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Synthesis and Metabolism of 5β-Cholestane-3α, 7α, 12α-triol-26-al

Bile Acids and Steroids 161

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The partial synthesis of 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid and 5β-cholestane-3α,7α,12α-triol-26-al is described. Tritium-labeled 5β-cholestane-3α,7α,12α-triol-26-al was readily converted into 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid by the mitochondrial, the microsomal, and the soluble fractions of rat liver homogenates.

Evidence has been obtained to indicate 5β-cholestane-3α,7α,12α-triol and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid as intermediates in the biological formation of cholic acid from cholesterol.[1-5] The conversion of 5β-cholestane-3α,7α,12α-triol into 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid has been shown to occur by means of the intermediate formation of 5β-cholestane-3α,7α,12α,26-tetrol.[6,7] No information is available concerning the possibility of 5β-cholestane-3α,7α,12α-triol-26-al as an intermediate in the oxidation of 5β-cholestane-3α,7α,12α,26-tetrol. Further studies on the mechanism of oxidation of 5β-cholestane-3α,7α,12α,26-tetrol required access to 5β-cholestane-3α,7α,12α-triol-26-al.

The present communication describes the synthesis of unlabeled and tritium-labeled 5β-cholestane-3α,7α,12α-triol-26-al and studies on the metabolism of this compound in homogenates of rat liver.

EXPERIMENTAL

3α,7α,12α-Trihydroxy-5β-cholest-24-en-26-oic acid. Cholic aldehyde,[8] 800 mg, and (α-carbethoxy-ethylidene)-triphenyl-phosphorane,[9] 1760 mg, were dissolved in 20 ml of dry benzene and the solution was refluxed for 6 h in an atmosphere of nitrogen. The reaction mixture was then washed with water and the solvent was evaporated at reduced pressure. The residue was dissolved in a solution of 10 % potassium hydroxide in methanol and

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the mixture was refluxed for 4 h. The reaction mixture was diluted with water, acidified with hydrochloric acid, and extracted with ethyl acetate. The ethyl acetate extract was washed with water until neutral and the solvent was evaporated. The residue was chromatographed on a column of 45 g of Hostalene GW (Farbwerke Hoechst, Germany) with phase system F 1.[10] Three ml of stationary phase was used per 4.5 g of support. The effluent was analyzed by means of thin layer chromatography using phase system S 7.[11] An acid with R_F-value of 0.547 in phase system S 7 was eluted between 150 and 300 ml of effluent (3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid has an R_F value of 0.552 in phase system S 7). These fractions were combined and the solvent was evaporated. The residue was crystallized from ethyl acetate yielding 650 mg of 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid, m.p. 200°; $\lambda_{max}^{ethanol}$ 216 mμ, ε = 14 100. (Found: C 72.2; H 9.9. Calc. for $C_{27}H_{44}O_5$: C 72.3; H 9.9). The infrared spectrum (taken in KBr with a Perkin-Elmer model 221 spectrometer) showed prominent bands at 1780 cm^{-1} and 1640 cm^{-1}, typical of an β-unsaturated acid (Fig. 1).

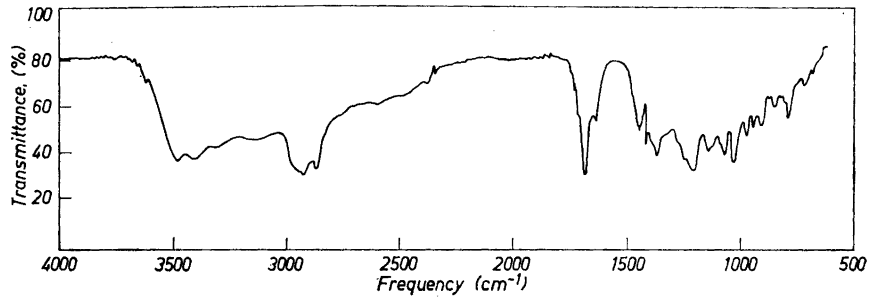

Fig. 1. Infrared spectrum of 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid (in KBr).

3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid. The foregoing material, 800 mg, was hydrogenated in acetic acid solution with platinum oxide as catalyst. The residue of the reaction mixture was crystallized from ethyl acetate, giving 750 mg of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid, m.p. 183°. A single spot with the same R_F-value as that of 3α,7α,12α-trihydroxy-5β-25α-cholestan-26-oic acid was obtained on thin layer chromatography with phase system S 7 and the infrared spectrum was identical with that of authentic 3α,7α,12α-trihydroxy-5β-25α-cholestan-26-oic acid.

28-Homo-5β-cholestane-3α,7α,12α,27,28-pentol. 3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid, 700 mg, was dissolved in 5 ml of acetic anhydride, anhydrous sodium acetate, 500 mg, was added and the mixture was kept at 100° for 20 h. Water was then added to the reaction mixture and the resulting suspension was allowed to stand at room temperature for a few hours. The reaction mixture was extracted with ether, and the ether extract was washed with water until neutral. The residue of the ether extract weighed 980 mg.

Crude 3α,7α,12α-triacetoxy-5β-cholestan-26-oic acid, 800 mg, was dissolved in 2 ml of thionyl chloride and the solution was allowed to stand at room temperature for 2 h. Excess of thionyl chloride was evaporated under reduced pressure. Attempts to crystallize the residue were unsuccessful.

The crude 3α,7α,12α-triacetoxy-5β-cholestanyl chloride was added in portions to a solution of diazomethane in ether. The reaction mixture was allowed to stand overnight and the solvent was then evaporated. The residue was treated with 8 ml of acetic acid at about 70°. When the evolution of gas had ceased, the solvent was evaporated. This crude 28-homo-5β-cholestane-3α,7α,12α,28-tetrol-27-one-3,7,12,28-tetraacetate was dissolved in 10 ml of dry tetrahydrofuran, a suspension of 0.5 g of lithium aluminum hydride in 10 ml of dry tetrahydrofuran was added dropwise with stirring, and the reaction was allowed to continue for 2 h. The reaction mixture was acidified and extracted with ethyl acetate. The residue of the ethyl acetate extract was chromatographed on a column of 45 g of

Hostalene GW with phase system F 1.[10] The effluent was analyzed by thin layer chromatography using phase system S 7.[11] The fractions collected between 150 and 400 ml of effluent contained material with properties expected of 28-homo-5β-cholestane-3α,7α,12α,27,28-pentol. These fractions were combined and the solvent was evaporated. Crystallization of the residue from a methanol-water mixture afforded 210 mg of *28-homo-5β-cholestane-3α,7α,12α,27,28-pentol*, m.p. 162°. (Found: C 70.0; H 10.7. Calc. for $C_{28}H_{50}O_5$, CH_3OH: C 69.8; H 10.9). The infrared spectrum is shown in Fig. 2.

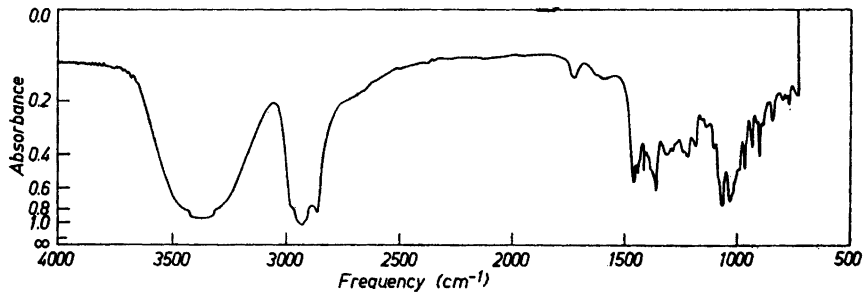

Fig. 2. Infrared spectrum of 28-homo-5β-cholestane-3α,7α,12α,27,28-pentol (in KBr).

5β-Cholestane-3α,7α,12α-triol-26-al. To a solution of 300 mg of lead tetraacetate in 10 ml of dry benzene, kept at 60°, 320 mg of 28-homo-5β-cholestane-3α,7α,12α,27,28-pentol in 10 ml of dry benzene were added dropwise. The reaction mixture was kept at 60° for an additional half-hour, and after this period of time an equal volume of water was added. The benzene layer was diluted with ethyl acetate and the solution was washed with water. The residue of the extract was chromatographed on a column of 18 g of Hostalene GW with phase system F 1. The fractions collected between 52 and 120 ml of effluent were combined and the solvent was evaporated. The residue was subjected to preparative thin layer chromatography using phase system EA-2.[12] With this phase system trihydroxycholestanoic acids do not move from the origin, whereas the corresponding alcohols and aldehydes do. The plate was sprayed with water and the zone that had moved from the origin was collected and extracted with methanol. The solvent was evaporated giving an oily residue. Attempts to crystallize the material from different solvents failed. The infrared spectrum (Fig. 3) showed the presence of a band corresponding to an aldehyde group (2820 cm^{-1}). The material gave a single spot upon thin layer chromatography with phase system S 7 and phase system EA-2. When kept in the solid state, the non-crystalline 5β-cholestane-3α,7α,12α-triol-26-al was gradually autoxidized to the acid but it was stable for several weeks in methanol solution.

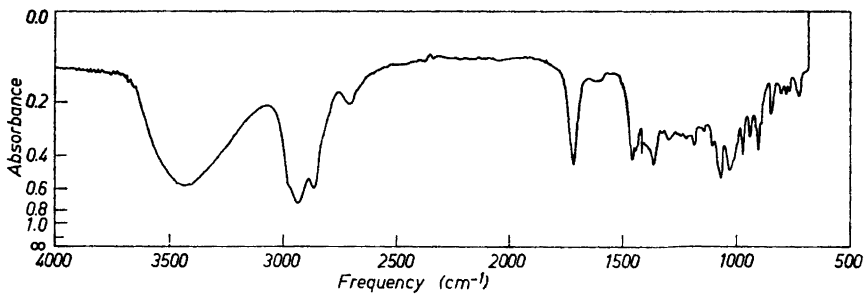

Fig. 3. Infrared spectrum of 5β-cholestane-3α,7α,12α-triol-26-al (in KBr).

Tritium-labeled 5β-cholestane-3α,7α,12α-triol-26-al. This compound was prepared exactly as described above, starting with tritium-labeled cholic acid. This acid was obtained by exposure of cholic acid to 2 C of tritium gas (Radiochemical Centre, Amersham, England) for 4 weeks.[13,14] The crude tritium-labeled cholic acid was hydrolyzed in a sealed steel tube at 110° for 12 h and was then purified by repeated chromatography with phase system C 1.[11] The specific activity of the tritium-labeled cholic acid used in the synthesis was about 20 μC and the specific activity of the 5β-cholestane-3α,7α,12α-triol-26-al obtained was 15 μC. The over-all yield in the synthesis was 5 % starting from 2 g of tritium-labeled cholic acid. The labeled aldehyde was kept in methanol solution and was freshly prepared from the tritium-labeled 28-homo-5β-cholestane-3α,7α,12α,27,28-pentol every third week.

Preparation of homogenates and analysis of incubation mixtures. White male rats of the Sprage-Dawley strain weighing about 200 g were used. Homogenates, 25 % (liver wet weight per volume), were prepared in a modified Bucher medium,[15] pH 7.4, with a Potter-Elvehjem homogenizer with a loosely fitting pestle. Fractionation of the homogenate was performed according to the procedure described by Wilgram and Kennedy.[16] The particulate fractions were suspended in the homogenizing medium and aliquots corresponding to 2 ml of whole homogenate were taken. NAD, 2.5 μmole was added to each incubation mixture. The tritium-labeled 5β-cholestane-3α,7α,12α-triol-26-al, 1 μmole, was added to the incubation mixture dissolved in 10 μl of methanol and the volume of the incubation mixture was adjusted to 3.2 ml by addition of water. Incubations were conducted at 37° for 30 min.

Incubations were terminated by addition of 10 ml of ethanol. The precipitate was filtered off, and the volume of the filtrate was reduced. The solution was acidified with hydrochloric acid and extracted with 10 ml of water-saturated butanol. The butanol extract was washed with water until neutral and the solvent was evaporated. The residue was dissolved in 0.5 ml of ethanol and 0.1 ml of the solution was subjected to thin layer chromatography on Kieselgel G (Merck, Germany) using phase system EA-2.[12] 5β-Cholestane-3α,7α,12α,26-tetrol and 5β-cholestane-3α,7α,12α-triol-26-al were used as reference compounds. The reference compounds were detected by spraying with concentrated sulfuric acid and the corresponding zones of the plate where the biological samples had been chromatographed as well as the material at the origin were eluted according to the technique of Matthews, Pereda, and Aguilera.[17] Radioactivity was determined on aliquots of the extracts using a methane gas flow counter. The extract of the material at the origin of the plate was rechromatographed with phase system S 7,[11] using 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid and cholic acid as reference compounds.

RESULTS AND DISCUSSION

The synthesis of 5β-cholestane-3α,7α,12α-triol-26-al was achieved by a Grundmann synthesis. This method has been used recently for the synthesis of cholic aldehyde.[8] The 5β-cholestane-3α,7α,12α-triol-26-al obtained could not by crystallized. It gave a single spot in different thin layer chromatographic systems and the infrared spectrum showed absorption bands typical of an aldehyde group. It was readily oxidized to 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid.

Table 1 summarizes the results of incubations of tritium-labeled 5β-cholestane-3α,7α,12α-triol-26-al with different subcellular fractions of a rat liver homogenate. The mitochondrial and the microsomal fractions as well as the 100 000 *g* supernatant fluid catalyzed the oxidation of 5β-cholestane-3α,7α,12α-triol-26-al into 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. In presence of the mitochondrial or the microsomal fraction appreciable amounts of 5β-cholestane-3α,7α,12α,26-tetrol were also formed. Addition of the 100 000 *g* supernatant fluid to the particulate fractions resulted in an increased forma-

Table 1. Metabolism of 5β-cholestane-3α,7α,12α-triol-26-al in intracellular fractions of rat liver. Incubations were as described in the experimental section. THAL, 5β-cholestane-3α,7α,12α-triol-26-al; TTHC, 5β-cholestane-3α,7α,12α,26-tetrol; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. The composition of products was calculated on the basis of the radioactivity present in the different zones of the thin layer chromatograms, and the total radioactivity recovered from these zones was put at 100 %.

Enzyme fraction	Products %			
	THAL	TTHC	THCA	Cpds more polar than THCA
Whole homogenate	14	16	4	66
Mitochondria	7	21	70	2
Microsomes	4	36	41	19
100 000 *g* supernatant	3	2	92	3
Mitochondria + 100 000 *g* supernatant	2	6	83	9
Microsomes + 100 000 *g* supernatant	6	6	72	16
100 00 *g* supernatant, boiled	71	13	4	12

tion of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. When the 100 000 *g* supernatant fluid was boiled for 5 min, most of the dehydrogenase activity was lost. However, not all of the 5β-cholestane-3α,7α,12α-triol-26-al added was recovered unchanged, and apparently some non-enzymatic transformations occurred under the conditions employed for the incubations.

The present experiments do not provide any evidence concerning the possible role of 5β-cholestane-3α,7α,12α-triol-26-al as an obligatory intermediate in the oxidation of 5β-cholestane-3α,7α,12α,26-tetrol to 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. Attempts have been made to demonstrate the accumulation of 5β-cholestane-3α,7α,12α-triol-26-al after incubation of tritium-labeled 5β-cholestane-3α,7α,12α,26-tetrol but have so far been unsuccessful. In view of the appreciable dehydrogenase activity in the 100 000 *g* supernatant fluid it might be possible to study these reactions with purified enzymes and work on the purification of the enzyme(s) catalyzing the oxidation of 5β-cholestane-3α,7α,12α,26-tetrol is in progress. In this connection it is of interest to mention that Herman and Staple [18] have reported in preliminary form the purification of an enzyme catalyzing the oxidation of 5β-cholestane-3α,7α,12α,26-tetrol to 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid.

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REFERENCES

1. Danielsson, H. *Advan. Lipid Res.* **1** (1963) 335.
2. Mendelsohn, D. and Staple, E. *Biochemistry* **2** (1963) 577.
3. Staple, E. and Rabinowitz, J.L. *Biochim. Biophys. Acta* **59** (1962) 735.
4. Carey, J.B. and Haslewood, G.A.D. *J. Biol. Chem.* **238** (1963) PC 855.
5. Carey, J.B. *J. Clin. Invest.* **43** (1964) 1443.
6. Danielsson, H. *Acta Chem. Scand.* **14** (1960) 348.
7. Suld, H.M., Staple, E. and Gurin, S. *J. Biol. Chem.* **237** (1962) 338.
8. Yashima, H. *J. Biochem. (Tokyo)* **54** (1963) 47.
9. Isler, O., Gutmann, H., Mantavon, M., Rüegg, R., Ryser, G. and Zeller, P. *Helv. Chim. Acta* **40** (1957) 1242.
10. Norman, A. and Sjövall, J. *J. Biol. Chem.* **233** (1958) 872.
11. Eneroth, P. *J. Lipid Res.* **4** (1963) 11.
12. Kazuno, T. and Hoshita, T. *Steroids* **3** (1964) 55.
13. Wilzbach, K.E. *J. Am. Chem. Soc.* **79** (1957) 1013.
14. Bergström, S. and Lindstedt, S. *Acta Chem. Scand.* **11** (1957) 1275.
15. Bergström, S. and Gloor, U. *Acta Chem. Scand.* **9** (1955) 34.
16. Wilgram, G.F. and Kennedy, E.P. *J. Biol. Chem.* **238** (1963) 2615.
17. Matthews, J.S., Pereda, A.L. and Aguilera, A. *J. Chromatog.* **9** (1962) 331.
18. Herman, R. and Staple, E. *Federation Proc.* **24** (1965) 661.

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