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On the Stereospecificity of 26-Hydroxylation of Cholesterol

Bile Acids and Steroids 155

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The stereospecificity of the 26-hydroxylation of cholesterol in mouse liver homogenates was studied. ^{14}C-Cholesterol labeled *inter alia* in one of the two terminal methyl groups of the side-chain was converted enzymically into cholest-5-ene-3β,26-diol. This sterol was oxidized to 3-keto-5α-cholestanoic acid which was decarboxylated. The isotope content of the carbon dioxide liberated was such as to demonstrate that the 26-hydroxylation occurs in a stereospecific manner.

Earlier studies on the conversion of cholesterol to bile acids have indicated that hydroxylation at the C-26 position is the first step in the degradation of the side-chain (*cf.* Ref. 1). The substrate for this reaction in cholic acid formation appears to be 5β-cholestane-3α,7α,12α-triol.[1,2] The further metabolism of the 26-hydroxylated derivative of this compound entails oxidation to 3α,7α,12α-trihydroxy-5β-cholestanoic acid followed by a "β"-oxidation process yielding cholic acid and propionic acid.[1,3,4] Several additional lines of evidence favour 3α,7α,12α-trihydroxy-5β-cholestanoic acid as an intermediate in the biosynthesis of cholic acid. Staple and Rabinowitz [5] and Carey and Haslewood [6,7] have isolated 3α,7α,12α-trihydroxy-5β-cholestanoic acid from human bile and have shown that it is formed from cholesterol. In the biosynthesis of chenodeoxycholic acid 26-hydroxylation could possibly be the first step.[1,8] The conversion of cholesterol into cholest-5-ene-3β,26-diol *in vitro* and the ready formation of chenodeoxycholic acid from the latter compound *in vivo* have been demonstrated.

Hydroxylation at C-26 gives an additional asymmetric carbon atom, *i.e.* C-25, in the C_{27} steroid, but it has not been completely established whether or not this reaction is stereospecific. Carey and Haslewood [6] reported that the methyl ester of the 3α,7α,12α-trihydroxy-5β-cholestanoic acid isolated from human bile did not depress the melting point of methyl 3α,7α,12α-trihydroxy-

5β-cholestanoate isolated from alligator bile. From earlier work by Haslewood and Bridgwater [9,10] it is known that the acid in alligator bile is the 25α-isomer. It is probable, therefore that only the 25α-isomer of 3α,7α,12α-trihydroxy-5β-cholestanoic acid is present in human bile, indicating that the initial 26-hydroxylation is stereospecific. On the other hand, both the 25α- and the 25β-isomers of 3α,7α,12α-trihydroxy-5β-cholestanoic acid have been isolated from frog bile.[11,12] The 26-hydroxylation is apparently not stereospecific in this species. It is conceivable, however, that racemization might have occurred during hydrolysis of the taurine conjugated bile acids.

The present communication deals with the stereospecificity of the 26-hydroxylation of cholesterol in mouse liver homogenates. Cholesterol-1,7,15,22,26-^{14}C, which has one of the two terminal methyl groups labeled with ^{14}C, was converted into cholest-5-ene-3β,26-diol and the presence of isotope in the 26-position of cholest-5-ene-3β,26-diol was determined. It was shown that the 26-hydroxyl group is introduced in a stereospecific manner.

EXPERIMENTAL

Cholesterol-1,7,15,22,26-^{14}C. This compound was prepared from 2-^{14}C-mevalonic acid (obtained from Radiochemical Centre, Amersham, England). Mevalonic acid-2-^{14}C, 0.1 mC (3.9 mg), was incubated with a homogenate of 15 g of rat liver as described by Wright and Cleland.[13] The nonsaponifiable fraction was isolated and was chromatographed on a column of 9 g of Hostalene GW (Farbwerke Hoechst, Frankfurt am Main, Germany) using phase system I.[14] The fractions eluted at the place characteristic of cholesterol were combined and were rechromatographed on a column of 10 g of aluminum oxide, grade II (Woelm, Eschwege, Germany). The column was eluted with increasing concentrations of benzene in hexane. About 95 % of the radioactivity was eluted with 90 % benzene in hexane. When part of this material was crystallized together with unlabeled cholesterol, the specific activity of the crystals remained constant through six recrystallizations from different solvents. Upon thin layer chromatography on Kieselgel G (Merck, Darmstadt, Germany) using benzene/ethyl acetate, 2:1, as moving phase, the radioactive material gave only one spot that could be detected with autoradiography. The spot coincided exactly with that of reference cholesterol. The total yield of labeled cholesterol from 0.1 mC 2-^{14}C-mevalonate was about 30 μC.

Conversion of cholesterol into cholest-5-ene-3β,26-diol. Labeled cholesterol, 30 μC, prepared as described above, was incubated with a fortified preparation of mouse liver mitochondria from 25 g of liver. The preparation of mitochondria and the addition of boiled liver juice and cofactors were as described by Danielsson.[15] After conclusion of the incubation, the incubation mixture was acidified and was extracted with water-saturated butanol. The residue of the butanol extract was chromatographed on a 9 g column of Hostalene GW using phase system I. Cholest-5-ene-3β,26-diol was isolated from the early part of the effluent and was purified as the diacetate by chromatography on aluminum oxide as described by Danielsson.[8] After hydrolysis the labeled cholest-5-ene-3β,26-diol was rechromatographed on a 4.5 g column of hydrophobic Hyflo SuperCel (Johns Manville & Co.) using phase system III.[14] The yield of labeled cholest-5-ene-3β,26-diol was about 0.35 μC. The purity was checked by thin layer chromatography on Kieselgel G using benzene/ethyl acetate, 2:3, as moving phase, and by cocrystallization with unlabeled cholest-5-ene-3β,26-diol (a generous gift of the late Dr. E. Mosettig).

Conversion of cholest-5-ene-3β,26-diol into 3-keto-5α-cholestanoic acid. Cholest-5-ene-3β,26-diol, 0.1 μC, was diluted with 5 mg of unlabeled material and was hydrogenated as described by Scheer *et al.*,[16] yielding 4.5 mg of 5α-cholestane-3β,26-diol, m.p. 179—180°, reported [16] 180—181°. Of this material 4 mg were oxidized with chromic acid according to Scheer *et al.*[16] The resulting 3-keto-5α-cholestanoic acid was isolated by preparative thin layer chromatography on Kieselgel G using benzene/ethyl acetate, 2:3, as moving phase. The yield of labeled 3-keto-5α-cholestanoic acid was 1.5 mg. This material had

exactly the same properties as authentic material in thin layer and gas chromatography, performed according to Sjövall.[17] Decarboxylation was done as decribed by Phares.[18]

Radioactivity determination. Radioactivity in all samples was measured in a Packard TriCarb Spectrometer after wet combustion of the samples as described by Jeffay and Alvarez.[19] The carbon dioxide liberated upon wet combustion or decarboxylation was trapped in a mixture of ethylene glycol and aminoethanol, 2:1. Deoxycholic acid-24-^{14}C was used as standard in the wet combustion as well as in the decarboxylation procedure.

RESULTS AND DISCUSSION

Table 1.

Compound	Radioactivity in c.p.m. Wet combustion	Decarboxylation
24-^{14}C-Deoxycholic acid	10 000	8350 (8010—8750) [a]
24-^{14}C-5β-Cholane-3α,12α,24-triol	10 000	210 (200—230) [b]
1,7,15,22,26-^{14}C-5α-Cholestan-3-one	10 000	220 (215—240) [b]
1,7,15,22,26-^{14}C-5α-Cholestane-3α,26-diol	10 000	240 (220—260) [b]
1,7,15,22,26-^{14}C-3-Keto-5α-cholestanoic acid	10 000	220 (190—240) [c]

Mean value and range of [a]) nine experiments, [b]) three experiments, [c]) six experiments.

Table 1 summarizes the results of the isotope determinations. The yield of labeled carbon dioxide upon decarboxylation of 24-^{14}C-deoxycholic acid was on an average 83.5 %. When the decarboxylation reaction was performed with 24-^{14}C-5β-cholane-3α,12α,24-triol (prepared by lithium aluminum hydride reduction of methyl 24-^{14}C-deoxycholate), the amount of labeled carbon dioxide formed was 2.1 %. Similar yields of labeled carbon dioxide, 2.4 and 2.2 %, were obtained upon "decarboxylation" of 1,7,15,22,26-^{14}C-5α-cholestane-3β,26-diol and 1,7,15,22,26-^{14}C-cholestan-3-one. The latter compound was prepared from 1,7,15,22,26-^{14}C-cholesterol in a manner analogous to that used for the preparation of 3-keto-5α-cholestanoic acid from cholest-5-ene-3β,26-diol.

Decarboxylation of three different samples of 1,7,15,22,26-^{14}C-3-keto-5α-cholestanoic acid yielded on an average 2.2 % of labeled carbon dioxide. This amount apparently represents the "background" in the decarboxylation reactions as similar amounts of labeled carbon dioxide were obtained from compounds lacking a carboxyl group. It is concluded, therefore, that the carbon atom which is hydroxylated in the conversion of 1,7,15,22,26-^{14}C-cholesterol into cholest-5-ene-3β,26-diol is the unlabeled one of the two terminal methyl carbons. Hence, the introduction of the 26-hydroxyl group occurs in a stereospecific manner.

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