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PROPERTIES OF 3α-HYDROXYSTEROID DEHYDROGENASE OF THE MALE RAT PITUITARY GLAND

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SUMMARY

The 3α-hydroxysteroid dehydrogenase activity was mainly concentrated in the cytosol fraction of the male rat anterior hypophysis. The product of 5α-dihydrotestosterone reduction at 3 position was identified as 5α-androstane-3α, 17β-diol. No formation of 3β,17β-isomer was detected in these experiments.

Some properties of the 3α-hydroxysteroid dehydrogenase in the soluble fraction from the male rat pituitary gland were examined. Optimal pH of the reaction was around 6·5–7·0. Its apparent K_m was $2{\cdot}0 \times 10^{-6}$ M. The activity of 3α-hydroxysteroid dehydrogenase required NADPH as cofactor. Whereas the 5α-reductase activity in the rat anterior hypophysis was increased by castration, the rate of 3α-reduction of 5α-dihydrotestosterone in this gland was not influenced by orchidectomy.

INTRODUCTION

The *in vivo* and *in vitro* metabolism of testosterone in the anterior pituitary has been studied in several laboratories [1–8]. In our laboratory, testosterone metabolism in the male rat pituitary and nature of the androgens bound to cytoplasmic and nuclear "receptors" have been studied [9, 12]. Thus, it is well established that in anterior hypophysis, testosterone is metabolized into 5α-dihydrotestosterone (DHT) and 5α-androstane-3α, 17β-diol (3α-androstanediol). These catabolites can act as physiologically active substances when added in prostatic organ culture [13] and a possibility exists that they may affect the secretion of gonadotrophins. In this respect, studies on the testosterone-converting enzymes are necessary. Recently, we have reported some properties of the testosterone 5α-reductase from rat anterior hypophysis, its increasing amount following castration and its hormonal control [14]. The present paper deals with some kinetic properties of the 3α-hydroxysteroid dehydrogenase (3α-ol dehydrogenase) responsible for the formation of 3α-androstanediol in the male rat pituitary.

MATERIALS AND METHODS

Chemicals

[^{3}H]-testosterone and [^{3}H]-dihydrotestosterone: (S.A, 42 Ci/mmol and 43 Ci/mmol respectively) were supplied by C.E.N. Belgium. Their radiochemical purity was tested by paper chromatography. For enzyme assay, the initial solutions were brought to dryness in test tube and then taken up by appropriate reagent mixture.

Androgens were purchased from Sigma Chemical Company, U.S.A., bovine serum albumin was supplied by Nutritional Biochemical Corp, Cleveland, U.S.A., NADPH and NADH were from Boehringer Mannheim, Germany; other products were from commercial sources.

Animals

Experiments were done with 80-day-old male Wistar rats provided by Elevage Janvier, Le Genest, France. Castration was performed via the abdominal route under ether anaesthesia. The animals were maintained on U.A.R. no. 103 diet and water *ad lib.* with light from 7 a.m. to 7 p.m.

Preparation and incubation of the soluble fraction

In most assays of 3α-ol dehydrogenase, anterior pituitary glands of 3 rats were used. The animals were decapitated and the pituitaries rapidly removed and homogenized in a Potter–Elvehjem type homogenizer with 2 ml of incubation medium (1 mM phosphate buffer pH 6·5–1 mM $MgCl_2$–0·32 M sucrose). The whole homogenate was first centrifuged at 10,000 *g* for 10 min, and the supernatant submitted to a centrifugation at 105,000 *g* for 60 min. The 105,000 *g* soluble fraction as well as the foregoing preparation were kept at 0°C–4°C. For each enzymatic assay, the soluble fraction (5 ml) prepared from organs of three rats, was diluted with medium up to 30 ml and 100 μl samples of the solution were incubated in conical tubes containing 30 μl of 3·3 mM NADPH solution and [^{3}H]-DHT (200,000 d.p.m. or $1{\cdot}62 \times 10^{-8}$ M). Unless otherwise stated, the incubations were carried out at 37°C for 30 min and the reaction stopped by addition of some drops of glacial acetic acid.

Extraction, separation and identification of steroids

The radioactive steroids were extracted from the incubation mixture with 10 vol. of diethyl ether and the

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organic fraction brought to dryness. The extracts were analyzed by t.l.c. on GF_{254} Merck Silica gel (250 μm-thick sheets activated at 100°C for 1 h before use). Development was performed at 30°C in the solvent system: chloroform–methanol (98:2, v/v) [15]. R_F values were 0·22, 0·36, 0·44, 0·63, 0·75 for androstanediols (α and β forms), testosterone, DHT, androstenedione and androstanedione respectively. Authentic testosterone and androstenedione were added to the extracts as carriers detectable under U.V. light. For routine estimations, radioactivity was determined in 4 zones of the chromatogram: starting zone (0–2 cm), zone of androstanediol, zone of testosterone and DHT, zone of androstenedione and solvent front zone. The gel of each area was then scrapped off and put into vials containing 1 ml methanol. Bray's solvent (10 ml) was then added to each vial for counting in a Mark I liquid scintillation spectrometer (Nuclear Chicago). After paper chromatography in the solvent system: heptane–benzene–methanol–water (5:1:5:1 by vol.), 3α-androstanediol (R_F 0·44) was separated from 3β-androstanediol (R_F 0·32) in order to confirm the identity of these metabolites.

Crystallization of radioactive androstanediol

The radioactive metabolite derived from [^{3}H]-DHT was eluted from the gel with diethyl ether and the extract brought to dryness. Its identity was confirmed by recrystallization to constant S.A. in purified methanol in the presence of authentic 3α-androstanediol as carrier.

Conversion of [^{3}H]-*testosterone*

In assays carried out with [^{3}H]-testosterone, the radioactive steroids were extracted from the incubation medium with diethyl ether and the extract taken to dryness. Separation and estimation of the metabolites were performed by paper chromatography in the solvent system of Kochakian and Stidworthy (benzene–cyclohexane–propanediol, 100:100:20, by vol.) [17]. Other experimental procedures were the same as previously described [14].

Preparation of citrate–phosphate–borate buffer

The mixed buffer was prepared following a method described by Theorell and Stenhagen[18].

RESULTS

1. *Fractionation and characterization of* 3α-*androstanediol*

After incubation of rat pituitary cytosol with [^{3}H]-DHT, the radioactive steroids were extracted with diethyl ether and routinely analysed by t.l.c. Two peaks of radioactivity were found on chromatogram scans, one corresponding to [^{3}H]-DHT, the other having the mobility of 3α-androstanediol. Further chromatography and recrystallization to constant S.A. confirmed the identity of this steroid (Table 1). Thus, it appears that 3α-androstanediol is the single metabolite produced during incubation of pituitary cytosol with [^{3}H]-DHT.

Table 1. Recrystallization of 3α-androstanediol to constant specific activity

Recrystallization	Specific radioactivity (dpm/mg carrier) Mother liquor	Crystal
Starting material	2116	
1st Crystallization	1999	1835
2nd Crystallization	1761	1820
3rd Crystallization	1767	1753

2. *Kinetic properties of* 3α-ol *dehydrogenase*

The results of an investigation of the time course of the enzyme activity in hypophysis of normal and castrated rats, are shown in Fig. 1. The enzymic activity appeared unchanged up to 40 min. Denaturation of 3α-ol dehydrogenase occurred on long-term incubation, a decrease of 20–25% of the initial activity was seen after 120 min.

The effect of substrate variation could be obtained by recasting the results in linear form, the substrate variation taken as a function of time:

$$S = S_0\, e^{-(V/K_m)t}, \text{ or } \ln \frac{S_0}{S} = \frac{V}{K_m} t \text{ (when } S_0 \ll K_m)$$

these plots show again a low decrease (15–20%) of enzyme activity after 120 min. Consequently an incubation time of 30 min has been chosen for all further subsequent investigations.

Determination of apparent K_m. Results of the study of the substrate concentration effect on the reaction velocity are shown in Fig. 2. DHT concentration was in the range of $4{\cdot}5 \times 10^{-7}$ M to $2{\cdot}5 \times 10^{-5}$ M. For the Lineweaver–Burk plot we used the modified method of Wilson *et al.*[19]. Values of (S) were then taken as the arithmetical mean of values of substrate concentrations during the incubation. The method was used because the reaction occurred to a large extent at

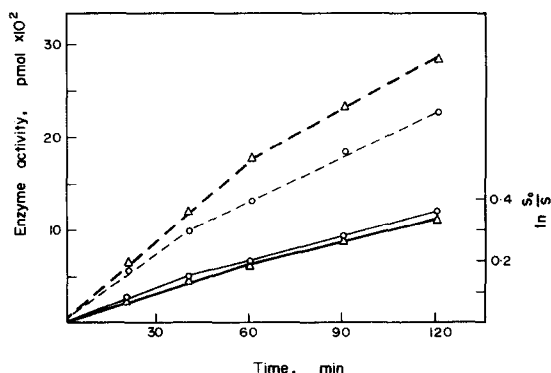

Fig. 1. Time course of 3α-ol dehydrogenase reaction. The soluble fractions prepared from organs of normal (ΔΔΔ) and 15-days castrated rats (OOO) were incubated under standard conditions. The enzyme activity was expressed as pmol × 10^{-2} of androstanediol formed per 30 min and per μg of cytosol proteins (dotted lines). Values of ln S_0/S are illustrated by solid lines.

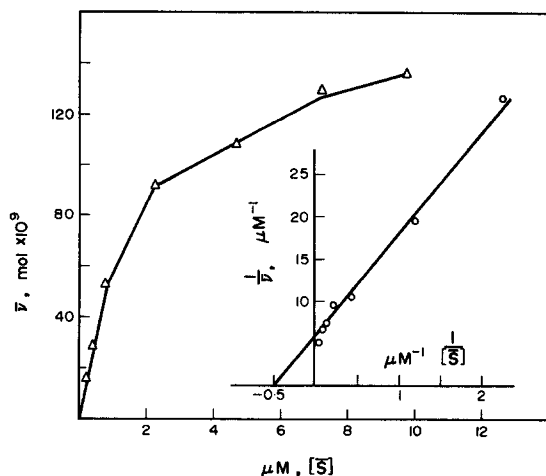

Fig. 2. Activity of 3α-ol dehydrogenase as a function of substrate concentration. The soluble fraction (5 ml) was obtained from organs of three normal rats and diluted up to 30 ml. Samples (100 μl) of the aliquot were incubated in conical tubes containing NADPH ($0{\cdot}8\ 10^{-3}$ M) 200,000 d.p.m. of [^{3}H]-DHT ($2{\cdot}11 \times 10^{-8}$ M) and varying concentrations of non-radioactive DHT. Velocity of the reaction (△——△) was expressed as mol × 10^{-9} of androstanediol produced per hour and per μg of cytosol proteins. O——O: Lineweaver–Burke plot.

low concentration of DHT, androstanediol formation went to the extent of 30% when $(S) \ll K_M/10$. Linear plotting of data gave a K_m value of 2·0 μM. A high affinity of DHT for 3α-ol dehydrogenase has thus been observed. For most investigations, enzymatic assays were carried out with $1{\cdot}62 \times 10^{-8}$ M of DHT (first order reaction) and results expressed as pmol × 10^{-2} of androstanediol formed per μg of cytosol proteins.

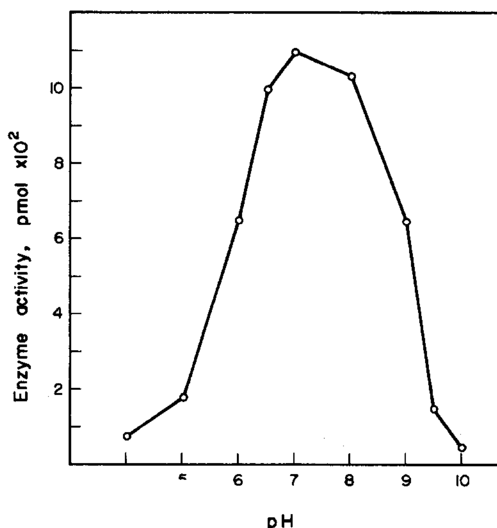

Fig. 3. Effect of pH on 3α-ol dehydrogenase activity. The cytosol from 3 hypophysis was prepared in 10^{-2} M NaCl–10^{-3} M $MgCl_2$ solution (no buffer). Samples (100 μl) were diluted (to 2 ml) in the mixed buffer at varying pH and fractions (150 μl) of the diluted preparations were incubated at 37°C during 30 min in conical tubes containing [^{3}H]-DHT (200,000 d.p.m.) and NADPH (50 μl of a solution 3·3 mM).

3. *Effects of pH and temperature*

The activity of 3α-ol dehydrogenase as a function of pH was determined in the citrate–borate–phosphate buffer containing quantities of NADPH and $MgCl_2$ previously used, over the range pH 4–10. The results are shown in Fig. 4. A rapid decrease in enzyme activity occurred when the incubation medium became acid. Maximum of activity was observed in the range pH 6·5–8·0 (Fig. 3). We chose pH 6·5 for routine assays in order to have a comparison with our previous study of 5α-reductase.

The results of varying the temperature are shown in Fig. 4. The optimum temperature of enzyme activity was between 37 and 40°C. A relatively low value of Q_{10} was noticed since the activity was about three fold higher at 37°C than at 0°C. High temperature caused denaturation of 3α-ol dehydrogenase, a decrease in enzyme activity 47% occurred in incubations carried out at 45°C (incubation time = 30 min), the enzyme thus showed a pronounced thermolability.

4. *Relationship between 5α-reduction and 3α-reduction in the pituitary gland*

Effect of NADPH concentration. Androstanediol formation was favored in enzyme assays with high concentration of NADPH. In this view, the 800 ***g*** supernatant of hypophysis homogenates was incubated with radioactive testosterone and varying amount of NADPH. In order to obtain high activity of 5α-reductase, and so a relatively great yield of DHT, organs of 30 days castrated animals were used. Results of these assays are summarized in Fig. 5. An enhancement of metabolism was observed with NADPH concentration increasing up to $5{\cdot}10^{-4}$ M, pronounced rise of androstanediol and DHT yields began at 10^{-5} M. With regard to the balance of the two metabolites, DHT was

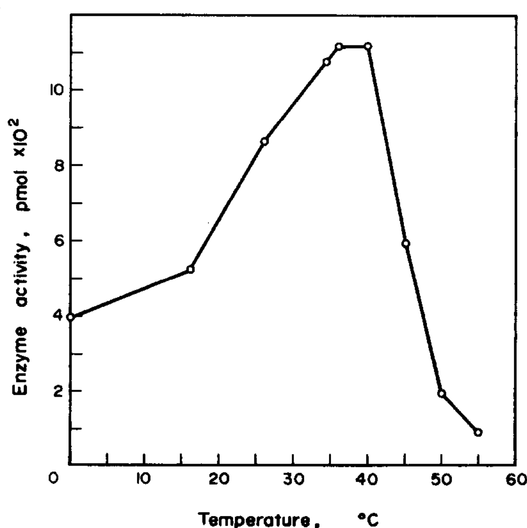

Fig. 4. Effect of temperature on 3α-ol dehydrogenase activity. Assays were performed at varying temperatures and under other standard conditions as described in Materials and Methods. Values reported in this figure and in Fig. 3 were the arithmetical mean of two independent determinations.

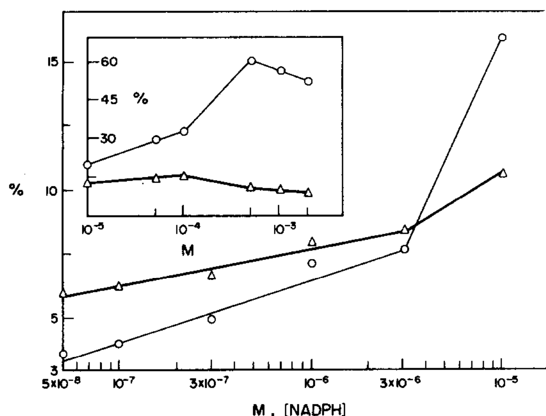

Fig. 5. Effect of NADPH concentration on the distribution pattern of testosterone metabolites. The 800 *g* supernatant (prepared in 350 μl of the extraction medium) was obtained from 8 organs of 30-days castrated rats. Samples (50 μl) of the aliquot were incubated in conical tubes containing varying concentration of NADPH and [^{3}H]-testosterone (200,000 d.p.m.). O——O: per cent of androstanediol Δ——Δ: per cent of DHT.

formed in greater extent at low concentration of NADPH, and androstanediol at high concentration. Consequently, NADPH appeared to be an important determinant of testosterone metabolism in hypophysis and the intracellular concentration of the cofactor in the gland would be $5 \cdot 10^{-8}$ M or lower.

Table 2. Effect of castration on 3α-ol dehydrogenase activity (mean of three determinations, ± S.E.M.)

	Protein concentration (μg/100 μl)	Specific activity (pmol × $10^{-2}/\mu$g of cytosol protein)
Intact rat	1·75 ± 0·85	10·7 ± 0·8
Castrated rat	2·13 ± 0·85	10·5 ± 1·5

The effect of castration on 3α-ol *dehydrogenase activity in cytosol prepared from organs of* 15 *days castrated rats.* This was determined under standard conditions and the results were compared with those obtained with intact animals (Table 2). A 12% increase of cytosol protein was observed in the castrates. This result is in agreement with previous observations concerning hypertrophy and hyperplasia of anterior pituitary gland induced by castration [20]. But the 3α-ol dehydrogenase activity appeared unchanged after castration. As shown in Fig. 2, similar values of 3α-ol dehydrogenase activity were found in normal and castrated rats. Interpretation of these observations, as compared to the results of the study on 5α-reductase previously reported [14], will be discussed below.

DISCUSSION

It was found that 3α-reductase activity of 5α-dihydrotestosterone was mainly located in the soluble fraction of rat pituitary gland. The 3α-hydroxysteroid dehydrogenase S.A. in mitochondria, microsomes and soluble fraction was found to be 0·75, 2·28 and 10·36 pmol × $10^{-2}/\mu$g protein respectively. Thus, most of this dehydrogenase was in the cytosol fraction. This suggests that 5α-dihydrotestosterone produced in the microsomal fraction is transferred to the soluble fraction for reduction of the 3-oxo group to 3α-hydroxyl. This intracellular distribution of the 3α-hydroxysteroid dehydrogenase is in agreement with previous works. Nozu and Tamaoki[21], Shimazaki *et al.*[22] have found that 5a-dihydrotestosterone reduction was mainly observed in the soluble fraction from rat ventral prostate. The same subcellular localization was reported by Arimasa and Kochakian in female rat liver [23].

The present investigation revealed that 5α-androstane-3α, 17β-diol is the main isomer accumulated in the soluble fraction from male rat anterior hypophysis. The formation of 3β,17β-isomer was not detected. This result is in agreement with previous reports. Indeed the 3α,17β-diol is the most common metabolite of testosterone found in rat ventral prostate [21, 22, 24], canine prostate [25], female rat liver [23] and human male scalp skin [26].

The 3α-hydroxysteroid dehydrogenase activity was 300 times higher than the 5α-reductase activity in anterior hypophysis preparations. Therefore, 3α-androstanediol would be the major metabolite produced after incubation of pituitary gland in the presence of testosterone. In fact, that is not the case [14]. Our results have demonstrated that DHT was formed to a large extent at low concentration of NADPH while at high concentration of cofactor androstanediol production was increased. It is suggested that testosterone metabolism in rat pituitary gland is dependent on the intracellular NADPH concentration which governs dihydrotestosterone or 5α-androstane-3α, 17β-diol production selectively.

Recently, we reported that 5α-reductase activity of rat pituitary gland was markedly increased by castration and reduced by androgen administration to castrated rats [14, 27]. These results are in agreement with those of McEwen *et al.*[28]. However, the rate of 3α-reduction of 5α-dihydrotestosterone seems to be unaffected by castration and 3α-hydroxysteroid dehydrogenase activity was unchanged in the rat ventral prostate after castration [22].

REFERENCES

1. Anderson K. and Liao S.: *Nature* **219** (1968) 277–279.
2. Jaffé R.: *Steroids* **14** (1969) 483–498.
3. Sholiton L. and Werk F.: *Acta endocr. Copenh.* **61** (1969) 641–648.
4. Perez-Palacios G., Castenada E., Gomez-Perez F., Perez A. and Gual C.: *Biol. Reprod.* **3** (1970) 205–335.
5. Rommerts F. and Van Der Molen H.: *Biochim. biophys. Acta* **246** (1971) 489–502.
6. Massa R., Stupnicka E., Kniewald Z. and Martini L.: *J. steroid Biochem.* **3** (1972) 385–399.
7. Monbon M., Loras B., Reboud J. P. and Bertrand J.: *Brain Res.* **53** (1973) 139–150.
8. Loras B., Genot A., Monbon M., Buscher F., Reboud J. P. and Bertrand J.: *J. steroid Biochem.* **5** (1974) 425–431.

9. Jouan P., Samperez S., Thieulant M. L. and Mercier L.: *C. r. hebd. Séanc. Acad. Sci., Paris* **272** (1971) 2368–2371.
10. Jouan P., Samperez S., Thieulant M. L. and Mercier L.: *J. steroid Biochem.* **2** (1971) 223–236.
11. Jouan P., Samperez S. and Thieulant M. L.: *J. steroid Biochem.* **4** (1973) 65–75.
12. Thieulant M. L., Samperez S. and Jouan P.: *J. steroid Biochem.* **4** (1973) 677–685.
13. Robel P., Lasnitzki I. and Baulieu E. E.: *Biochimie* **53** (1971) 81–96.
14. Nguyen C. T., Duval J., Samperez S. and Jouan P.: *Biochimie* **56** (1974) 899–906.
15. Bruchovsky N.: *Endocrinology* **89** (1971) 1212–1222.
16. Bray C.: *Analyt. Biochem.* **1** (1960) 279–285.
17. Kochakian Ch. and Stidworthy G.: *J. biol. Chem.* **199** (1952) 607–612.
18. Theorell and Stenhagen: Memento Scientifique. Geigy (1956) p. 101 (Edited by Geigy B.), Basle.
19. Hyun-Jae-Lee and Wilson I.: *Biochim biophys. Acta* **242** (1971) 519–522.
20. Nguyen T. P., Valotaire Y., Duval J. and Jouan P.: *C. r. hebd. Séanc. Acad. Sci.*, Paris **271** (1970) 1442–1445.
21. Nozu K. and Tamaoki B. I.: *Acta endocr., Copenh.* **73** (1973) 585–598.
22. Shimazaki J., Kato N., Nagai H., Yamanoka H. and Shida K.: *Endocr. japon* **19** (1972) 97–106.
23. Arimasa N. and Kochakian Ch.: *Steroids* **19** (1972) 325–355.
24. Bruchovsky N. and Wilson J.: *J. biol Chem.* **243** (1968) 2012–2021.
25. Harper M., Pierrepoint C., Fahmy A. and Griffiths J.: *Endocrinology* **49** (1971) 213–223.
26. Bingham K. and Shaw D.: *J. Endocr.* **57** (1973) 111–121.
27. Thieulant M. L., Nguyen C. T., Samperez S. and Jouan P.: *Biochimie* **55** (1973) 991–992.
28. Denef C., Magnus C. and McEwen B. J.: *Endocrinology* **59** (1973) 605–621.