Substrate and Inhibitor Specificity of the Cholesterol Oxidase in Bovine Adrenal Cortex

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(Received 25 April 1966)

1. Cholesteryl β -sulphate is oxidized *in vitro* by preparations of bovine adrenal-cortex mitochondria to pregnenolone sulphate and isocaproic acid (4-methyl-pentanoic acid) without hydrolysis of the ester linkage. 2. Free cholesterol is the preferred substrate for adrenal-cortex cholesterol oxidase; the apparent K_m for cholesteryl sulphate is $500\mu\text{M}$ and for free cholesterol $50\mu\text{M}$ under the same conditions. 3. Cholesteryl β -acetate is hydrolysed by bovine adrenal-cortex mitochondria *in vitro* to free cholesterol, which is subsequently oxidized to more polar steroids and isocaproic acid. Evidence was obtained that other cholesterol esters behave similarly. Cholesterol esters may thus act as precursors of steroid hormones. 4. Cholest-4-en-3-one is only poorly oxidized to isocaproic acid and more polar steroids and thus is probably not a significant precursor of steroid hormones. 5. Cholesteryl esters inhibit the oxidation of cholesterol competitively (K_i for cholesteryl phosphate $28\mu\text{M}$, for cholesteryl sulphate $110\mu\text{M}$, for cholesteryl acetate $65\mu\text{M}$) but pregnenolone esters do not inhibit this system. 6. Pregnenolone and 20α -hydroxycholesterol (both metabolites of cholesterol in this system) inhibit the oxidation of cholesterol non-competitively. K_i for pregnenolone is $130\mu\text{M}$ and K_i for 20α -hydroxycholesterol is $17\mu\text{M}$. 7. 25 -Oxo- 27 -norcholesterol inhibits cholesterol oxidation non-competitively (K_i $16\mu\text{M}$). A number of other Δ^5 - β -hydroxy steroids inhibit cholesterol oxidation and evidence was obtained that the β -hydroxyl group was necessary for inhibitory activity. 8. Pregnenolone, 20α -hydroxycholesterol and 25 -oxo- 27 -norcholesterol inhibit oxidation of cholesteryl sulphate by this system but their sulphates do not. 9. β -Hydroxychole-5-enoic acid, 3α -hydroxy- 5β -cholanolic acid and β -hydroxy- $22,23$ -bisanchol-5-enoic acid stimulated formation of isocaproic acid from cholesterol. 10. No evidence was obtained that phosphorylation or sulphation are obligatory steps in cholesterol oxidation by adrenal-cortex mitochondria. 11. The cholesteryl β -sulphate sulphatase of bovine adrenal cortex was found mostly in the microsomal fraction and was inhibited by inorganic phosphate.

It is currently believed that cholesterol is metabolized to pregnenolone (3β -hydroxypregn-5-en-20-one) in the adrenal cortex by the following stages:

Cholesterol $\rightarrow$ 20α -hydroxycholesterol $\rightarrow$ $20\alpha,22\beta$ -dihydroxycholesterol $\rightarrow$ pregnenolone + isocaproic acid

This and similar pathways of cholesterol oxidation in testis, ovary and placenta are reviewed by Dorfman & Ungar (1965) and by Hayano (1962). This sequence of reactions occurs in the mitochondria and requires molecular oxygen and NADPH

(Halkerston, Eichhorn & Hechter, 1961). Pregnenolone is subsequently oxidized in the appropriate organs via progesterone or 17α -hydroxypregnenolone or both to corticosteroids, androgens and oestrogens. Dorfman and his colleagues have produced evidence both that pregnenolone is an obligatory precursor of steroid hormones (Dorfman, 1959) and that it is not (Burstin & Dorfman, 1962; Gual, Lemus, Kline, Gut & Dorfman, 1962). The first hydroxylation of cholesterol (at C-20) may be rate-determining for the formation of pregnenolone and hence steroid hormone production; ACTH† may be involved in the regulation of pregnenolone formation (Dorfman & Ungar, 1965).

† Abbreviation: ACTH, adrenocorticotrophic hormone.

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Scheme 1. Metabolic pathway of cholesterol to pregnenolone. Broken lines indicate feed-back inhibition.

Koritz & Hall (1964*a,b,c*) have shown that both pregnenolone and 20 α -hydroxycholesterol non-competitively inhibit the oxidation of cholesterol to pregnenolone and isocaproic acid by an enzyme system extracted from an acetone-dried powder of bovine adrenal-cortex mitochondria (Scheme 1). They found that pregnenolone inhibits the production of 20 α -hydroxycholesterol but does not inhibit its metabolism to pregnenolone (Scheme 1). 20 α -Hydroxycholesterol was the more effective inhibitor but no quantitative data were quoted. Accordingly Koritz & Hall propose that pregnenolone production is subject to allosteric control by feed-back inhibition and that ACTH is involved in this mechanism, by controlling the permeability of the mitochondrial membrane to pregnenolone and cofactors (Hirshfield & Koritz, 1965).

About 80–90% of the total cholesterol of adrenals is esterified and both ACTH and 'stress' deplete the adrenal cortex of cholesteryl fatty acyl esters but do not affect the concentration of free cholesterol, suggesting that these esters may be precursors of steroid hormones (Riley, 1963; Griffiths, Grant & Symington, 1963; Goodman, 1965). Enzymes esterifying cholesterol and hydrolysing cholesteryl esters are found in adrenals and in other tissues (Kritchevsky, 1958; Shyamala, Lossow & Chaikoff, 1965).

Cholesteryl 3 β -sulphate has been isolated from bovine adrenals (Drayer, Roberts, Bandi & Lieberman, 1964), from human blood and gallstones (Drayer & Lieberman, 1965), and from several other human tissues (Moser, Moser & Orr, 1966). Wieland, de Courcy, Levy, Zala & Hirschmann (1965) have observed the presence of 17 α -hydroxypregnenolone 3 β -sulphate in human adrenal venous blood. Both cholesteryl sulphate and pregnenolone 3 β -sulphate are oxidized to dehydroepiandrosterone 3 β -sulphate *in vivo* and double-labelling of these esters with ^{14}C and ^{35}S has shown that the sulphate group was retained during oxidation of the steroid side chain (Roberts, Bandi, Calvin, Drucker & Lieberman, 1964; Calvin, Vande Wiele & Lieberman, 1963). Pregnenolone sulphate is hydroxylated *in vitro* at C-17 without hydrolysis of the sulphate group (Calvin & Lieberman, 1964). Endocrine organs metabolize certain steroid sulphates without hydrolysing the sulphate group (Baulieu & Carpechot,

1965; Sandberg, Gurpi & de Lieberman, 1965; Pasqualini, 1965; Baillie, 1965; Baillie & Griffiths, 1965). Enzymes sulphating steroids and hydrolysing steroid sulphates are found in adrenals and other tissues (Burstain & Dorfman, 1963; Wengle, 1964; Adams, 1964; Baulieu & Carpechot, 1965; Payne & Mason, 1965; Nichol & Roy, 1965; Warren & French, 1965).

It thus seems likely that there are alternative pathways of cholesterol metabolism and steroid hormone formation in which the steroids are esterified at C-3.

The object of this investigation was to examine the substrate specificity of the adrenal cholesterol-oxidase system and its specificity towards inhibitors, particularly those possibly concerned in its feed-back control. Enzyme activity was measured by the formation of [^{14}C]isocaproic acid (4-[^{14}C]methylpentanoic acid) from [26- ^{14}C]cholesterol derivatives and by the formation of ^{14}C -labelled C₂₁ steroids from [4- ^{14}C]cholesterol derivatives. Part of this work has been reported in a preliminary communication (Raggatt, Dean & Whitehouse, 1965).

EXPERIMENTAL

Bovine adrenals were kindly supplied by the Oxford Co-operative Wholesale Society Slaughterhouse. NAD and ATP were obtained from the Sigma Chemical Co. (London). Hexokinase (crystalline suspension) was obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Pregnenolone, pregnenolone 3 β -acetate, progesterone, cholesterol, cholesteryl 3 β -acetate and cholesteryl 3 β -oleate were obtained from British Drug Houses Ltd. (Poole, Dorset). Isocaproic acid, butan-2-one, 17 α -hydroxypregnenolone, 3 β -acetoxy-22,23-bisnorchol-5-enoic acid and palmitic acid were obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). 2,5-Diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were obtained from Thorn Electronics (Surrey). 25-Oxo-27-norcholesterol was obtained from the Schering Corp. (Bloomfield, N.J., U.S.A.). Pregn-5-ene-3 β ,20 α -diol was obtained from Schering A.-G. (Berlin, Germany). 3 β -Hydroxychol-5-enoic acid was a Rousel product (M.R.C. Reference Collection). Lithocholic acid and 22,23-bisnorchol-5-enoic acid were obtained from Mann Research Laboratories (New York, N.Y., U.S.A.). 3 β -Hydroxy-aetiocol-5-enoic acid was obtained from Zori Ltd. (Tel Aviv, Israel). A small sample of 20 α -hydroxycholesteryl 3 β -acetate was a gift from Dr V. Petrow (British Drug

Houses Ltd.). Silica H (Merck) was obtained from Camlab Ltd. (Cambridge).

Melting points were determined in thin-walled capillaries in an air bath and are uncorrected.

Steroids were detected after thin-layer chromatography by spraying with 20% (w/v) phosphomolybdic acid (British Drug Houses Ltd.) in ethanol and heating for 3–5 min. at 110°.

[¹⁴C] *Cholesterol*. [26-¹⁴C] Cholesterol (24.0 mc/m-mole) and [4-¹⁴C] cholesterol (34.0 mc/m-mole) were obtained from The Radiochemical Centre (Amersham, Bucks.). They were purified before use by chromatography on thin layers of silica H with ethyl acetate–hexane (3:7, v/v) and with butan-2-one–hexane (1:1, v/v) and suitably diluted with non-radioactive cholesterol (recrystallized from ethanol, m.p. 146–148°) just before use. [¹⁴C] Cholesterol was stored in dilute hexane solution at –15° in the dark.

Sodium cholesteryl 3β-sulphate. This was made by the method of Roy (1956) with pyridine–SO₃ (Sobel, Goodman & Blau, 1951). After recrystallization from butan-2-one–ethanol–water (approx. 5:5:1, by vol.), the product had m.p. 175–176° (Found: C, 66.4; H, 9.2; S, 6.3. C₂₇H₄₅NaO₄S requires C, 66.4; H, 9.2; S, 6.6%) and 50–100 μg. moved as a single spot on chromatography on thin layers of silica H with benzene–butan-2-one–ethanol–water (3:3:3:1, by vol.).

[¹⁴C] *Cholesteryl sulphate*. This was prepared similarly, from [26-¹⁴C]- and [4-¹⁴C]-cholesterol (10 μC, about 0.1 mg.) and a large excess of pyridine–SO₃. After the initial condensation (1½ hr.) the pH of the solution was adjusted to 8.0 with *n*-NaOH, pyridine and solvent were removed *in vacuo* and the residue was dissolved in ethanol–butan-2-one (2:1, v/v) and applied quantitatively as a streak to a 20 cm. × 20 cm. chromatoplate of silica H. After chromatography in butan-2-one, the band corresponding to cholesteryl sulphate (*R_F* 0.0–2) was removed and extracted with ethanol–butan-2-one (2:1, v/v) and rechromatographed with butan-2-one (this procedure removes all unchanged cholesterol and a yellow non-radioactive impurity). The product was further purified by thin-layer chromatography on silica H in benzene–butan-2-one–ethanol–water (3:3:3:1, by vol.), when it moved as a single narrow band (*R_F* 0.46) coincident with authentic non-radioactive cholesteryl sulphate. The overall yield was 20–25%. It was stored as a dry film under N₂ at –15° in the dark and used as soon as possible. Storage in ethanol–butan-2-one solution at –15° resulted in solvolysis; e.g. one 2 μC batch (0.04 mg.) in 5 ml. of ethanol–butan-2-one (1:1, v/v) was almost completely solvolyzed to cholesterol in 3 months (see also McKenna & Norymberski, 1957).

[26-¹⁴C] *Cholesteryl 3β-acetate*. [26-¹⁴C] Cholesterol (10 μC, 0.161 mg.) was dissolved in pyridine (0.5 ml.) and acetic anhydride (0.5 ml.) was added. After standing at 20° in the dark overnight, water (2 ml.) was added and the mixture was warmed at 50° for 5 min. After adjusting the pH to 8.0 with *n*-NaOH the cholesteryl acetate was extracted with benzene (4 × 3 ml.); the benzene solution was washed with *n*-HCl (1 ml.), water (1 ml.), 5% (w/v) NaHCO₃ (1 ml.) and water (1 ml.) and then dried with MgSO₄. The solution was concentrated by evaporation under N₂ at 50° and the product applied as a streak to a 20 cm. × 20 cm. chromatoplate of silica H. After chromatography in isopentyl acetate–hexane (1:9, v/v), the band corresponding to cholesteryl acetate was removed. The product was extracted from the

silica with ethyl acetate and rechromatographed in the same system. It was finally chromatographed on silica H in benzene–ethyl acetate (9:1, v/v). The radioactive product had the same mobility as authentic cholesteryl acetate and moved as a single narrow band on thin-layer chromatography. The overall yield was 81%. It was stored in benzene solution at –15° in the dark.

[26-¹⁴C] *Cholest-4-en-3-one*. This was made by Oppenauer oxidation of cholesterol (Serini, Köster & Strassberger, 1945). The cholesterol (14 μC, 0.322 mg.) was dissolved in dry xylene (0.1 ml.) and aluminium isopropoxide (1 mg.) was added. After heating at 95° for 5 min. dry cyclohexanone (0.1 ml.) was added and the mixture heated at 95° for 4 hr. in the dark. A few drops of water were added and then the solvents were removed *in vacuo*. The residue was extracted with diethyl ether, and applied to a 20 cm. × 20 cm. chromatoplate of silica H. After chromatography with ethyl acetate–hexane (3:7, v/v), the cholestenone band was removed, extracted with ether and rechromatographed in the same system, and finally purified by thin-layer chromatography in ethyl acetate–hexane (1:5, v/v). The radioactive product had exactly the same mobility as authentic non-radioactive cholestenone (prepared both by this method and independently) and in both these systems it moved as a single narrow band. The overall yield was 87%. It was stored in benzene solution at –15° in the dark.

27-*Norcholest-4-ene-3,25-dione*. This was made from 25-oxo-27-norcholesterol also by the Oppenauer oxidation and after crystallization from hexane and from aqueous acetone (yield 75%) had m.p. 129–130°, λ_{max}. 240–243 mμ, ε 1.76 × 10⁴ (authentic cholest-4-en-3-one had λ_{max}. 240–243 mμ and ε 1.69 × 10⁴). Selye (1942) reports the m.p. of this compound (supplied to him by the Schering Corp.) as 114–116°.

Dihydrogen cholesteryl 3β-phosphate. Cholesterol (2 g.) was dissolved in dry redistilled pyridine (10 ml.) and the solution added dropwise to freshly redistilled POCl₃ (2 ml.) in dry acetone (10 ml.) at –40°. The reaction mixture was left at room temperature in the dark overnight. The white precipitate that formed was collected and washed with acetone; this product contained pyridine and on treatment with water gave Cl[–] and H⁺ ions. Crystallization of this intermediate from acetic acid, as recommended by Venner (1960), gave predominantly *cholesteryl 3β-chloride* [m.p. 94–96° (from hexane), *R_F* 0.75 on silica H in the system ethyl acetate–hexane (3:7, v/v) (Found: C, 79.8; H, 11.2; Cl, 8.9. C₂₇H₄₅Cl requires C, 80.0; H, 11.2; Cl, 8.8%]. A small amount of white solid, m.p. 63–65° (from hexane–ethanol), probably dicholesteryl ether [m.p. 74° (Plimmer & Burch, 1929), 65° (Venner, 1960)], was also formed by this acetic acid treatment; little, if any, cholesteryl phosphate was formed. The white intermediate was heated with water (50 ml.) at 95° for 2 hr. and the white flocculent solid that formed was collected and washed with water by centrifugation. After recrystallization from ethanol–benzene mixtures, it softened at 160° and melted rapidly with decomposition at 194–196°; Venner (1960) gives m.p. 196°. Attempts to prepare and purify [¹⁴C]cholesteryl phosphate on a micro scale failed.

Sodium pregnenolone 3β-sulphate. This was made in the same way as cholesteryl sulphate; however, great care was needed to prevent solvolysis during crystallization. The yield was 67% [m.p. 197° (decomp.) (from ethanol–butan-2-one) (Found: C, 59.4; H, 7.6; S, 7.5. C₂₁H₃₁NaO₅S requires

C, 60.0; H, 7.5; S, 7.7%]; 50–100 μ g. of it moved as a single spot on thin-layer chromatography on silica H with benzene–butan-2-one–ethanol–water (3:3:3:1, by vol.).

Sodium 25-oxo-27-norcholesteryl β -sulphate. This was made in the same way as cholesteryl sulphate. The yield was 51% [m.p. 185–190° (decomp.) (Found: C, 58.9; H, 8.6; S, 6.3. $C_{26}H_{42}NaO_5S$ requires C, 59.6; H, 8.5; S, 6.5%)].

Pregnenolone β -palmitate. This was made by the method of Pinter, Hamilton & Muldray (1964). Palmitoyl chloride was made from palmitic acid (0.5 g.) and oxalyl chloride (1.0 ml.) and allowed to react with pregnenolone (0.3 g.) in di-isopropyl ether. The yield was 60% [m.p. 94–95° (from benzene–ethanol) (Found: C, 79.1; H, 11.0. $C_{37}H_{61}O_3$ requires C, 80.1; H, 11.1%]; 50–100 μ g. of it moved as a single spot on thin-layer chromatography on silica H with isopentyl acetate–hexane (1:9, v/v).

20 α -Hydroxycholesterol and 20 α -hydroxycholesteryl β -acetate. These were synthesized by the method of Petrow & Stuart-Webb (1956). The β -acetate had m.p. 156–157° (from methanol) (Found: C, 78.6; H, 10.9. $C_{29}H_{48}O_3$ requires C, 78.4; H, 10.8%) and 50–100 μ g. of it moved as a single spot (R_F 0.6) on thin-layer chromatography on silica H with ethyl acetate–hexane (3:7, v/v), identical with a sample of 20 α -hydroxycholesteryl β -acetate supplied by Dr V. Petrow. The acetate was hydrolysed by methanolic 0.2N-NaOH under N_2 in the dark overnight at room temperature (Bush, 1961). [The 20 α -hydroxycholesterol produced had m.p. 136–137° (Found: C, 80.5; H, 11.6. $C_{27}H_{46}O_2$ requires C, 80.6; H, 11.4%]; 50–100 μ g. of the product moved as a single spot (R_F 0.26) on thin-layer chromatography on silica H with ethyl acetate–hexane (3:7, v/v).]

The following compounds were prepared by Mr P. D. G. Dean of the Department of Biochemistry, University of Oxford.

25-Oxo-27-norcholesteryl β -acetate. This compound (m.p. 139–140°) was prepared by acetylation of 25-oxo-27-norcholesterol with acetic anhydride in pyridine.

25-Hydroxy-27-norcholesterol. This compound (m.p. 160–165°) was prepared by borohydride reduction of 25-oxo-27-norcholesterol β -acetate.

25-Hydroxycholesterol. This compound (m.p. 182–183°) was prepared by the method of Ryer, Gebert & Murrill (1950).

Enzyme preparation. All operations were performed at 0–5°. Whole adrenals were removed from cows (1–3 years old) at the slaughterhouse within 10 min. of death, chilled and transported to the laboratory on ice. The adrenals, when dissected free of fat and connective tissue, weighed 8–12 g. each. The cortex was dissected from the medulla and the cortical tissue scraped off the capsule; 4.5–6.0 g. wet wt. of cortex was obtained from each adrenal. The cortical tissue was suspended in 2.5 ml. of 'supplemented sucrose' (Halkerton *et al.* 1961) per g. of tissue and homogenized in a stainless-steel homogenizer. ['Supplemented sucrose' contains sucrose (0.44M), EDTA (1mM), sodium fumarate (5mM), nicotinamide (5mM) and potassium phosphate buffer (10mM) at pH 7.4.] The homogenate was centrifuged at 1200g for 30 min., the sediment discarded and the resulting supernatant centrifuged at 16000g for 20 min. The 'mitochondrial pellet' so obtained was washed with supplemented sucrose and recentrifuged at 16000g for 20 min. It was resuspended in supplemented sucrose for incubation. To prepare the 'microsomal fraction' the

16000g supernatant was centrifuged at 100000g for 30 min. The fatty layer was removed from the 100000g supernatant before use and the 'microsomal pellet' was resuspended in supplemented sucrose. The various pellets were resuspended in a loose-fitting all-glass homogenizer.

Acetone-dried powders. For the preparation of acetone-dried powders the homogenization and centrifugation were carried out as above in 0.1M-sodium phosphate buffer, pH 7.4. The mitochondrial preparation was poured into 10 vol. of acetone at –10° and agitated with a Waring Blendor. All subsequent operations were carried out at +1°. After standing for 10 min., the powder was filtered and washed with fresh acetone and dried *in vacuo* over $CaCl_2$ and anhydrous glycerol for 2 days. It was then pulverized, redried in the same way and stored over dry silica gel at –15°. Before use, it was homogenized with supplemented sucrose, kept at 0° for 30 min., rehomogenized and centrifuged at 600g for 15 min. The sediment was discarded.

Solubilization of steroids. Steroid solutions were measured out dissolved in a suitable volatile solvent, the solvent removed by evaporation at about 50° under a stream of N_2 and then the substances were dissolved in *NN*-dimethylformamide and 0.1 ml. of this solution was added to each 25 ml. incubation flask. The enzyme preparation (3 ml.) was then added, followed by 2 ml. of a cofactor mixture (see below). When the highest usual amount of cholesterol (0.04 μ mole) together with the highest usual amount of cholesteryl acetate (1 μ mole) (one of the most insoluble steroids used) were dissolved in 0.10 ml. of dimethylformamide and diluted with 3 ml. of supplemented sucrose and 2 ml. of the cofactor mixture (i.e. incubation conditions but without enzyme), a stable translucent suspension was produced that showed no tendency to sediment even after 24 hr. at 37°.

Incubation procedure. Cholesterol and steroids were measured into 25 ml. conical incubation flasks, dissolved in 0.10 ml. of dimethylformamide. Then 3 ml. of the enzyme preparation was added to this followed by 2 ml. of a cofactor mixture at pH 7.4, giving the following final concentrations: $MgCl_2$ (1.5 mM), sodium phosphate buffer (10 mM), disodium ATP (1.25 mM), NAD (1.25 mM) and sodium hydrogen malate (3 mM) (the final incubation volume was 5.1 ml.). This dilution produced final concentrations of the substances in the suspending medium as follows: sucrose (0.26 M), EDTA (0.59 mM), sodium fumarate (3 mM), nicotinamide (3 mM) and potassium phosphate buffer (5.9 mM). In some experiments, centre wells contained 0.2 ml. of N-NaOH. Incubations were for 10–180 min. at 37° in air in a shaking water bath in the dark. Controls contained enzyme denatured either by heat (30 min. in a boiling-water bath) or by acid (0.2 ml. of 18N- H_2SO_4) and converted only 0.03% of the [26- ^{14}C]cholesterol into [^{14}C]isocaproic acid in 20 min.

Extraction of [^{14}C]isocaproic acid from incubation mixtures. Incubations were terminated by adding 0.2 ml. of 18N- H_2SO_4 and cooling to –15°. After thawing, the incubation mixtures were transferred to separate centrifuge tubes and each incubation flask was rinsed with 1 ml. of 5N-NaOH containing 20 mg. of non-radioactive isocaproic acid as carrier; this was added to the appropriate tube. After mixing, the tube contents were acidified with 18N- H_2SO_4 (0.2 ml.), mixed well and centrifuged at 4800g for 10 min. at 10°. The clear supernatants were decanted, the sediments washed with water (1 ml.) and

the washings added to the supernatants. The residual cholesterol was removed from the supernatants by making alkaline with 16N-KOH (0.3 ml.) and extracting with methanol-ether (1:3, v/v) (5 ml.) and with ether (5 ml.). The aqueous layer was acidified with 18N-H₂SO₄ (0.2 ml.) and extracted with ether (3 × 3 ml.) to remove isocaproic acid. The ether extracts were made up to 9.0 ml. with ether and 3 ml. portions removed for liquid-scintillation counting.

Extraction and analysis of ¹⁴C-labelled steroids. After incubation with 4-¹⁴C-labelled steroids, the products were extracted by shaking with successive portions of suitable solvents (butan-1-ol for steroid sulphates, dichloromethane for free steroids) and the combined extract was washed with water and reduced in volume under a jet of N₂. The recovery of radioactivity was 80–90%. Carrier steroids were added and the extract was applied to 20 cm. × 20 cm. thin layers of silica H and chromatographed. The solvent systems were: I, for steroid fatty-acyl esters, isopentyl acetate-hexane (1:9, v/v); II, for free steroids, ethyl acetate-hexane (3:7, v/v); III, for steroid sulphates, benzene-butan-2-one-ethanol-water (3:3:3:1, by vol.). After location of marker compounds, appropriate bands of silica were removed, extracted with suitable solvents and portions of these extracts were assayed for radioactivity. The recovery of radioactive steroids from these thin layers was quantitative (>98%).

Liquid-scintillation counting. Portions (3.0 ml.) of [¹⁴C]isocaproic acid in ether were added to 4.0 ml. of a solution of 7.5 g. of 2,5-diphenyloxazole, 0.15 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 160 g. of naphthalene in 1 l. of dry toluene-dioxan (1:1, v/v).

Other solutions were added to sufficient of a solution of 5 g. of 2,5-diphenyloxazole, 0.1 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 80 g. of naphthalene in 1 l. of dry toluene-dioxan-methanol (5:5:3, by vol.) to give a total volume 7 ml.

Solutions of ¹⁴CO₂ in N-NaOH (0.2 ml.) were added to 7 ml. of a solution of 5 g. of 2,5-diphenyloxazole, 0.1 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 80 g. of naphthalene and 2 g. of Cab-O-Sil (Packard Instrument Co.; finely divided silica) in 1 l. of dry toluene-dioxan-methanol (5:5:3, by vol.) and shaken to produce a transparent gel.

The solutions were counted in an IDL Tritomat liquid-scintillation counter (Isotope Developments Ltd., Reading) for times sufficient to give at least 10⁴ observed counts. Efficiency was about 65% and scintillant-machine background about 1.5 counts/sec. with 7 ml. of scintillant.

Cholesterol content. The cholesterol content of the adrenal mitochondrial preparations was measured by the Lieberman-Burchard method (Cook, 1958). Free sterol (digitonin-precipitable) was in the range 30–65 μg/ml. with cholesterol as a standard.

Protein content. The protein content of the tissue preparations was measured by the biuret method (Gornall, Bardawill & David, 1949). The mitochondrial preparations contained 5–8 mg. of protein/ml. (with bovine serum albumen as a standard) and contained the equivalent of about 0.5 g. of adrenal cortex (wet wt.)/ml.

RESULTS

Substrate specificity of adrenal cholesterol oxidase

The ability of bovine adrenal-cortex mitochondrial preparations to cleave the side chains of [26-¹⁴C]cholesterol, [26-¹⁴C]cholesteryl 3β-sulphate, [26-¹⁴C]cholesteryl 3β-acetate and [26-¹⁴C]cholest-4-en-3-one was determined by measuring the [¹⁴C]isocaproic acid formed from these steroids in 1 hr. or 3 hr. incubations under identical conditions. Cholesterol was most readily oxidized (Table 1).

Apparent *K_m* values for cholesterol and cholesteryl sulphate were determined by measuring the [¹⁴C]isocaproic acid formed after incubation for 0, 10 and 20 min. by using three different concentrations of the 26-¹⁴C-labelled steroids in each experiment. The rate of isocaproic production decreased with time (product inhibition, see below) but was constant within experimental error for about 30 min. The initial rates and apparent *K_m* values were determined graphically (Lineweaver & Burk, 1934), neglecting endogenous substrate. For

Table 1. *Oxidation of cholesterol derivatives by bovine adrenal-cortex mitochondria compared with cholesterol*

Incubation conditions were as described in the text. In any one experiment specific activities and concentrations of various ¹⁴C-labelled substrates were identical.

Substrate	Expt. no.	Duration of incubation (hr.)	Percentage conversion into [¹⁴ C]isocaproic acid		Net percentage conversion compared with cholesterol in same experiment
			Denatured enzyme	'Active' enzyme	
[26- ¹⁴ C]Cholest-4-en-3-one	1	3	0.02	0.96	4.4
	2	3	0.11	2.01	15.0
	3	1	0.02	0.38	6.3
	4	1	0.10	1.28	9.3
[26- ¹⁴ C]Cholesteryl 3β-sulphate	1	1	0.09	2.98	13.5
	2	3	0.43	7.78	56.0
	3	1	0.05	3.75	24.0
[26- ¹⁴ C]Cholesteryl 3β-acetate	1	3	0.09	4.42	48.0
	2	1	0.10	3.70	32.0
	3	1	0.07	3.10	35.0

cholesteryl 3β -sulphate the apparent K_m was $500 \pm 50 \mu\text{M}$. The reported total cholesteryl sulphate content of bovine adrenals is equivalent to a concentration of about $1 \mu\text{M}$ in the incubation mixtures (Drayer *et al.* 1964). For cholesterol and with fresh mitochondrial preparations, the apparent K_m was $7 \pm 5 \mu\text{M}$ when the exogenous cholesterol concentration was in the range $2\text{--}8 \mu\text{M}$, but the apparent K_m was $50 \pm 5 \mu\text{M}$ when the exogenous cholesterol concentration was in the range $20\text{--}100 \mu\text{M}$. With an extract of acetone-dried powders (very little endogenous cholesterol) the apparent K_m for cholesterol was $20 \pm 10 \mu\text{M}$, but in the experiments with acetone-dried powders a variable time-lag of $10\text{--}20$ min. was observed before [^{14}C]isocaproic acid could be detected. Because of this variable time-lag, initial rates of cholesterol oxidation could not be measured as confidently with the acetone-dried powders as with the fresh mitochondrial preparations (no time-lag was observed with fresh mitochondrial preparations). The fresh mitochondrial preparation contained an appreciable amount of endogenous cholesterol (the total cholesterol content was equivalent to a final concentration in the incubation mixtures of $50\text{--}100 \mu\text{M}$). However, the degree of mixing of exogenous and endogenous cholesterol is unknown.

In the experiments in which the amount of exogenous cholesterol added was much less than the total endogenous sterol, a much greater variation in the value of the apparent K_m was observed than in those in which the amount of exogenous cholesterol was of the same order or greater than the total endogenous sterol. This is probably due to variation in the extent to which the added cholesterol was diluted by endogenous cholesterol. Consequently the values of the apparent K_m ($50 \mu\text{M}$) obtained at the high cholesterol concentrations are probably the more reliable and are supported by the values obtained with acetone-dried powders. However, as the entire range of values of apparent K_m for cholesterol ($2\text{--}55 \mu\text{M}$) was much smaller than the apparent K_m for cholesteryl sulphate ($500 \mu\text{M}$), it is clear that unesterified cholesterol is the preferred but not the only possible substrate.

Hydrolysis and oxidation of cholesteryl esters by adrenal mitochondrial preparations

[$4\text{--}^{14}\text{C}$]Cholesteryl 3β -acetate. This was incubated for 1 hr. with the mitochondrial preparation under the standard conditions, the products were extracted and chromatographed in system II and zones of the thin-layer chromatogram corresponding to marker compounds were assayed for radioactivity. In 1 hr. 50% of the cholesteryl acetate was converted into free sterols of similar mobility to cholesterol and 2.6% into more polar non-esterified

sterols. In parallel incubations with heat-denatured enzyme only 0.6% of the initial radioactive cholesteryl acetate was converted into free cholesterol and 97.5% was recovered as unchanged cholesteryl acetate; i.e. the adrenal-cortex mitochondrial preparation contains a cholesterol esterase. In parallel incubations with [$26\text{--}^{14}\text{C}$]cholesteryl acetate and active enzyme, 3.1% of the cholesteryl acetate was converted into [^{14}C]isocaproic acid.

A portion of the material extracted from the 'steroid acetate' zone was rechromatographed in system I. Only 0.5% of the initial radioactivity was in the pregnenolone 3β -acetate zone; after chromatography of the products of parallel incubations with denatured enzyme 0.4% of the initial radioactivity was in the pregnenolone acetate zone. Of the radioactive esterified sterols 97.3% was unchanged cholesteryl acetate. This indicates that cholesteryl acetate is not oxidized directly to pregnenolone acetate.

[$4\text{--}^{14}\text{C}$]Cholesteryl 3β -sulphate. This was incubated for 1 hr. with the mitochondrial preparation under the standard conditions (i.e. in the presence of inorganic phosphate, which inhibits steroid sulphatase; Roy, 1957) and the products were extracted, chromatographed in system III and assayed for radioactivity. Only 0.12% of the total activity was recovered as free sterols; in parallel incubations with heat-denatured enzyme 0.3% of the total activity was recovered as free sterols. Of the initial activity 98.1% was recovered as sterol sulphates, i.e. the adrenal-cortex mitochondrial preparation does not contain a cholesterol sulphatase active under these conditions.

A second part of the extract was treated with 5% (w/v) trichloroacetic acid in dry dioxan for 24 hr. at room temperature in the dark (Anderson, Briggs & Haslewood, 1964). Ethyl acetate was added and the mixture extracted with *N*-sodium hydroxide and with water. Separate control experiments established that this technique quantitatively solvolysed steroid sulphates without detectable side reactions. When the solvolysed product was chromatographed in system II, it was found that 6.7% of the total activity corresponded to pregnenolone and other more polar steroids; the rest was free cholesterol. Thus cholesteryl sulphate is converted into more polar steroid sulphates *in vitro*. Parallel incubations with heat-denatured enzyme, similarly extracted, solvolysed and chromatographed gave only 0.23% of the total activity outside the cholesterol zone. In parallel incubations with [$26\text{--}^{14}\text{C}$]cholesteryl sulphate the yield of [^{14}C]isocaproic acid was 7.5%.

Inhibitor specificity of cholesterol oxidase

Competitive inhibition by cholesteryl esters. The following cholesteryl esters at $180 \mu\text{M}$ inhibited the

formation of [^{14}C]isocaproic acid from [$^{26-14}\text{C}$]cholesterol ($15.5\mu\text{M}$) by 20–40%, in order of decreasing effectiveness: cholesteryl 3β -phosphate, cholesteryl 3β -acetate, cholesteryl 3β -sulphate, cholesteryl 3β -oleate. Cholesteryl 3β -chloride was without effect. Sodium lauryl sulphate at similar concentrations had no effect and therefore the effect of the sulphate and phosphate esters is probably not an unspecific detergent effect (also see the lack of effect of pregnenolone sulphate below).

Apparent K_i values were determined for some of these esters by measuring the yield of [^{14}C]isocaproic acid after 0, 10 and 20 min. incubations, at four different inhibitor concentrations and three different cholesterol concentrations in each experiment. Initial rates were measured and the apparent K_i and the nature of the inhibition found by the graphical method of Dixon (1953). A typical plot, as obtained with these cholesteryl esters, is shown in Fig. 1. In all cases the inhibition was competitive in character and the following K_i values were obtained: for cholesteryl 3β -sulphate, $110 \pm 10\mu\text{M}$; for cholesteryl phosphate, $28 \pm 5\mu\text{M}$; for cholesteryl acetate, $65 \pm 15\mu\text{M}$.

Inhibition by end products ('negative feed-back effect'). Both pregnenolone and 20α -hydroxycholesterol inhibited the production of [^{14}C]isocaproic acid from [$^{26-14}\text{C}$]cholesterol non-competitively; 20α -hydroxycholesterol was the more effective with an apparent K_i $17 \pm 5\mu\text{M}$, compared with $130 \pm 10\mu\text{M}$ for pregnenolone. A typical Dixon

Fig. 1. Competitive inhibition of cholesterol oxidation by cholesteryl esters (cholesteryl 3β -acetate, $K_i 65\mu\text{M}$). Substrate concentrations were: ●, $2.39\mu\text{M}$; ▲, $4.66\mu\text{M}$; ■, $7.68\mu\text{M}$. [I] is the concentration of added cholesteryl ester and v the initial rate of isocaproic acid formation.

Fig. 2. Non-competitive (feed-back) inhibition of cholesterol oxidation by its metabolites (pregnenolone, $K_i 130\mu\text{M}$). Substrate concentrations were: ●, $0.82\mu\text{M}$; ▲, $1.91\mu\text{M}$; ■, $2.93\mu\text{M}$. [I] is the concentration of added metabolite (pregnenolone) and v the initial rate of isocaproic acid formation.

plot for the effect of pregnenolone is shown in Fig. 2; it clearly indicates the non-competitive character of pregnenolone inhibition, in contrast with that of cholesteryl esters (Fig. 1).

Pregnenolone 3β -esters (acetate, sulphate and palmitate) at $190\mu\text{M}$ did not inhibit cholesterol oxidation significantly ($< 5\%$). 20α -Hydroxycholesteryl 3β -acetate caused only 25% inhibition at a concentration of $100\mu\text{M}$, but this effect is probably due to hydrolysis of the ester by the esterase present that can hydrolyse cholesteryl 3β -acetate (see above).

Inhibition by other Δ^5 - 3β -hydroxy steroids. Table 2 shows that other sterols also inhibited the enzymic oxidation and cleavage of the cholesterol side chain, the most potent being 25-oxo-27-norcholesterol. The apparent K_i for this compound was $16 \pm 4\mu\text{M}$ and the kinetic studies showed that the inhibition was non-competitive in character (for 20α -hydroxycholesterol, $K_i 17 \pm 5\mu\text{M}$, non-competitive). In Table 2, 20α -hydroxycholesterol appears to be less effective as an inhibitor than 25-oxo-27-norcholesterol but it is a substrate for the side-chain-cleaving enzyme, i.e. is metabolized. [The K_i values were determined from initial rates in short-time (≤ 20 min.) incubations whereas Table 2 shows the results of 1 hr. incubations.]

Apparent stimulation of cholesterol oxidation by steroid carboxylic acids. When [$^{26-14}\text{C}$]cholesterol was incubated for 1 hr. with adrenal-cortex mitochondrial preparations in the presence of certain steroid acids at concentrations of $100\mu\text{M}$, the yield of [^{14}C]isocaproic acid was consistently greater than that in parallel control incubations in the absence of

Table 2. *Inhibition of cholesterol oxidation by Δ^5 - 3β -hydroxy steroids*

Incubations were for 1 hr. under the conditions described in the text. All the added steroids were at a concentration of 100 μ M. Results are the averages of two separate experiments, each in duplicate.

Steroid added	Percentage inhibition of [14 C]isocaproic acid formation from [26- 14 C]cholesterol
3β -Hydroxypregn-5-en-20-one (pregnenolone)	19
Pregn-5-ene- 3β ,20 α -diol	24
3β ,17 α -Dihydroxypregn-5-en-20-one (17 α -hydroxypregnenolone)	20
Pregn-4-ene-3,20-dione (progesterone)	18
20 α -Hydroxycholesterol	34
20 α -Hydroxycholesteryl 3β -acetate	25
25-Oxo-27-norcholesterol	70
25-Oxo-27-norcholesteryl 3β -acetate	54
25-Oxo-27-norcholesteryl 3β -sulphate	8
25-Hydroxy-27-norcholesterol	3
27-Norcholest-4-ene-3,25-dione	11
25-Hydroxycholesterol	33

these acids. 3β -Hydroxychol-5-enoic acid (100 μ M) increased isocaproic acid formation by approx. 40% over the controls. Lithocholic acid (3 α -hydroxy-5 β -cholic acid) and 3β -hydroxy-22,23-bisnorchol-5-enoic acid also stimulated isocaproic acid production but to a smaller extent; 3β -acetoxy-22,23-bisnorchol-5-enoic acid and 3β -hydroxyaetiochol-5-enoic acid had no effect on cholesterol oxidation *in vitro* at concentrations of 100 μ M.

Inhibition of cholesteryl sulphate oxidation. Pregnenolone, 20 α -hydroxycholesterol and 25-oxo-27-norcholesterol inhibited oxidation of [26- 14 C]-cholesteryl sulphate to [14 C]isocaproic acid by the mitochondrial preparations in the presence of inorganic phosphate (to inhibit sulphatase) but pregnenolone sulphate and 25-oxo-27-norcholesteryl sulphate did not (Table 3). This suggests that cholesterol oxidase is not two enzyme systems, one for free cholesterol and one for cholesteryl sulphate, but one, as the inhibitor specificity was the same when free cholesterol was the substrate.

Oxidation of isocaproic acid by adrenal mitochondrial preparations

[26- 14 C]Cholesterol was incubated with adrenal-cortex mitochondria under the standard conditions in stoppered flasks with centre wells containing 0.2 ml. of N-sodium hydroxide. After 3 hr. 0.2 ml. of 18N-sulphuric acid was added to the incubation mixtures and the flasks were restoppered and re-

Table 3. *Inhibition of cholesteryl sulphate oxidation by Δ^5 - 3β -hydroxy steroids*

Incubations were for 1 hr. under the conditions described in the text. All added steroids were at a concentration of 150 μ M. Results are the averages of two separate experiments, each in duplicate.

Steroid added	Inhibition of [14 C]isocaproic acid formation from [26- 14 C]cholesteryl sulphate
Pregnenolone	36
Pregnenolone 3β -sulphate	0
20 α -Hydroxycholesterol	65
25-Oxo-27-norcholesterol	62
25-Oxo-27-norcholesteryl 3β -sulphate	3

incubated for 15 min. at 37°. Only 0.1% of the [26- 14 C]cholesterol was converted into $^{14}\text{CO}_2$ compared with 0.01% in parallel incubations with heat-denatured enzyme. In the same incubations 20.1% of the [26- 14 C]cholesterol was converted into [5- 14 C]isocaproic acid (compared with 0.03% by denatured enzyme).

When [1- 14 C]isocaproic acid was incubated for 1 hr. with the adrenal-cortex mitochondria under the usual conditions, 1.32% was converted into $^{14}\text{CO}_2$ (compared with 0.12% by denatured enzyme). In parallel incubations in the presence of 50 μ M-2,4-dinitrophenol (to inhibit fatty acid activation), 1.30% of the [1- 14 C]isocaproic acid was converted into $^{14}\text{CO}_2$. These experiments show that isocaproic acid is not readily metabolized under the conditions used and that measurements of isocaproic acid production do provide an index of cholesterol side-chain cleavage.

Effect of ATP, inorganic phosphate and inorganic sulphate on adrenal-cortex mitochondrial preparations

Omission of ATP or depletion of ATP by addition of hexokinase (0.1 mg.) and glucose (10 mm) caused some stimulation (12%) of [26- 14 C]-cholesterol oxidation to [14 C]isocaproic acid in the phosphate-rich medium. Addition of sodium phosphate buffer, pH 7.4, to mitochondrial preparations prepared in a phosphate-free medium caused slight stimulation (10%) of [26- 14 C]cholesterol oxidation to [14 C]isocaproic acid. Inorganic sulphate (10 mm) had no effect on oxidation of [26- 14 C]cholesterol or [26- 14 C]cholesteryl sulphate to [14 C]isocaproic acid.

These findings support the other evidence that phosphorylation or sulphation of cholesterol is not normally an obligatory step in the oxidation of free cholesterol by the adrenal-cortex mitochondria.

Intracellular distribution of cholesterol sulphatase

Subcellular fractions were prepared from adrenal cortex by homogenization, centrifugation, resuspension and incubation in a medium containing the standard substances at standard concentrations (described above) but with phosphate replaced by tris-hydrochloric acid at the same concentration. The 'mitochondrial fraction' was the usual 1200–16000g precipitate, the 'microsomal fraction' was the 16000–100000g precipitate and the 'supernatant' the 100000g supernatant. Each of these fractions was incubated in duplicate with [4-¹⁴C]-cholesteryl sulphate alone and separately with added sodium phosphate buffer, pH 7.4, to give a concentration of 10mM; heat-denatured controls for each fraction were also incubated. After 1 hr. at 37°, the incubation mixtures were transferred to stoppered tubes, the pH was adjusted to 9 and the tubes were extracted once with 3ml. of hexane-ethanol (9:1, v/v) and then twice with 3ml. of ether. Next 2mg. each of cholesterol and cholesteryl sulphate were added as carrier and the extracts were evaporated to dryness under a stream of nitrogen. The residue was dissolved in 2ml. of acetone-ethanol (3:1, v/v) and 3ml. of 0.5% digitonin in aq. 50% (v/v) ethanol was added (approx. threefold excess of digitonin). After mixing and standing overnight at room temperature in the dark, the digitonide was collected by centrifugation, washed with three separate portions of acetone-ethanol (1:1, v/v), dissolved in hot methanol-water (9:1, v/v) and quantitatively transferred to glass vials for counting. The radioactivity in the digitonide was taken as a measure of the production of free 3 β -hydroxy sterols from cholesteryl 3 β -sulphate, i.e. cholesteryl sulphate-sulphatase activity. The results were calculated per mg. of protein (biuret method).

The mitochondrial fraction showed some sulphatase activity but inorganic phosphate at 10mM completely inhibited the sulphatase. The microsomal fraction contained the most sulphatase activity and the supernatant contained no sulphatase activity (Table 4).

DISCUSSION

Substrate specificity of the adrenal-cortex cholesterol oxidase

These experiments show that cholesteryl sulphate is an alternative substrate for the adrenal cholesterol oxidase *in vitro*, in agreement with the experiments *in vivo* of Roberts *et al.* (1964) that demonstrated the production of [³H]pregnenolone [³⁵S]sulphate from [³H]cholesteryl [³⁵S]sulphate without change in the ³H/³⁵S ratio, i.e. without hydrolysis of the sulphate ester linkage. The products of cholesteryl sulphate oxidation *in vitro* were identified, though not rigorously, as pregnenolone sulphate and isocaproic acid. The yield (6.7%) of [¹⁴C]pregnenolone sulphate from [4-¹⁴C]cholesteryl sulphate was similar to the yield (7.5%) of [¹⁴C]isocaproic acid from [26-¹⁴C]cholesteryl sulphate of approximately the same specific activity, indicating that the pathway of cholesteryl sulphate oxidation is similar to the pathway of cholesterol oxidation. Evidence was obtained that this pathway of cholesteryl sulphate catabolism *in vitro* occurred without hydrolysis of the sulphate ester. No free [¹⁴C]-pregnenolone or other free [¹⁴C]steroids were detected amongst the products after incubating [4-¹⁴C]cholesteryl sulphate with the mitochondrial preparations, but after chemical hydrolysis of these products [¹⁴C]pregnenolone was detected.

The incubation medium normally used in these experiments contained inorganic phosphate, which

Table 4. *Cholesteryl sulphate sulphatase in subcellular fractions of adrenal cortex*

The subcellular fractions were prepared by the standard procedure (see the text) but with phosphate replaced by tris-HCl. The Table shows the production of digitonin-precipitable [¹⁴C]steroids from [4-¹⁴C]cholesteryl 3 β -sulphate in one of three similar experiments.

Subcellular fraction	Free cholesterol formed from cholesteryl sulphate	
	Counts/sec./mg. of protein	Percentage conversion
Mitochondrial fraction	2.6	0.9
Mitochondrial fraction + phosphate (10 mM)	2.0	0.7
Heat-denatured mitochondrial fraction	1.9	0.6
Microsomal fraction	6.8	2.3
Microsomal fraction + phosphate (10 mM)	2.3	0.8
Heat-denatured microsomal fraction	2.1	0.7
Supernatant fraction	0.94	0.3
Supernatant fraction + phosphate (10 mM)	1.0	0.3
Heat-denatured supernatant fraction	0.91	0.3

inhibits steroid sulphatases (Roy, 1957). In experiments with a phosphate-free medium, the mitochondrial fraction hydrolysed little cholesteryl sulphate and most of the cholesterol-sulphatase activity was in the microsomal fraction. In the normal phosphate medium no sulphatase activity was detected. Burstein & Dorfman (1963) reported the presence of steroid sulphatase in bovine adrenals but did not specify its intracellular location. These authors stated that the steroid sulphatases of liver and testis were associated with the microsomes.

The affinity of the cholesterol oxidase for cholesteryl sulphate appears to be much less than its affinity for free cholesterol and the apparent K_m for cholesteryl sulphate is about ten times that for free cholesterol. Admittedly, it is doubtful how significant are the absolute values of K_m obtained in these experiments with fresh particulate enzyme preparations that contain variable amounts of endogenous steroids and in which steroids may be separated in different functional compartments although they are supported by experiments with acetone-dried powders. Another uncertainty is the extent of mixing of the dispersed exogenous radioactive substrate with the endogenous substrate. (Therefore we refer to the kinetic constants as 'apparent' K_m and K_i values.)

However, we believe that a comparison of apparent K_m values for cholesterol and cholesteryl sulphate, obtained under identical conditions, does provide a measure of their relative efficiencies as substrates that is not available from simple incubations. Moreover, such experiments based on initial-rate measurements largely eliminate interference by the end-product inhibition to which this system is subject.

Further evidence that cholesteryl sulphate is an alternative substrate but not an intermediate in the oxidation of free cholesterol is provided by the competitive inhibition of cholesterol oxidation by cholesteryl sulphate. 20α -Hydroxycholesterol, a known intermediate in cholesterol oxidation, inhibited cholesterol oxidation non-competitively. This evidence, together with the observations of Lieberman's group that pregnenolone sulphate is oxidized both *in vivo* and *in vitro* to dehydroepiandrosterone sulphate without hydrolysis of the sulphate ester (Calvin *et al.* 1963; Calvin & Lieberman, 1964), suggests that there is a distinct pathway of steroid hormone formation in which the steroids are sulphated at C-3 while undergoing oxidation or hydroxylation elsewhere in the molecule. That one enzyme system is responsible for cleavage of the side chain of both cholesterol and cholesteryl sulphate is also suggested by the similar effects of a number of inhibitors (e.g. 20α -hydroxycholesterol) on oxidation of the two substrates.

The significance of this alternative pathway is at present uncertain. Calvin *et al.* (1963) suggested that steroid sulphates may serve as a steroid 'reservoir' from which free steroids may be released by steroid sulphatases when required. It is noteworthy that androgens, especially dehydroepiandrosterone sulphate, are reported as the main products of the metabolism of both cholesteryl sulphate and pregnenolone sulphate *in vivo*. Vande Wiele, MacDonald, Gupide & Lieberman (1963) have shown that the rate of secretion of dehydroepiandrosterone sulphate is considerably greater than the rate of secretion of free dehydroepiandrosterone. Baulieu & Carpechot (1965) have emphasized the quantitative importance of the metabolism of dehydroepiandrosterone sulphate into other steroid sulphates without hydrolysis. Thus it may be that the 'sulphate pathway' is of especial importance in the biosynthesis and metabolism of androgens.

We could obtain no definite evidence that cholesteryl phosphate could serve as a substrate for the adrenal cholesterol oxidase. Although cholesteryl phosphate inhibited cholesterol oxidation competitively, depleting the system of ATP did not affect the formation of isocaproic acid from cholesterol and attempts to isolate a labelled polar intermediate, e.g. cholesteryl phosphate, were unsuccessful.

Cholest-4-en-3-one was a poor substrate for the adrenal cholesterol oxidase. The adrenal cortex contains Δ^5 - 3β -hydroxy steroid dehydrogenase but Kowal, Forchielli & Dorfman (1964) have reported that this does not oxidize cholesterol. Shimizu (1964) has reported a very low rate of conversion of 20α -hydroxycholestenone into progesterone (0.73% in 2 hr.). Although it might be expected that some cholestenone would be converted into cholesterol and then metabolized, it is clear that this is not an important pathway. Cholestenone is, however, a good substrate for the hepatic cholesterol oxidase involved in the production of bile acids (Stevenson & Staple, 1962; Raggatt *et al.* 1965).

Cholesteryl acetate was metabolized by adrenal mitochondrial preparations but further experiments showed that it was probably hydrolysed to free cholesterol before the side chain was cleaved. No pregnenolone esters were detected and pregnenolone esters did not affect cholesterol oxidation. However, competitive inhibition of cholesterol oxidation by cholesteryl esters was observed; this may have been due to dilution of the radioactive cholesterol with non-radioactive cholesterol formed by rapid hydrolysis of the esters. Shyamala *et al.* (1965) reported that cholesterol esterase (hydrolase) of bovine adrenal is particulate. Thus it seems unlikely that steroid hormones are formed directly from cholesterol esters without prior hydrolysis to free cholesterol.

Inhibitor specificity of adrenal-cortex cholesterol oxidase

These kinetic studies of the non-competitive inhibition of adrenal cholesterol oxidation by its metabolites (20 α -hydroxycholesterol and pregnenolone) confirm the work of Koritz & Hall (1964*a,b,c*), who used an enzyme system extracted from acetone-dried powders and a different assay method, and agree with some related observations of Kahnt & Neher (1965). The inhibitory effect of other C₂₁ steroids suggests that the feed-back control is relatively unspecific. Removal of these inhibitory Δ^5 - β -hydroxy steroids from the mitochondria to the microsomes for β -dehydrogenation may perhaps be complementary to the feed-back inhibition in regulating cholesterol oxidation (Koritz & Hall, 1964*a*).

25-Oxo-27-norcholesterol was a more effective inhibitor of cholesterol oxidation than pregnenolone and like pregnenolone it inhibited non-competitively, suggesting that it mimics pregnenolone or some other intermediate and acts as a 'false' feed-back inhibitor. There is no evidence as yet that 25-oxygen derivatives of cholesterol are formed as intermediary metabolites in steroid hormone production, so 25-oxo-27-norcholesterol must be regarded as an effective drug, rather than as a natural intermediate or regulator, for regulating cholesterol oxidation *in vitro*. The experiments with derivatives of 25-oxo-27-norcholesterol suggest that both the β -hydroxyl and 25-ketone groups are necessary for the inhibitory effect. This compound may be so effective because it is a more lipophilic analogue of pregnenolone. The importance of lipophilic character in determining pharmacological activity among compounds with the requisite chemical groupings has been well documented (see Whitehouse, 1964; Parker, 1965; Sexton, 1963).

Although cholesterol oxidation is subject to feed-back inhibition by its products (e.g. pregnenolone) it is not inhibited by pregnenolone sulphate. However, oxidation of cholesteryl sulphate to pregnenolone sulphate is also inhibited by 20 α -hydroxycholesterol and pregnenolone but not by pregnenolone sulphate. Thus it seems that the 'sulphate pathway' is not subject to feed-back control by its products but is regulated, at least partly, by the concentration of free pregnenolone and other steroids. The production of androgen sulphates from cholesteryl sulphate (without hydrolysis) may thus be integrated with the production of unesterified hormonal steroids in the adrenal cortex.

It seems that the β -hydroxyl group is essential for inhibitory activity both in natural regulators (e.g. pregnenolone) and in 'false' regulators (e.g. 25-oxo-27-norcholesterol) and that inhibitor speci-

ficity is similar whether cholesterol or cholesteryl sulphate is the substrate. Thus the site of binding of regulators may be different from the substrate-binding site. Koritz & Hall (1964*a*) have called this 'allosteric' control, although the use of this term may be disputed.

We are grateful to Mr P. G. D. Dean and Dr V. Petrow for the gift of steroids, to Mr R. L. J. Gardener for technical assistance and to the Medical Research Council and Merck, Sharpe and Dohme Ltd. for financial support (to P.R.R.).

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