Journal of Steroid Biochemistry, Vol. 10, pp. 325 to 333.
Pergamon Press Ltd. 1979. Printed in Great Britain

BIOCHEMICAL CHARACTERIZATION OF THE NADPH:4-ENE-3-KETOSTEROID 5α-OXIDOREDUCTASE IN RAT OVARIAN SUSPENSION CULTURES

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(*Received* 26 *June* 1978)

SUMMARY

A method was devised to measure the NADPH:4-ene-3-ketosteroid 5α-oxidoreductase of ovarian cells, in suspension or in monolayer culture. The enzymatic activity was assayed by measuring initial conversion rates of [^{3}H]-17-epitestosterone to its 5α-reduced metabolites. With this substrate no 17-keto derivatives were formed, thus facilitating the chromatographic separation of the reaction products. Before and during the incubation with the tritiated substrate, aminoglutethimide (10^{-4} M) was added to the incubation medium in order to block the endogeneous steroid production.

The characteristics of the 5α-reductase in living cells were investigated and compared with those found in ovarian homogenates. In living cells the apparent K_M approximated 0.78 μM. The 5α-reductase of 17-epitestosterone was inhibited by 4-ene-3-keto-steroids without an oxygen substitution on carbon 11 but not by 5α-reduced steroids. 17β-Estradiol in high concentration functions as an uncompetitive inhibitor. In homogenates an apparent K_M of 0.57 μM was measured. The enzyme exhibited a pH optimum around 7 and required NADPH as cofactor. Inhibition of the enzyme activity by 17β-estradiol could not be demonstrated under these conditions. The characteristics of the 5α-reductase in the rat ovary are thus comparable to those described in androgen target cells.

The activity of the 5α-reductase in living cells was comparable with the activity found in homogenates supplemented with NADPH. Cofactor availability is thus not a limiting factor for 5α-reductase activity in ovarian cells.

INTRODUCTION

The 5α-reductase is a widespread enzyme activity transforming 4-ene-3-keto steroids into 5α-reduced steroids. Originally 5α-reduction was studied in the liver where it plays a catabolic role. Later on it was shown to be an activating mechanism in the androgen target cell and more recently this enzyme activity was also detected in steroidogenic organs such as the testis, the ovary and the adrenal [1]. Evidence is accumulating that 5α-reductase contributes to the regulation of steroidogenesis in the adrenal [2] and that 5α-reduced metabolites are involved in follicular maturation [3] and steroid hormone production [4–6] in the ovary.

In order to study the hormonal regulation of the rat ovarian 5α-reductase activity *in vitro* [7] a method had to be developed to assay this enzyme activity in living cells.

Since in a steroidogenic tissue both the function of the enzyme and the local steroid hormone concentrations are different from those in the liver or in androgen target tissues it seemed important to determine the characteristics of the 5α-reductase in the rat ovary. In order to mimic the *in vivo* situation as closely as possible this was done on living cells. The results are compared with those found in ovarian homogenates and with the biochemical characteristics described in the liver [8], and in androgen target organs such as the prostate [9], the kidney [10], the brain [11, 12] and the skin [13].

MATERIALS AND METHODS

Animals products and incubation media. Wistar rats (Pfd: Wist f), 60–90 days old, were obtained from the breeding center of the Catholic University of Leuven. They were housed in an environment of constant light–dark regimen and temperature, and they received a constant pellet diet.

[1,2-^{3}H]-17-Epitestosterone (50 Ci/mmol) and [1,2-^{3}H]-testosterone (50 Ci/mmol) were purchased from New England Nuclear and repurified on silica gel t.l.c. (chloroform–acetone, 35:5, V/V) before use. Unlabeled

Trivial names and abbreviations used: Dihydrotestosterone (DHT): 17β-hydroxy-5α-androstan-3-one; 17-Epidihydrotestosterone, (17-epiDHT), 17α-hydroxy-5α-androstan-3-one; 17-Epitestosterone, 17α-hydroxy-4-androsten-3-one; testosterone, 17β-hydroxy-4-androsten-3-one; 5α-Reductase, NADPH 4-ene-3-ketosteroid-5α-oxidoreductase (EC 1.3.1.99).

steroids were obtained from Sigma and Steraloids; 5α-androstane-3β,17α-diol was received from Schering Co., Belgium. Solvents were analytical grade from Merck and aminoglutethimide was a gift from Hoffmann–La Roche. Bovine pancreatic trypsin, soya bean trypsin inhibitor, deoxyribonuclease (DNAse), collagenase and glucose-6-phosphate dehydrogenase were purchased from Boehringer. Bovine serum albumin (BSA), NADPH and NADH were purchased from Sigma and minimum essential medium (MEM) and foetal calf serum from Flow laboratories.

The media used for the preparation of a cell suspension and for the assay of the 5α-reductase activity were Hanks buffered saline solutions pH 7.2, supplemented with potassium penicillin G (100 U/ml) and streptomycin (200 μg/ml). For tissue blocks BSA and Ca^{2+} were present at a concentration of 3 mg/ml and 1 mM respectively; for dissociated cells, the BSA concentration was raised to 5 mg/ml and the Ca^{2+} concentration was reduced to 0.1 mM. To prepare Ca^{2+} and Mg^{2+} free media, these salts were simply omitted.

Monolayer cultures were performed in MEM supplemented with potassium penicillin G (100 U/ml), streptomycin (200 μg/ml) and 10% foetal calf serum. All glassware was siliconized and all media were millipore filtered (GSWP, 0.22 μm pore diameter).

Cell dispersion; suspension and monolayer cultures. Ovarian cell suspensions were prepared using the technique described by Hopkins and Farquehar[14] with some modifications. Animals were killed by ether anaesthesia, the ovaries were removed aseptically, chilled immediately on ice and freed of fat tissue. After an incubation with collagenase (0.25%, 15 min) the corpora lutea and the largest follicles were removed by microdissection. The remaining tissue (mainly stromal tissue) was chopped with a scalpel into blocks of about 1 mm^3. The pooled blocks were washed three times and treated sequentially with collagenase (0.25%, 60 min), trypsin (0.1%, 10 min) trypsin inhibitor (0.1%, 10 min), DNAse (0.1%, 1 min), and Ca^{2+} and Mg^{2+} free medium. All incubations were performed in a shaking water bath at 37°C. Finally the tissue was mechanically dispersed using three conical glass pipettes of decreasing tip diameter (about 1 to 0.1 mm diameter). The cell suspension was freed of cell debris by centrifugation through a 4% BSA solution (250 g, 10 min, 0°C) and cell clumps were removed by filtration through a Nitex filter of 36 μm pore diameter. Routinely $2.6 \times 10^6 \pm 0.6 \times 10^6$ ($n = 43$) cells were obtained from one ovary and viability, as evaluated by trypan blue exclusion [14] was 85.6 ± 4% ($n = 37$). Monolayer cultures were performed in Petri dishes under an atmosphere of 5% CO_2 in air.

Homogenates and preparation of nuclei. Ovarian tissue after removal of the largest follicles and the corpora lutea (about 30 mg) was homogenized at 0°C by 10 strokes of a Potter–Elvehjem homogenizer in 10 ml of the medium used for the suspension cultures. A purified nuclear fraction was prepared as described by Verhoeven and De Moor[15].

Assay of the 5α-reductase. The 5α-reductase was assayed by measuring the initial conversion rate of [^{3}H]-17-epitestosterone to its 5α-reduced metabolites. Standard solutions of labeled and unlabeled steroids were evaporated to dryness under a nitrogen stream and solubilized in the incubation medium in a shaking water bath at 37°C for at least 10 min. Before the assay the cells were incubated with 10^{-4} M aminoglutethimide for 2 h, and during the incubation 10^{-4} M aminoglutethimide was present in the incubation mixture also. For the assay of the 5α-reductase 10^{-7} M 17-epitestosterone and about 0.05 μCi [^{3}H]-17-epitestosterone were used. When cell homogenates were used, the medium was supplemented with an NADPH generating system [15] consisting of glucose-6-phosphate (5 mM), NADP (3.10^{-4} M) and glucose-6-phosphate dehydrogenase (0.87 IU/ml). The volume of the incubation mixture was made up to 2 ml. The incubation was performed in a shaking water bath at 37°C, and the reaction was stopped by two drops of HCl 6 N. Steroids were extracted immediately with 5 vol. of a mixture of cyclohexane and ethylacetate (1:1, V/V). The extract was washed twice with HCl 1 N and twice with distilled water and about 40 μg of 17-epitestosterone, 17-epiDHT and 5α-androstane-3β,17α-diol were added. After the addition of reference steroids the extracts were evaporated to dryness under a nitrogen stream and chromatographed twice on precoated Silicagel F-254 glass plates in chloroform–acetone (35:5). Reference steroids were localized under U.V. at 240 μm or at 350 μm after spraying the plates with primulin, [16]. Radiometabolites were localized by means of a Packard radiochromatogram scanner. The zones corresponding to the unreacted substrate and the metabolites were scraped off, eluted directly in the scintillation fluid and counted in a Packard Tri-Carb liquid scintillation counter. The total radioactivity of each chromatogram was calculated by adding the radioactivity of the individual zones, and the radioactivity of a particular zone was expressed as a percent of the total. In this way more than 90% of the radioactivity of the extracts was routinely recovered.

Acetylation and oxidation of steroids; chromatographic systems used. For acetylation, steroids were evaporated to dryness under a nitrogen stream, 4 drops of pyridine and 1 drop of glacial acetic acid were added, and the mixture was left overnight in the dark at room temperature under an atmosphere of nitrogen. For oxidation steroids were evaporated to dryness, 0.1 ml of 2.5% solution of CrO_3 in glacial acetic acid was added for 10 min at 0°C; the reaction was stopped by 1 ml of a 20% ethanol solution in water and the oxidized steroids were extracted with 10 ml of methylene chloride.

Chromatography was performed on precoated F 254 silica-gel thin layer chromatography glass plates of 0.25 mm thickness. The chromatographic systems

used were (V/V): t.l.c.$_{I}$: chloroform–acetone (35:5, V/V); t.l.c.$_{II}$: methylene chloride–ether (70:30, V/V); t.l.c.$_{III}$: chloroform–ethanol (95:5, V/V); t.l.c.$_{IV}$: benzene–methanol (85:15, V/V); t.l.c.$_{V}$: hexane–ethylacetate (75:25, V/V).

The effect of acetylation on chromatographic mobilities was evaluated by calculating the ΔR_m value, which is the difference between the R_m $\{R_m = \ln [1/R_F - 1]\}$ value of the non acetylated and the acetylated molecule. The ΔR_m value has the property to depend only on the number of acetate groups introduced in the molecule and the chromatographic system used [17].

RESULTS

Identification of [^{3}H]-*17-epitestosterone metabolites*

The incubation of [^{3}H]-17-epitestosterone with an ovarian cell suspension or with an ovarian homogenate yielded four peaks on a radiochromatogram (Fig. 1) after extraction and two runs on t.l.c.$_{I}$.

No 17-keto derivatives were formed. Indeed no radioactivity was found to be associated with androstenedione ($R_F = 0.57$) or 5α-androstane-3,17-dione ($R_F = 0.66$). Incubation with [^{3}H]-testosterone on the contrary yielded large amounts of 17-keto derivatives.

All three metabolites of 17-epitestosterone were 5α-reduced. After CrO_3 oxidation the radioactivity of peaks I, III and IV had the same mobility as 5α-androstane-3,17-dione on t.l.c. in systems I, II, III and IV. No 5β-reduced metabolites were detected after separation of 5α- and 5β-androstane-3,17-dione on t.l.c.$_{V}$.

Acetylation and t.l.c.$_{II}$ yielded a ΔR_m of 1.2, 1.4, 2.5 and 2.8 for respectively peaks I, II, III and IV. Peak I and II thus consisted of monohydroxylated and peaks III and IV of bihydroxylated products since peak II is known to contain only one hydroxyl group.

Peak I was identified as 17-epiDHT since it was a 5α-reduced product of 17-epitestosterone with only one hydroxyl group, and since the polarity of peak I was comparable with that of 17-epiDHT both before and after acetylation on t.l.c.$_{I}$, t.l.c.$_{II}$ and t.l.c.$_{III}$. Moreover, in time course experiments it was shown to be an intermediate product (Fig. 2).

Peaks III and IV were identified as 17α-5α-androstanediols since they were 5α-reduced, bihydroxylated products. Peak III was identified as 5α-androstane-3β,17α-diol by crystallization to constant specific activity (Table 1) and peak IV was tentatively identified as 5α-androstane-3α,17α-diol. Indeed, peak IV was more polar than peak III on t.l.c.$_{I}$ and t.l.c.$_{II}$, and on t.l.c.$_{I}$ 5α-androstane-3α,17α-diol is known to be more polar than 5α-androstane-3β,17α-diol [18]. Crystallizations to constant specific activity or to constant $^{14}C/^3H$ ratio were not performed for 17-epiDHT and for 5α-androstane-3α,17α-diol since the necessary products could not be obtained.

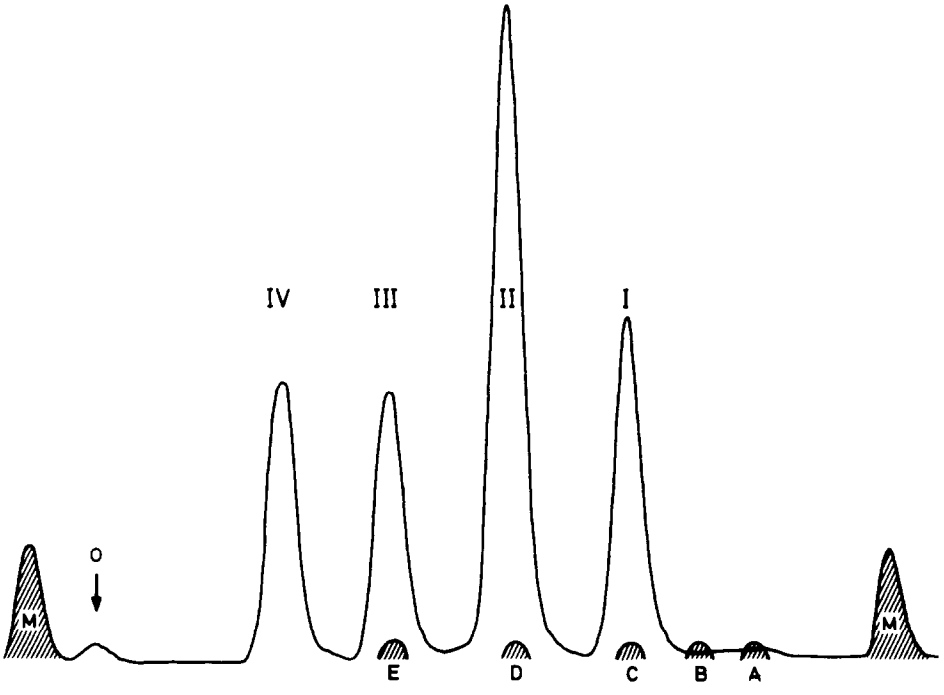

Fig. 1. t.l.c. Radiochromatogram of [^{3}H]-17-epitestosterone metabolites. [^{3}H]-17-Epitestosterone and 3×10^5 cells were incubated for 3 h at 37°C in 2 ml of Hanks medium supplemented with 5 mg BSA/ml and 10^{-4} M aminoglutethimide. The extract was chromatographed twice in chloroform–acetone (35:5, V/V). Peaks I, II, III and IV were identified as respectively 17-epiDHT, unreacted 17-epitestosterone, 5α-androstane-3β,17α-diol and 5α-androstane-3α,17α-diol. The markers (M) and the origin (0) are indicated, together with the localization of 5α-androstane-3,17-dione (A), androstenedione (B), 17-epiDHT (C) 17-epitestosterone (D) and 5α-androstane-3β,17α-diol (E).

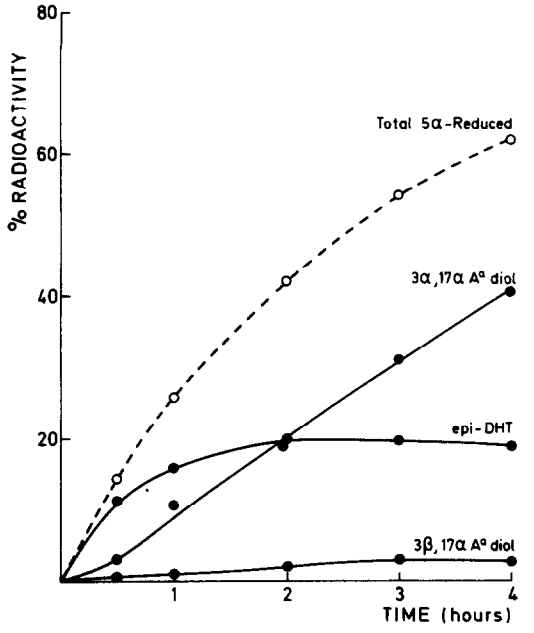

Fig. 2. 17-Epitestosterone metabolites as a function of time. Cells (3×10^5) were incubated with 17-epitestosterone as described in Fig. 1. The individual 5α-reduced metabolites (●) and the sum (○) of the 5α-reduced metabolites are indicated. Each point represent the mean of duplicate determinations.

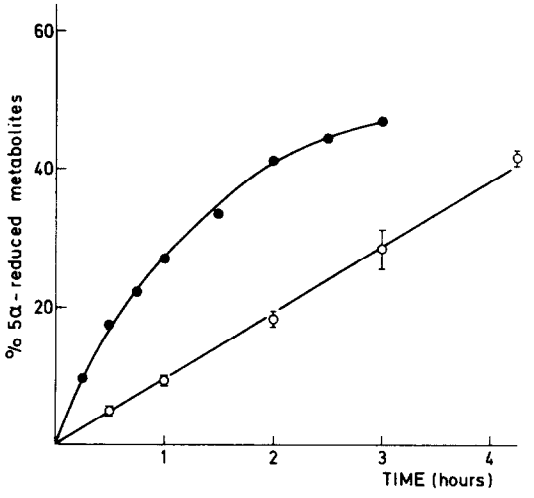

Fig. 3. 17-Epitestosterone metabolism by monolayer cultures (○) and by ovarian homogenates (●) as a function of time. 3×10^5 Cells, in monolayer culture for 24 h or an homogenate (about 0.3 mg wet wt.) were incubated with 17-epitestosterone as described in Fig. 1. Together with the homogenate an NADPH generating system was added to the incubation mixture. The mean of duplicate determination (●) and the mean ± S.D. of triplicate determinations (○) are indicated.

Assay conditions of the 5α-reductase

In all three systems used, suspension cultures, monolayer cultures and homogenates, the total amount of 5α-reduced metabolites formed was proportional to the duration of the incubation until about 30% of the 17-epitestosterone (10^{-7} M) incubated was reduced. With living cells the duration of incubation could be extended at least up to 3–4 h. With homogenates, however, the rate of formation of 5α-reduced products declined after one hour of incubation (Fig. 3). This could be due to thermal denaturation of the enzyme [9, 10].

The total amount of 5α-reduced metabolites formed was proportional to the number of cells incubated, irrespective of the amount of 17-epitestosterone incubated and of the duration of incubation at least up to 4 h (Fig. 4).

Since the half maximal conversion rate was obtained at about 10^{-6} M, 10^{-7} M 17-epitestosterone was routinely used to assay 5α-reductase. Bovine serum albumin was included in the incubation mixture in order to prevent aggregation of cells. As expected this slowed down the formation of 5α-reduced products, probably by aspecific binding of 17-epitestosterone. At the concentrations used this effect, however, was only minimal (Fig. 5).

Table 1. Specific activity of crystals during four subsequent crystallisations of [^{3}H]-5α-androstane-3β,17α-diol with authentic 5α-androstane-3β,17α-diol

Crystal	Specific activity (c.p.m./mg)
1. Ethanol–H_2O	6980
2. Methanol–H_2O	8307
3. Cyclohexane–ethylacetate	8457
4. Acetone–H_2O	7856

The use of aminoglutethimide to block endogeneous steroid formation

Aminoglutethimide in concentrations up to 10^{-4} M had no effect on the amount of 5α-reduced metabo-

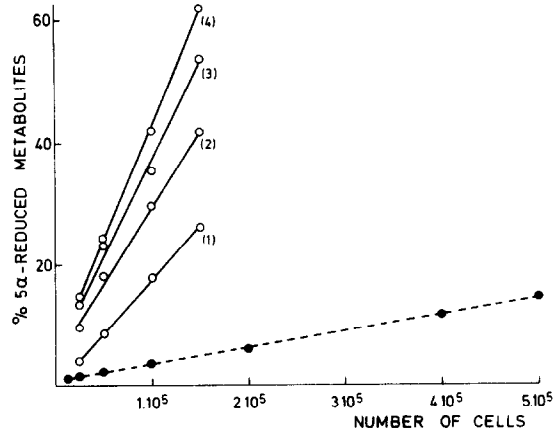

Fig. 4. 17-Epitestosterone metabolism by different numbers of freshly dispersed ovarian cells. From 0.125×10^5 to 5×10^5 cells/ml were incubated with 10^{-5} M 17-epitestosterone for 1 h (●——●) and from 0.25×10^5 to 1.5×10^5 cells/ml were incubated with 10^{-7} M 17-epitestosterone for respectively 1, 2, 3 and 4 h (○——○) as described in Fig. 1. Each point represents the mean of duplicate determinations.

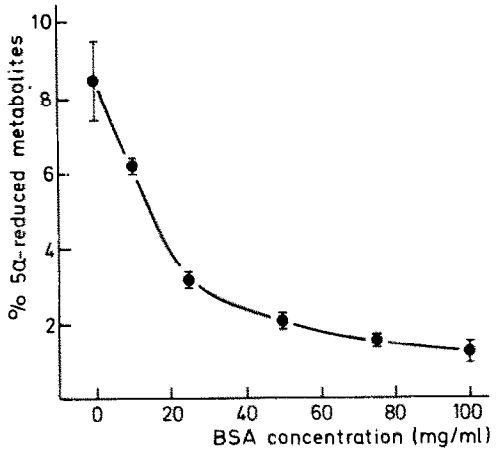

Fig. 5. Influence of BSA concentration upon the percentage of 5α-reduced metabolites formed. Ovarian homogenates were incubated for 30 min with varying concentrations of BSA and with 17-epitestosterone as described in Fig. 3. Means ± S.D. of triplicate experiments are depicted.

lites formed in cell homogenates and in freshly dispersed cells. When aminoglutethimide was added to monolayer cultures, however, the amount of 5α-reduced metabolites formed was increased when concentrations of 10^{-4}–10^{-5} M were used and was decreased at concentrations exceeding 10^{-3} M (Fig. 6a). When monolayer cultures were preincubated with 10^{-4} M aminoglutethimide for different periods of time, the initial conversion rate increased with the duration of pre-incubation and reached a plateau after 2 h of preincubation (Fig. 6b). This increase in conversion rate of 17-epitestosterone, however, was quantitatively variable and was not seen in all experiments.

The absorption spectrum of aminoglutethimide displays a peak at 205 and 238 nm, both in H_2O and in ethanol. Aminoglutethimide could thus be located easily by U.V. fluorescence on t.l.c. When [^{3}H]-17-epitestosterone (2.10^{-10} mol) was chromatographed directly in the presence of aminoglutethimide (2.10^{-7} mol) on t.l.c. in system I ($R_F = 0.15$) and II ($R_F = 0.15$) respectively 18.7 and 13.9% of the radioactivity was associated with the aminoglutethimide. This non-specific adsorption of [^{3}H]-17-epitestosterone to aminoglutethimide on t.l.c., which was also seen after incubation, could be prevented completely by washing out the aminoglutethimide from the extracts by 1 N HCl.

Partial characterization of the 5α-reductase

Using a *living cell* suspension, the 5α-reductase was measured at different substrate concentrations (Fig. 7a). The half maximal conversion rate was estimated from a Lineweaver–Burk plot, at 0.78 μM. With all restrictions imposed by the very low conversion rates, the 5α-reductase activity could not be saturated and a second apparent K_M of over 100 μM could be calculated (Fig. 7b). Various steroids were tested for an inhibitory effect upon the 5α-reduction of 17-epitestosterone. All 4-ene-3-keto steroids investigated inhibited this 5α-reductase activity, except those with an oxygen substitution on carbon 11. No marked product inhibition was found with various 5α-reduced steroids, while phenolic compounds inhibited the 5α-reductase activity at higher concentrations (Table 2). This effect of 17β-estradiol could be characterized as an uncompetitive inhibition [19] since on a Lineweaver–Burk plot the slopes obtained with different concentrations of 17β-estradiol remained constant (Fig. 8a).

In *homogenates* an apparent K_m of 0.57 μM was found (Fig. 8b), while 17β-estradiol had no effect upon the 5α-reduction of 17-epitestosterone. A pH optimum was determined in the presence of 0.5 mM NADPH using a Tris-citrate buffer (50 mM) with 1 M NaCl, 2.5 mM $CaCl_2$, 25 mM $MgCl_2$ and 0.25 mM EDTA. The pH varied from 4.5 to 9 and an optimum was found around pH 7 (Fig. 9). The cofactor specificity was evaluated using purified nuclei since in total homogenates the endogeneous NADPH would inter-

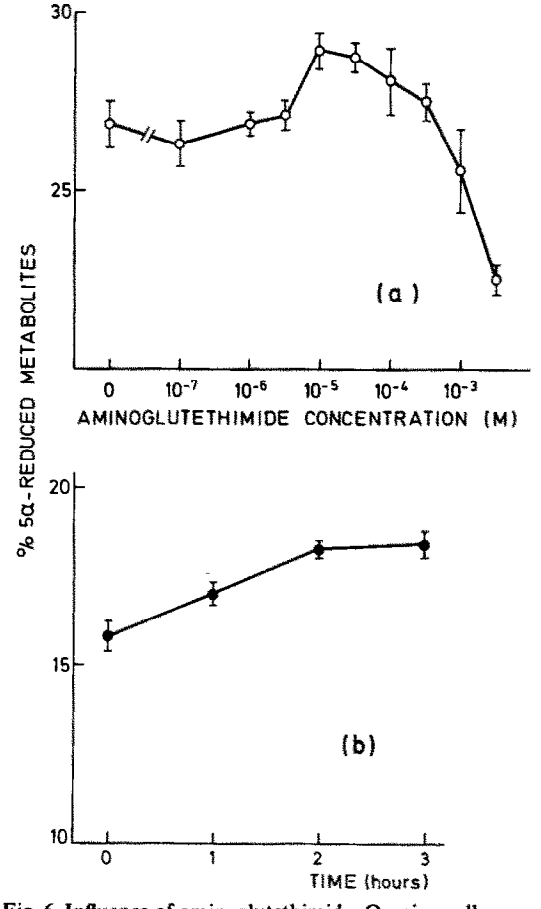

Fig. 6. Influence of aminoglutethimide. Ovarian cell monolayer cultures (3×10^5 cells, 24 h of culture) were incubated for 1.5 h as described in Fig. 1 with 17-epitestosterone and various concentrations of aminoglutethimide (a) or were preincubated with 10^{-4} M aminoglutethimide for various durations of time and incubated with 17-epitestosterone and 10^{-4} M aminoglutethimide for 1 h (b). Each point represents the mean ± S.D. of triplicate experiments.

Table 2. Percentual inhibition of the 5α-reduction of 17-epitestosterone by various steroids

Steroid	% Inhibition of the 5α-reduction of [^{3}H]-17-epitestosterone 10^{-6} M	5×10^{-6} M
4-ene-3-keto steroids		
17-Epitestosterone	51.1 ± 2.5	75.2 ± 1.3
Testosterone	21.9 ± 6.1	41.1 ± 3.6
4-Androsten-3,17-dione	11.1 ± 2.7	22.9 ± 2.8
4-Pregnen-3,20-dione	47.4 ± 3.7	69.3 ± 3.7
20α-Hydroxy-4-pregnen-3-one	12.3 ± 0.3	43.3 ± 1.6
17α-Hydroxy-4-pregnen-3,20-dione	49.0 ± 4.9	76.4 ± 8.0
11-Deoxy-cortisol	29.0 ± 0.5	37.1 ± 3.1
Cortisol	0.2 ± 3.9	2.3 ± 0.3
Corticosterone	0.6 ± 1.3	0.2 ± 0.5
5α-reduced Steroids		
DHT	−5.0 ± 8.0	−1.0 ± 7.6
5α-Androstane-3α,17β-diol	13.3 ± 2.0	12.0 ± 1.0
5α-Pregnane-3,20-dione	0.4 ± 1.0	0.2 ± 3.7
3α-Hydroxy-5α-pregnan-20-one	0.3 ± 1.5	2.9 ± 1.7
5α-Pregnane-3α,20α-diol	17.2 ± 11.4	25.5 ± 9.7
Phenolic Steroids		
17β-estradiol	7.0 ± 7.3	31.6 ± 3.2
Estrone	18.7 ± 1.7	33.9 ± 6.7

After 1 h of recovery 3×10^5 cells were incubated with 10^{-7} M 17-epitestosterone and 10^{-6} and 5×10^{-6} M of various steroids as described in Fig. 1. The mean ± S.E. of triplicate experiments is indicated.

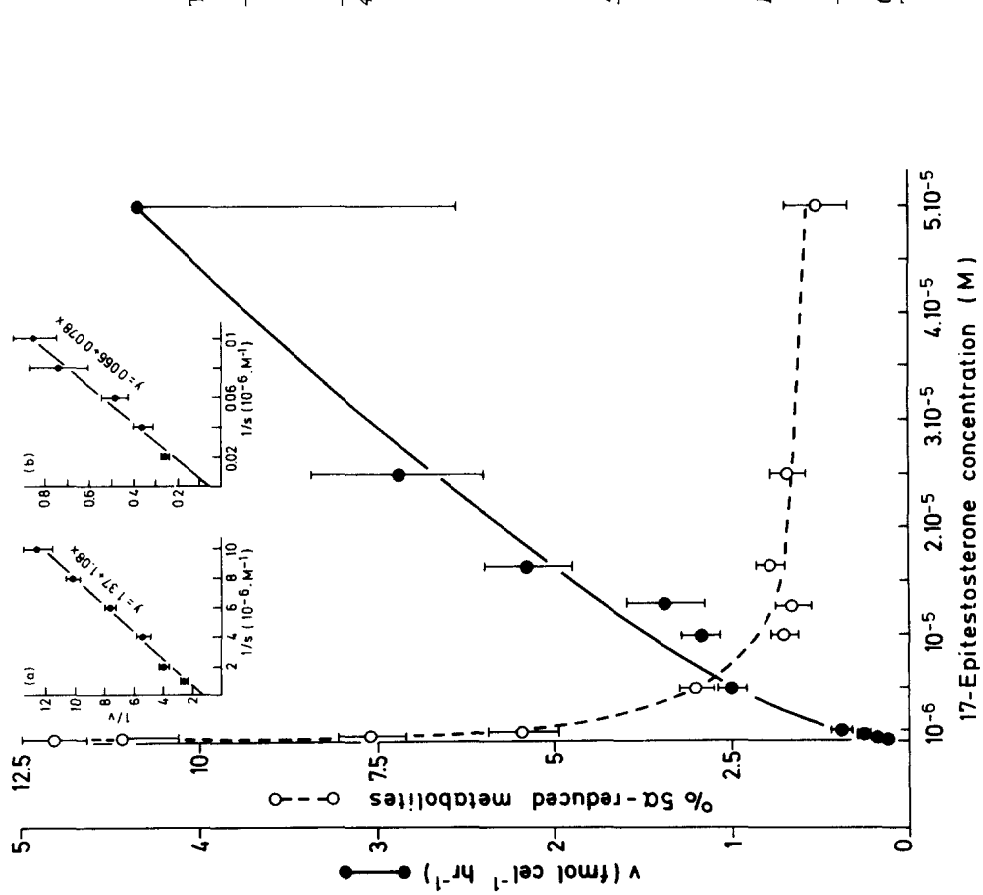

Fig. 7. Influence of 17-epitestosterone concentration. Cells (3×10^5) were incubated with 10^{-7} to 5×10^{-5} M 17-epitestosterone as described in Fig. 1. The % 5α-reduced metabolites (O) and the amount of 5α-reduced metabolites (●) formed are indicated. The Lineweaver–Burk plots are shown in the insert. Two apparent K_m values of respectively 0.78 μM (a) and more than 100 μM (b) are depicted.

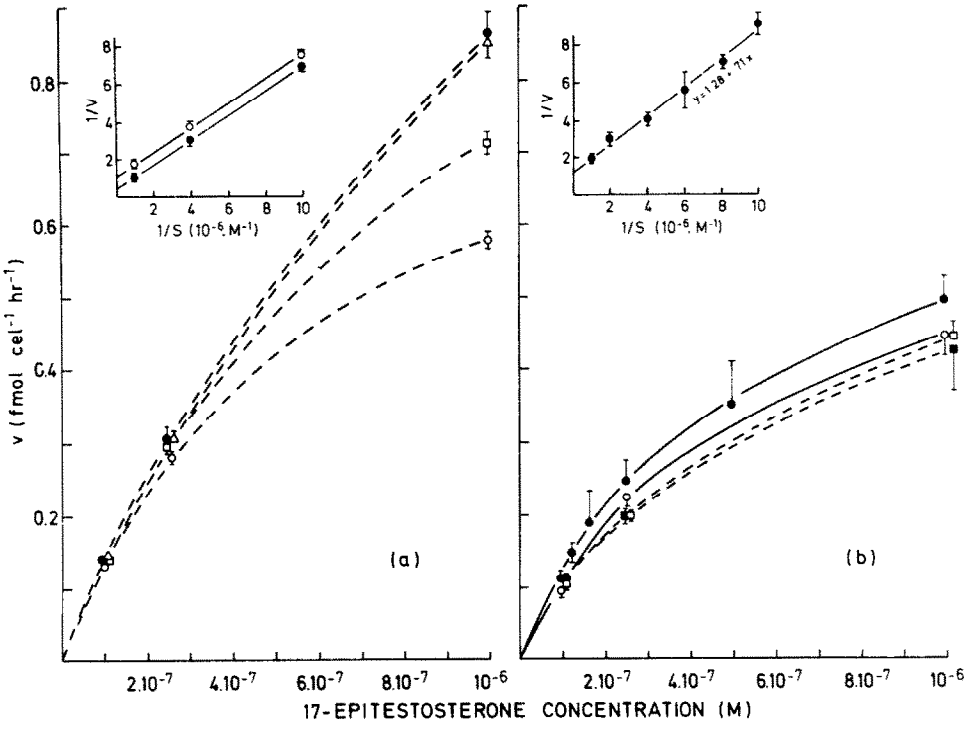

Fig. 8. Influence of 17β-estradiol concentration. In two separate experiments approximately 3×10^5 cells (a) or homogenates thereof (b) were incubated with different concentrations of 17-epitestosterone with (open symbols) and without 17β-estradiol (closed symbols) as described in Figs. 1 and 3. The intact cells (a) were incubated with respectively 10^{-6} M (△), 5.10^6 M (□) and 10^{-5} M (○) 17β-estradiol. The homogenates (b) were incubated with (●, ○) and without (■, □) an NADPH generating system. The Lineweaver-Bulk plots, depicted in the insert, show an uncompetitive inhibition of 17β-estradiol in intact cells (a) and an apparent K_m for 17-epitestosterone of 0.57 μM in homogenates (b). Each point represents the mean ± S.D. of triplicate experiments.

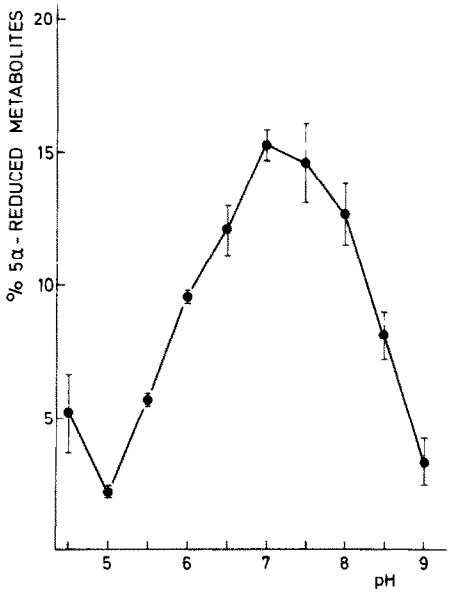

Fig. 9. Influence of pH upon 5α-reductase activity. Ovarian homogenates were incubated in a Tris–citrate buffer together with 0.5 mM NADPH as described in Fig. 3. The pH varied from 4.5 to 9. Each point represents the mean ± S.D. of triplicate experiments.

fere. The incubation of about 350,000 nuclei for 1 h with 10^{-7} M 17-epitestosterone in the presence of 0.5 mM NADH, 0.5 mM NADPH and an NADPH generating system yielded respectively 0.32 ± 0.09%, 3.78 ± 1.56% and 5.52 ± 0.60% 5α-reduced metabolites, demonstrating that NADPH was used as co-factor.

In order to compare the activity of the 5α-reductase in a living cell with the activity of the 5α-reductase in an homogenate about 3×10^5 cells were incubated with 17-epitestosterone, with and without prior

Table 3. The 5α-reductase activity was assayed in 3×10^5 cells with and without prior homogenisation as described in Fig. 1

	% 5α-Reduced metabolites	
	Living cell	Homogenate
Experiment 1	17.3 ± 0.4	20.0 ± 1.1
Experiment 2	14.0 ± 1.5	15.6 ± 0.6
Experiment 3	16.4 ± 0.03	17.2 ± 1.2
	15.9 ± 1.7	17.6 ± 2.2

Together with the homogenates an NADPH generating system was incubated. The mean ± S.D. (triplicates) of three separate experiments are indicated.

homogenization. The percentage of 5α-reduced metabolites formed was comparable in both systems, but slightly higher in an homogenate (Table 3). This increase in activity of the 5α-reductase in homogenates was comparable to the % trypan blue positive cells: respectively 18% in the first experiment, 13% in the second and 10% in the last experiment.

DISCUSSION

A system has been developed to assay 5α-reductase activity in living ovarian cells. This procedure offers the advantage that it permits the selection and culture of specific cells. Moreover, it permits the study of the regulation of the 5α-reductase activity by various hormones and humoral factors secreted by the pituitary or locally, in adjacent ovarian compartments, and the study of the role of the 5α-reductase activity in the regulation of steroidogenesis [2].

The assay of 5α-reductase

The use of [^{3}H]-17-epitestosterone to measure the 5α-reductase in the rat ovary is a definite improvement over the methods described up to now. Since no 17-keto derivatives are formed the number of metabolites is largely reduced. Furthermore, 5α-androstane-3α,17α-diol and 5α-androstane-3β,17α-diol are easily separated on t.l.c.

Theoretically in steroidogenic cells endogeneously formed 4-ene-3-keto steroids could inhibit competitively 5α-reductase. Therefore steroidogenesis should be inhibited when 5α-reductase activity is assayed. Aminoglutethimide was used for this purpose since it is an effective inhibitor of cholesterol side chain cleavage [20] and since it has no direct effect upon 5α-reductase as tested in homogenates.

No endogeneous inhibition of 5α-reductase could be demonstrated in freshly dispersed cells. In monolayer culture, however, aminoglutethimide caused sometimes an increase in 5α-reduction of 17-epitestosterone which is compatible with endogeneous inhibition. The inclusion of aminoglutethimide in the incubation mixture could thus be omitted in freshly dispersed cells, but becomes necessary in monolayer culture especially when the steroid hormone production is stimulated in order to study the hormonal regulation of the 5α-reductase activity.

The biochemical characterization of 5α-reductase

The biochemical characteristics of the 5α-reductase have been described in the liver [8] and in androgen target tissues such as the prostate [9], the kidney [10], the hypothalamus [11], the pituitary [12] and the skin [13]. The target tissue 5α-reductases have, a substrate specificity limited to 4-ene-3-keto steroids without an oxygen on carbon eleven [1]. The presence of NADPH as a cofactor is strictly required and the reaction has an apparent K_m around 1 μM although 10 times lower affinities have been described in human hair follicles [21] and human brain [22]. The liver 5α-reductase activity has a higher K_m value and a broader substrate specificity. This led to the hypothesis that several 5α-reductases with different properties are operating in the liver [8].

The characteristics of the rat ovarian 5α-reductase are comparable with those from target tissues such as prostate and kidney. The role of the ovarian 5α-reductase could however be completely different. In androgen target tissues the formation of active androgens as DHT depends completely on the 5α-reduction of testosterone. In the ovary, on the contrary, most of the 5α-reduced androgens formed could derive from 5α-reduced C_{21} steroids. Indeed, a pathway metabolizing 5α-reduced C_{21} steroids to C_{19} steroids has been described [23]. Moreover, progesterone and 20α-hydroxy-pregn-4-en-3-one, which must locally be present in very high concentrations and which are better substrates for the 5α-reductase may be expected to compete actively for the enzyme and could even "protect" testosterone and androstenedione against 5α-reduction.

The inhibition of the 5α-reduction of 17-epitestosterone by various steroids was comparable both qualitatively and quantitatively in living ovarian cells and in prostate [9] or kidney [10] nuclei, except for the inhibition of the 5α-reductase activity by 17β-estradiol in intact cells (Table 1). Since this inhibition was uncompetitive [19] and since it was not found in homogenates, we suggest that 17β-estradiol interferes with the membrane transport of 17-epitestosterone or becomes cytotoxic in high concentration. Although the estrogen concentrations used are very high, this could have physiologic implications since in the follicular fluid estrogen concentrations up to 5×10^{-6} M have been described [24].

The activity of the 5α-reductase

The activity of the ovarian 5α-reductase is very high (1–10 nmol/mg protein/h) which is compatible with a regulation of steroidogenesis [2]. The activity in living ovarian cells was not substantially different from that found in cell homogenates supplemented with optimal concentrations of NADPH. The slight increase in 5α-reduction of 17-epitestosterone in homogenates can be accounted for by the number of death cells in the cell suspension. Thus, *in vivo*, the ovarian 5α-reductase is maximally active and the intracellular NADPH availability is not a limiting factor.

Acknowledgements—The skilful technical assistance of Mrs. M. Mentens-Oost, Miss G. Lemmens and Mrs. B. Willems-Minten are greatly appreciated. The Schering Co. and the Hoffmann-La Roche Co. kindly donated 5α-androstane-3β,17α-diol and aminoglutethimide respectively.

REFERENCES

1. Wilson J. D.: Metabolism of testicular androgens. In *Handbook of Physiology* (Edited by D. W. Hamilton and R. O. Greep). American Physiological Society, Washington (1975) Section 7, Vol. V, pp. 491–508.

2. Colby H. D., Caffrey J. L. and Kitay J. I.: Interaction of growth hormone and ACTH in the regulation of adrenocortical secretion in rats. *Endocrinology* **93** (1973) 188–192.
3. Louvet J. P., Bayard F., Pujol A., Camerling M. and Boulard Cl.: Role of DHT in follicular atresia. In *Abstracts of the V Intern. Congress of Endocr.* Hamburg (1976) Abstract 625, p. 257.
4. Armstrong D. T. and Dorrington J. H.: Androgens augment FSH induced progesterone secretion by cultured rat granulosa cells. *Endocrinology* **99** (1976) 1411–1414.
5. Lucky A. W., Schreiber J. R., Hillier S. G., Schulman J. D. and Ross G. T.: Progesterone production by cultured preantral rat granulosa cells: stimulation by androgens. *Endrocrinology* **100** (1977) 128–133.
6. Nimrod A. and Lindner H. R.: A synergistic effect of androgens on the stimulation of progesterone secretion by FSH in cultured rat granulosa cells. *Molec. Cell. Endrocr.* **5** (1976) 315–320.
7. Koninckx Ph. and De Moor P.: The effect of prostaglandins E_2 and $F_{2\alpha}$ on 5α-reductase activity in rat ovarian interstitial tissue *in vitro*. *Fertil. Steril.* **28** (1977) 367.
8. McGuire J. S. and Tomkins G. M.: The heterogeneity of Δ^4-3-ketosteroid reductases (5α). *J. biol. Chem.* **235** (1960) 1634–1638.
9. Frederiksen D. W. and Wilson J. D.: Partial characterisation of the nuclear reduced nicotinamide adenine dinucleotide phosphate: Δ^4-3-ketosteroid 5α-oxidoreductase of rat prostate. *J. biol. Chem.* **246** (1971) 2584–2593.
10. Verhoeven G. and De Moor P.: Kinetic data on the formation of 17β-hydroxy-5α-androstan-3-one in renal nuclei from male and female rats. *Endocrinology* **91** (1972) 54–64.
11. Cheng Y. J. and Karavolas H. J.: Subcellular distribution and properties of progesterone (Δ^4-steroid) 5α-reductase in rat medial basal hypothalamus. *J. biol. Chem.* **250** (1975) 7997–8003.
12. Cheng Y. J. and Karavolas H. J.: Properties and subcellular distribution of Δ^4-steroid (progesterone) 5α-reductase in rat anterior pituitary. *Steroids* **26** (1975) 57–71.
13. Takayasu S. and Adachi K.: The conversion of testosterone to 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone) by human hair follicles. *J. clin. Endocr. Metab.* **34** (1972) 1098–1101.
14. Hopkins C. R. and Farquehar M. G.: Hormone secretion by cells dissociated from rat anterior pituitaries. *J. Cell Biol.* **59** (1973) 276–303.
15. Verhoeven G. and De Moor P.: Intranuclear metabolism of testosterone in kidneys of male and female rats. *Endrocrinology* **89** (1971) 842–847.
16. Wright R. S.: A reagent for the non-destructive location of steroids and some other lipophilic materials on silica gel thin layer chromatograms. *J. Chromatogr.* **59** (1971) 220–221.
17. Neher R.: In *Steroid Chromatography*. Elsevier, Amsterdam (1964).
18. Evans C. R. and Pierrepoint C. G.: Demonstration of a specific cytosol receptor in the normal and hyperplastic canine prostate for 5α-androstane-3α,17α-diol. *J. Endrocr.* **64** (1975) 539–548.
19. Mahler H. R. and Cordes E. H.: In *Biological Chemistry*. Harper and Row (1969).
20. Gower, D. B.: Modifiers of steroid-hormone metabolism: a review of their chemistry, biochemistry and clinical applications. *J. steroid Biochem.* **5** (1974) 501–523.
21. Takayasu S. and Adachi K.: The *in vivo* and *in vitro* conversion of testosterone to 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone) by the sebaceous gland of hamsters. *Endocrinology* **90** (1972) 73–80.
22. Denef C., Magnus C. and McEwen B. S.: Sex differences and hormonal control of testosterone metabolism in rat pituitary and brain. *J. Endocr.* **59** (1973) 605–621.
23. Karakawa T., Kurachi K., Aono T. and Matsumoto K.: Formation of 5α-reduced C_{19} steroids from progesterone *in vitro* by a pathway trough 5α-reduced C_{21} steroids in ovaries of late prepubertal rats. *Endocrinology* **98** (1976) 571–579.
24. McNatty K. P., Baird D. T., Bolton A., Chambers P., Corker C. S. and McLean H.: Concentration of oestrogens and androgens in human ovarian venous plasma and follicular fluid throughout the menstrual cycle. *J. Endrocr.* **71** (1976) 77–85.