The Chemical Synthesis and Biological Oxidation of 7 α -Hydroxy[26-¹⁴C]cholesterol, 7-Dehydro[26-¹⁴C]cholesterol and 26-Hydroxy[26-¹⁴C]cholesterol

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1. 26-Hydroxycholesterol was obtained by reducing the methyl ester of ($\pm$)-3 β -hydroxycholest-5-en-26-oic acid, which was synthesized from 25-oxonorcholesterol. 2. Methods for preparing 7 α -hydroxycholesterol and 7-dehydrocholesterol were modified to allow the micro-scale preparation of these [¹⁴C]sterols from [26-¹⁴C]-cholesterol. 3. 26-Hydroxycholesterol was oxidized more readily than 7 α -hydroxycholesterol, 7-dehydrocholesterol or cholesterol by mitochondrial preparations from livers of mice, rats, guinea pigs, common toads (*Bufo vulgaris*) and *Caiman crocodylus*. 4. ($\pm$)-3 β -Hydroxy[26-¹⁴C]cholest-5-en-26-oic acid was oxidized very rapidly to ¹⁴CO₂ by mouse and guinea-pig mitochondria without evident discrimination between the two optical isomers. 5. An enzyme system that oxidizes 26-hydroxycholesterol to 3 β -hydroxycholest-5-en-26-oic acid was identified in the soluble extract of rat-liver mitochondria. This enzyme could use NADP in place of NAD but was not identical with liver alcohol dehydrogenase (EC 1.1.1.1). 6. [26-¹⁴C]Cholesteryl 3 β -sulphate was not oxidized by fortified mouse-liver preparations that oxidized [26-¹⁴C]cholesterol to ¹⁴CO₂.

Cholesterol is oxidized in the liver of mammals, yielding bile salts, i.e. glycine- or taurine-conjugated hydroxycholic acids. There is evidence that this overall catabolic process is subject to end-product inhibition (Beher & Baker, 1959; Beher, Anthony & Baker, 1959; Bergström & Danielsson, 1958). Bergström (1959) suggested that bile salts present in the enterohepatic circulation may inhibit the 7 α -hydroxylation of cholesterol, believed to be one of the first points of attack on the cholesterol molecule. 7 α -Hydroxycholesterol (cholest-5-ene-3 β ,7 α -diol) is formed enzymically from cholesterol on incubation with liver microsomes (Danielsson, 1964). On the other hand, 26-hydroxycholesterol (cholest-5-ene-3 β ,26-diol) has been isolated from incubations of [¹⁴C]cholesterol with liver homogenates and liver mitochondria (Fredrickson & Ono, 1956; Danielsson, 1961) and was the principal hydroxylation product formed from cholesterol in these experiments.

We have attempted to resolve this question as to which hydroxylation reaction might be the early pacemaker in cholesterol catabolism and possibly subject to negative-feedback control. We compared the rates of oxidation of 7 α -hydroxycholesterol and 26-hydroxycholesterol, with that of cholesterol itself, by an enzyme system ('cholesterol oxidase'), present in mammalian liver mitochondria, which

cleaves the terminal isopropyl group of the sterol side chain and oxidizes the methyl groups to carbon dioxide (Whitehouse, Staple & Gurin, 1959). For this purpose it was necessary to prepare each of these hydroxycholesters labelled with ¹⁴C in the terminal carbon atom (C-26).

A new route for synthesizing ($\pm$)-26-hydroxycholesterol has been developed and is described here in some detail.

EXPERIMENTAL

All radioactive compounds and reactions were handled in the absence of direct light and under N₂ where possible. [¹⁴C]Sterols were stored in frozen benzene solution at -5° in the dark. Radioactivity was measured by using a Tritomat (Isotope Developments Limited, Beenham, Reading) liquid-scintillation counter after solution in Diotol (Herberg, 1960) containing 2% (w/v) of Cab-o-sil (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.). Counting conditions were arranged to give a standard error for the mean count of a sample of less than 0.7%.

The radiochemical purity of all sterol substrates was checked by radioautography. Thin-layer chromatograms of radioactive sterols and non-radioactive reference compounds were pressed on to Kodak X-ray film and left for 24 hr. The non-radioactive markers were then made visible as described below: in all cases only one radioactive spot was detected and corresponded exactly to the appro-

priate non-radioactive marker, or was exactly superimposable with the admixed non-radioactive compound.

Biochemical

Incubation of [26-¹⁴C]sterols with liver preparations. Mitochondrial fractions isolated from homogenates of liver from female guinea pigs and female mice were incubated with [26-¹⁴C]sterols, emulsified with Tween 20 (Atlas Powder Co., Wilmington, Del., U.S.A.), and the following cofactors: ATP, NAD, sodium citrate, MgCl₂, AMP, GSH and a boiled supernatant fraction ('SF'), exactly as described by Lee & Whitehouse (1963). Light was excluded during the period of incubation.

The ¹⁴CO₂ evolved was trapped in centre wells containing 0.2 ml. of 2N-KOH or 25% (v/v) triethanolamine in water.

Microsomes were collected by centrifuging at 100000g for 30 min. the supernatant layer obtained on removing the mitochondrial fraction of liver homogenates (by centrifugation at 8500g for 10 min.).

Extraction of 26-hydroxycholesterol dehydrogenase from mitochondria. Mitochondrial fractions isolated from liver from female rats as described by Lee & Whitehouse (1963) were dispersed in 0.25 M-tris hydrochloride buffer solution, pH 8.5 (at 0°), containing 10% (w/v) of sucrose, kept for 5 min. at 0° and then centrifuged at 8500g for 10 min. to remove the particulate fraction, which was then twice washed with the same hyperosmotic buffer-sucrose solution. The soluble extract, the washed mitochondrial particulate fraction and other liver fractions were each incubated with 26-hydroxy[26-¹⁴C]cholesterol, emulsified with Tween 20 in 0.25 M-tris hydrochloride buffer, pH 8.1 (at 37°), with NAD or NADP (1.5 mg./incubation mixture) in a final volume of 2 ml. for 1 hr. with shaking in air at 37°.

Other preparations. Rat-spleen and rat-kidney homogenates were prepared as for liver; heart-muscle homogenates were prepared from chopped rat hearts by prolonged (1 min.) homogenizing in a chilled stainless-steel homogenizer.

Mitochondrial fractions and boiled SF were prepared from toad and cayman livers by exactly the same procedure used to prepare mouse-liver 'mitochondria' and SF. Toads (*Bufo vulgaris*, weighing approx. 15 g.) were collected from the Norfolk Broads and fed on maggots until used; South American caymans (*Caiman crocodylus*), approx. 36 cm. long, were purchased from the Goldfish Bowl, Eastern Avenue, Oxford, and fed on uncooked cattle liver, live minnows or cooked meat purees (baby foods) until used.

Protein determinations were carried out by using a biuret reagent (Gornall, Bardawill & David, 1949).

Extraction and assay of steroid acids. [¹⁴C]Steroid acids were estimated by thin-layer chromatography, as follows. Incubations were terminated by the addition of 12N-H₂SO₄ (0.2 ml.) and extracted first with ethyl acetate (2 × 1 ml.) and then, after the addition of methanol (1 ml.), extracted again with chloroform (1 ml.). The organic extracts were combined, washed once with water (1 ml.) and concentrated by warming (to 45°) under N₂ to 50 μl. This concentrate was applied as a single spot on a thin-layer plate of either alumina (to assay the relative amounts of steroid and steroid acids) or silica gel H (to characterize the steroid acids). The alumina plates were developed to a height of 10 cm. with ethyl acetate-light petroleum (b.p. 60–80°) (1:1, v/v). The silica plates were developed with system

no. 1 (Hofmann, 1962). Areas corresponding to known steroids were transferred to glass vials and Ditol (4 ml.) was added for liquid-scintillation counting.

Other biochemical studies. Crystalline horse-liver alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1) (Dalziel, 1961), at 0.55 μg./ml., was incubated with 200 μg. of sterol/ml., added in 0.1 ml. of dimethylformamide-0.5 mM-NAD-44 mM (0.1*M*)-phosphate buffer, pH 8.0, and the enzyme activity assayed fluorimetrically (Dalziel, 1962).

Chemical

All melting points were determined on a Kofler block and are uncorrected.

Thin-layer chromatographic analyses were carried out by using 8 in. × 2 in. glass plates coated with silica gel grade H (nach Stahl). Then 5–10 μl. of a 1% solution of the compounds to be analysed was adsorbed on the plates and developed with one of the following solvent systems: A, ethyl acetate alone; B, ethyl acetate-light petroleum (b.p. 60–80°) (1:1, v/v); C, ethyl acetate-light petroleum (b.p. 60–80°) (3:7, v/v); D, ethanol-free chloroform-ethyl methyl ketone (4:1, v/v). Compounds were detected on the plates by spraying with 10% (w/v) phosphomolybdic acid solution in ethanol or by spraying with 5% (w/v) SbCl₃ solution in chloroform, and heating at 110° for 5 min. Ketones were detected by spraying with 5% (w/v) 2,4-dinitrophenylhydrazine in methanol-conc. H₂SO₄ (8:1, v/v) (Brady's reagent).

Infrared spectra and optical rotations were determined in chloroform solution, unless otherwise stated. The spectra were calibrated with a polystyrene strip.

Elemental analyses were carried out by Bernhardt and Co., Mulheim, Germany, or by Weiler and Strauss, Oxford.

25-Oxonorcholesterol (3β-hydroxy-27-norcholest-5-en-25-one) (I) was supplied by Schering Corp., Bloomfield, N.J., U.S.A. Sodium [¹⁴C]cyanide (specific activity 16.3 mc/m-mole), sodium [2-¹⁴C]propionate (specific activity 4.2 mc/m-mole) and [¹⁴C]methyl iodide (specific activity 29.5 mc/m-mole) were supplied by The Radiochemical Centre, Amersham, Bucks.

The nuclear-magnetic-resonance (n.m.r.) spectrum was determined by Mrs R. E. Richards, of the Dyson Perrins Laboratory, Oxford.

Dried pyridine. AnalaR pyridine was heated under reflux over KOH for 8 hr., then distilled from fresh KOH, with suitable precautions to protect the distillate from atmospheric moisture.

Thionyl bromide. Thionyl bromide, b.p. 38–40°/15 mm. Hg, was prepared by the method of Frazer & Gerrard (1954).

Preparation of 3β-hydroxycholest-5-en-26-oic acid (VIII) (Scheme 1). A solution of 25-oxonorcholesterol (I) (25 g.) in distilled pyridine (240 ml.) was treated with acetic anhydride (60 ml.). The solution was stirred and heated under reflux for 1 hr. The reaction mixture was allowed to cool and poured with stirring into ice and water (about 2 l.). The white precipitate that formed was filtered off at the pump and washed successively with dil. HCl and then with water, until free from pyridine. The precipitate was dried and recrystallized from ethanol-water; the yield was 28 g. (98%). A small sample was further recrystallized from the same solvent to give 25-oxonorcholesteryl acetate (II), m.p. 138–139° [Dauben & Bradlow (1950) give m.p. 138.5–139.5°]. It had *R_F* (system B) 0.49 (25-oxonorcholesterol

has R_F 0.68). Both compounds gave yellow spots when sprayed with Brady's reagent while adsorbed on chromatoplates.

A solution of this acetylated ketone (27g.) in 5% (v/v) methanol in ethanol (800ml.) was cooled to 10° and treated with a solution of NaBH_4 (2.7g.) in ethanol-water (1:1, v/v) (2ml.). The solution was rapidly stirred at room temperature for 0.5hr. The reaction mixture was poured into dil. HCl (500ml.) and the ethanol evaporated at 40° *in vacuo*. The mixture was then extracted with ether (3 × 500ml.). The combined ethereal extracts were washed with water (3 × 100ml.), dried over MgSO_4 and then evaporated, yielding a white solid; the yield was 24g. (85%). Recrystallization from ethanol-water gave (±)-25-hydroxynorcholesteryl acetate (III) as plates, m.p. 116–120°. Further recrystallization of a small sample raised the m.p. to 120–122°. It had R_F (system C) 0.51 (25-oxonorcholesteryl acetate has R_F 0.63) and $[\alpha]_{20}^{D} - 17.4 \pm 1^\circ$ (c 2 in chloroform).

A solution of this compound (20g.) in sodium-dried ether (500ml.) was cooled to -80°. Pyridine (3.8ml.) was added, followed by the dropwise addition of freshly prepared thionyl bromide (4.1ml.).

The solution was stirred and allowed to reach room temperature over 1hr. A white precipitate of pyridine hydrobromide separated. Benzene (500ml.) was added and the ether was removed *in vacuo*. The reaction mixture was heated under reflux for 3hr. and filtered after cooling. The precipitate was washed with ether and the washings were added to the benzene solution. The solvents were then removed *in vacuo* at 50° to give a pale-yellow solid; the yield was 20g. (88%). Recrystallization from light petroleum (b.p. 40–60°) gave needles, m.p. 105–107°. Recrystallization of a small sample gave (±)-25-bromonorcholesteryl acetate (IV) as needles, m.p. 106–108° (Found: Br, 16.9. $\text{C}_{28}\text{H}_{45}\text{BrO}_2$ requires Br, 16.2%). It had R_F (system C) 0.63 (25-hydroxynorcholesteryl acetate has R_F 0.27) and $[\alpha]_{20}^{D} - 16.8 \pm 1^\circ$ (c 2 in chloroform).

A solution of the bromoacetate (7g.) in ether (100ml.) was treated with a solution of methylmagnesium iodide (2.5 equiv.) in ether. The mixture was stirred at room temperature for 1hr. Water was added to destroy the excess of the Grignard reagent. The ethereal layer was treated with dil. HCl and washed with dil. Na_2CO_3 and then water. The ethereal solution was dried over MgSO_4 and the ether was evaporated to give a pale-yellow solid; the yield was 5.8g. (90%). Recrystallization of the latter from light petroleum (b.p. 40–60°) gave needles, m.p. 127–129°. Further recrystallization of a sample from the same solvent gave (±)-25-bromonorcholesterol (V) as needles, m.p. 128–

130°. It had R_F (system B) 0.46 [(±)-25-bromonorcholesteryl acetate has R_F 0.645].

The n.m.r. spectrum (Table 1) indicated that the bromine atom was at C-25, since the spectrum showed a single proton (i.e. secondary bromide) with an adjacent methyl group in addition to the C-20-proton interactions with the C-21-methyl group.

A solution of the bromonorcholesterol (1.8g.) in distilled 2,3-dihydropyran (6.5ml.) was treated with conc. HCl (2 drops) and the mixture shaken for 10min. and left to stand overnight. Ether (50ml.) was added and the solution was treated with solid KOH (about 3g.) for 10min., when the solution was filtered. The ether was evaporated *in vacuo* to give a white solid, which on recrystallization from di-isopropyl ether gave (±)-3β-tetrahydropyranyloxy-25-bromonorcholest-5-ene (VI) as beautiful needles, m.p. 156–158°. It had R_F (system B) 0.70 [(±)-25-bromonorcholesterol has R_F 0.46] (Found: C, 70.0; H, 9.83; Br, 15.8; $\text{C}_{31}\text{H}_{51}\text{BrO}_2$ requires C, 69.5; H, 9.6; Br, 14.9%).

A solution of bromonorcholesterol (1.0g.) in dimethyl sulphoxide (20ml.) was treated with dried KCN (1.0g.). The reaction mixture was heated under reflux, under N_2 , for 3.5hr. The solution was cooled and poured into water (200ml.). The precipitate that formed was filtered off and washed with water until the washings gave no precipitate with aqueous AgNO_3 solution. The precipitate was dried *in vacuo* for several hours and recrystallized from light petroleum (b.p. 40–60°), giving (±)-25-cyanonorcholesterol (VII) as needles, m.p. 110–117°. These crystals proved to be inseparable from small amounts of the starting material and were also contaminated with a by-product that had the same R_F as (±)-25-hydroxynorcholesterol, prepared by reduction of 25-oxonorcholesterol with NaBH_4 in ethanol. The R_F values of compounds in the mixture were 0.455, 0.56 and 0.62 [(±)-25-bromonorcholesterol has R_F 0.62; (±)-25-hydroxynorcholesterol has R_F 0.45]. Further purification did not prove necessary, however.

A solution of the product of the above reaction (1.0g.) in ethanol (10ml.) was treated with KOH (500mg.) and heated under reflux, under N_2 , for 18hr. The reaction mixture was cooled and poured into dil. H_2SO_4 (100ml.). The insoluble precipitate that formed was washed with water and dried. Recrystallization of the solid from acetic acid-water gave (±)-3β-hydroxycholest-5-en-26-oic acid (VIII) as plates, m.p. 176–178° [Kučera & Šorm (1958) reported plates, m.p. 173–175°]. It had R_F (system A) 0.17 (a sample kindly supplied by Professor Šorm had R_F 0.17).

Preparation of (±)-26-hydroxycholesterol (X) (Scheme 1). A solution of the hydroxycholestenic acid (0.5g.) in ether

Table 1. Proton resonances of 25-bromonorcholesterol

τ values of groups in deuterochloroform

CH_3 (C-18)	CH_3 (C-21)	CH_3 (C-19)	CH_3 (C-27)	$\text{CH}\cdot\text{OH}$ (C-3)	$\text{CH}_2\cdot\text{CHBr}\cdot\text{CH}_3$ (C-25)	$\text{C}\cdot\text{CH}\cdot\text{CH}\cdot\text{CH}_2$ (C-6)
9.31	9.08 (doublet)	8.99	8.295 (doublet)	8.1*	5.845 (sextet)	4.635 (ill-defined doublet)
	(J, 6 cyc./sec.)		(J, 6.6 cyc./sec.)		(J _{total} , 31.8 cyc./sec.)	(J, 3 cyc./sec.)

* This is the only peak quenched on the addition of deuterium oxide.

Scheme 1. Synthesis of 26-hydroxycholesterol. Ac, Acetyl; THP, tetrahydropyranyloxy.

(10 ml.) was treated with an excess of an ethereal solution of diazomethane and left to stand for 20 min. Dilute aq. acetic acid was added and the ethereal layer was washed with water and dried over MgSO_4 . The ether was evaporated to give a white solid. Recrystallization from methanol gave methyl $(\pm)$ -3 β -hydroxycholest-5-en-26-oate (IX) as needles, m.p. 100–103° [Kučera & Šorm (1958) reported needles, m.p. 102–103°]. It had R_F (system B) 0.52 (an authentic sample kindly supplied by Professor Šorm had R_F 0.52).

A solution of this ester (500 mg.) in sodium-dried ether (10 ml.) was treated with an excess of an ethereal solution of LiAlH_4 (about 1 g.) and the mixture heated under reflux for 0.5 hr. The excess of LiAlH_4 was destroyed by the careful addition of acetone diluted with ether, then acetone alone and finally dil. HCl. The mixture was extracted with ether (3 $\times$ 50 ml.) and the ethereal extracts were combined, washed with water and dried over MgSO_4 . The ether was evaporated to give a white solid, which on recrystallization from light petroleum (b.p. 60–80°)–ethyl acetate gave $(\pm)$ -26-hydroxycholesterol (X) as needles, m.p. 168–173° [Scheer, Thompson & Mosettig (1956) reported m.p. 177–178°]. It had R_F (system B) 0.35 (methyl hydroxycholestenolate has R_F 0.52; two samples of authentic 26-hydroxycholesterol kindly supplied by Dr Y. Sato and Dr E. Staple respectively had R_F 0.35).

Preparation of $(\pm)$ -3 β -hydroxy[26- ^{14}C]cholest-5-en-25-oic acid. A solution of $(\pm)$ -25-bromonorcholesterol (70 mg.) (prepared as above) in dimethyl sulphoxide (7 ml.) was treated with Na^{14}CN (1.5 mg.) and the solution was heated under reflux in an atmosphere of N_2 for 6 hr. The solution was then cooled and poured into water (7 ml.) and extracted with ether (3 $\times$ 5 ml.). The ethereal extracts were combined and the ether was evaporated in a stream of N_2 . The gummy residue was dissolved in ethanol (4 ml.), and aq. 3N-KOH (2 ml.) was added. The solution was heated under reflux in N_2 for 18 hr. The reaction mixture was poured into water (5 ml.) and extracted with light petroleum (b.p. 60–80°) (2 $\times$ 5 ml.). The aqueous phase was acidified with 4N- H_2SO_4 and extracted with ether (4 $\times$ 5 ml.). The ethereal extracts were combined and evaporated in a stream of N_2 to about 200 μl . The solution was then adsorbed on silica gel H plates (20 cm. $\times$ 20 cm.) and eluted with ethyl acetate. Bands corresponding to spots of authentic $(\pm)$ -3 β -hydroxycholest-5-en-26-oic acid were removed and extracted with methanol. This process was repeated twice. Finally the methanol solution was evaporated and the yield of radioactive acid from Na^{14}CN determined to be 150 μC (about 30%).

Preparation of $(\pm)$ -26-hydroxy[26- ^{14}C]cholesterol. A solution of $(\pm)$ -3 β -hydroxy[26- ^{14}C]cholest-5-en-26-oic acid (0.09 mc) in ether (1 ml.) was treated with excess of ethereal

diazomethane and the solution left to stand at room temperature for 0.5 hr. The solution was then evaporated at 10° in a stream of N₂, when excess of diazomethane was removed together with the ether. A solution of LiAlH₄ (10 mg.) in dry ether (1 ml.) was then added and the reaction mixture heated under reflux for 0.5 hr. The excess of LiAlH₄ was destroyed by adding acetone and the resulting suspension extracted successively with 2N-H₂SO₄, 2M-Na₂CO₃ and finally water. The ethereal layer was evaporated to 50 μ l. and ($\pm$)-26-hydroxy[26-¹⁴C]cholesterol was isolated by thin-layer chromatography, as described above, with 20 cm. $\times$ 20 cm. plates coated with silica gel H and developed with system B.

Preparation of 7 α -hydroxycholesterol. Cholesteryl benzoate (2.5 g.) was converted into 7 α -hydroxycholesteryl benzoate by using *tert.*-butyl perbenzoate and CuBr in acetic acid, as described by Starka (1962).

The crude benzoate was hydrolysed with excess of LiAlH₄ in dry ether for 1 hr. at room temperature. The excess of LiAlH₄ was cautiously destroyed by acetone. The resulting suspension was washed successively with dil. H₂SO₄, dil. Na₂CO₃ and water. The ethereal solution was dried over MgSO₄ and the ether was evaporated to give a yellow gum (2 g.), which was adsorbed on alumina (activity III on the Brockman scale) (100 g.) and eluted with benzene (3500 ml.), ether (1300 ml.) and methanol-ether (1:19, v/v) (300 ml.). Fractions of volume 100 ml. were collected and a small portion of each fraction was examined by thin-layer chromatography on silica gel H plates developed with system B (Table 2).

Fractions 37-47 were recrystallized from methanol to give 7 α -hydroxycholesterol as needles, m.p. 180-182° (literature m.p. 185°). It had *R_F* 0.57 (7 β -hydroxycholesterol has *R_F* 0.63). The yield was about 1 g. (50%).

Preparation of 7 α -hydroxy[26-¹⁴C]cholesterol. [26-¹⁴C]-Cholesterol (1.9 mg.) was dissolved in pyridine (0.5 ml.) and cooled to 0°. The solution was treated with cold benzoyl chloride (10 μ l.) and left at room temperature for 16 hr. Ice-cold water (1 ml.) and 2N-H₂SO₄ (1 ml.) were added and the solution was extracted with ether (3 $\times$ 2 ml.). The ethereal extracts were combined and washed with water (2 $\times$ 1 ml.). The ether was evaporated in a stream of N₂. Acetic acid (0.5 ml.) and CuBr (1 mg.) were added and the solution was heated under reflux, under N₂, by immersion in an oil bath at 120°. A solution of *tert.*-butyl perbenzoate (10 μ l.) in acetic acid (0.5 ml.) was added over 15 min. The solution was further heated under reflux at 125-130° for 10 min. and then cooled. Benzene (2 ml.) was added. The solution was extracted successively with water (1 $\times$ 2 ml.), 5% (w/v) Na₂CO₃ solution (1 $\times$ 2 ml.) and water (1 $\times$ 2 ml.).

Ether (2 ml.) was added and the solution treated with excess of LiAlH₄. After standing for 1 hr. at room temperature the excess of LiAlH₄ was destroyed with acetone and the ethereal suspension was extracted with water (1 ml.), dil. H₂SO₄ (1 $\times$ 2 ml.) and finally again with water (1 ml.). The ethereal solution was evaporated in a stream of N₂ to a small volume (50 μ l.). The latter solution was adsorbed on silica plates (20 cm. $\times$ 20 cm.) and developed with system B. Authentic non-radioactive 7 α -hydroxycholesterol was also chromatographed at the side and in the centre of these plates. Areas containing the radioactive 7 β -hydroxycholesterol (*R_F* 0.57) [revealed on spraying only the non-radioactive areas (*R_F* 0.57) with SbCl₃] were scraped off and extracted with methanol-ether (1:19, v/v). The chromatographic purification was repeated to constant specific activity. Three such purifications gave 7 α -hydroxy-[26-¹⁴C]cholesterol in 12-20% yield; 50% of the [26-¹⁴C]-cholesterol was recovered unchanged.

Preparation of 7-dehydrocholesterol. 7-Dehydrocholesterol was prepared according to the method of Hunzicker & Müllner (1958). Cholesteryl benzoate was used as the starting material and the second double bond was introduced by using 2,3-dibromo-5,5-dimethylhydantoin (bromantin) and trimethyl phosphite. The benzoyl group was removed reductively with LiAlH₄ instead of by KOH hydrolysis (used by Hunzicker & Müllner, 1958). The product was purified by chromatography on alumina and recrystallized from acetone to give 7-dehydrocholesterol as needles, m.p. 145° (literature m.p. 145-147°). It had *R_F* (system B) 0.66 and $\lambda_{\max}$ in methanol at 273, 283 and 295 m μ (ϵ 10800, 11500 and 6500 respectively).

Preparation of 7-dehydro[26-¹⁴C]cholesterol. [26-¹⁴C]-Cholesterol (1.5 mg., 0.1 mc) was benzoylated, as described above in the preparation of 7 α -hydroxycholesterol. The purified product was dissolved in heptane (b.p. 100°) and treated with 2,3-dibromo-5,5-dimethylhydantoin (1 mg.) in light petroleum (b.p. 100-120°) under N₂. The solution was heated under reflux at 100-120° with stirring for 10 min. The reaction mixture was cooled and centrifuged at 300g for 5 min. to remove precipitated 5,5-dimethylhydantoin. The supernatant was removed and the precipitate was washed with cold light petroleum (b.p. 100-120°) and further centrifuged until the supernatant was clear. The combined supernatants were evaporated to dryness and dissolved in dry xylene (3 ml.). This solution was added to a refluxing solution of trimethyl phosphite (10 μ l.) in xylene (1 ml.). The reaction mixture was further heated under reflux for 1.5 hr. at 125° and subsequently cooled. The solution was evaporated at 80° *in vacuo* to dryness. Dry ether was added and the solution was treated with LiAlH₄ (about 10 mg.) and

Table 2. *Purification of 7 α -hydroxycholesterol*

Fraction no.	Eluent	Volume (ml.)	Colour with SbCl ₃	m.p.	<i>R_F</i> (system B)
1-20	Benzene	2000	Blue		0.83
21-27	Benzene	700	—		—
28-33	Benzene	600	Pink		0.74
34-36	Ether	300	Blue-pink		0.60-0.74
37-47	Ether	1000	Blue	173-180°	0.57
48-51	Methanol-ether (1:19, v/v)	300	Blue	238-242°	0.30

left at room temperature for 12 hr. Acetone was cautiously added and the solution extracted with dil. HCl until clear. The ethereal solution was washed with water and evaporated to dryness under N₂, and the solid was purified by thin-layer chromatography as described above to give 7-dehydro-[26-¹⁴C]cholesterol (the yield was 10% from [26-¹⁴C]-cholesterol). It had *R_F* (system B) 0.66.

A sample of the above compound was added to 5 mg. of the pure 7-dehydrocholesterol and the mixture was examined by thin-layer chromatography. In one series of experiments the plate was examined under u.v. light and the fluorescent region was removed, extracted with ether and made up to a known volume with ethanol after the ether had been removed under N₂. Portions of this solution were assayed for ¹⁴C by liquid-scintillation counting; portions were also assayed spectrophotometrically for 7-dehydrocholesterol by measuring the extinction at 272, 282 and 294 mμ. Three successive purifications by thin-layer chromatography gave material of constant specific activity.

Cholesteryl 3β-O-monosulphate. Sodium [26-¹⁴C]cholesteryl 3β-O-monosulphate (24mc/m-mole) was synthesized from [26-¹⁴C]cholesterol and pyridine-SO₃ (Roy, 1956) and freed from cholesterol by thin-layer chromatography on silica with ethyl acetate-hexane (3:7, v/v). The immobile cholesteryl sulphate was then eluted and purified by thin-layer chromatography on alumina with methanol-water (9:1, v/v). Solvolysis with trichloroacetic acid and dioxan regenerated only cholesterol.

RESULTS

Oxidation of hydroxycholesterols by liver preparations

Oxidation of the terminal isopropyl group. Table 3 shows that the mitochondrial fraction of mouse livers contained the bulk of the sterol-oxidase activity and that added 26-hydroxy-[26-¹⁴C]cholesterol was oxidized to ¹⁴CO₂ much more readily than added 7α-hydroxy[26-¹⁴C]cholesterol, which in turn was oxidized considerably more rapidly than [26-¹⁴C]cholesterol or 7-dehydro-[26-¹⁴C]cholesterol.

Fig. 1 compares the relative rates of oxidation of

7α- and 26-hydroxycholesterol by mouse-liver mitochondria. Essentially the same difference in rates of oxidation of these two hydroxycholesterols was observed with guinea-pig mitochondrial preparations. The strain of guinea pig that we used (bred in this Laboratory) secretes chenodeoxycholic acid as the principal bile acid, which was identified by thin-layer chromatography (Hofmann, 1962) and colour reaction with ferric chloride and sulphuric acid (Anthony & Beher, 1964).

Thus although the mouse and the guinea pig secrete different bile salts (Haslewood, 1964) there is no obvious difference between liver mitochondrial preparations of these two mammals with regard to

Fig. 1. Comparative rates of oxidation of (±)-3β-hydroxycholest-5-en-26-oic acid (○), (±)-26-hydroxycholesterol (Δ), 7α-hydroxycholesterol (□) and cholesterol (●), determined as the yield of ¹⁴CO₂ by fortified mouse-liver mitochondria. Experimental details are given in the text.

Table 3. *Relative oxidation of [26-¹⁴C]sterols by mouse-liver preparations*

Supernatant fraction was present in all incubations. The final pH was 8.2 at 37°. The percentage oxidation was computed as ¹⁴CO₂ evolved × 100/[¹⁴C]sterol substrate.

Liver fraction	Percentage oxidation				
	Cholesterol	7-Dehydro-cholesterol	7α-Hydroxy-cholesterol	26-Hydroxy-cholesterol	Cholesteryl sulphate
Mitochondrial	4.0	6.0	18.5	60	0.2
Microsomal	0.3	0.2	5.5	1.0	0.01
Mitochondrial plus microsomal	0.2	0.3	2.1	18.7	0.1
Incubation time (hr.) 1		1	0.5	0.5	3
[¹⁴ C]Substrate added/incubation (μc)	0.025	0.05	0.01	0.01	0.05

Table 4. *Oxidation of hydroxycholesterols and 3 β -hydroxycholest-5-en-26-oic acid to carbon dioxide and steroid acids by mouse, English toad and South American cayman liver mitochondrial preparations*

Results are given as percentage conversions of [^{14}C]substrate (0.05 μC) into $^{14}\text{CO}_2$ or [^{14}C]steroid acids on incubating fortified mitochondrial preparations from equal weights of livers with homologous SF for 1 hr. at 37° in tris hydrochloride buffer, pH 8.2. N.D., Not determined.

Substrate	Product measured	Percentage conversion		
		Mouse	Cayman	Toad
26-Hydroxycholesterol	CO_2	60	1	5
7 α -Hydroxycholesterol	CO_2	19	0.6	0.7
3 β -Hydroxycholest-5-en-26-oic acid	CO_2	80	1	10
Cholesterol	CO_2	4	0	0
26-Hydroxycholesterol	Acids	N.D.	47	50
7 α -Hydroxycholesterol	Acids	N.D.	37	10
Cholesterol	Acids	N.D.	31	43

their ability to oxidize these two hydroxycholesterols.

Rat-heart, -kidney and -spleen homogenates did not yield significant amounts of $^{14}\text{CO}_2$ when incubated with 26-hydroxy[26- ^{14}C]cholesterol under conditions in which they appreciably oxidized [2- ^{14}C]octanoic acid and [2- ^{14}C]propionic acid to $^{14}\text{CO}_2$.

The supernatant fraction (SF) that is required for optimum cholesterol oxidation by rat- and mouse-liver preparations (Whitehouse, Staple and Gurin, 1961) stimulated the oxidation of both 7 α - and 26-hydroxycholesterol by mouse-liver mitochondria.

Since the percentage yield of $^{14}\text{CO}_2$ from incubations of 26-hydroxy[26- ^{14}C]cholesterol with mouse-liver mitochondria was usually above 50% after 1 hr. (e.g. 72% at 2 hr. in one experiment), there was evidently no ultimate discrimination between the two optical isomers of 26-hydroxycholesterol (due to the asymmetric C-25) by the mitochondrial steroid oxidase (cf. Berséus, 1965).

The mitochondrial fractions from toad and cayman livers were incubated with the hydroxycholesterols and other sterols, and both the $^{14}\text{CO}_2$ and the [^{14}C]steroid acids formed therefrom were determined. Table 4 compares the results obtained from these experiments and from parallel experiments with mouse-liver mitochondria. Both these non-mammalian liver preparations oxidized 26-hydroxycholesterol to both carbon dioxide and steroid acid(s) more readily than they oxidized 7 α -hydroxycholesterol. This acid fraction, assayed after thin-layer chromatography, did not include propionic acid, a potential metabolite of steroid acids.

Oxidation of related sterols. A racemic mixture of 3 β -hydroxy[26- ^{14}C]cholest-5-en-26-oic acid was very rapidly oxidized to $^{14}\text{CO}_2$ by mouse-liver

mitochondria, the initial rate being at least three times that of 26-hydroxycholesterol over the first 15 min. (Fig. 1). The relative yield of $^{14}\text{CO}_2$ was always greater than 50% after 20 min. incubation, indicating that there was again no ultimate discrimination by the mitochondrial oxidase between the C-25 optical isomers.

Cayman-liver mitochondria did not significantly oxidize the carboxylic acid group of this acid to $^{14}\text{CO}_2$ (Table 4), but toad-liver mitochondria had some definite capacity to do so. This finding confirms what is known about the distribution of the side-chain-cleaving enzymes (Whitehouse, Cottrell, Briggs & Staple, 1962; Haslewood, 1964). The cayman-liver mitochondria readily oxidized [2- ^{14}C]propionic acid to $^{14}\text{CO}_2$.

Cholesteryl sulphate has been implicated as a biological precursor of adrenal steroids (Roberts, Bandi, Calvin, Drucker & Lieberman, 1964). [26- ^{14}C]Cholesteryl 3-*O*-monosulphate was not significantly oxidized by mouse-liver mitochondria (Table 3), suggesting that it is not a precursor for bile acid formation.

26-Hydroxycholesterol dehydrogenase in mammalian liver. Table 5 shows the results of incubating 26-hydroxy[26- ^{14}C]cholesterol with various fractions isolated from rat livers and determining the [26- ^{14}C]steroid acid formed from the incubation mixture. A requirement for NAD was shown; in similar concentrations, NADP only partially substituted for NAD in this respect. Pretreatment of extracted sterols with moist silver oxide, before separation of the steroid acids by thin-layer chromatography, did not increase the yield of the ^{14}C -labelled acid fraction, indicating that the 26-aldehyde was not present in significant quantity in the incubation mixtures.

A comparison of the percentage conversion into the steroid acid (predominantly 3 β -hydroxycholest-

Table 5. *Subcellular distribution and properties of rat-liver 26-hydroxycholesterol dehydrogenase*

The ^{14}C -labelled acid formed was assayed after thin-layer chromatography and computed as percentage of ^{14}C -labelled acid $\times 100/[^{14}\text{C}]$ sterol substrate (0.05 μC).

Liver fraction	Cofactor added	Acid formed after 1 hr. (%)	Protein (mg./incubation)
Microsomal plus supernatant	NAD	26	170
Mitochondrial particulate	NAD	9	250
Soluble mitochondrial extract	NAD	54	90
	None	8	90
	NADP	35	90
'Killed' at zero time with acid	NAD	6	90

5-en-26-oic acid) per mg. of protein for each fraction showed that the particulate mitochondrial and microsomal fractions were not very efficient in oxidizing 26-hydroxycholesterol. On the other hand, the soluble fraction extracted from washed mitochondria converted at least 50% of the substrate into the 3β -hydroxycholest-5-en-26-oic acid within 1 hr. These mitochondrial extracts also oxidized ethanol. However, preparations of highly purified horse-liver alcohol dehydrogenase failed to oxidize 26-hydroxycholesterol.

pH optima for sterol oxidation. The optimum pH for $^{14}\text{CO}_2$ formation from each of these various [$^{26-14}\text{C}$]sterols and from [^{2-14}C]propionic acid by mouse-liver mitochondria was determined by using tris hydrochloride buffers in the pH range 7.0–9.0 (pH was measured at 37°). The following pH optima were found: for cholesterol, 8.3; for 7α -hydroxycholesterol, 8.5; for 26-hydroxycholesterol, 8.1; for 3β -hydroxycholest-5-en-26-oic acid, 7.7; for propionic acid, 7.3.

The pH optimum for the oxidation of 26-hydroxycholesterol to 3β -hydroxycholest-5-en-26-oic acid was approx. 8.1 in tris hydrochloride buffer.

DISCUSSION

Preparation of 3β -hydroxy[$^{26-14}\text{C}$]cholest-5-en-26-oic acid and 26-hydroxy[$^{26-14}\text{C}$]cholesterol. Previously described methods for the preparation of 26-hydroxycholesterol have involved the degradation of sapogenins (Scheer *et al.* 1956; E. Staple, unpublished work) and are not feasible for introducing a ^{14}C label specifically in one of the terminal three carbon atoms of the sterol. Various syntheses were therefore explored based on 25-oxo-(27)-norcholesterol as a starting material. The synthesis of hydroxycholestenoic acid from 25-oxonorcholes-

terol described by Kučera & Šorm (1958), involving cyanhydrin formation, gives rather poor yields of the acid and was not considered practicable on a micro-scale.

An alternative route from 25-oxonorcholesterol, outlined in Scheme 1, was therefore developed, involving the following conventional procedures. 25-Oxonorcholesteryl acetate was converted into 3β -acetoxy-25-hydroxynorcholest-5-ene by controlled reduction with sodium borohydride at room temperature. This alcohol was brominated with thionyl bromide (1.1 moles) and pyridine (1.0 mole) and the acetyl group then removed by treatment with a solution of methylmagnesium iodide in ether. The resulting 25-bromonorcholesterol was converted into the cyanide (^{14}C -labelled in the radioactive synthesis) with potassium cyanide or sodium cyanide in dimethyl sulphoxide, and the cyanide hydrolysed with potassium hydroxide in ethanol to give the required 3β -hydroxycholest-5-en-26-oic acid, which was readily separated from neutral material by extraction with alkali. Treatment of this acid with diazomethane in ether, before reduction with lithium aluminium hydride, improved the yield of 26-hydroxycholesterol: the latter compound was identical with authentic samples prepared by the late Dr Mosettig by another route.

While attempting to prepare 3β -acetoxy-25-bromonorcholest-5-ene it was found that phosphorus tribromide would not react smoothly with the 25-ol and gave resinous products from which was isolated a compound, m.p. $145\text{--}147^\circ$, which on analysis contained no bromine; this compound has not been further investigated.

It was found that removal of the 3-acetoxy group was necessary before introducing the cyanide group, since potassium cyanide reacted with 3β -acetoxy-25-bromonorcholest-5-ene to give a compound, m.p. $130\text{--}132^\circ$, which had a mobility on thin-layer

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plates identical with that of 3β -hydroxynorcholesterol-5-ene, m.p. 165–167°; this compound has not been further investigated.

Preparation of 7α -hydroxy[26- ^{14}C]cholesterol and 7-dehydro[26- ^{14}C]cholesterol. Routes to 7α -hydroxycholesterol include: (a) allylic bromination of cholesterol with *N*-bromosuccinimide (Bide, Henbest, Jones, Peevers & Wilkinson, 1948; Bernstein, Binovi, Dorfman, Sax & Subbarow, 1949); (b) photo-oxidation of cholesterol to the 5α -hydroperoxide and subsequent rearrangement to the 7α -hydroperoxide followed by reduction of the hydroperoxide to the alcohol (Schenck, Gollnick & Neumüller, 1957; Lythgoe & Trippett, 1959); (c) oxidation of cholesteryl benzoate by *tert*.-butyl perbenzoate in the presence of cuprous bromide and acetic acid followed by removal of the benzoyl group (Starka, 1962).

This last method was found to be the most suited to adaptation on the micro-scale. The only modification for micro-synthesis was in the final hydrolysis of the benzoate, achieved reductively with lithium aluminium hydride in ether, giving adequate yields of 7α -hydroxycholesterol. Starka (1962) also described the preparation of 7-dehydrocholesterol from 7α -hydroxycholesterol by an acid-catalysed elimination of water with hydrochloric acid in methanol. This reaction was not successful on the micro-scale.

7-Dehydro[26- ^{14}C]cholesterol was obtained by another route. Among the final products, a compound was detected (by thin-layer chromatography) that did not appear to be either 7α - or 7β -hydroxycholesterol but gave a vivid blue colour with antimony trichloride; this unknown compound had no absorption maximum in the u.v. region between 250 and 300 m μ and had a much higher R_f than either 7α - or 7β -hydroxycholesterol.

Mitochondrial oxidation of hydroxycholesterols. Recent work suggests that the hydroxylation of the cholesterol nucleus, which probably precedes cleavage of the side chain in bile acid formation (Danielsson, 1963), takes place in microsomes (Mendelsohn & Staple, 1963; Mitton & Boyd, 1965; Danielsson, 1960; Berséus, 1965). 26-Hydroxylation of sterols appears to be confined to mitochondria (Fredrickson & Ono, 1956; Danielsson, 1961; Suld, Staple & Gurin, 1961). 26-Hydroxycholesterol may therefore not be a normal intermediate in bile acid formation from cholesterol. This present study does, however, indicate that mammalian liver mitochondria can rapidly oxidize a 26-hydroxysterol and that oxidative degradation of the sterol side chain may be rather non-specific with regard to substrate, as suggested by Bergström (1963), any apparent specificity in cholic acid formation probably being determined by the nature of the microsomal hydroxylases.

If the 26-hydroxylase is really a mitochondrial enzyme, then it appears to be rather rate-limiting for the side-chain oxidation of sterols, since 7α -hydroxycholesterol was oxidized much less rapidly than 26-hydroxycholesterol by both mammalian and non-mammalian liver mitochondrial preparations.

The soluble enzyme contained in mitochondria that oxidizes 26-hydroxycholesterol to 3β -hydroxycholesterol-5-en-26-oic acid appears to be very similar to that described by Herman & Staple (1965), which oxidizes $3\alpha,7\alpha,12\alpha,26$ -tetrahydroxy- 5β -cholestane to the corresponding 26-carboxylic acid, although these authors did not identify the sub-cellular location of this enzyme. Like liver alcohol dehydrogenase (Dalziel & Dickenson, 1965), the 26-hydroxycholesterol dehydrogenase(s) can use NADP as the coenzyme in place of NAD. These two dehydrogenases are not identical however, as the purified alcohol dehydrogenase does not oxidize 26-hydroxycholesterol.

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REFERENCES

- Anthony, W. L. & Beher, W. T. (1964). *J. Chromat.* **13**, 567.
- Beher, W. T., Anthony, W. L. & Baker, G. D. (1959). *Proc. Soc. exp. Biol., N.Y.*, **102**, 317.
- Beher, W. T. & Baker, G. D. (1959). *Proc. Soc. exp. Biol., N.Y.*, **101**, 214.
- Bergström, S. (1959). In *Hormones and Atherosclerosis*, p. 32. Ed. by Pincus, G. New York: Academic Press Inc.
- Bergström, S. (1963). *Symp. biochem. Soc.* **24**, 73.
- Bergström, S. & Danielsson, H. (1958). *Acta physiol. scand.* **43**, 1.
- Bernstein, S., Binovi, L. J., Dorfman, L., Sax, K. J. & Subbarow, Y. (1949). *J. org. Chem.* **14**, 433.
- Berséus, O. (1965). *Acta chem. scand.* **19**, 325.
- Bide, A. E., Henbest, H. B., Jones, E. R. H., Peevers, R. W. & Wilkinson, P. A. (1948). *J. chem. Soc.* p. 1783.
- Dalziel, K. (1961). *Biochem. J.* **80**, 440.
- Dalziel, K. (1962). *Biochem. J.* **84**, 244.
- Dalziel, K. & Dickinson, F. M. (1965). *Biochem. J.* **95**, 311.
- Danielsson, H. (1960). *Acta chem. scand.* **14**, 846.
- Danielsson, H. (1961). *Ark. Kemi*, **17**, 373.
- Danielsson, H. (1963). *Advanc. Lipid Res.* **1**, 336.
- Danielsson, H. (1964). *Acta chem. scand.* **18**, 831.
- Dauben, W. G. & Bradlow, H. L. (1950). *J. Amer. chem. Soc.* **72**, 4248.
- Frazer, M. J. & Gerrard, W. (1954). *Chem. & Ind.* p. 280.
- Fredrickson, D. S. & Ono, K. (1956). *Biochim. biophys. Acta*, **22**, 183.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

- Haslewood, G. A. D. (1964). *Biol. Rev.* **39**, 537.
- Herberg, R. J. (1960). *Analyt. Chem.* **32**, 42.
- Hermann, R. & Staple, E. (1965). *Fed. Proc.* **24**, 661.
- Hofmann, A. F. (1962). *J. Lipid Res.* **3**, 127.
- Hunzicker, F. & Müllner, F. X. (1958). *Helv. chim. Acta*, **41**, 70.
- Kučera, J. & Šorm, F. (1958). *Coll. Czech. chem. Commun.* **23**, 116.
- Lee, M. J. & Whitehouse, M. W. (1963). *Biochem. J.* **86**, 48.
- Lythgoe, B. & Trippett, S. (1959). *J. chem. Soc.* p. 471.
- Mendelsohn, D. & Staple, E. (1963). *Biochemistry*, **2**, 577.
- Mitton, J. R. & Boyd, G. S. (1965). *Biochem. J.* **96**, 60P.
- Roberts, K. D., Bandi, L., Calvin, H. I., Drucker, W. D. & Liebermann, S. (1964). *J. Amer. chem. Soc.* **86**, 958.
- Roy, A. B. (1956). *Biochem. J.* **62**, 41.
- Scheer, I., Thompson, M. J. & Mosettig, E. (1956). *J. Amer. chem. Soc.* **78**, 4733.
- Schenck, G. O., Gollnick, K. & Neumüller, O. A. (1957). *Liebigs Ann.* **603**, 46.
- Starka, L. (1962). *Pharmazie*, **17**, 126.
- Suld, H. M., Staple, E. & Gurin, S. (1961). *Fed. Proc.* **20**, 284.
- Whitehouse, M. W., Cottrell, M. C., Briggs, T. & Staple, E. (1962). *Arch. Biochem. Biophys.* **98**, 305.
- Whitehouse, M. W., Staple, E. & Gurin, S. (1959). *J. biol. Chem.* **234**, 276.
- Whitehouse, M. W., Staple, E. & Gurin, S. (1961). *J. biol. Chem.* **236**, 68.