ethyl acetate-hexane yielded 32 mg of III, m.p. 157–160°[7]. Specific activity: 0.62 μC/mg. The physical properties of this compound agreed with those given in the reference cited.

Animal experiments. The sodium salt of 3α,12α-dihydroxy-Δ^6-cholenic acid was administered to bile duct cannulated rats[8], Sprague-Dawley strain (200 to 250 g), either by intraperitoneal or intercecal injection. The bile and feces were collected in ethanol for two days.

Isolation procedures and chromatographic methods. Bile acids were extracted from feces by refluxing three times for 3 h with 80 % aqueous ethanol. These extracts were taken to dryness, dissolved in 0.5 N NaOH, acidified and extracted with ether.

The bile was filtered, evaporated and hydrolyzed with 2 N NaOH in a sealed tube at 120°. The free bile acids were extracted with ether after acidification with hydrochloric acid.

The bile acids were separated with reversed phase column chromatography using the following solvent systems[9,10].

Solvent system	Moving phase (ml)	Stationary phase (ml)
C	Methanol-water 150:150	Chloroform-isooctanol 15:15
F1	Methanol-water 165:135	Chloroform-heptane 45:5
F2	Methanol-water 153-147	Chloroform-heptane 45:5

4 ml of the stationary phase were supported on Hydrophobic Super-Cel (Johns Manville, Comp.). When larger columns were needed these amounts were proportionally increased. Radioactivity was determined by counting of an infinitely thin layer in a methane gas-flow counter, (Frieseke-Hoepfner, FH 51).

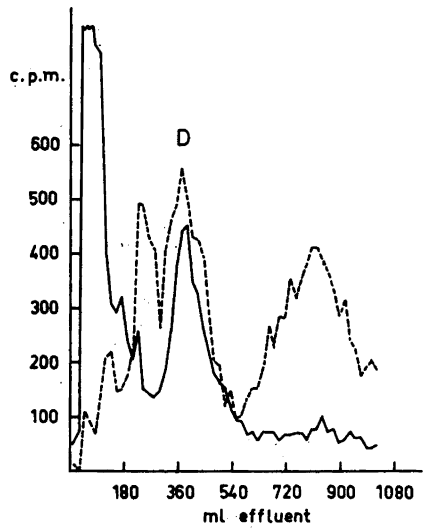

Fig. 1. Chromatographic separation of labelled products in feces after intracecal injection of 3*α*,12*α*-dihydroxy-Δ^6-cholenic acid-24-^{14}C into a bile duct cannulated rat. Solvent system F2. Column: 18 g. Inactive deoxycholic acid (10 mg) added as carrier (D). Solid line: Titration. Broken line: Radioactivity.

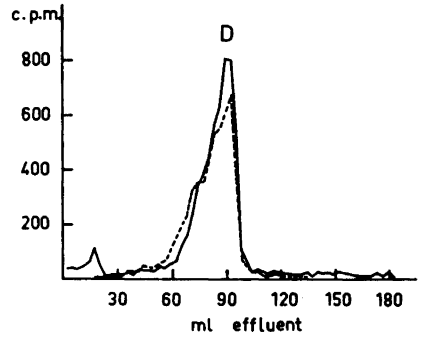

Fig. 2. Rechromatography of 320—450 ml effluent of the chromatography shown in Fig. 1. Solvent system: F2. Column: 4.5 g.

RESULTS

Administration of 3α,12α-dihydroxy-Δ^6-cholenic acid-24^{24}-C into cecum of bile duct cannulated rats. 3α,12α-dihydroxy-Δ^6-cholenic acid-24-^{14}C (2.5 mg) was injected into the cecal content of bile duct cannulated rats. Of the administered radioactivity about 30—40 % were excreted in feces within 48 h, the remainder being excreted with the bile. The radioactive compounds in feces represent the metabolites of 3α,12α-dihydroxy-Δ^6-cholenic acid formed in the intestine by microorganisms. In the bile radioactive compounds can be composed of the injected bile acid or metabolites of it formed in the liver, furthermore intestinal metabolites of the injected acid and finally liver metabolites of the derivatives formed in the intestine.

Radioactive compounds in feces. The radioactive bile acids excreted in feces together with 10 mg of inactive deoxycholic acid were chromatographed with solvent system F2, which was found to be the most suitable system for separation of deoxycholic acid from 3α,12α-dihydroxy-Δ^6-cholenic acid (Fig. 1). The radioactive material eluted from 320—450 ml effluent was rechromatographed with solvent system F2 where the radioactivity coincided with the titration peak of the added carrier (Fig. 2). The identity of this metabolite with deoxycholic acid was finally established by isotope dilution. The radio-

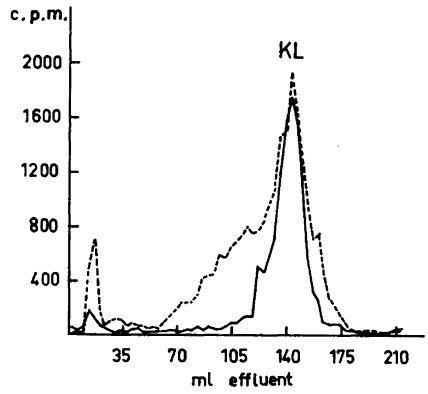

Fig. 3. Rechromatography of 600—1 000 ml effluent of the chromatography shown in Fig. 1. Solvent system: F1. Column: 4.5 g. Inactive 12-ketolithocholic acid (10 mg) added as carrier (KL).

active material (190—300 ml effluent) being eluted immediately before deoxycholic acid was identified as unchanged 3α,12α-dihydroxy-Δ^6-cholenic acid by rechromatography with the inactive acid and by isotope dilution. The broad band (600—1 000 ml effluent) of radioactivity which appears after deoxycholic acid was chromatographed together with 12-ketolithocholic acid (3α-hydroxy-12-ketocholanic acid) with solvent system F1 (Fig. 3). A minor part of the radioactive material is then eluted before 12-ketolithocholic acid whereas most of it coincides with this acid. Isotope dilution established the identity of this latter material. The nature of the more hydrophilic compound has not been investigated.

The radioactive material remaining in the column amounted to 10—15 % of the chromatographed radioactivity.

Radioactive compounds in bile. The free bile acids obtained after hydrolysis of the bile were chromatographed with solvent system F2 after addition of inactive deoxycholic acid (Fig. 4). The main part of the isotope was present as unchanged 3α,12α-dihydroxy-Δ^6-cholenic acid, proved by isotope dilution. The radioactive compound eluted at the same rate as inactive deoxycholic acid (370—500 ml effluent) was identified by isotope dilution.

The front band was rechromatographed with solvent system C (Fig. 5). The titration peak caused by the inactive cholic acid present in rat bile coincided with the main isotope peak. The identity was established by isotope dilution. The nature of the two more hydrophilic radioactive bands was not investigated.

Intraperitoneal administration of 3α,12α-dihydroxy Δ^6-cholenic acid -24-^{14}C to bile duct cannulated rats. This experiment was designed to differentiate between the reactions occurring in the intestine and in the liver. The sodium salt of 3α,12α-dihydroxy-Δ^6-cholenic acid-24-^{14}C was injected intraperitoneally into bile duct cannulated rats and the radioactive material excreted in the bile was separated by chromatography. About 85—90 % of the administered ^{14}C was excreted during the first 24 h. Hydrolysis of this material and chromatographic separation with solvent system F2 revealed that only unchanged 3α,12α-dihydroxy-Δ^6-cholenic acid was present.

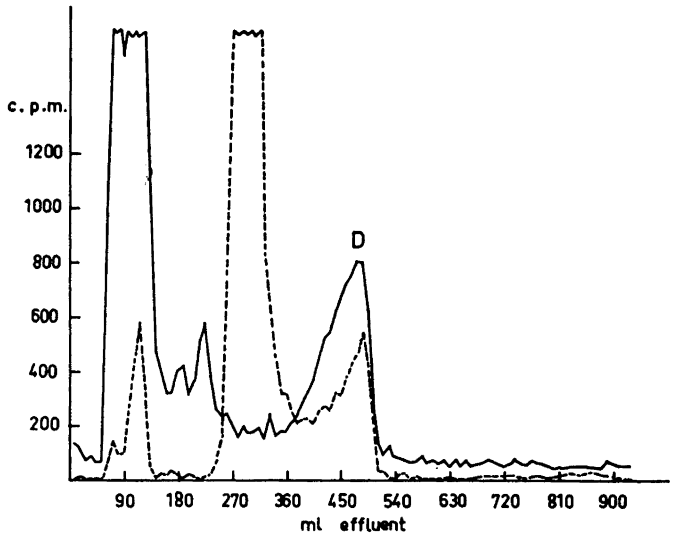

Fig. 4. Chromatographic separation of labelled products excreted in bile after intracecal injection of 3*α*,12*α*-dihydroxy-Δ^6-cholenic acid-24-^{14}C into a bile duct cannulated rat. Solvent system: F2. Column: 18 g. Inactive deoxycholic acid (10 mg) added as carrier (D). Solid line: Titration. Broken line: Radioactivity.

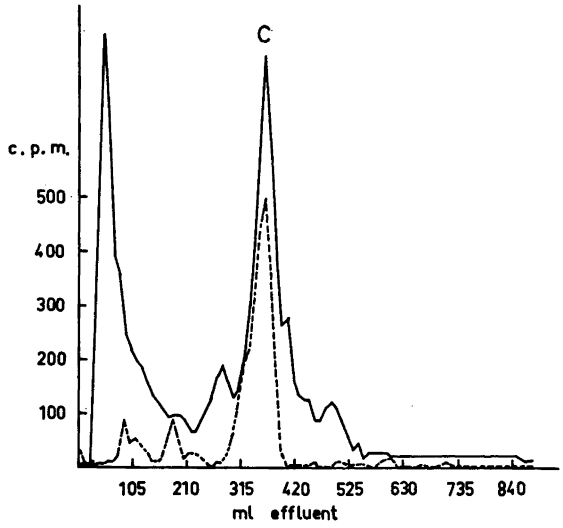

Fig. 5. Rechromatography of 50—140 ml effluent of the chromatography shown in Fig. 4. Solvent system: C. Colunm: 18 g. Cholic acid (C).

DISCUSSION

The identified metabolites of 3α,12α-dihydroxy-Δ^6-cholenic acid formed in the intestine consist of deoxycholic acid and 12-ketolithocholic acid. The former acid is formed by reduction of the unsaturated acid and the latter derivative by oxidation at C-12 of deoxycholic acid. Of the metabolites found in the bile deoxycholic acid is formed primarily in the intestine and a part of it is then 7α-hydroxylated to cholic acid in the liver. The cholenic acid was not attacked during the passage through the liver. The results obtained thus support the earlier postulated biogenesis of deoxycholic acid, although it has not been possible to isolate 3α,12α-dihydroxy-Δ^6-cholenic acid as a metabolite of cholic acid. Similar experiments with 3α,12α-dihydroxy-Δ^7-cholenic acid have shown that this acid is not reduced to deoxycholic acid in the intestine [11].

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