Occurrence and Metabolism of Chenodesoxycholic Acid in the Rat. Bile Acids and Steroids 13

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Byers and Biggs [1] and Bergström [2] have recently shown that cholesterol is the precursor of cholic acid in the rat, as had earlier been demonstrated in the dog by Bloch, Berg and Rittenberg [3]. Cholic acid is the main bile acid present in rat bile but it has been observed [2,4] that small amounts of an acid regularly occurred in the chromatograms at the place where dihydroxycholanic acids appeared. This material also became labelled when cholesterol-4-^{14}C had been administered as shown in the curves published earlier [2,4].

The main part of this fraction has now been found to be chenodesoxycholic acid. In order to investigate if this $3\alpha,7\alpha$-dihydroxy-cholanic acid is an intermediate in the conversion of cholesterol into $3\alpha,7\alpha,12\alpha$-trihydroxy-cholanic acid (cholic acid) we have also studied its metabolism with the aid of chenodesoxycholic acid-24-[14]C.

A general survey of the bile salts present in rat bile has also been made by paper chromatography.

EXPERIMENTAL

Chenodesoxycholic acid-24-^{14}C was prepared according to Bergström, Rottenberg and Voltz [5].

The chromatographic procedures used have been described in several earlier publications [4,6,7,8].

The systems of phases used have been prepared by mixing the volumes indicated below:

System A: Methanol 180; Water 120; Chloroform 45; Heptane 5.
 B: Methanol 150; Water 150; Sec. octanol 15; Chloroform 15.

Of the less hydrophilic phase 4 ml was taken for 4.5 g of hydrophobic Hyflo Supercel. The maximum amount of material for a column of this size is 30—50 mg of bile acids.

The technique of insertion of a bile fistula for the collection of the bile, followed by injection of labelled material intraperitoneally, and finally alkaline hydrolysis and isolation of the free bile acids has also been described earlier [4,9]. Paper chromatography of free and conjugated bile acids has been described by Sjövall [14,15] and by Haslewood and Sjövall [16].

RESULTS

Fractionation of normal rat bile. The combined bile that had been collected in ethanol from different rats, corresponding to the bile secreted from one rat during thirty days, was filtered and evaporated to dryness. Of this crude material 20 mg was dissolved in one ml of water for paper chromatography. A paper chromatogram was run with *iso*propylether: heptane 75/25 as moving phase and 70 % acetic acid as stationary phase in which glycine conjugates are separated. A strong spot appeared at the starting line where taurine conjugates stay and a very weak spot was found at the position of glycocholic acid. No free bile acids could be detected. In work with bile acid conjugation in rat liver slices indications of a slight conjugation of desoxycholic acid with glycine have also been obtained [17].

100 γ of the crude bile salts were run in the butanol system for taurine conjugated acids [15]. After spraying two spots were detected, the stronger one running at the same speed as taurocholic acid and the weaker one at the same speed as taurochenodesoxycholic acid or taurodesoxycholic acid.

The main part of the crude bile salts was hydrolyzed in 1 N sodium hydroxide at 110° for 5 hours. Acidification and extraction with ether yielded 1.3 g of residue. 20 mg of this residue was dissolved in 1 ml alcohol and used for paper chromatography.

Ascending chromatography of 100 γ hydrolyzed bile acids with *iso*propyl ether : heptane 60 : 40 as moving phase gave two spots, the weaker one corresponding to chenodesoxycholic acid or desoxycholic acid and the stronger one appearing at the same place as cholic acid. When more than 100 γ was run a very weak spot appeared between the two just mentioned. The compound causing this is as yet unknown.

In order to differentiate between desoxycholic acid and chenodesoxycholic acid 400 γ of hydrolyzed bile acids were run descending for 20 hours with *iso*propyl ether : heptane 20 : 80 as moving phase. Spots appeared at the same places as cholic acid and chenodesoxycholic acid. There was a very weak coloration at the position of desoxycholic acid. It is uncertain whether any substance moving as desoxycholic acid is present. If there is, it must be less than 10 % of the chenodesoxycholic acid (see below) corresponding to 1 or 2 % of the total bile acids present.

For the further purification of the hydrolyzed bile acids the fatty acids were removed from the main part of the hydrolysate. The material was subjected to a two stage countercurrent extraction between light petroleum and 70 % ethanol, with 50 ml of each phase. The combined ethanolic phases yielded a residue of 1.0 g.

For a separation on a preparative scale 500 mg of this material was subjected to reversed phase partition chromatography in phase system A on a column containing 85 g of hydrophobic Supercel. The titration curve of this run is shown in Fig. 1.

The main band (150—272 ml effluent) contained mainly cholic acid that was obtained pure after one crystallisation from aqueous acetic acid and one from aqueous ethanol. Melting point 195—197°, no depression with authentic cholic acid. Infra-red spectrum identical with that of cholic acid.
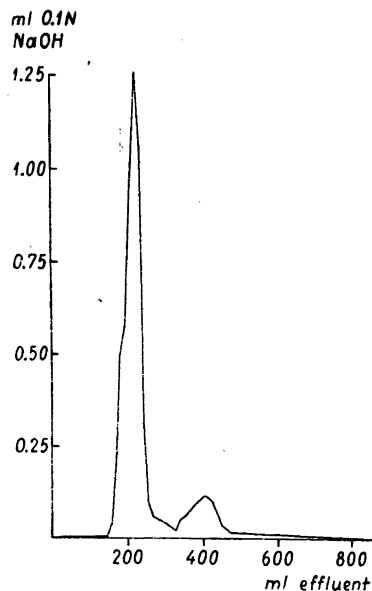
ml 0.1N
NaOH

Fig. 1. Chromatography of acids (0.5 g) from hydrolysed rat "crude bile salts". Column 85 g hydrophobic Supercel. Phases: Type A.

The fractions of the second band (325 to 500 ml effluent) were combined, most of the methanol was evaporated off on the water bath. The remaining aqueous solution was acidified with hydrochloric acid and extracted three times with three volumes of ether. The combined ether solutions were washed with water until free from hydrochloric acid and the indicator (bromothymol blue) and then evaporated to dryness. The residue was dissolved in 1 ml of ethyl acetate and was left at 5°, when the gelatinous appearing clusters of crystals formed that are typical for chenodesoxycholic acid. One further crystallization from ethyl acetate and light petroleum yielded 15 mg of crystals melting at 140—42°, undepressed by admixture of authentic chenodesoxycholic acid. For analysis a sample was dried at 110° at 0.001 mm over P_2O_5 for 12 h. (Found: C 73.2; H 10.27; Eq.wt. 393. Calc. for $C_{24}H_{40}O_4$: C 73.4; H 10.27; Eq.wt. 392.6). The mother liquor was subjected to paper chromatography and only showed one spot at the position of chenodesoxycholic acid.

Professor Einar Stenhagen has kindly compared the long X-ray spacing of the isolated material with that of authentic chenodesoxycholic acid and found them to be identical. The IR spectrum of corresponding material was also found to be identical with that of chenodesoxycholic acid.

As mentioned above, it has earlier been found in this laboratory that when ringlabelled cholesterol was administered to rats, some labelled material regularly appeared in the "dihydroxycholanic acid band" [2,4] which has now been found to consist mainly of chenodesoxycholic acid.

That the labelled compound also was chenodesoxycholic acid has been shown by isotope dilution. When labelled material from the "dihydroxy band" was diluted with unlabelled desoxycholic acid the activity was rapidly lost on
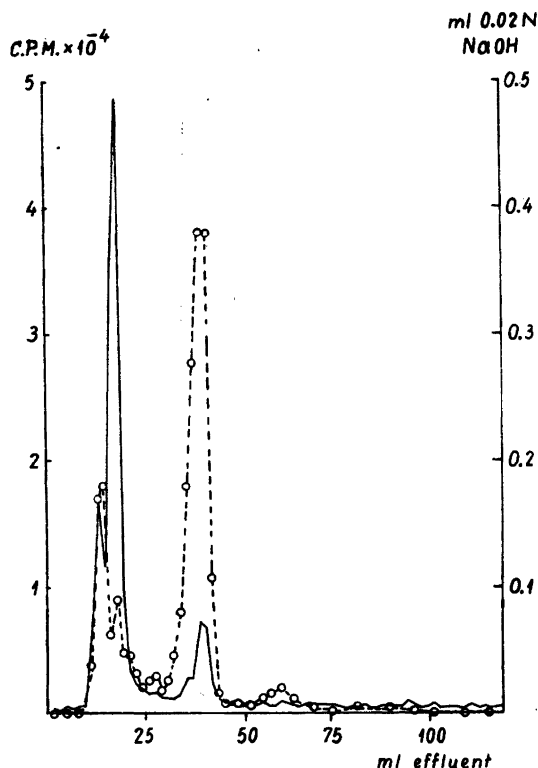
Fig. 2. Chromatography of acids from hydrolysed bile, excreted after intraperitoneal admi-nistration of chenodesoxycholic acid-24-[14]C. Column: 4.5 hydrophobic Supercel. Phases: Type A.

recrystallization. When it was diluted with chenodesoxycholic acid the speci-fic activity of the original mixture remained constant during extensive crystal-lizations from aqueous ethanol, acetic acid and ethyl acetate.

Metabolism of intraperitoneally administered chenodesoxycholic acid-24-[14]C. When 2.5 mg of chenodesoxycholic acid-24-^{14}C was injected intraperitoneally into rats with a bile fistula the isotope was excreted in the bile as rapidly as when similar experiments were performed with labelled lithocholic [9] or cholic acid [10], *i. e.* more than two thirds in the three hours following the injection, while practically all of it had appeared in 24 hours. As with the other acids Norman has found that practically all of the labelled material is excreted in the bile conjugated with taurine [11]. Hydrolysis of the bile and chromatography of the free bile acids in system A is shown in Fig. 2. Although most of the activity appeared at the position of chenodesoxycholic acid, about one quarter of the activity has been shifted to the place where cholic acid and other more polar acids appear. The band shows some evidence of inhomogeneity. The material in this first band was then dissolved from the planchets, acidified and extracted
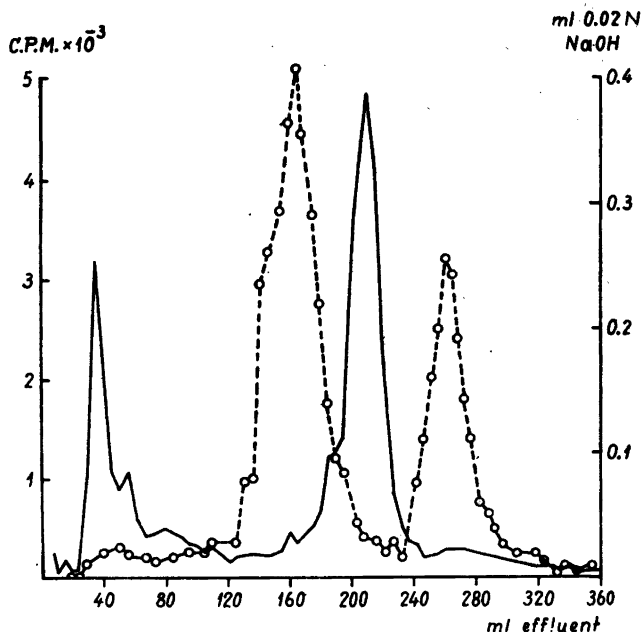
Fig. 3. *Sample of the first band (Fig. 2) rerun with unlabelled cholic acid. Column 9 g hydrophobic Supercel. Phases: Type B.*

with ether and the residue from the washed ether extract chromatographed in phase system B together with some added cholic acid. The curves shown in Fig. 3 were obtained. It is seen that the main part of the activity is contained in two bands of which one appears before and one after the titrated cholic acid band. In an earlier paper by Bergström and Norman [4] it was found that after administering ring-labelled cholesterol, cholic and chenodesoxycholic acids were the most strongly labelled acidic compounds in the bile. However, two minor components were observed which also appeared just before and after cholic acid at the same place as the two metabolic products found in this work (cf. Fig. 4 in [4]). It appears possible that the former also contain secondary metabolic products formed from the chenodesoxycholic acid. The structure of these metabolic compounds has not yet been determined.

The metabolic transformations of chenodesoxycholic acid take place decidedly more slowly than those of lithocholic [9], desoxycholic [10] or even cholanic acid which is presumably the reason why some chenodesoxycholic acid is present in the bile.

It has resently been shown that the rat transforms desoxycholic acid into cholic acid, i. e. a 7α-hydroxyl is introduced whe the $3(\alpha)$- and $12(\alpha)$-hydroxyl are present. The work reported here indicates that if the $3(\alpha)$- and 7α-hydroxyls are present, a 12α-hydroxyl is not introduced, but that other metabolic changes transforming chenodesoxycholic acid into more hydrophilic compounds take place.

Thus chenodesoxycholic acid does not appear to be an intermediate in the normal conversion of cholesterol into cholic acid in the rat. Possibly some of the chemical changes in the ring system occur before the cholesterol side chain has been completely degraded into that of the bile acids.

It might finally be pointed out that in all these experiments [9,10] the labelled acids have been injected intraperitoneally so that they have not been in contact with the intestinal microorganisms before being excreted in the bile fistula. The complicated metabolic changes of the bile acids that take place in the intestinal content are being studied [4,12,13].

SUMMARY

Chenodesoxycholic acid has been identified as a minor component of the bile acids normally present in rat bile. The labelled material appearing in this band after administering cholesterol-24-^{14}C has also been found to be chenodesoxycholic acid.

With the aid of chenodesoxycholic acid-24-^{14}C it has been found that this acid is transformed into more hydrophilic acids by the tissues of the rat. These products are not identical with cholic acid and it thus appears unlikely that chenodesoxycholic is an intermediate in the conversion of cholesterol into cholic acid.

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