Subcellular Localization and Properties of Mouse Adrenal C₁₉-Steroid 5 β -Reductase

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The localization and some characteristics of mouse adrenal C₁₉-steroid 5 β -reductase were determined by the incubation of subcellular fractions of mouse adrenal tissue with [7 α -³H]androst-4-ene-3,17-dione. This enzyme was present only in the soluble fraction and was NADPH-dependent, although a small activity in the presence of NADH was also detected. The soluble fraction also contained 3 α -, 3 β - and a small amount of 17 β -hydroxy steroid dehydrogenase. These and other steroid-metabolizing enzymes present in the remaining subcellular fractions are also described briefly. To measure 5 β -androstane-3,17-dione production by the mouse adrenal soluble fraction, all 5 β products first had to be oxidized to 5 β -androstane-3,17-dione, and the recovery of radioactivity between the substrate androst-4-ene-3,17-dione and product 5 β -androstane-3,17-dione of $96.1 \pm 3.2\%$ validated this technique. C₁₉-steroid 5 β -reductase has a pH optimum of 6.5 and at low substrate concentrations the K_m and V_{max} for 5 β reduction of [7 α -³H]androst-4-ene-3,17-dione was $2.22 \times 10^{-6} \pm 0.48 \times 10^{-6}$ M and 450 ± 53 pmol/min per mg of protein respectively. At high substrate concentration, inhibition of the reaction occurred, which was shown to be due to increasing product concentration.

Studies *in vitro* on the mouse adrenal gland have shown that it principally forms 5 β -reduced steroids from dehydroepiandrosterone (3 β -hydroxyandrost-5-en-17-one) (Maynard & Cameron, 1972), but the presence of a C₁₉-steroid 5 β -reductase enzyme in this tissue cannot be readily explained. The C₁₉ 5 β -reduced steroids appear to have little or no androgenic activity (Segaloff & Gabbard, 1962) and it is possible that the C₁₉-steroid 5 β -reductase is part of a control mechanism within the gland regulating the amount of androgenic material present. The guinea-pig adrenal also possesses a steroid 5 β -reductase activity localized in the 105 000 g_{av} . supernatant, which is capable of metabolizing C₁₉ and C₂₁ steroids (Brown-Grant *et al.*, 1960).

The current investigation was concerned with the elucidation of some of the characteristics of mouse adrenal C₁₉-steroid 5 β -reductase and the establishment of a method for its measurement. The localization of a number of other steroid-metabolizing enzymes was also determined.

Materials and Methods

Materials

Animals. Mice of the Balb/c strain were obtained from The Laboratory Animals Centre, MRC

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Laboratories, Carshalton, Surrey, U.K., and bred and maintained conventionally.

Reagents. All reagents, including ethanol, methanol and acetone, were of analytical grade. Other solvents were of laboratory grade and were redistilled before use. Acetone used in the steroid oxidation procedure was further purified by refluxing with successive small quantities of KMnO₄ until the violet colour persisted. It was then dried with anhydrous K₂CO₃, filtered and redistilled.

Kieselgel HF_{254/366} nach Stahl (Typ 60) was obtained from Anderman and Co. London S.E.1, U.K. ATP, NADPH, NADH and bovine serum albumin were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. 2,5-Diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and NaBH₄, Tris (Aristar grade) and Suprasorb (80–100 mesh) were from BDH, Poole, Dorset, U.K. OV-1 is a product of Supelco Inc., Bellefonte, Pa., U.S.A.

Radioactive substrate. [7 α -³H]Androstenedione (androst-4-ene-3,17-dione; specific radioactivity, 8.3 Ci/mmol and 4.2 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Radiochemical purity of the steroids was checked by reverse isotope dilution by using t.l.c. on silica gel (Kieselgel HF_{254/366}) in a number of systems before and after derivative formation.

Methods

Preparation of subcellular fractions of mouse adrenal tissue. All preparative procedures were carried out at 0–4°C. Mice were killed by cervical dislocation and the adrenal glands removed and placed in a Petri dish on ice. A 10% (w/v) homogenate in 0.25 M-sucrose containing 3 mM-MgCl₂ was prepared in a glass homogenizer with steel pestle (Philpot & Stanier, 1956). Subcellular fractions were obtained by differential centrifugation carried out in a Beckman L65B preparative ultracentrifuge with either the SW 50 (3 × 5 ml) swinging-bucket rotor ($r_{av.}$ = 7.3 cm) or the SW 50.1 (6 × 5 ml) swinging bucket rotor ($r_{av.}$ = 8.35 cm). The homogenate was centrifuged at 700 $g_{av.}$ for 10 min to sediment nuclei and whole cells. The supernatant was re-centrifuged at 27 000 $g_{av.}$ for 20 min to give a mitochondrial pellet and a further supernatant, which was separated by centrifugation at 105 000 $g_{av.}$ for 1 h into the particulate pellet and the soluble fraction. The mitochondrial and particulate pellets were washed by resuspension and re-centrifugation. The nuclear fraction was purified further by gently resuspending the pellet in 0.25 M-sucrose containing 3 mM-MgCl₂ (1.1 ml) and layering on a discontinuous gradient of 0.88 M-sucrose (1.1 ml), 1.80 M-sucrose (1.1 ml) and 2.2 M-sucrose (1.6 ml) solution, all three containing 3 mM-MgCl₂. The gradient was centrifuged at 70 000 $g_{av.}$ for 1 h. Protein was determined in each subcellular fraction by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Incubation conditions. The incubation medium was similar to that of Griffiths & Glick (1966). Equal volumes of subcellular fraction and incubation medium were mixed. The concentration of incubation components, after mixing, were: Tris-HCl buffer, pH 7.4, 43.02 mM; KCl, 42.82 mM; MgSO₄, 3.16 mM; ATP, 0.94 mM; NADPH or NADH were added as indicated.

The radioactive precursor dissolved in ethanol was placed in the incubation vessel together with propylene glycol (0.1 ml) and the ethanol evaporated in a stream of air. Incubation medium was added to the vessel and allowed to reach 37°C. When the subcellular fraction had similarly attained 37°C this was also added and the reaction allowed to proceed in a shaking water bath. The reaction was terminated by the addition of ethanol-acetone (1:1, v/v) (5 ml) containing carrier steroids as indicated.

Extraction of steroids. The incubation vessel was centrifuged to sediment precipitated material and the supernatant layer removed. The precipitate was washed with a further 5 ml of ethanol-acetone (1:1, v/v) and re-centrifuged. The combined extracts were evaporated to dryness in a stream of air and the residue was partitioned between water (2 ml) and diethyl ether (2 × 5 ml). After evaporation of the

combined ether extracts, the residue was dissolved in ethanol in preparation for t.l.c.

T.l.c. of steroids. Identification and purification of steroids before determination was done by t.l.c. Compounds were chromatographed on Kieselgel HF_{254/366} in the following solvent systems: I, chloroform-acetone (37:3, v/v); II, cyclohexane-ethyl acetate (1:1, v/v); III, cyclohexane-ethyl acetate (11:9, v/v); IV, cyclohexane-ethyl acetate (7:3, v/v); V, cyclohexane-ethyl acetate (4:1, v/v); VI, chloroform-methanol (19:1, v/v); VII, cyclohexane-chloroform-acetone-diethyl ether (140:10:1:50, by vol.).

Derivative formation and steroid measurement. Derivatives were prepared by oxidation, reduction and acetylation as described by Griffiths *et al.* (1963), except for the hydrolysis of C₁₉-steroid acetates, where the method of Pierrepont *et al.* (1969) was used.

The measurement of Δ^4 -3-oxo steroids was carried out by determining their absorption at 240 nm on a Zeiss spectrophotometer PMQ II. The Δ^5 -3 β -hydroxy steroids and ring-A reduced steroids were determined on a Pye 104 gas chromatograph with flame-ionization detection. The column was of glass (150 cm × 0.4 cm) and packed with 3% OV-1 on Supasorb (80–100 mesh). Internal standardization was used for steroid calibration on the g.l.c. system.

Radioactivity was measured in a Nuclear-Chicago mark I liquid-scintillation spectrometer. A portion of the steroid in ethanol was placed in a counting vial and the ethanol evaporated in a stream of air. The residue was dissolved in scintillation fluid (5 ml) containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene. Counting efficiency determined by counting [³H]toluene standard solutions of known radioactivity prepared in scintillation fluid, was 48% for ³H in samples counted under single isotope-counting conditions. The identity and radiochemical purity of a steroid was judged to be proved if the steroid and its derivatives have the same mobilities as authentic steroids chromatographed in several solvent systems, and if the specific radioactivities differed by less than 10%.

Results

Localization of steroid-metabolizing enzymes

To determine the subcellular location of C₁₉-steroid-metabolizing enzymes and their cofactor requirement, subcellular fractions of mouse adrenal gland were incubated with [7 α -³H]androstenedione in the presence of either NADPH or NADH. A control series containing neither cofactor was also set up.

Adrenal tissue (0.6 g) from female Balb/c-mice was homogenized and subcellular fractions were

obtained as described above. The fractions were made up to the following volumes (so that the protein content of each would be similar): nuclear, 3.0 ml; mitochondrial, 10.0 ml; particulate, 3.0 ml; supernatant, 25.0 ml. A portion of the whole homogenate (0.2 ml) was made up to 3.0 ml.

A portion (0.5 ml) of each sample was incubated with 2.1 μ Ci of [7α - 3 H]androstenedione (specific radioactivity, 8.3 Ci/mmol) for 2 h. A portion (100 μ g) of NADPH or NADH was added to each incubation flask per hour. When the reaction was terminated, the following carrier steroids (200 μ g of each) were added to each incubation mixture: androstene-3,17-dione, testosterone, 5 β -dihydrotestosterone (17 β -hydroxy-5 β -androstan-3-one), 5 α -dihydrotestosterone (17 β -hydroxy-5 α -androstan-3-one), 5 β -androstanedione (5 β -androstan-3,17-dione), 5 α -androstanedione (5 α -androstan-3,17-dione), aetiocholanolone (3 α -hydroxy-5 β -androstan-17-one), 3-epiaetiocholanolone (3 β -hydroxy-5 β -androstan-17-one), androsterone (3 α -hydroxy-5 α -androstan-17-one), 3-epiandrosterone (3 β -hydroxy-5 α -androstan-17-one), 5 α -androstan-3 α ,17 β -diol, 5 α -androstan-3 β ,17 β -diol, 5 β -androstan-3 α ,17 β -diol and 11 β -hydroxyandrostenedione (11 β -hydroxyandrost-4-ene-3,17-dione). Radioactive metabolites were detected and identified by t.l.c. Radioactivity associated with a steroid was detected on a thin-layer plate with a Berthold Thin-layer Scanner II (model no. LB 2722). Derivatives of radioactive metabolites were prepared and chromatographed with authentic steroids.

Whole homogenate. The major metabolites formed by whole homogenate (155 μ g of protein), with NADPH as cofactor, were aetiocholanolone and 11 β -hydroxyandrostenedione. To a lesser extent 5 β -androstanedione, 5 β -dihydrotestosterone, 3-epiaetiocholanolone and 5 β -androstan-3 α ,17 β -diol were produced. Traces of 5 α -androstanedione, androsterone, 3-epiandrosterone and 5 α -androstan-3 β ,17 β -diol were also detected. With NADH a very small quantity of an unidentified compound principally formed by the particulate fraction was seen (see below).

Nuclear fraction. In the presence of NADPH, the nuclear fraction (162 μ g of protein) formed mainly 3-epiandrosterone. Some 5 α -androstanedione was produced and also a trace of 11 β -hydroxyandrostenedione. No metabolism was detected when NADH was the cofactor.

Mitochondrial fraction. 11 β -Hydroxyandrostenedione was the major metabolite of this fraction (195 μ g of protein) when incubated with NADPH. A small amount of 5 α -androstanedione was also produced. As with the nuclear fraction, no metabolite was detected when the mitochondrial fraction was incubated with NADH as cofactor.

Particulate fraction. Metabolism by this fraction (105 μ g of protein) was slight, although incubation

with NADPH produced some 5 α -androstanedione and trace amounts of 11 β -hydroxyandrostenedione and 3-epiandrosterone. Where NADH was used, a metabolite was formed which was only tentatively identified. The compound was more polar than the substrate androstenedione and formed a derivative on acetylation which chromatographed as a monoacetate. Oxidation of the free compound gave a product which had a chromatographic mobility identical with that of androstenedione, and reduction of this oxidized derivative gave a further derivative which had a chromatographic mobility identical with that of testosterone. The original compound was reduced and this derivative ran with androst-4-ene-3 β ,17 β -diol on several t.l.c. systems. As the original compound did not chromatograph with testosterone, it is suggested that the compound was 3 β -hydroxyandrost-4-en-17-one. However, the lack of authentic material prevented complete characterization.

Soluble fraction. Metabolism was greatest by this fraction (122 μ g of protein) in the presence of NADPH. The substrate androstenedione was almost exhausted and large amounts of aetiocholanolone and 3-epiaetiocholanolone were formed, aetiocholanolone being formed in greater quantity than the latter compound. A smaller but still considerable quantity of 5 β -androstanedione was present, and 5 β -dihydrotestosterone and 5 β -androstan-3 α ,17 β -diol were detected in smaller amounts. When incubated with NADH, a small amount of 5 β -androstanedione was detected, but in the control series, where neither NADPH nor NADH were included, no metabolism of androstenedione was detected.

Time-course study of soluble-fraction 5 β -reductase

The measurement of soluble-fraction 5 β -reductase activity was slightly complicated by the presence of 3 α -, 3 β - and a trace of 17 β -hydroxy steroid dehydrogenases. Thus 5 β -androstanedione produced from androstenedione as a result of 5 β -reductase activity would be further metabolized to aetiocholanolone, 3-epiaetiocholanolone, and traces of 5 β -androstan-3 α ,17 β -diol and 5 β -dihydrotestosterone. To overcome this problem, the incubation products, after extraction, were oxidized so that all 5 β products were converted into 5 β -androstanedione.

The time-course of 5 β -reduction of androstenedione was followed by a discontinuous sampling technique. The soluble fraction from 36.6 mg of adrenal tissue was made up to 2.5 ml of 0.25 M-sucrose containing 3 mM-MgCl₂ and a portion (2.0 ml; 1018.0 μ g of protein) incubated with incubation medium (2.0 ml) containing 67.7 μ Ci of [7α - 3 H]androstenedione (specific radioactivity, 8.3 Ci/mmol) and 120 nmol of unlabelled androstenedione. NADPH (2 mg) was added and samples (0.6 ml) were removed at intervals into 5 ml of ethanol-acetone (1:1, v/v)

Table 1. Identification of androst-4-enedione and 5 β -androstenedione isolated at times indicated, during incubation of mouse adrenal soluble fraction with androstenedione

Mouse adrenal soluble fraction (2.0ml) prepared from 29.3mg of tissue was incubated with incubation medium (2.0ml) containing 67.7 μ Ci of [7 α - 3 H]androstenedione (specific radioactivity, 8.3 Ci/mmol), 120nmol of androstenedione and 2mg of NADPH. Samples (0.6ml) were removed at the times indicated.

Time (min)	Specific radioactivities (d.p.m./nmol)			
	Steroid isolated Androstenedione	Derivative Testosterone	Steroid isolated 5 β -Androstenedione	Derivative 5 β -Androstane-3 α ,17 β -diol
5	20467	22467	813	816
10	19441	20308	1595	1685
20	18648	19010	3151	3432
40	15754	16791	6068	6701
80	10546	11142	12376	12001
120	6383	6952	16927	17410

Fig. 1. Time-course of 5 β -reduction of [7 α - 3 H]androstenedione by mouse adrenal soluble fraction

For experimental details see the text. Values for the amount of steroid present in the incubation at each time were calculated from the results in Table 1.

containing carrier steroids: androstenedione, 300 μ g; 5 β -androstenedione, 260 μ g; aetiocholanolone, 20 μ g; 3-epiaetiocholanolone, 20 μ g. After extraction, steroids were oxidized, acetylated and chromatographed in system III. Only two radioactive peaks associated with androstenedione and 5 β -androstenedione could be detected by scanning the chromatogram. The steroids were eluted and re-chromatographed in system I. After elution a portion of each was reduced. The 5 β -androstane-3 α ,17 β -diol formed from 5 β -androstenedione was chromatographed in system VI and the testosterone formed from androstenedione was chromatographed in system I. The specific radioactivities were determined and are shown in

Table 1. Agreement of the specific activities to within 10% was taken as evidence for radiochemical purity. The time-course for the reaction is shown in Fig. 1. For each time-interval, the total d.p.m. recovered for the two steroids isolated was summed and the mean percentage recovery of radioactivity (with standard deviation) for the six samples was $96.1 \pm 3.2\%$. From Fig. 1 the times 5 and 20 min were chosen for sampling in the following experiments.

Effect of pH on mouse adrenal soluble-fraction 5 β -reductase activity

The soluble fraction from 46.3mg of mouse adrenal tissue was made up to 9.0ml with 0.25M-sucrose containing 3mM-MgCl₂. A portion of this (1.0ml; 240 μ g of protein) was incubated with incubation medium (1.0ml) containing 5.6 μ Ci of [7 α - 3 H]androstenedione (specific radioactivity, 8.3 Ci/mmol), 20.7nmol of unlabelled androstenedione and 0.5mg of NADPH. A series of incubation media was prepared with a Tris-HCl buffer, pH range from 5.0 to 9.0. Samples (0.9ml) were removed after 5 and 20 min and run into 5ml of ethanol-acetone (1:1, v/v) containing: androstenedione, 150 μ g; 5 β -androstenedione, 130 μ g; aetiocholanolone, 10 μ g; 3-epiaetiocholanolone, 10 μ g. The steroids were isolated and identified as described and the specific radioactivity of 5 β -androstenedione and velocity of 5 β -reduction is shown in Table 2.

Since pH 6.5 is optimal for the enzyme under the incubation conditions used, this pH was used in all subsequent experiments. The time-course of 5 β -reduction of androstenedione by the soluble fraction was repeated with the incubation medium at pH 6.5, all other experimental details being identical with those at pH 7.4. The shape of the time-course curve of 5 β -androstenedione production was the same at pH 6.5 as at pH 7.4 (Fig. 1), and over the linear

Table 2. Identification of 5 β -androstenedione produced and velocity of 5 β -reduction during incubation of mouse adrenal soluble fraction with androstenedione, at various pH values

The soluble fraction (1.0ml; 240 μ g of protein) from 5.1 mg of mouse adrenal tissue was incubated with incubation medium (1.0ml) containing 5.6 μ Ci of [7 α -³H]androstenedione (specific radioactivity, 8.3 Ci/mmol), 20.7 nmol of androstenedione and 0.5 mg of NADPH. Incubation media of pH from 5.0 to 9.0 were used. Samples (0.9 ml) were removed at 5 and 20 min.

Time (min)	pH	Specific radioactivities (d.p.m./nmol)		Reaction velocity (pmol/min per mg of protein)
		Steroid isolated 5 β -Androstenedione	Derivative 5 β -Androstane-3 α ,17 β -diol	
5	5.00	103	95	157
	5.93	189	193	304
	6.54	206	201	324
	7.00	197	187	306
	7.60	148	154	266
	8.05	132	121	188
	8.51	94	102	155
	9.07	92	96	148
	5.00	300	330	124
20	5.93	759	717	295
	6.54	777	735	322
	7.00	639	615	295
	7.60	393	405	253
	8.05	267	261	162
	8.51	178	170	113
	9.07	145	166	109

Table 3. Identification of 5 β -androstenedione produced during incubation of mouse adrenal soluble fraction with androstenedione in the presence of various concentrations of NADPH

The soluble fraction (1.0ml, 400 μ g of protein) from 8.6mg of adrenal tissue was incubated with incubation mixture (1.0ml) containing 5.6 μ Ci of [7 α -³H]androstenedione (specific radioactivity, 8.3 Ci/mmol) and 20.7 nmol of androstenedione, NADPH added as indicated. Samples (0.9ml) were removed at 5 and 20 min. —, no d.p.m. significantly above background observed in steroid isolated.

Time (min)	NADPH concentration (μ g/incubation)	Specific radioactivities (d.p.m./nmol)	
		Steroid isolated 5 β -Androstenedione	Derivative 5 β -Androstane-3 α ,17 β -diol
5	0	—	—
	5	297	270
	10	444	406
	50	597	549
	100	603	561
	200	582	569
	500	595	550
	0	—	—
20	5	749	828
	10	1096	1199
	50	1998	2201
	100	2278	2456
	200	2201	2398
	500	2297	2237

portion (0–50 min) a 10% increase in 5 β -androstenedione production was evident. Hence further experiments were still sampled at 5 and 20 min while being incubated at pH 6.5.

Effect of NADPH concentration on soluble fraction 5 β -reductase

The soluble fraction from 69.0mg of mouse adrenal tissue was made up to 8.0ml with 0.25 M-

sucrose containing 3 mM-MgCl₂. A portion (1.0 ml; 400 µg of protein) was incubated with an equal volume of incubation mixture containing 5.6 µCi of [7α-³H]-androstenedione (specific radioactivity, 8.3 Ci/mmol) and 20.7 nmol of unlabelled androstenedione. NADPH was added as indicated. Samples (0.9 ml) were removed at 5 and 20 min and added to 5 ml

of ethanol-acetone (1:1, v/v), containing: androstenedione, 100 µg; 5β-androstenedione, 80 µg; aetiocholanolone, 10 µg; and 3-epiaetiocholanolone, 10 µg. The steroids were isolated and identified as described and the specific radioactivity of 5β-androstenedione is shown in Table 3. The effect of NADPH concentration on 5β-reductase activity is shown in Fig. 2. Further experiments were carried out with NADPH at a concentration of 200 µg/incubation.

Fig. 2. Effect of NADPH concentration on the 5β-reduction of [7α-³H]androstenedione by mouse adrenal soluble fraction

For experimental details see the text. Enzyme activities were calculated from the results in Table 3. ○, 5 min incubation; ▲, 20 min incubation.

Relationship between concentration of soluble-fraction 5β-reductase and the rate of 5β-reduction

The soluble fraction from 61.5 mg of mouse adrenal tissue was made up to 7.0 ml in 0.25 M-sucrose containing 3 mM-MgCl₂. Portions (0.1, 0.2, 0.4, 0.6 and 1.0 ml) were made up to 1.0 ml with 0.25 M-sucrose containing 3 mM-MgCl₂ and incubated with incubation medium (1.0 ml) containing 5.78 µCi of [7α-³H]androstenedione (specific radioactivity, 8.3 Ci/mmol), 20.7 nmol of unlabelled androstenedione and 200 µg of NADPH. A control tube containing no protein (only 0.25 M-sucrose solution) was also incubated as for the protein-containing samples. Samples (0.8 ml) were removed at 5 and 20 min intervals and added to 5 ml of ethanol-acetone (1:1, v/v) containing: androstenedione, 100 µg; 5β-androstenedione, 80 µg; aetiocholanolone, 10 µg; 3-epiaetiocholanolone, 10 µg. The steroids were isolated and identified as described and the specific radioactivity of 5β-androstenedione is shown in Table 4. The relationship between soluble fraction concentration and velocity of 5β-reduction is shown in Fig. 3: up to 600 µg of soluble-fraction protein,

Table 4. Identification of isolated 5β-androstenedione produced during incubation of androstenedione with various quantities of mouse adrenal soluble fraction

The soluble fraction, in amounts stated, was incubated with incubation medium (1.0 ml) containing 5.78 µCi of [7α-³H]-androstenedione (specific radioactivity, 8.3 Ci/mmol), 20.7 nmol of androstenedione and 200 µg of NADPH. Samples (0.8 ml) were removed at 5 and 20 min. —, No d.p.m. significantly above background observed in isolated steroid.

Time (min)	Protein concentrations (µg/incubation)	Specific radioactivities (d.p.m./nmol)	
		Steroid isolated 5β-Androstenedione	Derivative 5β-androstane-3α,17β-diol
5	0	—	—
	100.5	108	100
	201	206	204
	402	439	417
	603	656	648
	1005	948	924
20	0	—	—
	100.5	397	428
	201	823	837
	402	1612	1655
	603	2496	2386
	1005	3590	3601

Fig. 3. Effect of soluble fraction concentration on the 5 β -reduction of [7 α -³H]androstenedione by mouse adrenal

For experimental details see the text. Enzyme activities were calculated from the results in Table 4. ○, 5 min incubation; ▲, 20 min incubation.

the enzyme velocity is proportional to protein concentration.

Determination of K_m and V_{max} for 5 β -reduction of androstenedione by mouse adrenal soluble fraction

The soluble fraction from 99.0 mg of mouse adrenal tissue was made up to 12.0 ml in 0.25 M-sucrose containing 3 mM-MgCl₂. Portions (1.0 ml; 398 μ g of protein) were incubated with incubation mixture (1.0 ml) containing 5.25 μ Ci of [7 α -³H]androstenedione (specific radioactivity, 4.2 Ci/mmol), unlabelled androstenedione to give a final concentration indicated and 200 μ g of NADPH. Samples (0.8 ml) were removed at 5 and 10 min and added to 5 ml of ethanol-acetone (1:1, v/v) containing: androstenedione, 100 μ g; 5 β -androstenedione, 80 μ g; aetiocholanolone, 10 μ g; 3-epiaetiocholanolone, 10 μ g. This experiment was carried out in duplicate. The steroids were isolated and identified as described and the specific radioactivity of 5 β -androstenedione and the amount formed is shown in Table 5. The $1/v$ against $1/s$ plots (Lineweaver & Burk, 1934) are shown in Fig. 4. An inhibition of reaction velocity occurs at higher substrate concentrations, therefore K_m and V_{max} were calculated for lower substrate concentrations (0.63–3.13 μ M-androstenedione). K_m and V_{max} were determined from the velocity data in Table 5 by a linear-regression programme by using a weighted Lineweaver & Burk (1934) equation (Cleland, 1967). The value for K_m (at low substrate concentrations) with standard error of the four measurements was $2.22 \times 10^{-6} \pm 0.48 \times 10^{-6}$ M and for V_{max} was 450 ± 53 pmol/min per mg of protein.

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Effect of reaction products on 5 β -reduction of androstenedione

The soluble fraction from 69.4 mg of adrenal tissue was made up to 9.0 ml with 0.25 M-sucrose containing 3 mM-MgCl₂. Portions (1.0 ml; 370 μ g of protein) were incubated with incubation medium (1.0 ml) containing 5.46 μ Ci of [7 α -³H]androstenedione (specific radioactivity, 4.2 Ci/mmol) unlabelled androstenedione to give a final concentration of 1.15 μ M, 200 μ g of NADPH, and a mixture of aetiocholanolone and 3-epiaetiocholanolone as indicated. This final concentration of androstenedione was used, as no inhibition was observed at this substrate concentration in the previous experiment. Samples (0.8 ml) were removed at 5 and 10 min and added to 5 ml of ethanol-acetone (1:1, v/v) containing: androstenedione, 100 μ g; 5 β -androstenedione, 80 μ g; aetiocholanolone, 10 μ g; 3-epiaetiocholanolone, 10 μ g. The steroids were isolated and identified as described above and the specific radioactivity of 5 β -androstenedione and the reaction velocity are shown in Table 6.

Discussion

Incubation of whole homogenate from Balb/c-mouse adrenal tissue with [7 α -³H]androstenedione and NADPH results in the formation of a number of 5 β -reduced C₁₉-steroids. Conversions were similar to those observed previously in LACA mice (Maynard & Cameron, 1972).

Subcellular fractions in their metabolism with [7 α -³H]androstenedione preferred NADPH to NADH as cofactor, although the microsomal

Fig. 4. Double-reciprocal plot for 5 β -reduction of androstenedione by mouse adrenal soluble fraction

For experimental details see the text. Values for $1/v$ and $1/s$ were calculated from results in Table 5. K_m (at substrate concentrations 0.63–3.13 μ M-androstenedione) was $2.22 \times 10^{-6} \pm 0.48 \times 10^{-6}$ M and V_{max} was 450 ± 53 pmol/min per mg of protein. Expt. 1: ○, 5 min incubation; ▲, 10 min incubation. Expt. 2: □, 5 min incubation; ■, 10 min incubation.

Table 5. *Identification of 5 β -androstanedione isolated after incubation of mouse adrenal soluble fraction with various concentrations of androstenedione*

Mouse adrenal soluble fraction (1.0ml; 398 μ g of protein) prepared from 8.3 mg of tissue was incubated with incubation mixture (1.0ml) containing 5.25 μ Ci of [7 α - 3 H]androstenedione (specific radioactivity, 4.2 Ci/mmol), unlabelled androstenedione to give the final concentration indicated and 200 μ g of NADPH. Samples (0.8ml) were removed at 5 and 10 min.

Expt. no.	Time (min)	Substrate concentration (μ M)	Specific radioactivities (d.p.m./nmol)		Reaction velocity (pmol/min per mg of protein)
			Steroid isolated 5 β -Androstenedione	Derivative 5 β -Androstane-3 α ,17 β -diol	
1	5	0.63	2537	2285	0.113
		0.88	2402	2176	0.150
		1.13	2129	1943	0.171
		1.63	1704	1588	0.200
		2.13	1487	1399	0.229
		3.13	1338	1210	0.298
		5.63	741	675	0.298
		10.63	367	339	0.281
		*20.63	165	149	0.243
		30.63	89	83	0.197
	10	0.63	4397	3967	0.098
		0.88	4258	4002	0.135
		1.13	3677	3533	0.152
		1.63	3117	2891	0.183
		2.13	2998	2774	0.229
		3.13	2404	2198	0.269
		5.63	1368	1276	0.278
		10.63	666	658	0.263
		*20.63	328	300	0.244
		30.63	167	151	0.183
2	5	0.63	2020	1988	0.094
		0.88	1875	1765	0.119
		1.13	1677	1519	0.134
		1.63	1455	1327	0.169
		2.13	1382	1308	0.214
		3.13	1107	1013	0.248
		5.63	638	588	0.258
		10.63	317	287	0.240
		*20.63	153	141	0.227
		30.63	100	94	0.223
	10	0.63	3889	3595	0.087
		0.88	3294	3158	0.106
		1.13	3200	2960	0.130
		1.63	2657	2395	0.153
		2.13	2435	2371	0.191
		3.13	1858	1680	0.207
		5.63	1155	1089	0.236
		10.63	550	526	0.214
		*20.63	263	247	0.197
		30.63	189	175	0.208

* These points omitted from the double reciprocal plot Fig. 4 for the sake of clarity.

fraction appeared to contain a NADH-dependent Δ^4 -3 β -hydroxy steroid dehydrogenase. 3 β -Hydroxy steroid dehydrogenase activity (NADPH-dependent) was seen in the nuclear, particulate and soluble fractions, the greatest activity being in the soluble fraction. The nuclear 3 β -hydroxy steroid dehydrogenase activity was greater than that of the parti-

culate fraction, and so contamination from this latter source is unlikely. Further, the major soluble-fraction enzyme 5 β -reductase was not seen in the nuclear and particulate fractions, and the presence of nuclear and particulate 3 β -hydroxy steroid dehydrogenase was therefore unlikely to have been due to contamination with a soluble-fraction enzyme. This

Table 6. *Effect of aetiocholanolone and 3-epiaetiocholanolone (reaction products) on the 5 β -reduction of androstenedione by mouse adrenal soluble fraction*

Mouse adrenal soluble fraction (1.0ml; 370 μ g of protein) was incubated with incubation medium (1.0ml) containing 5.46 μ Ci of [7 α -³H]androstenedione (specific radioactivity, 4.2Ci/mmol), unlabelled androstenedione to give a final concentration of 1.15 μ M, 200 μ g of NADPH and a mixture of aetiocholanolone and 3-epiaetiocholanolone at the concentrations indicated. Samples (0.8ml) were taken at 5 and 10min.

Time (min)	Final concns. of aetiocholanolone and 3-epiaetiocholanolone (μ M)	Specific radioactivity (d.p.m./nmol)		Reaction velocity (pmol/min per mg of protein)
		Steroid isolated 5 β -Androstanedione	Derivative 5 β -Androstane-3 α ,17 β -diol	
5	0	1727	1593	147
	0.5	1551	1441	133
	1.0	1569	1411	133
	2.0	1382	1322	120
	3.0	1140	1052	99
	4.0	1153	1043	99
	5.0	925	879	79
	10	3203	2987	137
10	0.5	3017	2851	129
	1.0	2891	2841	128
	2.0	2653	2523	114
	3.0	2192	2082	95
	4.0	1950	1878	84
	5.0	1699	1561	74

enzyme has been shown to be present in rat liver particulate and soluble fractions (Hoff & Schriefers, 1973) and in ox adrenal particulate fraction (Beyer & Samuels, 1956). Although 3 β -hydroxy steroid dehydrogenase has been detected in rat adrenal mitochondria (McCune *et al.*, 1970) and bovine adrenal mitochondria (Cowan *et al.*, 1971), it was not detected in the mouse adrenal mitochondria in this study.

A 3 α -hydroxy steroid dehydrogenase (NADPH-dependent) was observed in the soluble fraction, its activity being greater than the activity of the 3 β -hydroxy steroid dehydrogenase. The difference in activity between the two dehydrogenases is greater in the whole homogenate than the soluble fraction. Whether this is due to a decrease in 3 α -hydroxy steroid dehydrogenase activity because of fractionation causing increased lability, or to the separation of the enzyme from a cofactor in another fraction, or even an increase in 3 β -hydroxy steroid dehydrogenase by separating the enzyme from an inhibitor, is not certain. A 3 α -hydroxy steroid dehydrogenase (NADPH-dependent) has been described as being localized in the soluble fraction of rat liver with a 3 α -/3 β -hydroxy steroid ratio in the soluble fraction of 10 (Arimasa & Kochakian, 1972).

Although the major 11 β -hydroxylase activity was found in the mitochondria, slight traces were also found in the nuclear and particulate fractions. These traces were probably due to slight contamination by the mitochondrial fraction.

17 β -Hydroxy steroid dehydrogenase was located

in the soluble fraction, but only in a very small quantity. This enzyme has been reported in the cytosol fractions of rat adrenal (Maynard & Cameron, 1973) and human liver (Littman *et al.*, 1971).

The nuclear, mitochondrial and particulate fractions all showed NADPH-dependent 5 α -reductase activity, nuclear activity being greater than that of the mitochondrial and particulate fractions. A similar distribution of 5 α -reductase activity has been shown to exist in rat ventral prostate (Roy, 1971). The same report showed that rat liver also possesses mitochondrial and microsomal 5 α -reductases, and microsomal 5 α -reductase (NADPH-dependent) has been described in rat adrenal tissue (Kitay *et al.*, 1971; Maynard & Cameron, 1973) and prostatic tissue (Bonne & Raynaud, 1973).

Only in the soluble fraction were 5 β -reduced metabolites detected. The 5 β -reductase responsible was NADPH-dependent though a small quantity of 5 β -androstenedione was produced when NADH was used as cofactor.

Therefore either a separate NADH-dependent 5 β -reductase exists in the soluble fraction, or the one enzyme can utilize both cofactors, but with a much decreased reaction velocity with NADH. The presence of an NADH kinase in the soluble fraction might also explain the formation of 5 β -androstenedione in the presence of NADH. Soluble-fraction 5 β -reductase activity has also been described in guinea-pig adrenal (Brown-Grant *et al.*, 1960) and pig liver (Van Doorn & Clarke, 1973).

The soluble fraction C₁₉-steroid 5 β -reductase

had a pH optimum of 6.5, at which all further studies were performed. This pH optimum compares with pH 6.4 for rat adrenal microsomal 5α -reductase (Kitay *et al.*, 1971) and pH 5.5 for guinea-pig adrenal soluble-fraction 5β -reductase (Brown-Grant *et al.*, 1960).

At high substrate concentrations, the 5β -reduction of $[7\alpha\text{-}^3\text{H}]$ androstenedione showed a decrease in the reaction velocity observed at lower substrate concentrations (Table 5). This inhibition was due either to substrate inhibition, as shown for 3β -hydroxy steroid dehydrogenase (Marcus & Talalay, 1963), or to product inhibition. Table 6 shows the inhibition of 5β -reduction of $[7\alpha\text{-}^3\text{H}]$ androstenedione by mouse adrenal soluble fraction in the presence of increasing concentrations of aetiocholanolone and 3-epiaetiocholanolone, the two major products of the reaction. Whether this product inhibition is due to reversibility of the reaction or to the formation of an inactive product-enzyme complex is not yet known.

The K_m value for the 5β -reduction of $[7\alpha\text{-}^3\text{H}]$ androstenedione (at low substrate concentration) by mouse adrenal soluble fraction is similar to that obtained for the microsomal 5α -reductase of rat adrenal tissue (Maynard & Cameron, 1973), whereas the V_{max} for mouse adrenal soluble-fraction 5β -reduction is 20 times that for the rat adrenal microsomal 5α -reductase.

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