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On the Formation of Cholic Acid from Cholest-4-ene-3α,7α, 12α-triol and Cholest-4-ene-3β,7α,12α-triol

Bile Acids and Steroids 166

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The synthesis of 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol and 3α-^{3}H-4-^{14}C-cholest-4-ene-3β,7α,12α-triol is described. Both compounds were transformed efficiently into cholic acid in the bile fistula rat. The ratio of tritium to ^{14}C in the cholic acid isolated from bile was such as to demonstrate that the formation of cholic acid from these sterols occurred mainly through a pathway including a 3-keto steroid as an intermediate.

In the conversion of cholesterol to the primary bile acids, cholic and chenodeoxycholic acids, the 3β-hydroxyl group is inverted into the 3α-position and the Δ^5-double bond is saturated.[1] Studying the formation of bile acids from 3α-^{3}H-cholesterol and 4β-^{3}H-cholesterol in bile fistula rats, Gréen and Samuelsson[2] have shown that the transformation of the 3β-hydroxy-Δ^5 configuration into the 3α-hydroxy-5β configuration probably occurs by means of the intermediate formation of a 3-keto-Δ^4 compound. Recent work[3,4] indicates that cholest-4-en-7α-ol-3-one might be a key intermediate in the biosynthesis of cholic and chenodeoxycholic acids. This compound is converted *in vivo* into cholic acid and chenodeoxycholic acid. *In vitro*, it is efficiently hydroxylated in the 12α-position to yield cholest-4-ene-7α,12α-diol-3-one, a probable intermediate in the formation of cholic acid.[5] A recent investigation of the conversion of cholest-4-ene-7α,12α-diol-3-one into 5β-cholestane-3α,7α,12α-triol *in vitro* indicates that the saturation of the double bond precedes the reduction of the keto group.[5] Analogous sequences of reactions are well known in the formation and metabolism of steroid hormones. However, it is conceivable that additional pathways for the formation of cholic and chenodeoxycholic acids might exist. The capacity of rat liver to convert a number of oxygenated C_{27} steroids into bile acids has been well established.[1] For instance, it is possible that 3α- and 3β-hydroxy-Δ^4 sterols might be formed from cholest-4-en-7α-ol-3-one and cholest-4-ene-7α,12α-diol-3-

one, each of which probably is an intermediate in the formation of cholic acid. Recently, it has been shown that enzymes present in several tissues, including liver, catalyze the reduction of some C_{19} and C_{21} steroids with a Δ^4-3-keto configuration into the corresponding Δ^4-3α- and 3β-hydroxy compounds.[6-8]

The present communication describes studies on the formation of cholic acid from 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol and 3α-^{3}H-4-^{14}C-cholest-4-ene-3β,7α,12α-triol.

EXPERIMENTAL

Cholest-4-ene-3α,7α,12α-triol and cholest-4-ene-3β,7α,12α-triol. These compounds were prepared by sodium borohydride reduction of cholest-4-ene-7α,12α-diol-3-one and the ratio between the epimeric alcohols obtained was about 9:1. Cholest-4-ene-7α,12α-diol-3-one, 400 mg with m.p. 225—226° (reported [5] m.p. 228—229°), was dissolved in 400 ml of 70 % aqueous methanol and treated with 200 mg of sodium borohydride at room temperature for 1 h. The reaction mixture was acidified with dilute hydrochloric acid and extracted with ether. The ether extract was washed with a 5 % solution of sodium carbonate and then with water until neutral. The residue of the ether extract was chromatographed on a column of 50 g of aluminium oxide, grade IV, eluting with increasing concentrations of methanol in ethyl acetate. Methanol (2.5 %) in ethyl acetate eluted 324 mg of cholest-4-ene-3β,7α,12α-triol. Two crystallizations from a methanol-water mixture yielded 236 mg, m.p. 219—222°, $[\alpha]_D^{23}$ + 57° (*c*, 1.0 in chloroform). (Found: C 77.5; H 11.1. Calc. for $C_{27}H_{46}O_3$: C 77.5; H 11.0).

Methanol (5 %) in ethyl acetate eluted 50 mg of a mixture of cholest-4-ene-3β,7α,12α-triol and cholest-4-ene-3α,7α,12α-triol. This mixture was subjected to preparative thin layer chromatography with ethyl acetate as moving phase giving 21.5 mg of cholest-4-ene-3α,7α,12α-triol. Crystallization from a methanol-water mixture yielded 19.2 mg, m.p. 211—214°, $[\alpha]_D^{23}$ + 127° (*c*, 1.0 in chloroform). (Found: C 77.1; H 11.1. Calc. for $C_{27}H_{46}O_3$: C 77.5; H 11.0).

3β-³H-4-¹⁴C-Cholest-4-ene-3α,7α,12α-triol and 3α-³H-4-¹⁴C-cholest-4-ene-3β,7α,12α-triol. The 4-C-^{14}labeled compounds were prepared from cholic acid-4-^{14}C. 4-^{14}C-Cholesterol, 100 μC, was administered to a bile fistula rat and bile was collected for 9 days. Cholic acid was isolated by chromatographying the hydrolysed bile with phase system C 1.[9] Two crystallizations from a methanol-water mixture afforded 324 mg, m.p. 195—197° (reported [10] m.p. 193—195°) with a total activity of 12 μC. This material was electrolyzed with isovaleric acid as described by Bergström and Krabisch.[11] The ether extract of the reaction mixture was chromatographed on a column of 30 g of aluminium oxide, grade IV. Ethyl acetate (40 %) in benzene eluted 138 mg of 4-^{14}C-5β-cholestane-3α,7α,12α-triol with m.p. 186—188° (reported [11] m.p. 184—185°) and with a specific activity of 0.043 μC per mg. This material was oxidized with aluminium *t*-butoxide and the resulting 4-^{14}C-5β-cholestane-7α,12α-diol-3-one was isolated as described previously.[5] Crystallization from a methanol-water mixture yielded 60.3 mg with m.p. 204—206° (reported [5] m.p. 209—210°) and with a specific activity of 0.043 μC per mg. This material was oxidized to 4-^{14}C-cholest-4-ene-7α,12α-diol-3-one as described previously,[5] and 31 mg were obtained having m.p. 224—226° (reported [5] m.p. 228—229°) and a specific activity of 0.044 μC per mg. This material was reduced with sodium borohydride as described above and the epimeric alcohols were separated by preparative thin layer chromatography with ethyl acetate as moving phase. The cholest-4-ene-3β,7α,12α-triol obtained was oxidized with manganese dioxide (10 mg in 0.2 ml of chloroform per mg sterol) for 3 h at room temperature. The reaction mixture was filtered, and the filtrate evaporated to dryness. The residue, almost pure cholest-4-ene-7α,12α-diol-3-one according to thin layer chromatography, was reduced with sodium borohydride and the reduction products were separated by means of thin layer chromatography. After repeated oxidation and reduction of the 3β-epimer, 4 mg of 4-^{14}C-cholest-4-ene-3α,7α,12α-triol, m.p. 210—212°, and 12 mg of 4-^{14}C-cholest-4-ene-3β,7α,12α-triol, m.p. 220—221°, were obtained. Both compounds had a specific activity of 0.044 μC per mg.

Cholest-4-ene-7α,12α-diol-3-one, 50 mg with m.p. 228—229°, was reduced with tritium-labeled sodium borohydride (Radiochemical Centre, Amersham, England). The epimeric

alcohols were isolated by aluminium oxide chromatography and preparative thin layer chromatography as described above, yielding 32.4 mg of 3α-^{3}H-cholest-4-ene-3β,7α,12α-triol, m.p. 219—222°, and 1.5 mg of 3β-^{3}H-cholest-4-ene-3α,7α,12α-triol, m.p. 210°. Both compounds had a specific activity of 2 μC per mg.

Specificity of tritium label. Samples of 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol and 3α-^{3}H-4-^{14}C-cholest-4-ene-3β,7α,12α-triol were diluted with unlabeled material and oxidized with managanese dioxide. The keto-compounds formed were isolated by preparative thin layer chromatography and the isotope content was determined. The amount of tritium retained was 1.9 % and 2.3 %, respectively.

Animal experiments. Male rats of the Sprague-Dawley strain weighing about 200 g were used. Bile fistulas were prepared in the usual manner. The labeled compounds were administered intraperitoneally in an emulsion stabilized with serum albumin. Bile was collected in 24-hours' portions. Hydrolysis of bile was carried out with 1 M sodium hydroxide in 50 % aqueous ethanol in sealed steel tubes at 110° for 12 h. The hydrolysed mixture was acidified with hydrochloric acid and extracted with ether. The residue of the ether extract was chromatographed on hydrophobic Hyflo Super-Cel with phase system C 1.[9]

Radioactivity assay. Simultaneous determination of tritium and ^{14}C was performed in a Packard Tri-Carb model 314 spectrometer. The bile acids were converted to methyl esters with diazomethane before addition of the phosphor solution (4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per 1000 ml of toluene). The discriminator-ratio method described by Okita *et al.*[12] was used, and the discriminator settings were 10 to 40 (first channel) and 10 to 100 (second channel). Photomultiplier voltage was 1150 V. Internal standards of tritium and ^{14}C were used to correct for quenching.

RESULTS

After administration of 1—2 mg of 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol and 3α-^{3}H-4-^{14}C-cholest-4-ene-3β,7α,12α-triol to bile fistula rats, 50—70 % of administered ^{14}C was excreted in bile during the first 24 h. The main part (70—90 %) of the radioactivity excreted in bile was identified as cholic acid by chromatography with phase system C 1 (*cf.* Fig. 1 and Fig. 2) and by crystallization to constant specific activity after addition of unlabeled cholic

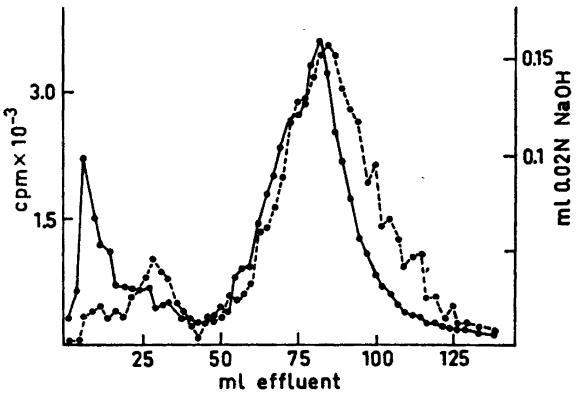

Fig. 1. Chromatogram of first 24-hours' portion of hydrolysed bile from bile fistula rat injected intraperitoneally with 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol. Column, 4.5 g of hydrophobic Hyflo Super-Cel; phase system C 1.———, titration values; — — —, radioactivity.

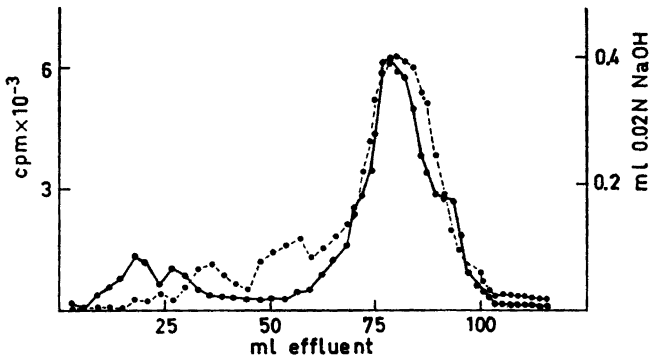

Fig. 2. Chromatogram of first 24-hours' portion of hydrolysed bile from bile fistula rat injected intraperitoneally with 3α-^{3}H-4-^{14}C-cholest-4-ene-3β,7α,12α-triol. Chromatographic conditions and symbols as in Fig. 1.

acid. The ratios of tritium to ^{14}C in the administered sterols and in the cholic acid isolated from bile are given in Table 1 and Table 2. Almost all of the tritium in 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol as well as in 3α-^{3}H-4-^{14}C-cholest-4-ene-3β,7α,12α-triol was lost during the formation of cholic acid.

DISCUSSION

Recent investigations on the mechanism of the *in vitro* conversion of cholesterol to 5β-cholestane-3α,7α,12α-triol indicate that cholest-5-ene-3β,7α-diol, cholest-4-en-7α-ol-3-one, cholest-4-ene-7α,12α-diol-3-one, and 5β-

Table 1. Conversion of 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol to cholic acid.

Compound	^{3}H, cpm	^{14}C, cpm	^{3}H/^{14}C	Per cent ^{3}H retained
Administered 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol	4864	728	6.68	100
Cholic acid isolated from rat 1	649	1220	0.53	8
Cholic acid isolated from rat 2	765	1499	0.51	8

Table 2. Conversion of 3α-^{3}H-4-^{14}C-cholest-4-ene-3β,7α,12α-triol to cholic acid.

Compound	^{3}H, cpm	^{14}C, cpm	^{3}H/^{14}C	Per cent ^{3}H retained
Administered 3α-^{3}H-4-^{14}C-cholest-4-ene-3β,7α,12α-triol	3790	836	4.53	100
Cholic acid isolated from rat 3	1508	11 210	0.13	3
Cholic acid isolated from rat 4	764	8 660	0.09	2

cholestane-7α,12α-diol-3-one are intermediates in this process and thereby in the biosynthesis of cholic acid.[1,3-5,13-15] The present investigation has demonstrated that cholest-4-ene-3α,7α,12α-triol and cholest-4-ene-3β,7α,12α-triol are readily converted into cholic acid *in vivo*, and that the formation of cholic acid from both sterols occurs predominantly with a 3-keto compound as an intermediate. The structure of this intermediate can not be deduced from the present *in vivo* experiments, but it is conceivable that the first step in the conversion of cholest-4-ene-3α,7α,12α-triol and cholest-4-ene-3β,7α,12α-triol to cholic acid might be an oxidation of the 3-hydroxyl group yielding, in both cases, cholest-4-ene-7α,12α-diol-3-one. The retention of tritium in the cholic acid formed from 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α,12α-triol was somewhat higher than that in the cholic acid formed from 3α-^{3}H-4-^{14}C-cholest-4-ene-3β,7α,12α-triol, indicating that cholest-4-ene-3α,7α,12α-triol might be transformed into cholic acid to some extent through a pathway involving direct saturation of the Δ^{4}-double bond. Preliminary experiments with 3β-^{3}H-4-^{14}C-cholest-4-ene-3α,7α-diol indicate that this compound is converted into chenodeoxycholic and cholic acids to some extent by direct saturation of the double bond.

The possible function of cholest-4-ene-3α,7α,12α-triol and cholest-4-ene-3β,7α,12α-triol in the biosynthesis of cholic acid can not be assessed from the present experiments and work on the formation and metabolism of these compounds *in vitro* is in progress.

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