341. *The Components of Wool Wax. Part III.* 7-Oxocholesterol and the Alleged Presence of Cholestanol.*

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Schoenheimer's alleged cholestanol,[1] isolated by fission of the digitonin complex obtained after the wool-wax alcohols have been brominated, contains mainly 7-oxocholesterol with some cholesterol dibromide and smaller and more variable amounts of lanostenol. Cholesta-3 : 5-dien-7-one is present only in samples obtained by decomposition of the digitonin complex under unsuitable conditions : it arises by dehydration of 7-oxocholesterol.

In 1930 Schoenheimer [1] described a method for determining what he considered to be the saturated congeners of cholesterol. The method is founded on the observation that cholesterol dibromide does not form an insoluble complex with digitonin, the percentages of sterol that could be precipitated with digitonin before and after treatment with bromine being determined. Treatment of the saturated sterols with sodium in boiling xylene causes coprostanol to epimerise and so to lose its ability to react with digitonin whereas cholestanol is, to a large extent, unaffected.[2] Schoenheimer used this observation to distinguish between the two natural isomers of dihydrocholesterol.[3]

Cholesterol concentrates from different sources were examined, and for wool wax it was found that 14—19% of the total digitonin-precipitable material (4—7% of the alcoholic fraction) was apparently saturated.[4] Although the identity of the saturated wool-wax sterols was not investigated further, the epimerisation test indicated that cholestanol was the main component of some samples from other sources. Later workers [5,6] have accepted the implication that cholestanol occurs in wool wax. However, chromatographic separations of the wool-wax alcohols in this laboratory led to the conclusion that the presence of cholestanol, in more than traces, was unlikely. Schoenheimer's experiments have, therefore, been repeated and extended, and completely different results have been obtained.

Wool-wax alcohols were brominated at 0° in ethanol and, after the insoluble dibromides had been filtered off, those compounds which still reacted with digitonin were precipitated. Subsequently, the digitonides were split by Bergmann's [7] or Dam and Schoenheimer's method.[8] In agreement with Schoenheimer's observations, the yield of "saturated" sterol obtained from the wool-wax alcohols varied from 4·8 to 6·4%.

Initially a number of samples of the "saturated" sterols (hereinafter called the S-fraction) were examined by different methods. Later the entire S-fraction was chromatographed on alumina in order to determine the approximate amounts of each component.

Elementary analysis of the S-fraction showed the presence of bromine. A change in the optical rotation from [α] +14° to +24° during three days indicated the presence of dibromocholesterol which is first precipitated as the 5α : 6β-isomer, [α] −44°, but slowly isomerises to the more stable 5β : 6α-isomer,[9] [α] +47°. Proof of the presence of cholesterol dibromide was obtained by debromination and identification of cholesterol. Contrary to Schoenheimer's generalisation from other observations it was found that more thorough washing of the digitonide from the brominated wool-wax alcohols did not yield an S-fraction containing appreciably less bromine. Cholesterol dibromide alone does not form an insoluble digitonide so that, in these circumstances, it must be occluded in, or co-precipitated with, the other insoluble digitonides.

* Part II, *J.*, 1954, 3344.

[1] Schoenheimer, *Z. physiol. Chem.*, 1930, **192**, 77.
[2] Windaus and Uibrig, *Ber.*, 1914, **47**, 2384; 1916, **49**, 1724.
[3] Schoenheimer, *Z. physiol. Chem.*, 1930, **192**, 86.
[4] Behring, Hummel, and Schoenheimer, *ibid.*, p. 93.
[5] Daniel, Lederer, and Velluz, *Bull. Soc. Chim. biol.*, 1945, **27**, 218.
[6] Truter, *Quart. Reviews*, 1951, **5**, 390.
[7] Bergmann, *J. Biol. Chem.*, 1940, **132**, 471.
[8] Dam and Schoenheimer, *Z. physiol. Chem.*, 1933, **215**, 59.
[9] Barton and Miller, *J. Amer. Chem. Soc.*, 1950, **72**, 1066.

Treatment of the S-fraction with 2 : 4-dinitrophenylhydrazine followed by adsorption analysis disclosed 7-oxocholesterol and cholesta-3 : 5-dien-7-one. The presence of the latter in the S-fraction was surprising because it does not form a digitonin complex. Subsequently, two lines of evidence showed that it arose by dehydration of 7-oxocholesterol. First, if the digitonide was decomposed at room temperature cholestadienone could not be detected in the subsequent chromatographic fractionation. Secondly, the ultraviolet spectrum of the fresh digitonide showed a single absorption maximum at 238 mμ, but after the digitonide had been split with *hot* pyridine the resulting S-fraction showed absorption maxima at 238 and 280 mμ. The second maximum indicated that cholestadienone had been formed during the process.

Lanost-8-enol was identified after acetylation of the S-fraction and chromatography on alumina. It forms a moderately insoluble digitonide [10] so that its presence is not unexpected.

From preliminary elementary analyses the percentage of dibromide in fraction S was calculated, on the assumption that all the bromine was present as cholesterol dibromide; lanosterol dibromide may also be present but this has been ignored because lanosterol was not isolated from the products of debromination. Chromatography was then used to separate the known components of the mixture. The percentages, based upon the amounts of *purified* material, are as follows :

	Hartolan *	Laboratory wax
Lanost-8-enol (%)	20	—
7-Oxocholesterol (%)	38	70
Dibromide (calc. from the Br analysis; and includes 3% of cholesterol dibromide)	26	15
Total (%)	84	85
S-fraction (% of total alcohols)	4·8	6·3
Material not accounted for (% of total alcohols)	0·8	0·9

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No lanosterol was isolated from the laboratory wax although a fraction which amounted to 11% of the total gave the characteristic Liebermann–Burchardt colour reaction.

The Table shows that, as applied to wool-wax alcohols, Schoenheimer's technique gives an S-fraction which, as Schoenheimer himself claimed, comprises about 5% of the total. The composition of more than 80% of this material has now been elucidated but no evidence has been found to support the allegation that cholestanol is present—if it is, the quantity will not amount to 1%.

Experimental

Optical rotations were measured in $CHCl_3$, and ultraviolet spectra in EtOH.

Isolation of the " Saturated " Sterols.—A *ca.* N-solution of bromine in ethanol was slowly added to a vigorously stirred solution of wool-wax alcohols (20·05 g.) in absolute ethanol (450 ml.) cooled in ice, until a slight excess was present (starch–iodide). After 2 hr. the suspension was filtered and the residue was washed with ethanol (100 ml.). The crude cholesterol dibromide (4·91 g.) had $[\alpha]_D$ −38·5° (*c* 2·3) and m. p. 113°. By the addition of water to the filtrate the concentration of ethanol in it was adjusted to 96%. A 1% solution of digitonin in 96% ethanol (450 ml.) containing a slight excess of bromine was added, and the mixture was kept in the dark while the slight excess of bromine was maintained by occasional additions. After 48 hr. the precipitate was filtered off, washed with 96% ethanol (50 ml.) and with ether (2 × 50 ml.) (this is " the standard way ") and dried at 80°. The product (4·77 g.) was very pale yellow.

In another experiment identical quantities of reagents were used, but the digitonide was washed by removing it from the sintered crucible and shaking it with ethanol (50 ml.), filtering the suspension, and repeating the treatment. This procedure was then repeated with ether (2 × 50 ml.). The bromine content of the digitonide washed in the standard manner was 1·71% while from the more carefully washed digitonide it was 1·73%.

[10] Ruzicka, Denss, and Jeger, *Helv. Chim. Acta*, 1945, **28**, 759.

Recovery of Sterols from the Digitonide.—In the initial experiment the digitonide (4·64 g.) was dissolved in pure pyridine (75 ml.) on the water-bath (1 hr.). The solvent was then removed under reduced pressure and the residue was extracted (Soxhlet) with dry ether.[7] The residue (3·5 g.) was dissolved in boiling 85% ethanol (350 ml.) and after 24 hr. at room temperature the insoluble material was filtered off and treated with pyridine and ether as already described. Evaporation of the combined ethereal extracts yielded a yellow glass (1·23 g.), $[\alpha]_D$ −6°.

In subsequent experiments the digitonide (4·51 g.) was dissolved in dry pyridine (50 ml.) at room temperature, and the digitonin was precipitated by addition of ether (200 ml.). The precipitate (3·3 g.) was filtered off and dissolved in 85% ethanol (330 ml.) and the solution was set aside for 24 hr. The white deposit was filtered off and treated with pyridine and ether as indicated above. The combined ethereal extracts were washed with water, dilute hydrochloric acid, and then water, and dried (Na_2SO_4). Evaporation of the solvent afforded a pale yellow glass.

Separation of the Phenylhydrazones.—One crystallisation of the yellow glass (1·23 g.) from ethyl acetate–methanol (1 : 1; 5 ml.) gave a white powder (0·78 g.), m. p. 138°. Because the optical rotation of this material became progressively more dextrorotatory with time it was thought to contain cholesterol dibromide. The yellow glass obtained by evaporation of the mother-liquor was treated with a solution of 2 : 4-dinitrophenylhydrazine and cooled in ice. After 30 min. the precipitate (278 mg.) was filtered off and adsorbed on alumina (Brockmann No. 4; 1·5 × 50 cm.). 9 : 1 Light petroleum–benzene eluted the 2 : 4-dinitrophenylhydrazone (100 mg.) of cholesta-3 : 5-dien-7-one. Benzene eluted a further 44 mg. of unidentified material and finally benzene containing 1% of ethanol eluted the 2 : 4-dinitrophenylhydrazone of 7-oxocholesterol.

Cholesta-3 : 5-dien-7-one. Recrystallisation of the crude hydrazone from benzene–ethanol gave orange needles of cholesta-3 : 5-dien-7-one, m. p. and mixed m. p. 224—225° (Found: C, 70·8; H, 8·2; N, 9·7. Calc. for $C_{33}H_{46}O_4N_4$: C, 70·5; H, 8·2; N, 9·95%), $\lambda_{max.}$ 265 (log ε 4·3) and 397 mμ (log ε 4·5) [lit.: $\lambda_{max.}$ 265 (log ε 4·3) and 397 mμ (log ε 4·5)], $[\alpha]_D$ −380° (*c* 0·382). An attempt to split the 2 : 4-dinitrophenylhydrazone by Robinson's method [11] using 99% formic acid and copper carbonate yielded a tar.

7-Oxocholesterol. The 2 : 4-dinitrophenylhydrazone of 7-oxocholesterol was obtained as yellow needles (from chloroform–methanol), m. p. and mixed m. p. 246—247°, $[\alpha]_D$ −607° (*c* 0·28), $\lambda_{max.}$ 258 (log ε 4·24) and 385 mμ (log ε 4·47) (Found: C, 67·9; H, 8·35; N, 9·65. Calc. for $C_{33}H_{48}O_5N_4$: C, 68·2; H, 8·3; N, 9·65%).

Lanostenol. A portion of the S-fraction (0·692 g.) was crystallised from methanol (5 ml.) and then from ethyl acetate–methanol (3 ml.) to give white needles (80 mg.), m. p. 145°, $[\alpha]_D$ +60° (*c* 0·41), of lanost-8-enol (Found: C, 84·25; H, 12·0. Calc. for $C_{30}H_{52}O$: C, 84·0; H, 12·2%). The acetate (needles, from ethyl acetate–methanol after purification on alumina) had m. p. 120°, $[\alpha]_D$ +58° (*c* 0·38) (Found: C, 81·95; H, 11·2. Calc. for $C_{32}H_{54}O_2$: C, 81·7; H, 11·5%). The 3 : 5-dinitrobenzoate had m. p. 207° (lit., m. p. 208°), and the benzoate had m. p. 192—192·5°, $[\alpha]_D$ +69° (*c* 1·03) (lit., m. p. 194°, $[\alpha]_D$ +68°).

Composition of the S-Fraction derived from Hartolan.—The S-fraction (1·475 g.) was chromatographed on acid-washed and reactivated alumina (1 × 20 cm.). Six fractions were eluted: E1 (light petroleum), 343 mg.; E2 (1 : 4 benzene–light petroleum), 44 mg.; E3 (4 : 1 benzene in light petroleum), 106 mg.; E4 (benzene), 376 mg.; E5 (ether), 435 mg., and E6 (1% of methanol in ether), 114 mg.

Lanostenol. Fraction E1 was further purified by adsorption on alumina and recrystallisation from ethyl acetate–methanol, to give needles (294 mg.), m. p. and mixed m. p. 144°, $[\alpha]_D$ +61°. The benzoate (needles from methanol) had m. p. 193°, $[\alpha]_D$ +69° (*c* 1·83) (Found: C, 83·0; H, 10·6. Calc. for $C_{37}H_{56}O_2$: C, 83·3; H, 10·7%).

Cholesterol. Fractions E2, E3, and E4 contained bromine. They were combined, dissolved in ethanol, and debrominated by refluxing with sodium iodide for 3 hr. The cold solution was then diluted with ether, and the ethereal solution washed with aqueous sodium sulphite, then with water, and dried (Na_2SO_4). Distillation of the ether and crystallisation of the residue from methanol gave cholesterol (50 mg.), m. p. 147·5°, $[\alpha]_D$ −39° (*c* 1·31) (Found: C, 83·9; H, 11·9. Calc. for $C_{27}H_{46}O$: C, 84·0; H, 11·9%). It gave an acetate, m. p. and mixed m. p. 114° (Found: C, 81·1; H, 10·9. Calc. for $C_{29}H_{48}O_2$: C, 81·2; H, 11·2%), and a benzoate, m. p. (to an anisotropic liquid) 149°, $[\alpha]_D$ −14·4° (*c* 1·13) (Found: C, 82·9; H, 10·0. Calc. for

[11] Robinson, *Nature*, 1954, **173**, 541.

$C_{34}H_{50}O_2$: C, 83·2; H, 10·2%). The original S-fraction contained 8·02% of bromine which corresponds to a cholesterol dibromide content of 26%.

7-Oxocholesterol. Fractions E5 and E6 were combined and recrystallised to give 7-oxocholesterol (white needles from aqueous methanol), m. p. and mixed m. p. 170°, $[\alpha]_D$ −102° (*c* 0·505), λ_{max} 238 mμ (log ε 4·1) (Found: C, 80·5; H, 10·8. Calc. for $C_{27}H_{44}O_2$: C, 80·8; H, 11·0%). Its acetate had m. p. and mixed m. p. 155°, $[\alpha]_D$ −95° (*c* 0·42) (Found: C, 78·7; H, 10·3. Calc. for $C_{29}H_{46}O_3$: C, 78·6; H, 10·45%).

Composition of the S-Fraction derived from a Laboratory-extracted Wax.—Wax was extracted from Australian merino wool (64s) by means of peroxide-free ether, and was hydrolysed at room temperature by sodium methoxide. After the alcohols (13 g.) had been isolated they were processed as indicated above, and yielded a brominated fraction (2·9 g.) and an S-fraction (0·82 g.). The S-fraction was chromatographed on neutral alumina (1 × 20 cm.). Three main fractions were obtained: F1 (27 mg.) was eluted with 3 : 7 benzene–light petroleum; F2 (67 mg.) with benzene; and F3 (456 mg.) with ether.

Fraction F2 contained bromine. It did not crystallise even after purification on alumina. The bromine analysis of the original S-fraction (Br, 4·34%) suggests a dibromosterol content of 15%.

Fraction F3 was 7-oxocholesterol (white needles from methanol; 418 mg.), m. p. and mixed m. p. 170°, $[\alpha]_D$ −103° (*c* 0·5). The 2 : 4-dinitrophenylhydrazone had m. p. 247°, $[\alpha]_D$ −592° (*c* 0·3) (Found: C, 68·25; H, 8·2. Calc. for $C_{33}H_{48}O_5N_4$: C, 68·2; H, 8·3%).

Cholesta-3 : 5-dien-7-one was synthesised by Marker and Whittle's method.[12] Cholesterol (55 g.) was dissolved in thionyl chloride (55 ml.) and set aside for 24 hr. The product was stirred with acetone and filtered and the residue recrystallised from acetone, to give pale yellow needles of cholesteryl chloride (37·5 g.), m. p. 96·5°.

7-Oxocholesteryl chloride. During 1 hr. a solution of chromium trioxide (26 g.) in 50% aqueous acetic acid (36 ml.) was added to a vigorously stirred solution of cholesteryl chloride (30 g.) in acetic acid (600 ml.) at 55°. After 2 hr. the excess of chromium trioxide was destroyed by ethanol, the solution concentrated to one-third of its volume, and water (15 ml.) added. The crystalline chloro-ketone (8 g.) deposited from the cold solution had m. p. 144—145°.

Cholesta-3 : 5-dien-7-one. 7-Oxocholesteryl chloride (7·5 g.) was heated under reflux for 2 hr with potassium hydroxide (7N; 240 ml.) in 94% ethanol. After removal of the ethanol, water was added and the ketone was extracted with ether. Evaporation of the ether gave a brown residue which was purified by adsorption on alumina. The first fraction to be eluted with light petroleum was the desired ketone (cream-coloured prisms from ethanol; 2·3 g.), m. p. 114° (Found: C, 84·7; H, 11·1. Calc. for $C_{27}H_{42}O$: C, 84·8; H, 11·0%). The 2 : 4-dinitrophenylhydrazone (orange needles from chloroform–methanol) had m. p. 225—226° (Found: C, 70·7; H, 8·1; N, 10·0. Calc. for $C_{33}H_{46}O_4N_4$: C, 70·5; H, 8·2; N, 9·95%).

7-Oxocholesterol. During 2 hr. chromium trioxide (9·1 g.) in 50% aqueous acetic acid (12 ml.) was added to a vigorously stirred solution of cholesteryl acetate (13 g.) in acetic acid (140 ml.) at 55°. After a further 2 hr. the excess of trioxide was destroyed by ethanol, and the solution was concentrated to one-third of its bulk. On cooling, the crude keto-ester was deposited; it was recrystallised twice from ether (m. p. 156; 2 g.). Hydrolysis of the acetate (1·4 g.) at room temperature with sodium methoxide afforded 7-oxocholesterol (white needles from aqueous methanol; 0·5 g.), m. p. 170°. The 2 : 4-dinitrophenylhydrazone (yellow needles from chloroform–methanol) had m. p. 246°, $[\alpha]_D$ −610° (*c* 0·2) (Found: C, 67·9; H, 8·4; N, 9·8. Calc. for $C_{33}H_{48}O_5N_4$: C, 68·2; H, 8·3; N, 9·65%).

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[12] Marker and Whittle, *J. Amer. Chem. Soc.*, 1937, **59**, 619.