

74. METABOLISM OF STEROIDS

2. THE ISOLATION OF CHOLESTANE-3:5:6-TRIOL AND OTHER SUBSTANCES FROM OX LIVER EXTRACTS

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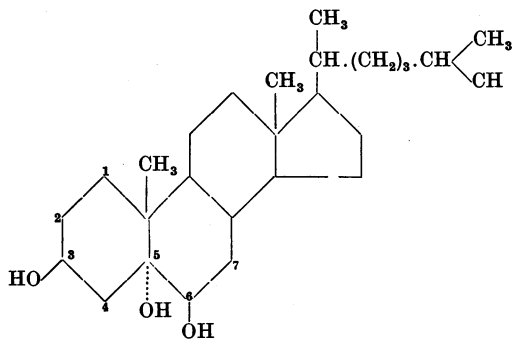
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IN Part I [Haslewood, 1939], the isolation from ox liver of α -7-hydroxycholesterol was reported. Since that time, a ' β -7-hydroxycholesterol' has been mentioned as isolated from hog liver [MacPhillamy, 1940], and β -7-hydroxycholesterol has been obtained from pregnant mares' serum [Wintersteiner & Ritzmann, 1940]. Wintersteiner & Bergström [1941] report further that cholesterol can be converted into a mixture containing 7-hydroxy- and 7-keto-cholesterol by treatment at an alkaline reaction with oxygen at 85°. As Wintersteiner and his colleagues ably and correctly point out, all this work does not answer the question of the authenticity of the oxygenated cholesterols as metabolites, since it would seem possible that such oxidation products are formed from cholesterol during the processes leading to their isolation. Oxidation of cholesterol appears to take place readily at the double bond, as well as at position C₇, as would be expected on chemical grounds, but whether the products are actually those taking part in metabolism can only be decided by many types of experiment, including, perhaps, such work as the treatment of cholesterol with enzymic preparations.

The present work was carried out on the same fraction of ox liver as was previously used, and from this source there has been obtained an incompletely purified *alcohol*, probably C₂₄H₄₆O₃, characterized as the *monoacetyl derivative*, C₂₄H₄₅O₂(O.CO.CH₃). This substance is not of the steroid type.

α -7-Hydroxycholesterol (identified as the dibenzoate) has been obtained from the extract directly as crystals melting at 174–176°: the compound is difficult to crystallize [cf. Wintersteiner & Ritzmann, 1940].

There has also been isolated from the liver extract cholestane-3:5:6-triol, identified as the diacetate. This triol is obtained by oxidation of cholesterol or its acetate with H₂O₂ [Pickard & Yates, 1908] and has been called 'triol I' by Ellis & Petrow [1939], who assign to it the structure:



Many authors have stressed the analogy between biological oxidations and those brought about by H_2O_2 ; nevertheless the foregoing remarks about the origin of substances isolated apply to cholestane triol, especially since ether was used in its preparation from liver.

As already reported [MacPhillamy, 1940; Haslewood, 1941], the 'hepatols' have been derived from digitonin used in their preparation. One of them, 'hepatol A', is apparently digitogenin, $C_{27}H_{44}O_5$, as stated by MacPhillamy [1940]. Its confusion with a C_{21} compound arose because of the difficulty of obtaining an accurate measurement of its molecular weight. The problem was solved when the diacetate was oxidized with cold chromium trioxide to a monocarboxylic acid, probably $C_{30}H_{46}O_7 \cdot COOH$, which was titrated. This acid was obtained in good yield; its formation is explicable in terms of the formula assigned to digitogenin by Marker & Rohrmann [1939], although it is difficult to understand why digitogenin (with hydroxyl groups at C_2 , C_3 and C_6) should not easily be completely acetylated.

The cholestane triol was first obtained as the digitonide. This was decomposed with pyridine and acetic anhydride and from the acetylated mixture the diacetate of the triol was readily crystallized.

EXPERIMENTAL

Analyses were microanalyses by Dr A. Schoeller (S) and Dr G. Weiler (W). All melting points are uncorrected.

Starting material was the evaporated methyl-alcoholic mother liquors (fraction A) from the crystallization of the 'sterols' from the unsaponifiable ether-soluble fraction of ox-liver 'marc' [Haslewood, 1941].

The alcohol (?) $C_{24}H_{46}O_3$. Fraction A (20 g.), on standing in solution in about 20 ml. of ether, or éther in light petroleum, slowly deposited a semi-crystalline solid (0.1 g.) which was collected and crystallized from acetone. It formed colourless globules of small crystals, m.p. $93-95^\circ$, not soluble in alkali, readily forming a 'gel' from dilute alcohol and giving negative tests for N and P. The substance gave no precipitate with digitonin or with 2:4-dinitrophenylhydrazine in dilute alcoholic solution. Found (W): C, 74.85, H, 12.4%, mol. wt. = 353. $C_{24}H_{46}O_3$ requires C, 75.4, H, 12.0%, mol. wt. = 382.

This material (50 mg.) in pyridine (0.2 ml.) with acetic anhydride (0.2 ml.) was heated at 100° for 15 min. The precipitate collected after dilution with water was recrystallized from acetone and separated as clumps of white needles, m.p. $103-105^\circ$. Found (W): C, 73.8, H, 11.3%, mol. wt. = 428. $C_{24}H_{46}O_2(O.CO.CH_3)$ requires C, 73.6, H, 11.3%, mol. wt. = 424. A sample from the mother liquors had m.p. $107-108^\circ$, while another obtained after six crystallizations from acetone of the 'sterols', acetylated with acetic anhydride, melted at $100-111^\circ$, both samples being saponified with dilute alcoholic NaOH to the above alcohol. These facts suggest that the material has not been completely purified, though all samples of the acetate had sharp melting points.

Separation of hydroxylated cholesterols. Fraction A (16 g.) was dissolved by shaking with 100 ml. of 90% (by vol.) aqueous methyl alcohol and 100 ml. of light petroleum (B.P. $40-60^\circ$). The alcoholic layer was separated and washed in a funnel with two portions each of 50 ml. of fresh petroleum. The combined petroleum on evaporation gave 14.3 g. of *fraction B*, while evaporation of the alcohol yielded *fraction C* (1.7 g. of an orange gum). *Fraction C* was dissolved in 25% (by vol.) benzene in light petroleum and allowed to run through a cylindrical column (length 25 cm., diam. 0.8 cm.) of Al_2O_3 (Hopkin & Williams).

The column was then washed with solvents as follows, the eluates being collected separately and evaporated, and the weights of the residues being obtained. In this way, fractions were collected as below:

Fraction	Solvent	Weight g.	Description of residue
I	Up to 100 ml. of 25% benzene in light petroleum	0.2	Mobile oil
II	100 ml. of benzene	0.15	"
III	100 ml. of ether	0.8	Stiff gum
IV	100 ml. of warm alcohol	0.2	"
V	100 ml. of alcohol acidified with HCl and warmed with the disintegrated column; product isolated with ether, after dilution	0.17	"

Fraction III (0.8 g.) in benzene was adsorbed on a fresh column (20 × 0.8 cm.) and the column eluted with 100 ml. of 50% (by vol.) benzene in ether (residue (0.55 g.): fraction IIIa) and then with 100 ml. of alcohol (residue (0.25 g.): fraction IIIb).

Fraction IIIa (0.55 g.) in benzene was adsorbed on a fresh column (10 × 0.8 cm.) and was eluted with benzene up to 500 ml. Evaporation of the solvent gave solid α -7-hydroxycholesterol, which, from benzene in light petroleum, formed long white needles, m.p. 174–176° decomp. Yield, ca. 10 mg. 5 mg. of this product on benzylation with PhCOCl (1 drop) and pyridine (2 drops) for 2 hr. at room temperature gave on dilution a gum which was crystallized from methyl alcohol to give white needles of α -7-hydroxycholesterol dibenzoate, m.p. 170–171°, not depressed by authentic material, m.p. 170–172°. The hydroxycholesterol and its dibenzoate gave the characteristic colour and precipitation reactions.

Fraction IIIb (0.25 g.) was warmed with a solution of digitonin (0.2 g.) in 20 ml. of 80% alcohol. A crystalline digitonide separated rather slowly and after 24 hr. was collected, washed with alcohol and ether and dried. The product (50 mg.) in pyridine (0.5 ml.) with acetic anhydride (0.5 ml.) was heated for 20 min. at 100°. The precipitate obtained after dilution with water was collected and dissolved in warm alcohol. When the warm filtered solution was diluted to faint cloudiness and allowed to stand, needles separated and were collected and recrystallized from dilute alcohol, from which separated long glistening needles (8 mg.), m.p. 163–165°, not depressed by cholestane triol I diacetate, m.p. 167–168°, prepared by acetylation by the above method of the purified triol. Found (W): C, 73.4, H, 10.6%, M = 453, 480. C₃₁H₅₂O₅ requires C, 73.8, H, 10.3%, mol. wt. = 504. Saponification gave cholestane triol (m.p. 235–237°) identical with authentic material. 40 mg. of authentic triol (m.p. 233–235°), obtained from cholesterol with H₂O₂, together with 0.1 g. of digitonin, were dissolved by warming with 15 ml. of 80% alcohol. After standing for 24 hr., the crystalline digitonide (80 mg.) was collected, washed, dried and acetylated as above. The acetate obtained had m.p. 163–165°, not depressed by the specimen from liver.

'Hepatol A' from digitonin. Digitonin (0.2 g.), with a few drops of a dilute solution of Br₂ in alcohol, was refluxed for 2 hr. with 2 ml. of xylene. The xylene was evaporated and the residue purified by washing with light petroleum, sublimation and crystallization as previously described. White needles, m.p. 275–278°, not depressed by 'hepatol A', were obtained.

The acid $C_{30}H_{45}O_7 \cdot COOH$. The diacetate (10 mg. of m.p. 235–238°, and prepared as previously described) of the above compound was dissolved in glacial acetic acid (0.2 ml.) and the mixture allowed to stand at room temperature for 16 hr. with 0.2 ml. of a solution made by dissolving 10 g. of CrO_3 in the minimal amount of water and making up to 100 ml. with acetic acid. After dilution with water, the precipitate was collected and recrystallized from dilute alcohol, from which it formed white needles, m.p. 263–264°, decomp. Yield, 7 mg. Found (S): C, 65.7, H, 8.2%, mol. wt. (titration) = 540, 519. $C_{30}H_{45}O_7 \cdot COOH$ (one carboxyl group) requires C, 66.2, H, 8.2%, mol. wt. = 562. The above compound was easily soluble in warm dilute $NaHCO_3$ solution, from which it was precipitated on acidification. It showed no alteration after treatment with acetic anhydride and pyridine and gave no obvious reactions with ketone-detecting substances. With diazomethane, a partially purified methyl ester, m.p. 184–186°, was formed. Found (S): C, 66.3, H, 8.5%. $C_{30}H_{45}O_7 \cdot COOCH_3$ requires C, 66.7, H, 8.3%.

SUMMARY

In addition to crystalline α -7-hydroxycholesterol, m.p. 174–176°, there has been obtained from the unsaponifiable fraction of an ether extract of ox liver cholestane-3:5:6-triol I, and an alcohol, m.p. ca. 95°, characterized as a monoacetyl derivative. 'Hepatol A' is digitogenin: its diacetate has been oxidized to a monocarboxylic acid.

Experiments of the type now reported do not finally establish the authenticity of isolated substances as intermediates in metabolism.

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