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On the Metabolism of Coprostane-3 α, 7 α-diol in Mouse Liver Homogenates

Bile Acids and Steroids 130

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Mouse liver mitochondria were found to oxidize tritium labeled coprostane-3α,7α-diol to coprostane-3α,7α,26-triol. Fortification of the mitochondrial preparations with 100 000 × *g* supernatant fluid of mouse liver homogenate resulted in the additional formation of smaller amounts of one unknown neutral sterol and of steroid acids.

Tritium labeled coprostane-3α,7α,26-triol was metabolized by the bile fistula rat mainly into chenodeoxycholic acid and two compounds with chromatographic properties as those of the two 6β-hydroxylated metabolites of chenodeoxycholic acid, while cholic acid was a minor metabolite.

As an extension of the work of Bergström and Lindstedt (*cf.* Ref.[1]) on the mechanism of the conversion of cholesterol to bile acids a series of investigations of the metabolism *in vitro* of cholesterol and other neutral C_{27}-sterols has been initiated in this laboratory [2-4]. The results so far obtained have shown that the main reaction that occurs when cholesterol or 7α-hydroxycholesterol are incubated with liver mitochondrial preparations is an hydroxylation at the C_{26}-position. No evidence for hydroxylation at C_{12} could be obtained. The metabolites formed, 26-hydroxycholesterol and Δ^5-cholestene-3β,7α,26-triol, were converted mainly into chenodeoxycholic acid when administered to the bile fistula rat. These and earlier results (*cf.* Ref.[1]) indicate that in the conversion of cholesterol to cholic acid hydroxylation at C_{12} precedes hydroxylation at C_{26}.

The order in which the hydroxyl groups at C_7 and C_{12} are introduced has not been established. While 7α-hydroxycholesterol is converted to cholic acid in good yield in the bile fistula rat,[5] 12α-hydroxycholesterol is a comparatively inefficient precursor of cholic acid in the bile fistula rabbit,[6] results indicative hydroxylation at C_7 precedes hydroxylation at C_{12}. If such is the case the possible structures of the substrate for the 12α-hydroxylase should

be limited to a few sterols carrying oxygen groups at C_3 and C_7: 7α-hydroxycholesterol, coprostane-3α,7α-diol and intermediates involved in the conversion of the 3β-hydroxy-Δ^5- into the 3α-hydroxy-5β-hydrogen-configuration.

As mentioned above, no hydroxylation at C_{12} of 7α-hydroxycholesterol was obtained *in vitro*. It was of interest, therefore, to investigate if this reaction could be demonstrated to occur *in vitro* using as substrate coprostane-3α,7α-diol, a sterol that has been shown to be converted into cholic acid *in vivo* [7].

The present communication describes the results of studies of the metabolism of tritium labeled coprostane-3α,7α-diol in mitochondrial preparations from mouse liver.

EXPERIMENTAL

Tritium labeled coprostane-3α,7α-diol. This compound was prepared from tritium labeled chenodeoxycholic acid and unlabeled isovaleric acid. Chenodeoxycholic acid was exposed for 3 weeks to 2 C of tritium gas according to the method of Wilzbach [8,9] and then diluted with unlabeled chenodeoxycholic acid and purified by repeated chromatography with phase system F1 [10]. 500 mg of the tritium labeled chenodeoxycholic acid were electrolyzed with isovaleric acid using the procedure described by Bergström and Krabisch [11]. The reaction mixture was diluted with water, extracted twice with ether and the combined ether extracts washed with sodium carbonate and water. The residue of the ether extracts was chromatographed on a 50 g column of aluminum oxide, (Woelm, Eschwege, W-Germany), grade III, eluting with increasing concentrations of ethyl acetate in benzene. Coprostane-3α,7α-diol was eluted with 30 % ethyl acetate in benzene. The tritium labeled diol thus obtained was rechromatographed twice on aluminum oxide columns to give material with a specific activity of 10 μC/mg.

25-DL-Coprostane-3α,7α,26-triol. This compound was prepared in a manner analogous to that recently described for the synthesis of 25-DL-Δ^5-cholestene-3β,7α,26-triol [4]. 450 mg of chenodeoxycholic acid was electrolyzed with 4 g of methyl-2DL-methyl-3-carboxypropionate. The reaction mixture was diluted with water and extracted twice with ether. The combined ether extracts were washed with sodium carbonate and water and taken to dryness. The residual oil was chromatographed on a column of 50 g aluminum oxide, Woelm, grade III, eluting with increasing concentrations of ethyl acetate in benzene. Small aliqiots of each fraction were subjected to thin-layer chromatography in a system described by Eneroth [12] consisting of heptane/trimethylpentane/acetic acid: 10/10/4. As reference subtance authentic methyl 3α,7α-dihydroxycoprostanoate isolated from crocodile bile [13] was used. The fractions obtained with 55 % ethyl acetate in benzene as eluent were found to contain material with chromatographic properties identical to those of authentic methyl 3α,7α-dihydroxycoprostanoate. These fractions were combined and weighed 85 mg. Attempts to crystallize this material were unsuccessful but the identity was further strengthened by masspectrographic analysis. 80 mg of the methyl 25-DL-3α,7α-dihydroxycoprostanoate were disssolved in 25 ml dry ether and treated with 80 mg lithium aluminum hydride for 3 h at room temperature. The reaction mixture was acidified, extracted twice with ether and the combined ether extracts washed with sodium carbonate and water. The residue was chromatographed on a 10 g column of aluminum oxide, grade V, eluting with increasing concentrations of ethyl acetate in benzene. Elution with 20 % ethyl acetate in benzene gave 55 mg of 25-DL-coprostane-3α,7α,26-triol. Crystallization three times from acetone/petroleum ether and once from acetone/water afforded 25 mg of 25-DL-coprostane-3α,7α,26-triol with m.p. 152–154°. (Found: C 77.2; H 11.8. Calc. for $C_{27}H_{48}O_3$: C 77.1; H 11.4).

Preparation of homogenates. White male mice of the Danish Serum Institute strain weighing approximately 25 g, were used. Homogenates (33 % wet weight/volume) were prepared in 0.25 M sucrose containing 3.6 mg nicotinamide per ml with a tight fitting Teflon pestle. Fractionation of the homogenates was carried out in a Spinco preparative ultracentrifuge in the usual manner. The mitochondrial fraction was washed twice with homogenizing medium. Mitochondria and microsomes were resuspended in the sucrose-medium by homogenization with a loose-fitting pestle for 10–20 sec.

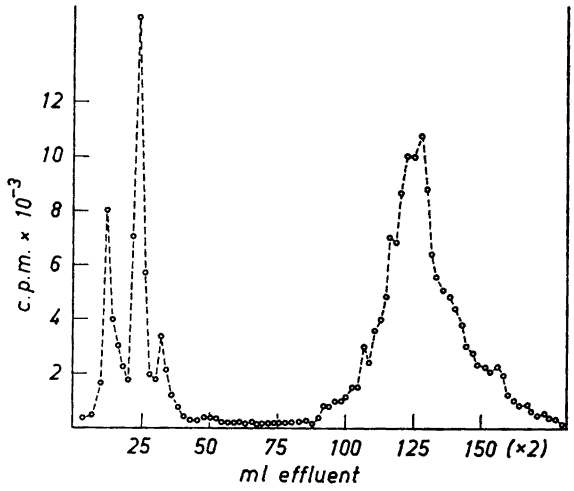

Fig. 1. Chromatogram of butanol extract of an incubation of 0.1 mg tritium labeled coprostane-3α,7α-diol with mouse liver mitochondria fortified with 100 000 × *g* supernatant. Column: 9 g hydrophobic Hyflo Supercel. Phase system III.

The following amounts of unfractionated and fractionated homogenate were used per incubation: Whole homogenate, 6 ml (corresponding to about 3 g of liver); mitochondria 6 ml (from about 8 g of liver); microsomes 6 ml (from about 8 g of liver); 100 000 × *g* supernatant fluid, 6 ml (from about 3 g of liver). Boiled liver juice was prepared as earlier described [2]. To each incubation 2 ml 0.05 M tris(hydroxymethyl)aminomethane-hydrochloric acid buffer, pH 7.4, were added. Incubations were run for 2 h at 37°.

Analysis of incubation mixtures. Incubations were terminated by addition of 4 volumes of ethanol, then filtered and the filtrate reduced to a small volume which was diluted with water, acidified with hydrochloric acid and extracted twice with butanol. The combined butanol extracts were washed with water until neutral and then taken to dryness. The residue was subjected to reversed phase partition chromatography in phase system III[14].

Analysis of bile from bile fistula rats. The strain of animal used and the procedures for analysis of bile were the same as those recently described [15].

RESULTS

Metabolism of tritium labeled coprostane-3α,7α-diol in vitro. Incubations of 100—200 μg quantities of tritium labeled coprostane-3α,7α-diol with whole homogenates of mouse liver resulted in the conversion of about 20 % of added isotope into more polar compounds. A similar yield (about 30 %) of the same more polar compounds was obtained in incubations with mitochondria fortified by addition of 100 000 × *g* supernatant fluid. A typical chromatogram of an extract of such an incubation is shown in Fig. 1. The more polar products formed appear in three distinct bands with peaks at 12, 24, and 32 ml of effluent, peaks A, B, and C, respectively. Unchanged coprostane-3α,7α-diol is eluted between 100 and 160 ml of effluent. No isotope was retained in the stationary phase indicating that no labeled esterified compounds were formed.

In incubations with mitochondria alone or with mitochondria fortified by addition of boiled liver juice the conversion to more polar products was about 20 %. Chromatography of extracts of these incubations showed that the main product formed had properties as peak B, while the amount of isotope eluted as peaks A and C was much smaller than that seen in Fig. 1.

The material eluted as peak A, Fig. 1, was combined with material with the same chromatographic properties obtained from a number of incubations and chromatographed with phase system F1 [10] together with unlabeled chenodeoxycholic acid. The isotope was distributed in three peaks, one located close to the solvent front, one shortly before and one shortly after the titration peak of chenodeoxycholic acid. The labeled material eluted close to the solvent front was rechromatographed together with unlabeled cholic acid on phase system C1 [10] but the radioactivity did not coincide with the titration peak of cholic acid. The labeled material eluted after chenodeoxycholic acid was rechromatographed with phase system F2 [10] together with unlabeled 3α,7α-dihydroxycoprostanic acid but was found to be somewhat more polar than this acid.

The labeled material eluted as peak B, Fig. 1, was combined with material with the same chromatographic properties obtained from several incubations and rechromatographed on phase system III [14]. The isotopic material was again eluted as one main peak with maximum at 25 ml of effluent. Part of this material was crystallized together with 15 mg unlabeled 25DL-coprostane-3α,7α,26-triol. The specific activity remained constant through four crystallizations.

The identity of the labeled material eluted as peak C, Fig. 1, has not yet been established. On paper chromatography it behaved as a homogeneous compound, somewhat less polar than coprostane-3α,7α,12α-triol.

Metabolism of tritium labeled coprostane-3α,7α,26-triol in the bile fistula rat. Tritium labeled coprostane-3α,7α,26-triol obtained as described above was injected intraperitoneally into a bile fistula rat. About 40 % of the administered isotope was excreted in bile during the first 24 h. The hydrolyzed bile was separated by chromatography with phase system F1 [10] into a cholic acid and a chenodeoxycholic acid fraction, each containing about 45 % of the

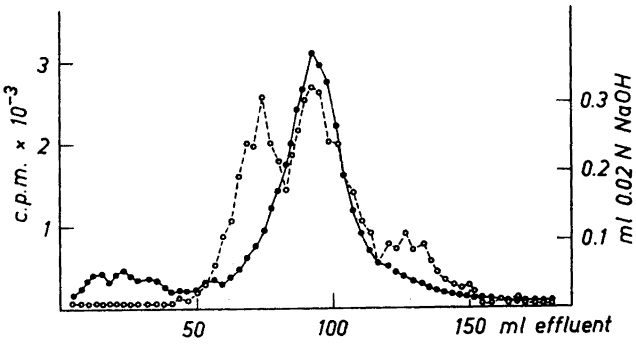

Fig. 2. Rechromatography of the cholic acid fraction of the first 24 h portion of bile from bile fistula rat injected with coprostane-3α,7α,26-triol. Column: 4.5 g hydrophobic Hyflo Supercel. Phase system C1. Solid line: titration values. Broken line: radioactivity.

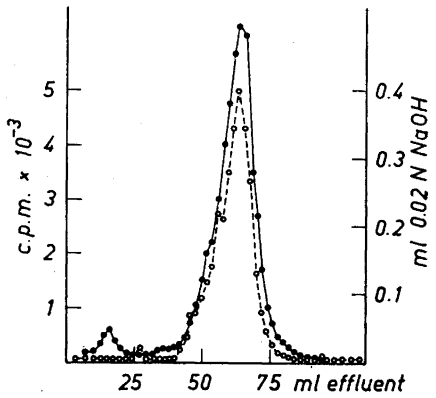

Fig. 3. Rechromatography of the chenodeoxycholic acid fraction of the first 24 h portion of bile from bile fistula rat injected with coprostane-3α,7α,26-triol. Column: 4.5 g hydrophobic Hyflo Supercel. Phase system F1. Solid line: titration values. Broken line: radioactivity.

extracted isotope. The cholic acid fraction was rechromatographed with phase system C1 [10]. This chromatogram is shown in Fig. 2. The radioactivity was distributed in three main peaks, one shortly before, one shortly after and one within the titration peak of cholic acid. The two first-mentioned peaks have the same retention volumes as those of 3α,6β,7α-trihydroxy- and 3α,6β,7β-trihydroxycholanic acids, respectively. Part of the labeled material eluted within the cholic acid band was identified as cholic acid by crystallization to constant specific activity. Of the total radioactivity excreted about 15 % was accounted for as cholic acid. The chenodeoxycholic acid fraction was rechromatographed on phase system F1 [10]. As shown in Fig. 3 the radioactivity coincided with the titration peak of chenodeoxycholic acid and the identity established as above.

DISCUSSION

The present investigation has shown that mouse liver mitochondrial preparations oxidize coprostane-3α,7α-diol to coprostane-3α,7α,26-triol. Under the experimental conditions employed coprostane-3α,7α,26-triol was the main metabolite. No evidence for the formation of a compound carrying a 12α-hydroxyl group was obtained. These results are similar to those obtained in studies of the metabolism *in vitro* of cholesterol [2,3] and 7α-hydroxycholesterol [4]. Thus, in all cases the main reaction has been found to be an hydroxylation at position C_{26}. This reaction has been implicated as the first step in the oxidation of the C_{27}-side-chain [16,17]. Previous and present work indicates that the formation of chenodeoxycholic acid does not require that oxidation at position C_{26} should occur at a specific stage. Thus, 26-hydroxycholesterol, Δ^5-cholestene-3β,7α,26-triol, and coprostane-3α,7α,26-triol are converted by the bile fistula rat predominantly into chenodeoxycholic acid and the 6β-hydroxylated metabolites of chenodeoxycholic acid. In the formation of cholic acid, however, the possible structures of the substrate of the 26-hydroxylase are apparently more limited and it seems likely that the substrate is a trihydroxysterol with hydroxyl groups at positions C_3, C_7 and C_{12}.

As is evident from the results so far obtained in *in vitro* studies of the biosynthesis of bile acids, the preparation of a system capable of carrying out 12α-hydroxylation is essential to a more complete understanding of the biosynthetic pathway of cholic acid.

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