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On the Oxidation of 3α,7α,12α-Trihydroxycoprostane by Mouse and Rat Liver Homogenates

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HENRY DANIELSSON

Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

3α,7α,12α-Trihydroxycoprostane has been found to be hydroxylated to 3α, 7α, 12α, 27-tetrahydroxycoprostane in whole homogenates as well as washed mitochondria of rat liver. The same reaction occurs also in mouse liver homogenates, which in addition transform 3α, 7α, 12α-trihydroxycoprostane into acidic material, part of which has been identified as 3α, 7α, 12α-trihydroxycoprostanic acid. Mouse liver mitochondria alone effect mainly the 27-hydroxylation, while addition of 100 000 × *g* supernatant increases the formation of acidic material 3- to 4-fold. 3α, 7α, 12α, 27-Tetrahydroxycoprostane is a more efficient precursor of the coprostanic acid than 3α, 7α, 12α-trihydroxycoprostane.

The intermediate steps in the degradation of cholesterol to bile acids are not wholly known. The probable sequence of reactions has been proposed by Bergström and Lindstedt [1–4] on basis of their studies on the formation of cholic acid in bile-fistula rats from different possible intermediates. According to these investigations the steroid nucleus is hydroxylated prior to the completion of the side-chain oxidation, and one of the earliest steps appears to be the introduction of the hydroxyl-group of C_7 of the steroid molecule.

The use of *in vitro*-systems seems a reasonable approach to further investigations on the individual steps in the degradation of cholesterol to bile acids. Horning *et al.* [5,6] and Gurin *et al.* [7,8] studied the oxidation of 26-^{14}C-cholesterol to $^{14}CO_2$ in mitochondrial preparations of mouse and rat liver and characterized the cofactor requirements for maximal $^{14}CO_2$-production. Gurin *et al.* [7] also provided evidence for the formation of labeled 25-dehydrocholesterol together with a labeled C_{27}-steroid aldehyde and acid from 26-^{14}C-cholesterol in these experiments. Using the above-mentioned mitochondrial system Fredrickson [9] investigated the products formed from 4-^{14}C-cholesterol and was able to isolate labeled 25- and 26-hydroxycholesterol, neither of which was converted to cholic acid in the bile-fistula rat [10]. In addition there were formed several acidic products not identical with cholic or deoxycholic acid.

Currently the metabolism of 4-^{14}C-cholesterol in mouse and rat liver mitochondrial systems is being studied in this laboratory. With the use of reversed phase partition chromatography [11] some 10 compounds formed from cholesterol in these systems have been isolated and partly identified [12,13]. Some of these substances undoubtedly arise through autoxidation of the added cholesterol, but at least two enzymically formed labeled products have been isolated. Both these compounds, which are transformed into normal bile acids in the bile-fistula rat, are neutral steroids retaining the C_{27}-side-chain, but the detailed structures are as yet not known. In these experiments *in vitro* with 4-^{14}C-cholesterol very limited degradation of the side-chain has so far been obtained.

To study the oxidation of the side-chain we then turned to 3α,7α,12α-trihydroxycoprostane. Bergström *et al.* [14] have shown, that when this compound is injected into a bile-fistula rat, it is rapidly excreted in bile mainly as cholic acid. We have found that both rat and mouse liver homogenates are able to oxidize this compound in good radiochemical yield (50—70 %).

EXPERIMENTAL

White male mice (Danish State Serum Institute strain) weighing approx. 25 g and white male rats (Sprague-Dawley strain) weighing approx. 150 g were used. Homogenates (33 % w/v) were prepared in 0.25 M sucrose containing 3.6 mg nicotinamide/ml using a tight-fitting Potter-Elvehjem pestle. Fractionations of homogenates were carried out in a Spinco preparative ultracentrifuge in the usual manner. The mitochondrial and microsomal pellets were resuspended in the above-mentioned sucrose medium by homo-

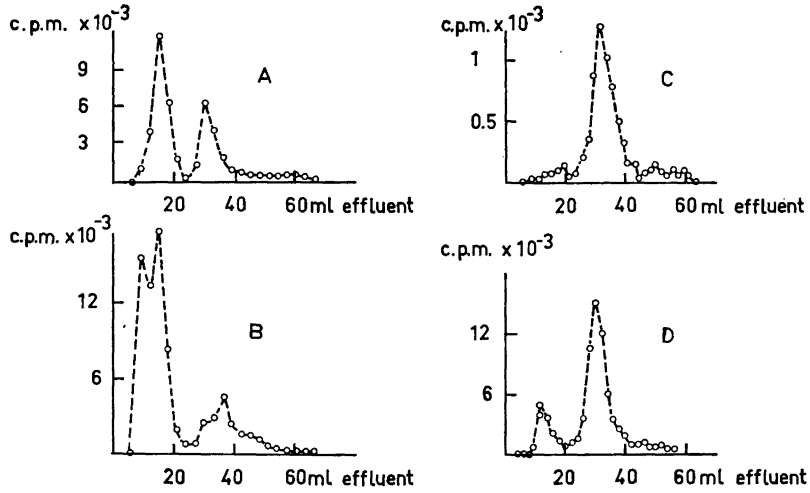

Fig. 1. Chromatograms of incubations of 3α,7α,12α-trihydroxycoprostane with mouse liver mitochondria (curve A) and with ditto plus supernatant (curve B). 4.5 g Hostalene columns. Phase system III. Curves C and D show the rechromatograms of 9—21 ml of effluent of the corresponding chromatograms (curves A and B) with phase system IV. 4.5 g hydrophobic Hyflo columns.

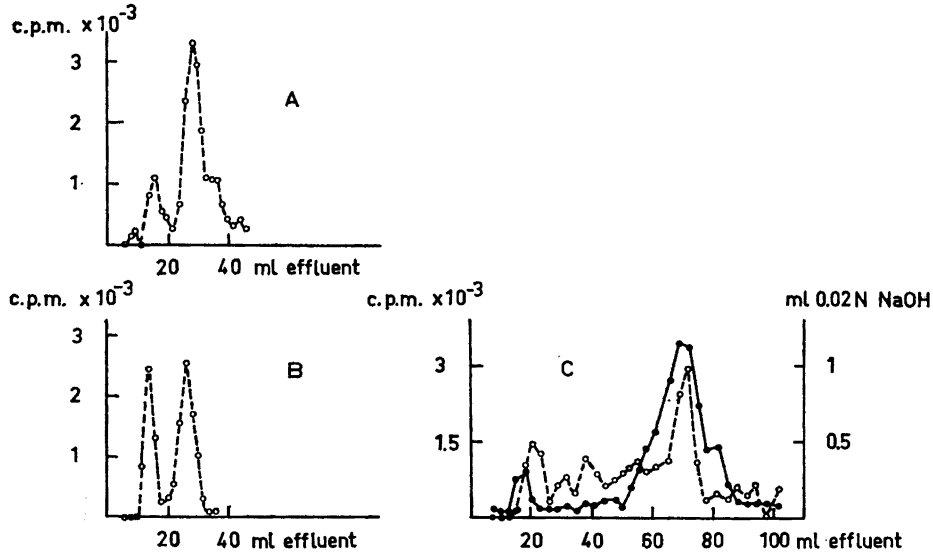

Fig. 2. Chromatograms of incubations of 3*a*,7*a*,12*a*,27-tetrahydroxycoprostane with mouse liver mitochondria (curve A) and with ditto plus supernatant (curve B). 4.5 g hydrophobic Hyflo columns. Phase system IV. Curve C shows the chromatogram of the saponified acidic material of the previous columns together with unlabeled 3*a*,7*a*,12*a*-trihydroxycoprostanic acid. 4.5 g hydrophobic Hyflo column. Phase system: 50 % aqueous methanol as moving phase, 10 % heptane in chloroform as stationary phase.

genization with a loose-fitting pestle for 10—20 sec. Incubations were run aerobically for 1 h at 37° with constant shaking.

The 3*a*,7*a*,12*a*-trihydroxycoprostane used was randomly labeled with tritium according to the method of Wilzbach [15] in the apparatus described by Bergström and Lindstedt [16]. The tritium-labeling was kindly carried out by Dr. B. Samuelsson of this laboratory. To each incubation there were added 0.8—2 μC (60—150 μg) of the labeled 3*a*,7*a*,12*a*-trihydroxycoprostane as an emulsion prepared by dissolving the compound in 0.1 ml of ethanol and diluting with 0.9 ml of a 1 % solution of bovine serum albumin in water.

The following amounts of unfractionated and fractionated homogenate were used per incubation: 3 ml whole homogenate (corresponding to approx. 1 g of liver), 3 ml mitochondria (from approx. 4 g of liver), 3 ml microsomes (from approx. 4 g of liver) and 3 ml 100 000 × *g* supernatant (from approx. 1 g of liver). To each incubation 1 ml of 0.1 M Tris-HCl buffer, pH 7.6, was added.

Incubations were terminated by addition of ethanol and after filtration and evaporation the aqueous solution was acidified and extracted twice with ether. The ether-extracts were chromatographed in the reversed phase systems described previously [11]. In addition a modification of system III was employed using 45 % aqueous *iso*propanol as moving phase (phase-system IV). Hostalene [17] or hydrophobic Hyflo Supercel [11] were used as supporting material for the stationary phase.

RESULTS AND DISCUSSION

Incubations with whole homogenates of mouse liver resulted in the conversion of about 50 % of the 3*a*,7*a*,12*a*-trihydroxycoprostane into more polar material, that could be separated into one acidic fraction and one neutral

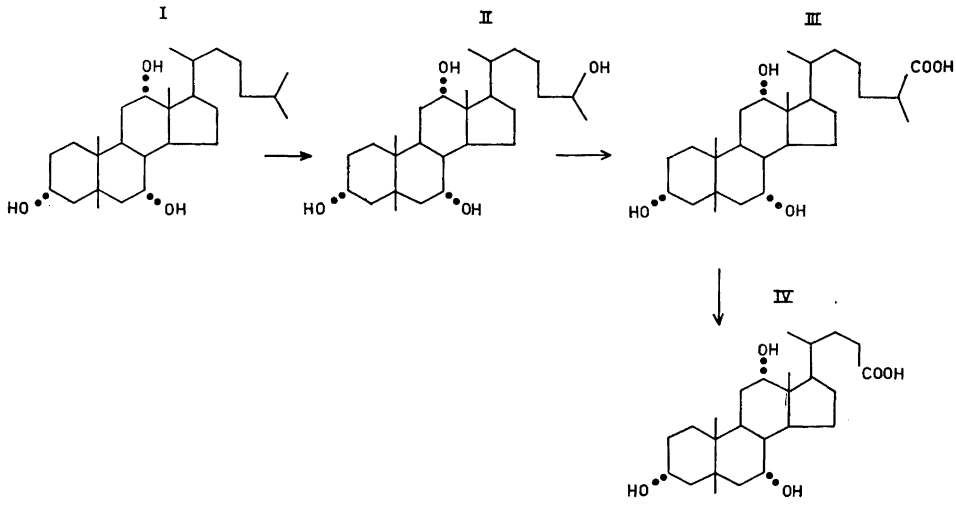

Fig. 3. Postulated sequence for the oxidation of the C_{27}-side-chain: 3α,7α,12α-trihydroxycoprostane (I), 3α,7α,12α,27-tetrahydroxycoprostane (II), 3α,7α,12α-trihydroxycoprostanic acid (III) cholic acid (IV).

tetrahydroxyl compound. Use of the whole homogenate of rat liver resulted in the formation of the tetrahydroxy fraction only. Washed mitochondria of mouse and rat liver effected mainly the formation of the tetrahydroxy compound and addition of 100 000 × *g* supernatant (hereafter called supernatant) to mouse liver mitochondria but not to rat liver mitochondria resulted in the additional formation of acidic material. Hardly any change (0—5 % conversion to more polar material) of the 3α,7α,12α-trihydroxycoprostane was observed in presence of microsomes and/or supernatant from either species.

Fig. 1 shows chromatograms using phase system III of incubations with mouse liver mitochondria (curve A) and with mouse liver mitochondria plus supernatant (curve B). The peak at 30 and 36 ml of effluent, respectively, represents unchanged 3α,7α,12α-trihydroxycoprostane. The material eluted between 9 and 21 ml of effluent in both chromatograms was rerun with phase system IV (curves C and D, Fig. 1). In the presence of mitochondria alone (curve C, Fig. 1) there is one main peak at 31 ml of effluent appearing at the position of a neutral tetrahydroxylated C_{27}-steroid and a small amount of isotope (5 % of total isotope eluted) is eluted earlier representing the acidic fraction. The addition of supernatant (curve D, Fig. 1) increases considerably the amount of isotope in the acidic fraction (18 % of total isotope eluted). The labeled material in the main peak was identified as 3α,7α,12α,27-tetrahydroxycoprostane by cocrystallization to constant specific activity with unlabeled material (m.p. 204°), obtained by $LiAlH_4$-reduction of 3α,7α,12α-trihydroxycoprostanic acid (m.p. 170—171°, reported 172—174° [18]) isolated from frog bile.

Incubation of labeled 3α,7α,12α,27-tetrahydroxycoprostane, biosynthesized *in vitro* from 3α,7α,12α-trihydroxycoprostane, with mouse liver mitochondria demonstrated that this compound was a more efficient precursor of the acidic fraction than 3α,7α,12α-trihydroxycoprostane. Fig. 2 shows the chromatograms with phase system IV of the products of these incubations. There was a 15 % conversion to acidic material by mouse liver mitochondria (curve A, Fig. 2); the addition of supernatant increased the yield of acidic material to 50 % (curve B, Fig. 2). The acidic peaks from these experiments were combined, saponified and chromatographed with unlabeled 3α,7α,12α-trihydroxycoprostanic acid (curve C, Fig. 2). Part of the radioactivity coincides with the titration peak of the added carrier and the identity was confirmed by isotope dilution. The peaks appearing earlier in the chromatogram were not identified, but it is known from the work of Bergström *et al.*[19], that 3α,7α,12α-trihydroxycoprostanic acid is converted to cholic acid also *in vitro*.

A preliminary scheme for the oxidation of the C_{27}-steroid side-chain would then entail hydroxylation of one of the terminal methylgroups and subsequent oxidation of this hydroxyl to a C_{27}-acid, which in turn is oxidized to the C_{24}-acid (*cf.* Fig. 3). It cannot be stated at present, whether the C_{24}-acid is formed by direct oxidation of the C_{27}-acid or whether a series of C_{26}-intermediates occur.

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