ACTA CHEMICA SCANDINAVICA 15 (1961) 242–248

Synthesis and Metabolism of Δ^4-Cholestene-7α-ol-3-one

Bile Acids and Steroids 108

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A method of preparing Δ^4-cholestene-7α-ol-3-one is described. This compound can be obtained in low yield (4–5 %) from 3β-benzoxy-7α-tetrahydro-2′-pyranyloxycholesterol by hydrolysis of the 3β-benzoxy group, Oppenauer oxidation at this position followed by reduction with sodium borohydride yielding a 3-hydroxy-7-pyranyl ether with the double bond in the 4-position. Hydrolysis of this product followed by selective oxidation at the 3-position with manganese dioxide yielded Δ^4-cholestene-7α-ol-3-one.

Randomly tritium-labelled Δ^4-cholestene-7α-ol-3-one, prepared biosynthetically by incubation of labelled 7α-hydroxycholesterol with mouse liver homogenates, has been administered to bile-fistula rats, and the labelled products in the bile have been separated with chromatography and partly identified. Δ^4-Cholestene-7α-ol-3-one is metabolized in part to chenodeoxycholic acid and to cholic acid. In addition several unidentified bile acids of polarity similar to that of cholic as well as of chenodeoxycholic acid are formed.

7α-Hydroxycholesterol has been shown to be converted to both cholic and chenodeoxycholic acid in the rat *in vivo* [1] and this compound has been suggested as a possible intermediate in the formation of bile acids from cholesterol. The metabolism of 7α-hydroxycholesterol in mouse liver homogenates has recently been studied in this laboratory [2]. One of the products formed was found to be somewhat less polar than 7α-hydroxycholesterol, suggesting the presence of a keto group instead of a hydroxyl in this compound. This metabolite was not identical with 7-ketocholesterol and the possibility that oxidation had occurred at the 3-position with the formation of Δ^4- or Δ^5-cholestene-7α-ol-3-one appeared likely. While this work was in progress Yamasaki *et al.*[3] reported that 7α-hydroxycholesterol was metabolized in rat liver homogenates to a compound, which on grounds of its spectrographic properties was suggested to be Δ^4-cholestene-7α-ol-3-one. Any further characterization of the metabolite was not reported.

Greenhalgh *et al.*[7] have reported the synthesis of Δ^4-cholestene-7β-ol-3-one and also some early steps in the synthesis of the 7α-epimer, but these

authors were not able to obtain any Δ^4-cholestene-7α-ol-3-one because of its ready dehydration in the final steps of the synthesis. In this communication will be described the synthesis of Δ^4-cholestene-7α-ol-3-one and the metabolism of this compound in the bile-fistula rat.

EXPERIMENTAL

All rotations were taken in chloroform solutions. Melting points are uncorrected.

7α-Hydroxycholesteryl benzoate. This compound was prepared from cholesteryl benzoate using the method of Bide *et al.*[4] for synthesis of 7α-bromo-cholesteryl benzoate and that of Schaltegger and Müllner [5] for conversion of the 7α-bromo- into the 7α-hydroxycompound.

The *7α-bromo-cholesteryl benzoate* synthesized had m.p. 139—140°, $[\alpha]_D^{23}$° −175° (*c*, 1.0); reported [4] m.p. 140°, $[\alpha]_D$ −172° and the *7α-hydroxycholesteryl benzoate* had m.p. 167—168°, $[\alpha]_D^{23}$° −50° (*c*, 1.0); reported [6] m.p. 167—168°, $[\alpha]_D$ −50.5°.

3β-Benzoxy-7α-tetrahydro-2′-pyranyloxycholesterol. 4 g of 7α-hydroxycholesteryl benzoate were dissolved in 40 ml of dihydropyran and 8 drops of phosphorus oxychloride added as described by Greenhalgh *et al.*[7] The solution was kept at room temperature for 2 h. Afterwards it was made alkaline with methanolic potassium hydroxide, diluted with water and extracted twice with ether The ether extracts were washed with water until neutral and taken to dryness Crystallization twice from acetone/water gave 3.6 g of *3β-benzoxy-7α-tetrahydro-2′-pyranyloxycholesterol*, m.p. 140—141°, $[\alpha]_D^{23}$° −106° (*c*, 1.0). (Found: C 79.0; H 9.7. Calc. for $C_{39}H_{58}O_4$: C 79.25; H 9.9.)

7α-Tetrahydro-2′-pyranyloxycholesterol. The foregoing benzoxypyranyl ether (3.6 g) was dissolved in 50 ml of methanol and treated with 50 ml of 10 % potassium hydroxide in methanol under reflux for 30 min. Isolation by ether extraction and crystallization from methanol/water gave 2.8 g 7α-tetrahydro-2′-pyranyloxycholesterol, m.p. 138°, $[\alpha]_D^{23}$° −138° (*c*, 1.1); reported [7] m.p. 137—140°. (Found: C 77.1; H 11.2. (The material had been dried *in vacuo* (0.1 mm Hg) over phosphorus pentoxide 24 h at room temperature.) Calc. for $C_{32}H_{54}O_3$: C 78.95; H 11.2; Calc. for $C_{32}H_{54}O_3 + CH_3OH$: C 76.5; H 11.1.)

Δ^4-Cholestene-7α-ol-3-one. 2.5 g of foregoing hydroxy-pyranyl ether in 50 ml of dry benzene were heated under reflux for 2 h with 20 ml of a 25 % solution of aluminum *tert.*-butoxide in toluene and 36 ml of acetone as described by Greenhalgh *et al.*[7] The mixture was then acidified with 0.2 N hydrochloric acid, diluted with water and extracted twice with ether. The ether extracts were washed neutral and evaporated to dryness. To the residue were added a few ml of xylene and the solution was again evaporated to dryness. The resultant product showed two absorption maxima, at 243 mμ and at 285 mμ. The ratio of the absorption at 243 to that at 285 mμ was 3.2. Assuming that all material obtained were the desired product, 7α-tetrahydro-2′-pyranyloxy-cholest-4-en-3-one, the molar extinction coefficient at 243 mμ was 8 900. An ε of 15 000—16 000 was expected of this compound [7,12] and the content of it in the oxidation mixture would then be about 60 %. Attempts to crystallize the mixture failed. In an effort to remove unchanged starting material and the material giving rise to the absorption at 285 mμ, presumably $\Delta^{4,6}$-cholestadiene-3-one, the mixture was chromatographed on a 90 g column of Hostalene (Farbwerke Hoechst G.m.b.H., W-Germany) using phase system I [8]. The purification obtained was not very satisfactory. Although much of the starting material could be removed, the compounds giving rise to the absorption at 243 and 285 mμ, respectively, showed similar chromatographic properties in this system. The molar extinction coefficient at 243 mμ of the ultraviolet-absorbing material had increased to 11 000 after chromatography, but the ratio of absorption at 243 to 285 mμ had decreased to 2.6. No crystalline material could be obtained. Efforts to separate the α,β-unsaturated ketone from the cholestadienone with other phase systems [8] were not successful. Chromatography on aluminum oxide, either Merck (Merck A.G., W-Germany) or Woelm (M. Woelm, Eschwege, W-Germany), grade I, caused dehydration of the α,β-unsaturated ketone and mainly material absorbing at 285 mμ was recovered. Chromatography on weaker aluminum oxide was not tried at this stage.

As found by Greenhalgh *et al.*[7] hydrolysis with dilute hydrochloric acid of the α,β-unsaturated ketone to remove the protecting pyranyl ether group causes dehydration at the 7α-position with formation of $\Delta^{4,6}$-cholestadiene-3-one. It appeared possible to overcome this difficulty by removing the protecting pyranyl ether group after reduction of the ketone to the alcohol and then obtain the desired Δ^4-cholestene-7α-ol-3-one by selective oxidation of the allylic alcohol group at the 3-position.

The chromatographed material (1.7 g) was reduced with 1.7 g of sodium borohydride in 200 ml of methanol for 1 h at room temperature. The solution was then cooled, acidified with 0.2 N hydrochloric acid and extracted with ether. No crystalline material could be obtained. Attempts to separate the dienol, formed from the cholestadienone, from the hydroxy-pyranyl ether by reversed phase partition chromatography [8] were unsuccessful.

1 g of the reduction mixture was hydrolyzed with 100 ml of 0.08 % hydrochloric acid in absolute ethanol for 1 h at room temperature *. The hydrolysis mixture was then made alkaline with methanolic potassium hydroxide, diluted with water and extracted with ether. The washed ether extracts were taken to dryness, the residue dissolved in 200 ml of chloroform and then stirred at room temperature for 24 h with 10 g of manganese dioxide, prepared as described by Mancera *et al.*[9] The solution was then filtered, evaporated to dryness and the residue chromatographed on a 45 g column of hydrophobic Hyflo Supercel (Johns Manville and Co., USA) using phase system III [8]. After elution with 3 000 ml of mobile phase only one main peak of ultraviolet-absorbing material, appearing at 2 000 ml of effluent, had been eluted. This material, which had its absorption maximum at 243 mμ and no absorption at 285 mμ, was rechromatographed on a 13.5 g column using phase system I. The main fraction weighed 77 mg and had a molar extinction coefficent of 15 200. Crystallization from methanol/water afforded 42 mg as needles of Δ^4-cholestene-7α-ol-3-one, m.p. 183—184°; $[\alpha]_D^{23°}$ + 67° (*c*, 0.82); λ_{max}, 242.5 mμ ε = 16 100. (Found: C 81.1; H 10.9. Calc. for $C_{27}H_{44}O_2$: C 80.95; H 11.1.) Further recrystallization from acetone/water and hexane did not change the melting point or the optical rotation.

Δ^4-Cholestene-7α-ol-3-one acetate. 28 mg of foregoing hydroxy-ketone were dissolved in 1 ml of dry pyridine and 0.5 ml of acetic anhydride and left at room temperature for 24 h. The mixture was worked up in the usual manner and crystallized from methanol/water yielding 20 mg, m.p. 136—137°. Recrystallization from hexane afforded 10 mg as needles of *Δ^4-cholestene-7α-ol-3-one acetate*, m.p. 144—145°, $[\alpha]_D^{23°}$ + 20° (*c*, 0.82); λ_{max}, 238 mμ ε = 16 300. (Found: C 78.4; H 10.2. Calc. for $C_{29}H_{46}O_3$: C 78.65; H 10.5.)

Δ^4-Cholestene-7β-ol-3-one. This compound was prepared exactly as described by Greenhalgh *et al.*[7] and had m.p. 182—183°, $[\alpha]_D^{23°}$ + 62° (*c*, 0.52); reported [7] m.p. 183.5—184°, $[\alpha]_D$ + 63°.

Animal experiments. Randomly tritium-labelled Δ^4-cholestene-7α-ol-3-one was prepared by incubating tritium-labelled 7α-hydroxycholesterol with mouse liver homogenates [2] and was purified by chromatography with phase system III [8]. The identity of the labelled compound was established by chromatography together with unlabelled material, synthesized as described above, and crystallization to constant specific activity both as free sterol and as acetate.

White male rats (Sprague-Dawley, weighing about 250 g) were provided with bile fistula in the usual manner. The bile was saponified with 1 N potassium hydroxide over night at 110°. The free bile acids were extracted with ether and then chromatographed with phase system F 1 [10] to separate the cholic and the chenodeoxycholic acid fraction. The cholic acid fraction was rechromatographed with phase system C 1 [10] and the chenodeoxycholic acid fraction with phase system F 1.

* Exposure for longer times, 3 h to 6 days, gave with time lower and lower yields as measured by the amount of Δ^4-cholestene-7α-ol-3-one formed after oxidation of the hydrolysis mixture with manganese dioxide.

RESULTS AND DISCUSSION

Chemical synthesis of Δ^4-cholestene-7α-ol-3-one. The physical properties of Δ^4-cholestene-7α-ol-3-one and Δ^4-cholestene-7β-ol-3-one were found to be strangely similar with respect to melting point and optical rotation. The 7α-epimer synthesized as described above had m.p. 183—184° and $[\alpha]_D$ + 67° while the 7β-epimer has m.p. 183.5—184° and $[\alpha]_D$ + 63° [7]. The same physical constants were obtained in repeated syntheses of Δ^4-cholestene-7α-ol-3-one, and synthesis of the 7β-epimer according to Greenhalgh *et al.*[7] gave a product with the same properties as the one described by these authors. That the two compounds were not identical could be shown by mixed melting point determination, chromatography and preparation of the acetates. There was a substantial depression of the melting point on admixture of the epimer (155—165°), the two compounds separated completely on thin layer chromatography (see Fig. 1) and the physical constants of the acetate of the 7α-epimer were

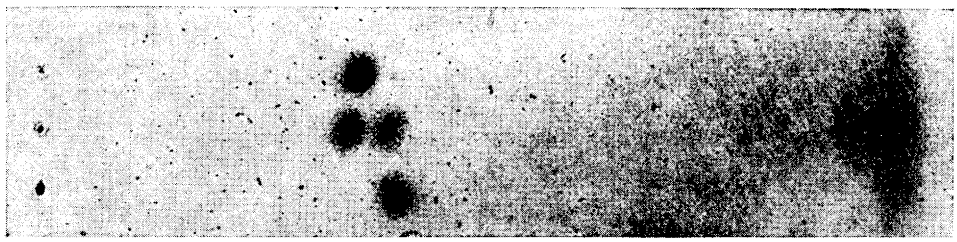

Fig. 1. Thin layer chromatography of Δ^4-cholestene-7β-ol-3-one (uppermost), Δ^4-cholestene-7α-ol-3-one (lower) and a mixture of these (middle) according to the method of Van Dam *et al.*[11] Mobile phase: benzene/ethyl acetate 2:1 (v/v).

quite different from those of the 7β-epimer. In addition, oxidation of 7β-tetrahydro-2′-pyranyloxycholesterol under the same conditions used for the 7α-epimer proceeded much slower and yielded preponderantly a cholestadienone [12].

Readings of the optical rotation of samples of the 7α-compound crystallized from different solvents and obtained from different syntheses all gave values for $[\alpha]_D$ between + 67° and + 70°. This value is worth noting as it could be expected to be *less* positive than that of the 7β-epimer. In spite of the large "positive" contribution to the M_D by the shift of the double bond from the 5- to the 4-position [13], the large difference in rotation of 7α-hydroxycholesterol ($[\alpha]_D$ —92°) and 7β-hydroxycholesterol ($[\alpha]_D$ + 7°) was thought to be noticeable also in the epimeric 7-hydroxy-Δ^4-cholestene-3-ones. In fact, the $[\alpha]_D$ of the 7α-acetate was substantially less positive (+ 20°) than that of the 7β-acetate (+ 77°).

Metabolism of Δ^4-cholestene-7α-ol-3-one. The labelled Δ^4-cholestene-7α-ol-3-one was prepared biosynthetically by incubation of tritium-labeled 7α-hydroxycholesterol with mouse liver homogenates [2]. The identity of the isolated

product with the compound synthesized chemically was established by co-chromatography and isotope dilution.

Tritium-labelled Δ^4-cholestene-7α-ol-3-one in an emulsion stabilized with serum albumin was injected intraperitoneally into a bile fistula rat. After 24 h 35 % of the injected dose had been excreted in bile. In this connection it should be pointed out that a considerable part of the tritium label can be expected to be located in the terminal *iso*propyl group of the injected compound [14], as it was prepared from 7α-hydroxycholesterol which in turn had been synthesized from cholesterol labelled with tritium according to Bloch and Rittenberg [15]. This tritium will be lost in the degradation of the side-chain to the C_{24}-length, hence the per cent radioactivity excreted in bile can be regarded as a minimum figure.

After preliminary chromatographic purification of the saponified bile, the cholic acid fraction was rechromatographed with phase system C 1. As is seen in Fig. 2 the radioactivity is distributed in three main peaks, one appear-

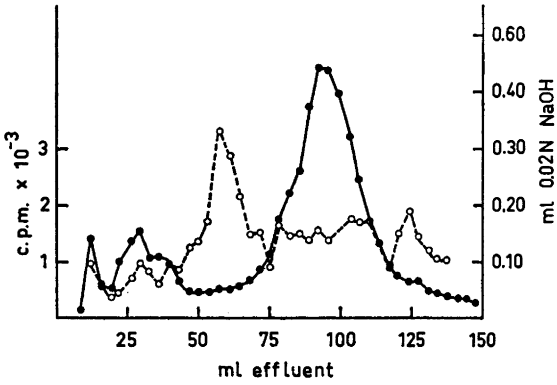

Fig. 2. Chromatography of the cholic acid fraction of the first 24 h portion of bile from bile fistula rat injected with Δ^4-cholestene-7α-ol-3-one. Column: 4.5 g hydrophobic Hyflo Supercel. Phase system C 1. Broken line: radioactivity. Solid line:titration values.

ing before the titration peak of cholic acid, one within the cholic acid peak and one shortly after. The main part of the cholic acid peak was rerun on a column with phase system C 1 and the radioactivity coincided with the titration peak (Fig. 3). The identity of this labelled material with cholic acid was established by isotope dilution. The radioactive material eluted shortly after cholic acid (Fig. 2) has the same elution volume as one of the metabolites of chenodeoxycholic acid, 3α-,6β-,7β-trihydroxycholanic acid, while no clue is at present available to the structure of the radioactive acid eluted before the cholic acid.

The chenodeoxycholic acid fraction was rechromatographed with phase system F 1 (Fig. 4). Part of the radioactivity coincided with the titration peak of chenodeoxycholic acid and the identity was established by isotope dilution. The structures of the radioactive acids eluted before and after chenodeoxycholic acid are not known and have not yet been further investigated.

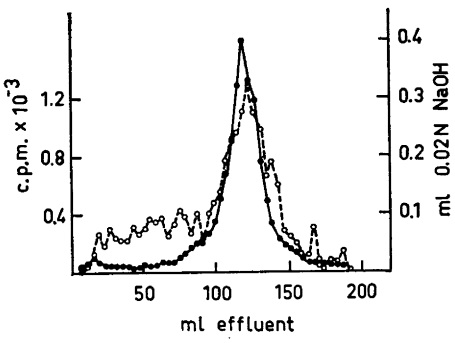

Fig. 3. Rechromatography of the cholic acid peak, Fig. 2. Column: 4.5 g hydrophobic Hyflo Supercel. Phase system C 1.

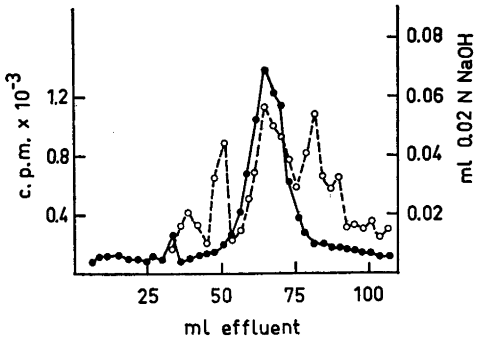

Fig. 4. Chromatography of the chenodeoxycholic acid fraction of the first 24 h portion of bile. Column: 4.5 g hydrophobic Hyflo Supercel. Phase system F 1.

In repeated experiments the same results were obtained and the radioactive acid which is more polar than cholic acid accounted for somewhat more of the activity excreted in bile than did cholic acid. Also, the radioactive acid eluted after chenodeoxycholic acid contained more radioactivity than did the chenodeoxycholic acid.

The findings of Lindstedt [1] and Bergström and Lindstedt [16] that 7*α*-hydroxycholesterol and 3*α*-,7*α*-dihydroxycoprostane are converted to both chenodeoxycholic and cholic acid in the rat have led Yamasaki *et al.*[3] to suggest these sterols as intermediates in the degradation of cholesterol to bile acids and that 7*α*-hydroxycholesterol is converted into 3*α*-,7*α*-dihydroxycoprostane *via* the intermediate formation of Δ^4-cholestene-7*α*-ol-3-one. At present, there is no definite proof available that 7*α*-hydroxycholesterol or 3*α*-,7*α*-dihydroxycoprostane are intermediates [1] nor has the postulated conversion of 7*α*-hydroxycholesterol to 3*α*-,7*α*-dihydroxycoprostane been shown. The results obtained on the metabolism of Δ^4-cholestene-7*α*-ol-3-one in the bile fistula rat should be interpreted with caution in relation to the possibility that this compound might be an intermediate in the conversion of cholesterol to bile acids. It does not appear unlikely that the enzymes involved in the catabolism of cholesterol to bile acids are arranged in an orderly manner passing their substrates along to next enzyme. When a sterol is administered extrahepatically and when no sizeable pool of this compound is present in the liver, the quantitative relations between its different metabolites might be quite different from those found when the compound is formed and metabolized *in situ*.

Cholic acid and chenodeoxycholic acid are present in rat bile in the proportion of about 8:2 and when labelled cholesterol is administered to a bile fistula rat these acids will be labelled to this proportion. When labelled 7*α*-hydroxycholesterol and 3*α*-,7*α*-dihydroxycoprostane are administered approximately 50 % of the radioactivity excreted in bile is present as chenodeoxycholic acid. In this investigation it was found that Δ^4-cholestene-7*α*-ol-3-one was metabolized to both cholic and chenodeoxycholic acid but also to other unidentified

acids. However, as discussed above, it cannot be excluded on these grounds that Δ^4-cholestene-7α-ol-3-one, 7α-hydroxycholesterol and 3α-,7α-dihydroxycoprostane might be intermediates in the degradation of cholesterol to bile acids. To clarify these points, the conversion of cholesterol to these compounds should be established.

Acknowledgements. The valuable technical assistance of Miss Birgitta Holmberg and Miss Marie-Louise Reinius is gratefully acknowledged. Thanks are also due to Dr. Peter Eneroth for help with thin layer chromatography.

This work is part of investigations supported by *The National Heart Institute*, Bethesda, Md., USA, (Grant H-2842) and has also been supported by a grant from *Karolinska Institutets Reservationsanslag.*

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Received January 4, 1961.