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On the Biosynthesis and Metabolism of Allodeoxycholic Acid in the Rat

Bile Acids and Steroids 175*

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After intracecal administration to bile fistula rats of tritium-labeled 12α-hydroxy-3-keto-5β-cholanoic acid, 12α-hydroxy-3-keto-5α-cholanoic acid, and 12α-hydroxy-3-ketochol-4-enoic acid, labeled deoxycholic acid as well as allodeoxycholic acid were isolated from bile and feces. After intraperitoneal administration of 12α-hydroxy-3-keto-5β-cholanoic acid and 12α-hydroxy-3-keto-5α-cholanoic acid the products isolated from bile had retained the configuration at C-5. After intraperitoneal administration of 12α-hydroxy-3-ketochol-4-enoic acid both deoxycholic acid and allodeoxycholic acid were isolated from bile. After intracecal administration of tritium-labeled allodeoxycholic acid labeled deoxycholic acid could be isolated from bile and feces. The possible mechanism of the interconversion of deoxycholic and allodeoxycholic acids are discussed.

The conversion of deoxycholic acid into allodeoxycholic acid during the enterohepatic circulation of bile has been demonstrated recently in the rat [1] as well as in the rabbit [2] and evidence has been obtained indicating that this reaction is catalyzed by microbial enzymes. The mechanism of this interconversion has not been established but it is possible that 12α-hydroxy-3-keto-5β-cholanoic acid, 12α-hydroxy-3-ketochol-4-enoic acid, and 12α-hydroxy-3-keto-5α-cholanoic acid are intermediates in the formation of allodeoxycholic acid from deoxycholic acid.

This communication reports on the formation of allodeoxycholic acid from tritium-labeled 12α-hydroxy-3-keto-5β-cholanoic acid, 12α-hydroxy-3-ketochol-4-enoic acid, and 12α-hydroxy-3-keto-5α-cholanoic acid as well as further studies on the metabolism of allodeoxycholic acid.

* In accordance with the recommendation of the IUAPC, the following systematic names are given for the compounds referred to in the text: deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; allodeoxycholic acid, 3α,12α-dihydroxy-5α-cholanoic acid.

The following abbreviations are used: GLC, gas-liquid chromatography; TLC, thin layer chromatography; dpm, disintegrations per minute.

EXPERIMENTAL

Melting points are uncorrected

Tritium-labeled deoxycholic acid. Methyl deoxycholate, 500 mg, was exposed to 2 C of tritium gas (Radiochemical Centre, Amersham, England) for three weeks according to the method of Wilzbach [3] in the apparatus described by Bergström and Lindstedt.[4] The tritiated methyl deoxycholate was hydrolyzed with 2 M potassium hydroxide at 120° for 6 h. After ether extraction of the acidified hydrolysis mixture, the deoxycholic acid was purified by reversed phase partition chromatography using phase system F 1.[5] The radioactivity peak was pooled, diluted with 5 g of unlabeled deoxycholic acid and crystallized to constant specific activity from acetic acid-water. The specific radioactivity of the tritium-labeled deoxycholic acid was 21×10^6 dpm/mg.

Tritium-labeled methyl 12α-hydroxy-3-keto-5β-cholanoate. Tritium-labeled deoxycholic acid was methylated in methanol-sulfuric acid and crystallized from methanol. Tritium-labeled methyl deoxycholate, 5 g, was oxidized with aluminum *t*-butoxide according to Jones *et al.*[6] After acidification, the reaction mixture was extracted with ether. The residue of the ether extract was chromatographed on a column of 250 g of aluminium oxide, grade III (Woelm, Eschwege, Germany). The column was eluted with increasing amounts of ethyl acetate in benzene. Methyl 12α-hydroxy-3-keto-5β-cholanoate was eluted with 5 % ethyl acetate in benzene and crystallized from methanol-water. The yield was 2.1 g with m.p. 141—143°, reported 142—145°,[6] and with a specific activity of 18×10^6 dpm/mg.

Tritium-labeled 12α-hydroxy-3-ketochol-4-enoic acid. Bromine, 0.3 ml in 50 ml of glacial acetic acid, was added under vigorous stirring to a solution of 2 g of methyl 12α-hydroxy-3-keto-5β-cholanoate in 100 ml of glacial acetic acid. The reaction mixture was poured into ice-cold saline and extracted three times with ether. The combined ether extracts were washed with a 10 % solution of sodium bicarbonate and with water until neutral. The residue was dissolved in 50 ml of freshly distilled pyridine and refluxed for 4 h. The reaction mixture was poured into 2 M hydrochloric acid and extracted with ether. The ether was evaporated and the residue was chromatographed on a column of 100 g of aluminium oxide, grade III. Ethyl acetate, 7 % in benzene, eluted material with a UV-spectrum typical of an α,β-unsaturated ketone, with an absorption maximum at 242 mμ. This material contained a significant amount of methyl 12α-hydroxy-3-keto-5β-cholanoate as shown by TLC with phase system S 11[5] and was further purified, after hydrolysis, by reversed phase partition chromatography with phase system F 1.[6] The fractions containing the unsaturated ketone, as measured by UV, were analyzed on TLC and those which were found to be devoid of the saturated ketone were combined and crystallized from acetone. The yield was 300 mg with m.p. 237—238°, reported 240°,[8] λ_{max} 242 mμ (methanol), $\varepsilon = 14\,000$. Reported for methyl ester λ_{max} 241.5 mμ, $\varepsilon = 14\,470$.[9] The specific activity was 18×10^6 dpm/mg.

Tritium-labeled 12α-hydroxy-3-keto-5α-cholanoic acid. Lithium, 5 mg, dissolved in 20 ml of liquid ammonia, was added dropwise to a vigorously stirred suspension of 50 mg of tritium-labeled 12α-hydroxy-3-ketochol-4-enoic acid in 15 ml of liquid ammonia. Care was taken to exclude moisture from the reaction vessel. The dissolved lithium was added until the blue color remained in the reaction mixture. Then, 100 mg of ammonium chloride were added and the ammonia was evaporated. The residue was dissolved in water, acidified and extracted with ether. After evaporation of the solvent the residue was methylated with diazomethane and purified by chromatography on a column of 8 g of aluminium oxide, grade III. Methyl 12α-hydroxy-3-keto-5α-cholanoate was eluted with 5 % ethyl acetate in benzene and crystallized from ether-petroleum ether. The yield was 17 mg with a melting point of 133—135°, reported 134—136°,[2] and a specific activity of 18×10^6 dpm/mg.

The tritium-labeled methyl 12α-hydroxy-3-keto-5α-cholanoate contained not more than 0.5 % each of methyl 12α-hydroxy-3-ketochol-4-enoate and methyl 12α-hydroxy-3-keto-5β-cholanoate as shown by GLC (Fig. 1).

In the metabolic experiments the free acid, which was obtained by hydrolysis with 2 M methanolic potassium hydroxide at room temperature, was used.

Tritium-labeled allodeoxycholic acid. Methyl allodeoxycholic acid, 10 mg, prepared as described previously [2,10] was exposed to 2 C of tritium gas for three weeks at room temperature. The material was then worked up and purified in the manner described previously.

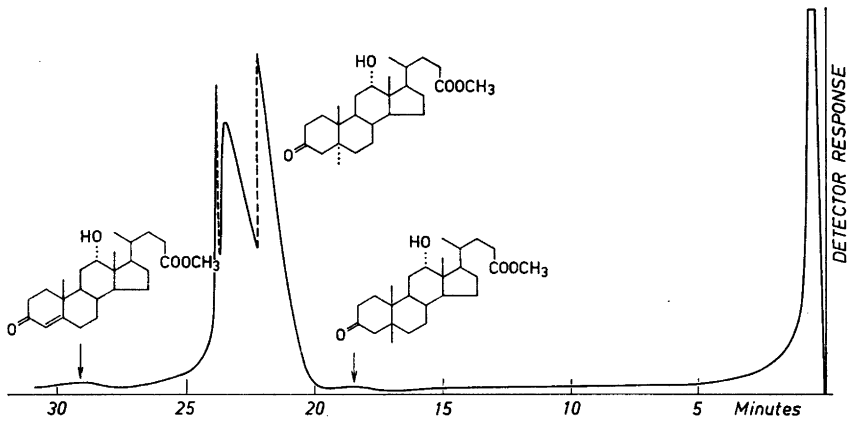

Fig. 1. Gas-liquid chromatogram of methyl 12α-hydroxy-3-keto-5α-cholanoate. Column: 6 foot × 5 mm, 3 % QF-1 on Gas-Chrom P.[11,12] Column temperature was 245° and argon pressure 1.8 kg/cm². Hatched part of peak indicates a decrease in amplification gain of 33 %.

Animal experiments. White male rats of the Danish State Serum Institute strain V.S. weighing about 250 g were used. Bile fistulas were prepared in the usual manner. The labeled bile acids, in doses of 15—20 × 10^6 dpm, dissolved in 0.5 ml of 0.02 M potassium hydroxide, were administered intraperitoneally or intracecally to bile fistula rats. Bile and feces were analyzed as described in the preceding report.[1]

RESULTS

After intraperitoneal administration of tritium-labeled 12α-hydroxy-3-keto-5α-cholanoic acid, 12α-hydroxy-3-keto-5α-cholanoic acid, and 12α-hydroxy-3-ketochol-4-enoic acid, 50—70 % of the radioactivity was recovered from the first 24 h portion of bile. After intracecal administration 30—40 % of the radioactivity was recovered from the first 24 h portion of bile and 10—20 % from feces collected during the first 24 h.

Extracts of bile and feces were analyzed by reversed phase partition chromatography and thin layer chromatography. Final identification of the metabolites formed was carried out by crystallization to constant specific activity after the addition of unlabeled material. The reliability of this procedure to separate deoxycholic acid and allodeoxycholic acid was tested by crystallizing a trace amount of radioactive allodeoxycholic acid with inactive deoxycholic acid and a trace amount of radioactive methyl deoxycholate with methyl allodeoxycholate. Three crystallizations from acetic acid-water and methanol-water removed 93 and 92 %, respectively of the added radioactivity.

Metabolism of tritium-labeled 12α-hydroxy-3-keto-5β-cholanoic acid (I). After intraperitoneal as well as intracecal administration of this acid, trihydroxy- and dihydroxycholanoic acids were isolated from bile (Fig. 2).

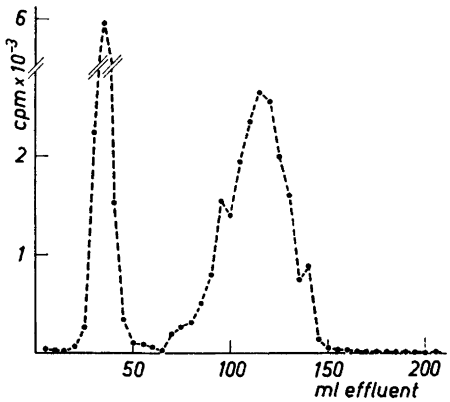

Fig. 2. Chromatogram of hydrolyzed bile after intraperitoneal administration of 12α-hydroxy-3-keto-5β-cholanoic acid. Column: 9 g of hydrophobic Hyflo Super-Cel. Phase system: Moving phase 157.5 ml of methanol, 142.5 ml of water, stationary phase: 45 ml of chloroform and 5 ml of heptane.

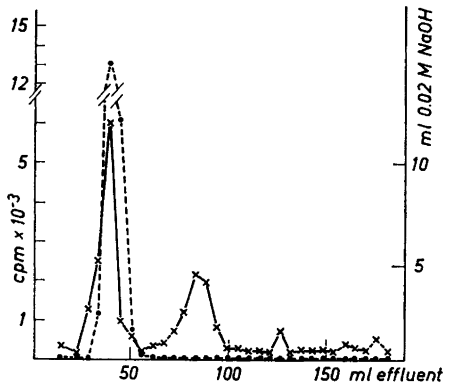

Fig. 3. Chromatogram of hydrolyzed bile after intracecal administration of 12α-hydroxy-3-ketochol-4-enoic acid. Conditions as in Fig. 2. Inactive deoxycholic acid, 5 mg, was added. (Radioactivity - - - and titration values ——).

The radioactivity recovered from feces appeared mainly in the dihydroxycholanoic acid fraction. About 10 % of the radioactivity put on the column was retained in the stationary phase. The identity of this labeled material was not established.

The dihydroxycholanoic acid fractions were further analyzed by preparative TLC. The dihydroxycholanoic acid fraction isolated from bile after intraperitoneal administration of I contained almost all of the radioactivity in the deoxycholic acid zone (Table 1). After elution of the TLC-zone with 2 % acetic acid in ether the identity of the labeled material with deoxycholic acid was established by recrystallization to constant specific activity after addition of unlabeled deoxycholic acid. The dihydroxycholanoic acid fractions isolated from feces and bile after intracecal administration of I, both contained radioactive deoxycholic and allodeoxycholic acids (Table 1).

Metabolism of tritium-labeled 12α-hydroxy-3-keto-5α-cholanoic acid (II). The distribution of radioactivity between trihydroxy- and dihydroxycholanoic acids and less polar compounds obtained after intraperitoneal and intracecal administration of II was similar to that obtained after administration of I. After intracecal administration of II both deoxycholic and allodeoxycholic acids were isolated by preparative TLC, whereas after intraperitoneal administration of II no inversion of the A/B ring junction could be demonstrated (Table 1).

Metabolism of tritium-labeled 12α-hydroxy-3-ketochol-4-enoic acid (III). After intraperitoneal administration of III, trihydroxycholanoic acids as well as dihydroxycholanoic acids were isolated from bile. After intracecal

Table 1. Composition of the dihydroxycholanoic acid fractions isolated after administration of tritium-labeled 12α-hydroxy-3-keto-5β-cholanoic acid (I), 12α-hydroxy-3-keto-5α-cholanoic acid (II), 12α-hydroxy-3-ketochol-4-enoic acid (III), allodeoxycholic acid (IV).

Acid	Mode of administration	Deoxycholic acid %*	Allodeoxycholic acid %*	Source
I	intraperitoneal	98	2	bile
I	intracecal	80	20	feces
I	intracecal	88	12	bile
II	intraperitoneal	2	98	bile
II	intracecal	50	50	feces
II	intracecal	41	59	bile
III	intraperitoneal	55	45	bile
III	intracecal	65	35	feces
IV	intracecal	34	66	bile
IV	intracecal	47	53	feces

* The radioactivity recovered from the zones of the thin-layer chromatographic plates was crystallized to constant specific activity after dilution with unlabeled material. The sum of the labeled material identified as deoxycholic acid and allodeoxycholic acid, respectively, was put at 100 %.

administration, however, no radioactivity was present in bile that chromatographed like dihydroxycholanoic acids (Fig. 3). The radioactivity in the fecal extract appeared predominantly in the dihydroxycholanoic acid region (Fig. 4). As shown in Table 1, the dihydroxycholanoic acid fractions, isolated from bile after intraperitoneal administration and from feces after intracecal administration, contained radioactive allodeoxycholic acid as well as radioactive deoxycholic acid.

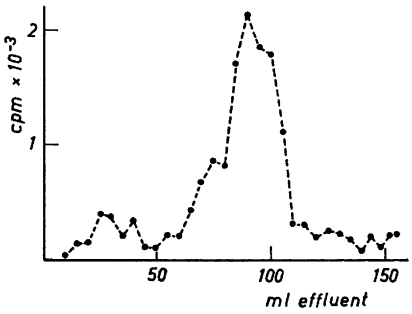

Fig. 4. Chromatogram of bile acids obtained from feces after intracecal administration of 12α-hydroxy-3-ketochol-4-enoic acid. Conditions as in Fig. 2.

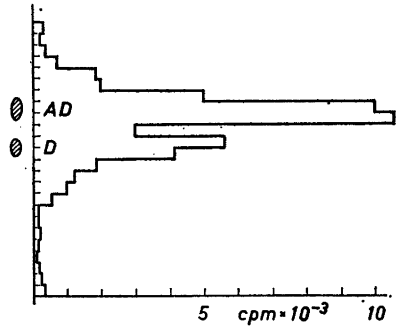

Fig. 5. Thin layer chromatography of the dihydroxycholanoic acid fraction of bile after intracecal administration of allodeoxycholic acid. Reference compounds: allodeoxycholic acid (AD); deoxycholic acid (D).

Metabolism of tritium-labeled allodeoxycholic acid (IV). After intracecal administration of IV, radioactivity was obtained in the trihydroxy- and dihydrocholanoic acid fractions in bile. On chromatography of the fecal extract radioactivity appeared in the dihydroxycholanoic acid region only. The dihydroxycholanoic acid fractions were further analyzed by preparative TLC and the identity of the active zones established by crystallization to constant specific activity as described above. As shown in Table 1 and Fig. 5, the main part of the radioactivity was located in the allodeoxycholic acid region but a significant amount represents deoxycholic acid.

DISCUSSION

Previous investigations on the formation and metabolism of allodeoxycholic acid have shown that allodeoxycholic acid is formed mainly from deoxycholic acid and by means of reactions catalyzed by intestinal microorganisms.[1,2] In an attempt to obtain evidence for the participation of keto acids in this reaction sequence the formation of allodeoxycholic acid from 3β-^{3}H-24-^{14}C-deoxycholic acid was studied.[1] However, it was found that the deoxycholic acid as well as the allodeoxycholic acid isolated from bile and feces had lost the tritium label indicating the existence of an equilibrium between deoxycholic acid and 12α-hydroxy-3-keto-5β-cholanoic acid. Thus, these experiments do not yield any further information with respect to the mechanisms of allodeoxycholic acid formation. The metabolism of probable intermediates in the conversion of deoxycholic acid into allodeoxycholic acid was therefore investigated. The present report describes results of experiments on the metabolism in the bile fistula rat of 12α-hydroxy-3-keto-5β-cholanoic acid, 12α-hydroxy-3-ketochol-4-enoic acid, and 12α-hydroxy-3-keto-5α-cholanoic acid, all probable intermediates in allodeoxycholic acid formation. When administered intracecally all these three ketonic bile acids were found to be metabolized to allodeoxycholic acid as well as to deoxycholic acid. The inversion of the A/B-ring configuration in the formation of allodeoxycholic acid from 12α-hydroxy-3-keto-5β-cholanoic acid and of deoxycholic acid from 12α-hydroxy-3-keto-5α-cholanoic acid was catalyzed by enzymes in the intestinal microorganisms as these transformations were not observed after intraperitoneal administration.*

In a previous investigation it was found that, after intracecal administration to bile fistula rats, allodeoxycholic acid was not transformed into deoxycholic acid. The results on the metabolism of 12α-hydroxy-3-keto-5α-cholanoic acid prompted a reinvestigation of the metabolism of allodeoxycholic acid. It was found that, in contrast to previous results, allodeoxycholic acid was transformed in part to deoxycholic acid. The strain of rats used in the present investigation was the Danish State Serum Institute strain V.S. whereas in the previous study on allodeoxycholic acid metabolism Sprague-Dawley rats

* It should be mentioned that this enzymatic activity is attributed to the intestinal microorganisms on the basis of the probability thereof and in cognizance of the fact that the possibility of the reactions being partly or wholly catalyzed by enzymes in the intestinal wall is not excluded by the experiments.

were used. Repeating the experiments with Sprague-Dawley rats it was found that deoxycholic acid could be isolated after intracecal administration of allodeoxycholic acid. The discrepancy between these results and those reported previously might be attributed to differences in the intestinal flora.

The finding that 12α-hydroxy-3-keto-5β-cholanoic acid and 12α-hydroxy-3-keto-5α-cholanoic acid are metabolized to allodeoxycholic acid and deoxycholic acid does not provide conclusive evidence that these keto acids are intermediates in the interconversion of deoxycholic acid and allodeoxycholic acid, as it is possible that the keto acids are reduced to the corresponding 3α-hydroxy acids prior to inversion of the configuration at C-5. The finding that 12α-hydroxy-3-ketochol-4-enoic acid is converted to allodeoxycholic acid as well as to deoxycholic acid by enzymes in the intestine might be considered as a further indication that an inversion of the configuration at C-5 proceeds by means of the formation of 3-keto acids. However, the experiments do not definitely exclude the possibility that 12α-hydroxy-3-ketochol-4-enoic acid is reduced to either deoxycholic acid or allodeoxycholic acid prior to inversion of the A/B ring juncture.

It is interesting to mention that 12α-hydroxy-3-ketochol-4-enoic acid was found to be metabolized to deoxycholic acid as well as to allodeoxycholic acid by liver enzymes. The possible role of these enzymes in the formation and metabolism of bile acids in the liver is not known at present. Work is in progress to study these enzymes further.

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REFERENCES

1. Kallner A. *Acta Chem. Scand.* **21** (1967) 87.
2. Danielsson, H., Kallner, A. and Sjövall, J. *J. Biol. Chem.* **238** (1963) 3846.
3. Wilzbach, K. E. *J. Am. Chem. Soc.* **79** (1957) 1013.
4. Bergström, S. and Lindstedt, S. *Acta Chem. Scand.* **11** (1957) 1275.
5. Eneroth, P. *J. Lipid Res.* **4** (1963) 11.
6. Norman, A. and Sjövall, J. *J. Biol. Chem.* **233** (1958) 872.
7. Jones, A. S., Webb, H. and Smith, F. *J. Chem. Soc.* **1949** 2164.
8. Matumoto, K. *J. Biochem. (Tokyo)* **36** (1944) 173.
9. Riegel, B. and McIntosh, A. V. *J. Am. Chem. Soc.* **66** (1944) 1099.
10. Kallner, A. *Acta Chem. Scand.* **18** (1964) 1502.
11. Sjövall, J. *Acta Chem. Scand.* **16** (1962) 1761.
12. Sjövall, J., Meloni, C. R. and Turner, D. A. *J. Lipid Res.* **2** (1961) 317.

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