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On the Metabolism of Chenodeoxycholic Acid in the Rat

Bile Acids and Steroids 85

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Chenodeoxycholic acid-7β-^{3}H and randomly tritium labelled 3α, 6β, 7α-(Acid II) and 3α, 6β, 7β- (Acid I) trihydroxycholanic acids have been prepared. Acid II is converted into Acid I when injected into a bile fistula rat, whereas the reverse reaction does not occur. The inversion of the 7α-hydroxyl group has been studied with 7β-tritio, 24-^{14}C labelled chenodeoxycholic acid. The tritium label is completely lost in this reaction. The mechanism for the inversion and the course of the metabolism of chenodeoxycholic acid are discussed.

Chenodeoxycholic acid constitutes a minor component of the bile acids normally present in rat bile[1,2]. In 1954 Bergström and Sjövall showed that chenodeoxycholic acid is transformed in the rat liver into two more polar acids which are not identical with cholic acid [2]. The same metabolites had been observed earlier by Bergström and Norman after administration of cholesterol-4-^{14}C to bile fistula rats [3]. These metabolites have recently been identified by Doisy *et al.* as 3α,6β-7β- (Acid I) and 3α,6β,7α-trihydroxycholanic acid (Acid II) [4,5].

The aim of the present investigation was to study the mechanism of the conversion of chenodeoxycholic acid into Acid I and II with chenodeoxycholic acid 7β-^{3}H, 24-^{14}C and randomly tritium labelled Acid I and Acid II.

EXPERIMENTAL

Syntheses

Chenodeoxycholic acid-7-^{3}H. 60 mg of 7-ketolithocholic acid (M. p. 200–202°) prepared through oxidation of methyl 3α-cathyloxy-7α-hydroxycholanate with sodium dichromate,[6] were dissolved in 10 ml of "diglyme" (Diethyleneglycoldimethylether, Fluka A. G., Buchs/S.G., Switzerland, refluxed over calciumhydride and distilled from lithium-aluminium hydride) and 40 mg of sodium borohydride were added. The latter compound had been labelled through exposure to an athmosphere of tritiumgas [7] (2C, 95 % pure, 200 mm Hg) for ten days at room temperature. After 12 h at room temperature the reaction mixture was diluted with water, acidified with 2 N hydrochloric acid and extrac-

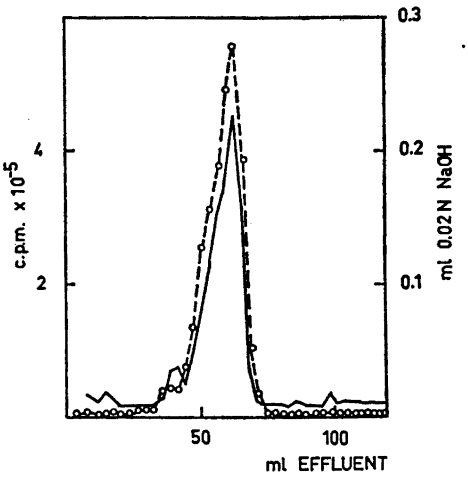


Fig. 1. Chromatographic separation of the reaction product after NaB^3H_4 reduction of 7-ketolithocholic acid. Column: 4.5 mg hydrophobic Supercel. Phase system: Type F. Solid lines: Titration values. Broken line: Radioactivity.

ted with ether. After evaporation *in vacuo* the residue was chromatographed with phase system F[10]. The single peak (Fig. 1) appeared at the place of chenodeoxycholic acid, which with this system separates clearly from the 7β-isomer, ursodeoxycholic acid. The radioactive material was crystallized from ethylacetate/light petroleum yielding 47 mg of chenodeoxycholic acid-7β-^{3}H, m. p. 141—143°. Mixed melting point with authentic chenodeoxycholic acid showed no depression. Specific activity: ~0.5 μC/mg (1 μC ^{3}H is equivalent to approximately 6×10^5 c.p.m. when counted in an infinetely thin layer in a Tracerlab gas flow counter). Dilution of a sample of this acid with inactive chenodeoxycholic acid and extensive recrystallizations gave no depression of the specific activity. 25 mg of this diluted acid was oxidized with chromic acid in aqueous acetic acid to 3,7-diketocholanic acid (m. p. 152—154°). The diketoacid contained 2.5 % of the amount of tritium label present in the acid before the oxidation.

A stock solution of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C was made by dissolving 10 mg of chenodeoxycholic acid-7β-^{3}H (0.5 μC/mg) and 0.25 mg of chenodeoxycholic acid-24-^{14}C (10 μC/mg) in 10 ml of acetone.

Acid I and Acid II were prepared by the methods of Doisy *et al.*[4,5] They were labelled with tritium by exposing 5 mg of the acid to tritium gas (2 C, 200 mm Hg, 95 % pure) for 6 days at room temperature [7]. The tritium labelled products were diluted with 20 mg of the corresponding inactive acid and chromatographed with phase system C[3]. Acid I was crystallized from ethylacetate yielding 17 mg, m. p. 226—227°. Specific activity ~50 μC/mg.

Acid II was crystallized from acetone/light petroleum. Yield 13 mg, m. p. 201—202°. Specific activity: ~35 μC/mg.

Ursodeoxycholic acid was prepared according to Samuelsson [8]. M. p. 201—202°.

Chenodeoxycholic acid-24-^{14}C was prepared according to the method of Bergström *et al.*[9] Specific activity: 10 μC/mg.

Animal experiments

The labelled bile acids were injected intraperitoneally as the sodium salt in 0.9 % sodium chloride solution into 200—250 g white male rats of the institute stock. The bile duct was cannulated 12 h before the administration. The rats had free access to white bread and oats and 0.9 % sodium chloride during the experimental period. The bile was collected daily in ethanol and hydrolyzed with 1.5 N NaOH in a sealed tube for 6 h at 120°. The free acids were extracted from the acidified solution with ether.

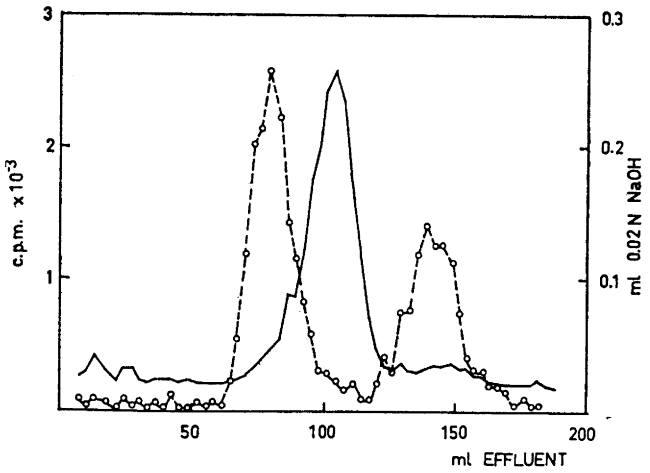


Fig. 2. Chromatographic separation of acids from hydrolyzed bile, excreted during 24 hours following intraperitoneal administration of 2 mg of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C. Column: 4.5 g hydrophobic Supercel. Phase system: Type C. Solid line: Titration. Broken line: Radioactivity.

Chromatographic separations

The free acids were chromatographed on hydrophobic Supercel as described by Bergström, Norman and Sjövall[3,10]. The following solvent systems were used.

System	Moving phase	ml	Stationary phase	ml
F[10]	Methanol: water	165:135	Chloroform: heptane	45:5
C[3]	Methanol: water	150:150	Chloroform: isooctanol	15:15

4 ml of the stationary phase were used per 4.5 g of hydrophobic Supercel. All the chromatograms were run at a constant temperature of +23°.

Isotope determinations

Suitable aliquots of the titrated fractions of the chromatographies were plated on aluminium planchets and counted in a Tracerlab gas flow counter, when tritium labelled compounds were chromatographed.

The radioactivity of the products obtained after injection of chenodeoxycholic acid-7β-^{3}H,24-^{14}C was determined in a Tracerlab end window counter, in which only the ^{14}C activity is recorded. In this way it was possible to get a quantitative picture in spite of the fact that some of the metabolites had lost their tritium label. The ^{3}H and ^{14}C activity in the administered and isolated acids were determined by gas phase counting after combustion to $^{14}CO_2$ and ^3H_2O and conversion of the latter to tritio butane by the methods of Glascock [11].

RESULTS

Metabolism of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C. 2 mg of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C was injected into each of two bile fistula rats. The bile excreted during the first 24 h following the injection was hydrolyzed

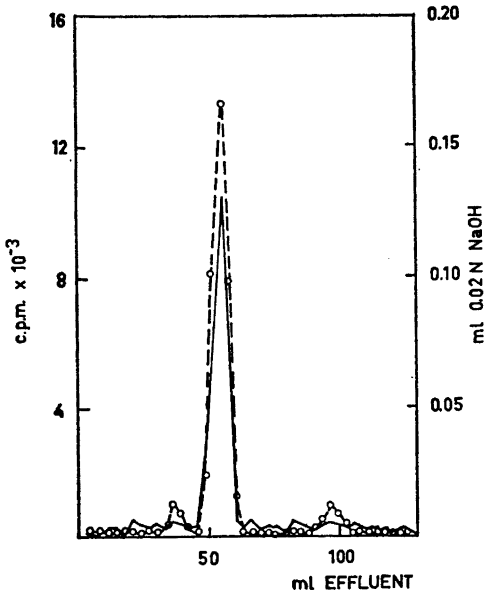


Fig. 3. Chromatographic separation of acids remaining in the stationary phase of the chromatogram shown in Figure 2. Column: 4.5 g hydrophobic Supercel. Phase system Type F. Solid line: Titration values. Broken line: Radioactivity.

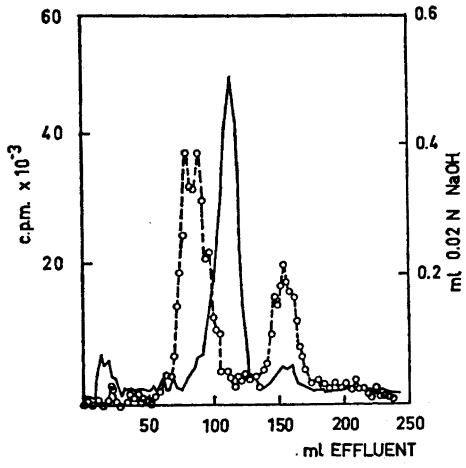


Fig. 4. Chromatographic separation of acids from hydrolyzed bile, excreted during 24 hours following intraperitoneal administration of 0.2 mg of Acid II. Column: 4.5 g hydrophobic Supercel. Phase system: Type C. Solid line: Titration values. Broken line: Radioactivity.

and chromatographed with phase system C (Fig. 2). The material remaining in the stationary phase was separated by chromatography with phase system F. The chromatogram is shown in Fig. 3.

As found earlier [2] the two main metabolites are eluted with phase system C (Fig. 2). One of them (Acid II) appears before the inactive cholic acid (100 ml) and the other (Acid I) after this acid. Acid I and II were diluted, with the corresponding inactive acid to suitable specific activity for the $^{3}H/^{14}C$ determinations and crystallized from ethylacetate and acetone/light petroleum respectively.

In the chromatogram shown in Fig. 3, most of the activity is eluted at the place of chenodeoxycholic acid, but two small radioactive bands also occur, one before (ursodeoxycholic acid) and one after (7-ketolithocholic acid) chenodeoxycholic acid. The radioactive material in these two peaks were rechromatographed with the corresponding inactive acid and were finally identified by isotope dilution.

The percentage composition of the recovered ^{14}C is shown in Table 1. The ^{3}H and ^{14}C content was determined in the administered doubly labelled chenodeoxycholic acid and in the isolated metabolites (Table 2).

Table 1. Percentage composition of the ^{14}C recovered in the bile during 24 h following intraperitoneal injection of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C.

Compound	Per cent	
	Rat I	Rat II
Chenodeoxycholic acid	48.3	53.1
7-Ketolithocholic acid	5.3	4.9
Ursodeoxycholic acid	4.0	3.7
Acid II (3α, 6β, 7α)	17.3	15.5
Acid I (3α, 6β, 7β)	24.0	21.0
Total	98.9	98.2

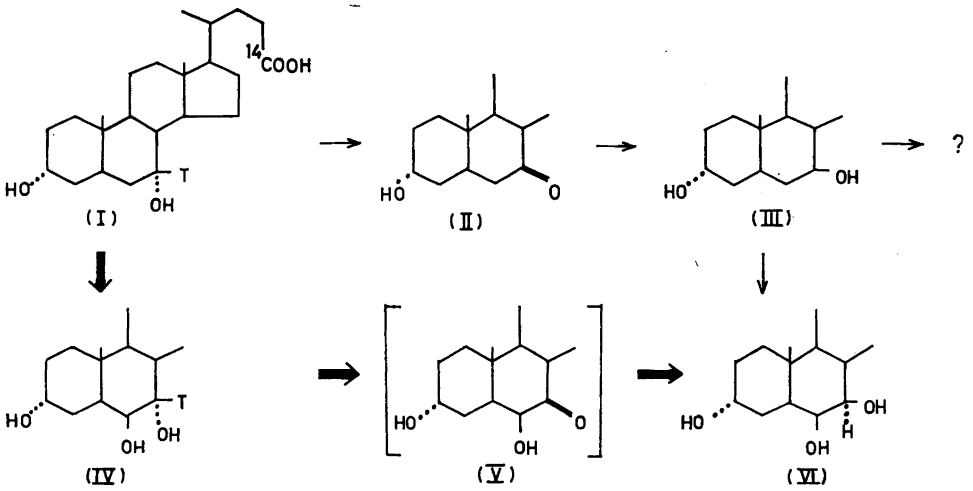
7-Ketolithocholic acid, ursodeoxycholic acid and Acid I only contained traces of ^{3}H whereas this isotope was retained in Acid II.

Metabolism of ^{3}H-labelled Acid II. 0.2 mg of randomly tritium labelled Acid II was injected intraperitoneally into a bile fistula rat and the bile excreted during 24 h following the injection was chromatographed with phase system C after hydrolysis. (Fig. 4). About 35 % of the chromatographed activity was eluted as Acid I, which was identified by isotope dilution.

Metabolism of ^{3}H labelled Acid I. Chromatography of the hydrolyzed bile excreted during 24 h following administration of 0.2 mg of tritium labelled Acid I to a bile fistula rat showed only unchanged Acid I.

Table 2.

Compound	^{3}H c.p.m./mg	^{14}C c.p.m./mg	$^{3}H/^{14}C$	Per cent ^{3}H retained
Administered chenodeoxycholic acid-7β-^{3}H, 24-^{14}C	253	356	0.71	
7-Ketolithocholic acid Rat I and II	3	319	0.01	1
Ursodeoxycholic acid Rat I and II	8	382	0.02	3
Acid II (3α, 6β, 7α)				
Rat I	154	237	0.65	92
Rat II	202	298	0.68	96
Acid I (3α, 6β, 7β)				
Rat I	3	244	0.01	2
Rat II	4	242	0.02	2
Acid I (after administration of acid II-7β-^{3}H, 24-^{14}C)	5	209	0.02	3



Metabolism of biosynthetically formed doubly labelled Acid II. Acid II, which had been isolated from the bile after administration of chenodeoxycholic acid-7β-^{3}H, 24-^{14}C was injected into a bile fistula rat and the excreted bile separated with phase system C after hydrolysis. About 8 % of the injected Acid II had been transformed into Acid I. Determination of ^{3}H and ^{14}C in Acid I showed that the tritium label is completely lost during this transformation (Table 2).

DISCUSSION

Based on results obtained in this and previous investigations [2,4,5,12] the course of the metabolism of chenodeoxycholic acid in the rat liver may be formulated as in scheme 1. The main pathway leads from chenodeoxycholic acid to Acid I (VI) *via* Acid II (IV). Acid II is formed from chenodeoxycholic acid in good yield through direct 6β-hydroxylation, and as was shown with tritium labelled Acid II this acid is readily transformed into Acid I when a tracerdose is injected. The proportion between Acid II and Acid I after injection of chenodeoxycholic acid is about 1:0.7 and after injection of Acid II 1:0.6.

The experiments with 7β-tritiochenodeoxycholic acid showed that Acid I is formed with loss of the tritium label and it was further established with biosynthetic 7β-tritio Acid II, that the tritium loss occurs in the reaction Acid II $\rightarrow$ Acid I, *i.e.* the inversion of the hydroxyl at C7 from α to β position.

Concerning the mechanism of the inversion it is most likely that the 7α-ol is dehydrogenated to a ketone, which is reduced stereospecifically to the equatorial 7β-ol in Acid I. The steroid alcohol dehydrogenases show a very high degree of stereospecificity for the substrate [13], and there are presumably two separate enzymes which carry out the inversion. The following equilibrium might then be obtained:

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

The presence of both 7α- and 7β-hydroxydehydrogenases active in the bile acid series has been demonstrated in the rat liver through metabolic studies of 7-ketohydroxycholanic acids. As mentioned above 7-ketolithocholic acid is formed in small amounts from chenodeoxycholic acid and the former acid is mainly reduced to ursodeoxycholic acid in the liver [12,14]. About equal parts of cholic acid (7α-ol) and 3α,7β,12α-trihydroxycholanic acid are obtained when 7-ketodeoxycholic acid is administered [15]. A mechanism consisting of the formation of a double bond through dehydration of the 7α-ol and a rehydration to the opposite steric position can be excluded by the isotope experiments. The above-mentioned oxidation-reduction reactions which were suggested for the inversion are usually linked to DPN or TPN[13]. The requirement of DPN for the lactate racemase described by Kaufman *et al.*[16] has recently also been found for the β-hydroxybuturyl CoA racemase by Wakil[17]. The latter author also shows that the racemization is effected by two different dehydrogenases (D(—) and L(+) β-hydroxy buturyl-CoA-dehydrogenase) with acetoacetyl CoA as an intermediate. A problem similar to the transformation of Acid II into Acid I is the epimerization of the hydroxyl at C4 in the conversion of uridinediphosphogalactose to uridinediphosphoglucose. The mechanism of this reaction has been studied extensively with ^3H_2O and $H_2{}^{18}O$ in the medium and also with tritium labelled DPN or DPNH [18]. Of the originally proposed mechanisms essentially only one involving a dehydrogenation remains. This reaction, however, is considered to be carried out by one enzyme bearing two specific groups, the epimerization consisting of a shift of a hemiacetal between the 4-carbonyl group and these groups under the influence of a certain catalyst [18].

A minor pathway (I→II→III→VI) for the metabolism of chenodeoxycholic acid consists of the formation of 7-ketolithocholic acid, reduction of the 7-carbonyl group to ursodeoxycholic acid and a 6β-hydroxylation of this acid to Acid I.[19] Another metabolite of ursodeoxycholic acid was also isolated but the structure of this acid has not been determined.[19] It is probable that a direct 6β-hydroxylation of 7-ketolithocholic acid also can take place, as the proportion between ursodeoxycholic acid, Acid I and the unidentified metabolite after administration of essentially the same amount of a tracerdose of 7-ketolithocholic acid and ursodeoxycholic acid, respectively, are 1:0.75:0.05 and 1:0.07:0.03 [12].

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REFERENCES

1. Bergström, S. and Borgström, B. *Ann. Rev. Biochem.* **25** (1956) 177.
2. Bergström, S. and Sjövall, J. *Acta Chem. Scand.* **8** (1954) 611.
3. Bergström, S. and Norman, A. *Proc. Soc. Exptl. Med.* **83** (1953) 71.
4. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **226** (1957) 667.
5. Hsia, S. L., Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A. Jr., Thayer, S. A. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 573.

6. Fieser, L. F., Herz, J. E., Klohs, M. W., Romero, M. A. and Utne, T. *J. Am. Chem. Soc.* **74** (1952) 3309.
7. Wilzbach, K. E. *J. Am. Chem. Soc.* **79** (1957) 1013.
8. Samuelsson, B. *To be published.*
9. Bergström, S., Rottenberg, M. and Voltz, J. *Acta Chem. Scand.* **7** (1953) 481.
10. Bergström, S. and Sjövall, J. *Acta Chem. Scand.* **5** (1951) 1267.
11. Glascock, R. *Isotopic Gas Analysis for Biochemists*, Academic Press N.Y. 1954.
12. Samuelsson, B. *Acta Chem. Scand.* **13** (1959) 236.
13. Talalay, P. *Physiol. Rev.* **37** (1957) 362.
14. Mahowald, T. A., Mao Wu Yin, Matschiner, J. T., Hsia, S. L., Doisy, E. A., Jr., Elliott, W. H. and Doisy, E. A. *J. Biol. Chem.* **230** (1958) 581.
15. Norman, A and Sjövall, J. *To be published.*
16. Kaufman, S., Korkes, S. and del Campillo, A. *J. Biol. Chem.* **192** (1951) 301.
17. Wakil, S. J. *Biochim. et Biophys. Acta* **18** (1955) 314.
18. Kalckar, H., *Advances in Enzymol.* **20** (1958) 111.
19. Samuelson, B. *Acta Chem. Scand.* **13** (1959) 970.

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