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ANDROGEN AND ESTROGEN RECEPTORS IN THE CYTOSOL FROM MALE RAT ANTERIOR HYPOPHYSIS: FURTHER CHARACTERISTICS AND DIFFERENTIATION BETWEEN ANDROGEN AND ESTROGEN RECEPTORS

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SUMMARY

Further characteristics of androgen and estrogen receptors from male rat pituitary cytosol were studied.

Following ultra-centrifugation in a linear sucrose gradient (5–20%) two radioactive peaks for testosterone were observed, one at the top of the gradient, and one in the 7–8 *S* region. For dihydrotestosterone there were also two peaks one in the 3–4 *S* region, the other in the 7–8 *S* region. Only the 7–8 *S* peaks were specific for androgens. Under the same conditions, estradiol-17β exhibited only one peak in the 8–10 *S* region.

When rats were previously castrated, an increase of the 7–8 *S* peak for testosterone and for dihydrotestosterone was observed. Thus, orchidectomy did not abolish androgen receptors in pituitary cytosol.

It was likely that androgen and estrogen receptors were proteins. Indeed, the hormone–receptor binding was abolished to a great extent by proteolytic enzymes action.

Taking into account some characteristics of these receptors: dissociation constants, specific binding capacity, sedimentation constants, sensitivity to heat or to enzyme action, it seemed easy to differentiate androgen receptors from estrogen receptors. Thus, it was concluded that pituitary cytosol from male rats contained two kinds of receptors, one for androgens, and one for estrogens. Contrarily, it is not certain at the moment that there are two androgen receptors, one for testosterone and one for dihydrotestosterone, or that there is only one receptor for both hormones.

INTRODUCTION

In previous papers we demonstrated the presence of specific receptors for testosterone and dihydrotestosterone in the pituitary cytosol from prepubertal male rats [1, 2]. Likewise, Kato and Onouchi [3, 4] and more recently Naess *et al.* [5] also described the presence of binding components for testosterone and dihydrotestosterone in the same organ. At the same time, other authors found an 8 *S* receptor for estrogen in the cytosol from male pituitary [6, 10]. Therefore we can assume that in the male anterior hypophysis there are receptors for androgen together with a receptor for estrogen, and it is very likely that these binding macromolecules could play a part in the regulation of gonadotropins secretion.

The purpose of our present work is: (1) to investigate some further characteristics of androgen and estrogen receptors, in male rat anterior pituitary; (2) to attempt to distinguish between testosterone receptor and dihydrotestosterone receptor; (3) to point out differences between androgen and estrogen receptors.

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MATERIALS AND METHODS

Steroids and chemicals. [1,2-^{3}H]-Testosterone [S.A. 46 Ci/mmol], [1,2-^{3}H]-Dihydrotestosterone [DHT, S.A. 43 Ci/mmol] were purchased from CEN (Mole, Belgium). [6,7-^{3}H]-Estradiol [S.A. 60 Ci/mmol] was obtained from CEA [Gif-sur-Yvette, France). 5α[1,2-^{3}H]-androstane-3α,17β-diol [3α-Diol, S.A. 44 Ci/mmol] was from New England Nuclear Chemicals (Dreieichenhain, Germany). Their radiochemical purity was checked before use by paper chromatography in the solvent system of Kochakian and Stidworthy [11].

Unlabelled steroids were supplied by Sigma Chemical Company (U.S.A.). Sephadex G-25 and Dextran T_{70} were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Pronase, Trypsin, chymotrypsin A, RNAse A and RNAse T_1 were supplied by Boerhinger (Mannheim, Germany).

Animals. Male Wistar rats, from Elevage Janvier (LeGenest, France), 35–37 days old were used in our experiments. When castration was needed, it was performed by abdominal route. Castrated animals were killed 2 days or 15 days later.

Preparation of subcellular fractions. Animals were killed by decapitation without anaesthesia, and anterior pituitaries were quickly removed and homogenized in a small vol. of 0.05 M phosphate buffer pH 7.4. The homogenate was then centrifuged at 800 *g* and the supernatant was recentrifuged at 105,000 *g* for 60 min in the Rotor SW 50 of a Beckman L_4 ultra-centrifuge. All operations were carried out at 4°C. The 105,000 *g* supernatant (cytosol) was used subsequently in our experiments.

Incubations. For the study of androgens binding, cytosol fractions (440 μl, protein concentration 9800 μg/ml) were incubated for 6 h at 0°C, in the presence of 2.10^{-10}M tritiated testosterone, or DHT, or 3α-Diol. For competition assays 2.10^{-7}M unlabelled steroids were added in the incubation medium.

When estradiol binding was studied cytosol fractions (150 μl, protein concentration 2920 μg/ml) were incubated for 4 h at 0°C with tritiated estradiol (4.10^{-10}M). Competition assays were carried out using 4.10^{-8}M unlabelled steroids.

Enzymes action. Cytosol fractions (1.6 ml, proteins concentration 2920 μg/ml) were incubated at 0°C for 6 h with tritiated testosterone or DHT ($1.5 \cdot 10^{-9}$M). Then, aliquots (300 μl) were reincubated at 37°C for 30 min in the presence of various enzymes dissolved in 0.05 M phosphate buffer: trypsin, chymotrypsin A, RNAse A (250 μg), pronase (100 μg), RNAse T_1 (50 μg). Following incubation, cytosol fractions were submitted to gel-filtration through a column of Sephadex G-25 medium in order to separate bound from unbound radioactivity.

For the study of estrogen receptor, the procedure was the same as the previous one except that cytosol fractions were 150 μl. Enzymes concentration was the same as described above. Controls were carried out in the same conditions but enzymes were omitted.

Heat-sensitivity. Following incubations at 0°C, cytosol fractions were incubated at 37°C for various periods of time. Then, they were submitted to gel-filtration and bound radioactivity was measured.

Dissociation by Dextran coated charcoal. Norit A charcoal (500 mg) and Dextran T_{70} (50 mg) were mixed together in 100 ml of 0.05 M phosphate buffer pH 7.4. Pituitary cytosol (500 μl) from rats (100 days old) castrated 48 h before, was incubated at equilibrium (16 h at 0°C) with [^{3}H]-testosterone or [^{3}H]-DHT at various concentrations (see Fig. 6). Then it was filtered through Sephadex G-25 column and bound radioactivity was collected by fractions. 2 ml of Dextran coated charcoal were then added to 2 ml of bound radioactive fraction and they were incubated at 0°C for various periods of time (from 10 to 180 min). Following incubation, aliquots (300 μl) were centrifuged at 5000 rev./min for 5 min and the radioactivity of the supernatant was measured.

Gel-filtration. Separation of bound from unbound radioactivity was accomplished using gel-filtration through a column (27 × 1.5 cm.) of Sephadex G-25 medium. Gel was equilibrated in 0.05 M phosphate buffer as previously described (2).

Sucrose gradient centrifugation. Sedimentation coefficients of receptors were determined according to the method of Martin and Ames [12], using BSA and

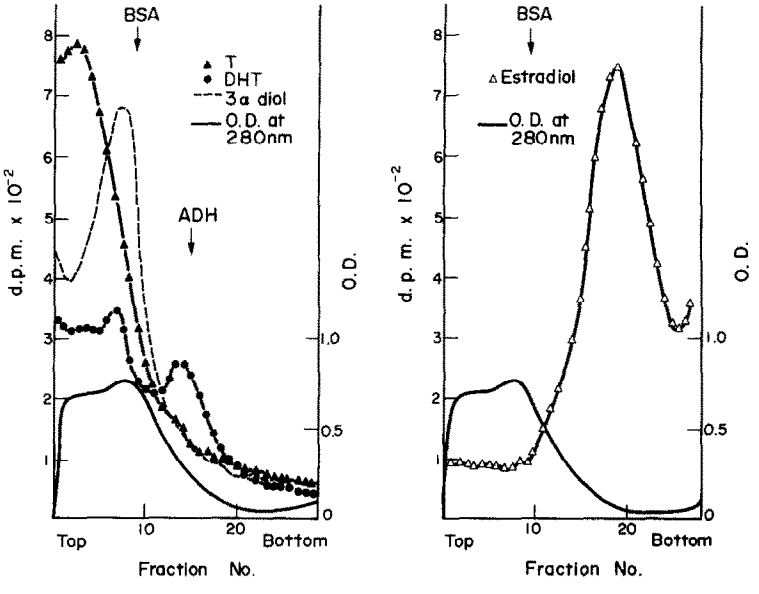

Fig. 1. Sedimentation pattern of cytoplasmic binding components in a linear 5–20% sucrose gradient (see materials and methods). Cytosol was prepared from 24 anterior hypophysis in 0.05 M phosphate buffer (protein concentration: 9.8 mg/ml), and was incubated at 0°C with 2×10^{-10} M of the following tritiated steroids: (a) testosterone (—▲—), 5α-DHT (—●—), 5α-androstane-3α,17β-diol (– – –), for 6 h; (b) estradiol-17β (—△—), for 4 h. Aliquots (400 μl) were then layered over preformed sucrose gradients and centrifuged at 39,000 rev./min for 14 h at 4°C in a SW-50 rotor of a Beckman ultracentrifuge.

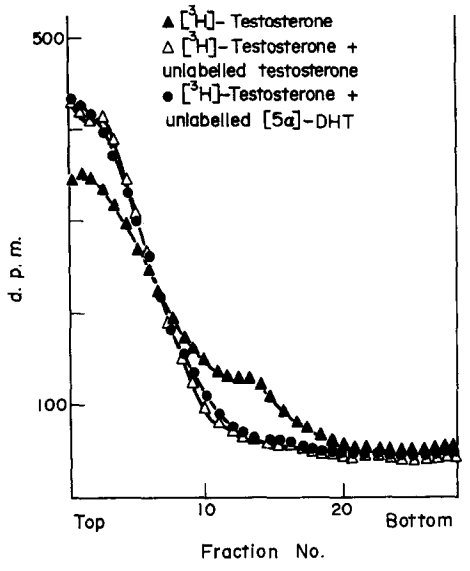

Fig. 2. Competition between [^{3}H]-testosterone and unlabelled steroids. Cytosol was prepared and sucrose gradient centrifugation was performed as given in the legend to Fig. 1. —▲— control: [^{3}H]-testosterone 1×10^{-10}M; —△— [^{3}H]-testosterone 1×10^{-10} M + unlabelled testosterone 1×10^{-7}M; —●— [^{3}H]-testosterone 1×10^{-10}M + unlabelled 5-DHT 1×10^{-7}M.

yeast alcohol dehydrogenase as marker proteins. Cytosols were incubated at 0°C in the presence of labelled steroids (2.10^{-10}M). When equilibrium was reached (6 h for androgens, 4 h for estrogens) aliquots (400 μl) were layered over preformed 5–20% sucrose gradients (sucrose was dissolved in 0.05 M phosphate buffer pH 7.4). Gradients were centrifuged for 14 h at 39,000 rev./min. at 4°C in a SW_{50} rotor of a Beckman L_4 ultra-centrifuge. Fractions (6 drops) were collected using a density gradient fractionator (ISCO, Lincoln U.S.A.). Proteins were determined at 280 nm with the help of a U.V. analyser (ISCO, UA-2 model).

Other analytical procedures. Proteins were determined according to the method of Lowry *et al.* [13] using bovine serum albumin as standard.

Radioactivity was measured using an Isocap 300 spectrometer (Nuclear Chicago). Cytosols and eluates from gel-filtration were dissolved in Bray's mixture [14]. Aliquots of sucrose gradients were counted in Instagel.

RESULTS

1. *Sucrose gradient profiles.* Following incubation of cytosol at equilibrium in the presence of tritiated steroids, androgen and estrogen receptors were analysed by ultra-centrifugation in a linear sucrose gradient. Representative sedimentation patterns are shown in Fig. 1. It could be shown that in pituitary cytosol from prepubertal male rats, there were two radioactive peaks for testosterone: one at the top of the gradient, the other in the 7–8 *S* region. DHT exhibited also two peaks, the first with 3.8 *S* constant, the second 7.5 *S*. In contrast 3α-Diol presented only one peak in the 4 *S* region.

In case of estradiol, there was only one peak in the 8–10 *S* region.

2. *Specificity of binding.* In order to determine the specificity of the macromolecular binding of androgens or of estrogens, incubations were carried out at equilibrium in the presence of tritiated hormones (2.10^{-10}M) and of unlabelled steroids (1.10^{-7}M). Aliquots were then submitted to centrifugation in a linear sucrose gradient. We could observe that the 7 *S* radioactive peak of testosterone and of DHT was reduced by unlabelled hormones (Figs. 2 and 3). The 3.8 *S* peak was not modified. In addition, corticosterone was without effect on the 7 *S* peak. Unlabelled 3α-Diol had no competitive action on the 4 *S* peak of tritiated 3α-Diol (Fig. 4).

The radioactive peak of estradiol-17β was highly specific since only unlabelled estradiol-17β, estrone or diethylstylbestrol had a competitive effect towards these binding sites. Androgens and corticosterone were without any effect.

3. *Effect of castration.* Prepubertal male rats, 35 days old were castrated and sacrificed 15 days later. Analysis of cytosol bindings by sucrose gradient showed that the 7 *S* peak observed in case of testosterone or of DHT was increased by castration (Fig. 5). This fact was specially clear in case of DHT.

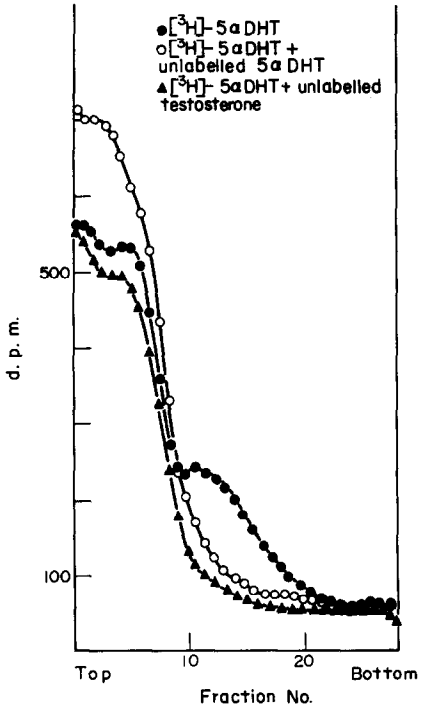

Fig. 3. Competition between [^{3}H]-DHT and unlabelled steroids. Cytosol preparation and sucrose gradient centrifugation as given in the legend to Fig. 1. —●— control: [^{3}H]-5α-DHT (2×10^{-10}M); —○— [^{3}H]-5α-DHT (2×10^{-10}M) + unlabelled 5α-DHT (2×10^{-7}M); —▲— [^{3}H]-5α-DHT (2×10^{-10}M) + unlabelled testosterone (2×10^{-7}M).

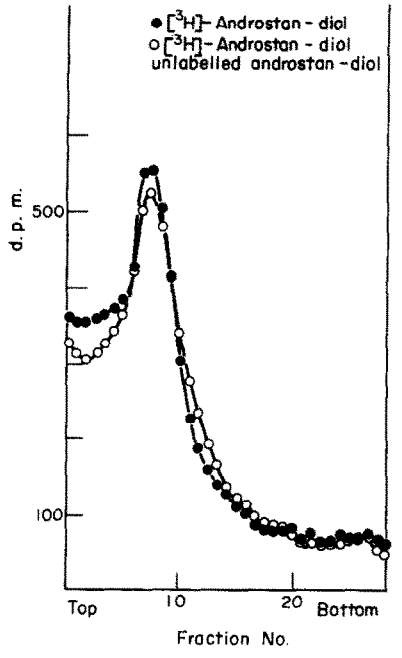

Fig. 4. Competition between [^{3}H]-5α-androstane-3α,17β-diol and unlabelled 5α-androstane-3α,17β-diol. —●— control: [^{3}H]-5α-androstane-3α,17β-diol (2×10^{-10}M); —○— [^{3}H]-5α-androstane-3α,17β-diol (2×10^{-10}M) + unlabelled 5α-androstane-3α,17β-diol (2×10^{-7}M).

4. *Effects of various enzymes.* The macromolecular binding of testosterone and of DHT was considerably decreased by action of proteolytic enzymes (Table 1). In contrast, pancreatic ribonuclease or ribonuclease T_1 had no action.

The estradiol-17β-receptor binding was completely abolished by proteolytic enzymes (Table 1). Furthermore, it was also decreased by action of the two ribonucleases (Table 1).

5. *Sensitivity to heat.* When pituitary cytosol was incubated at 0°C in the presence of labelled steroids, the hormone–receptor complex was stable for at least 24 h. But, when cytosol was heated at 37°C, after it was incubated at 0°C with tritiated hormones, the S.A. of the binding decreased, more obviously in case of estrogens, the estradiol–receptor complex was almost completely abolished by heating at 37°C (Table 2).

6. *Dextran coated charcoal assays.* Under our experimental conditions, the Dextran coated charcoal technique did not allow us to completely exclude the non-specific binding of androgens. However, by means of this method, we could measure the dissociation rate constants according to the equation of Mester and Robertson [15]. The following results were obtained: for testosterone $k_{-1} = 2.3 \cdot 10^{-5} \cdot S^{-1}$ and for DHT $K_{-1} = 3 \cdot 10^{-5} \cdot S^{-1}$ (Fig. 6).

DISCUSSION

All experiments we described here were carried out at equilibrium. When pituitary cytosol from prepubertal male rats was incubated at 0°C for periods of time varying from 5 min to 24 h in the presence of tritiated hormones, equilibrium was seen to be reached at 5 h for androgens and at 1 h only for estrogens. This first observation substantiates the evidence for a real difference between androgen and estrogen receptors.

When pituitary cytosol was centrifuged in a linear sucrose gradient following *in vitro* administration of [^{3}H]-testosterone of or [^{3}H]-DHT, two radioactive

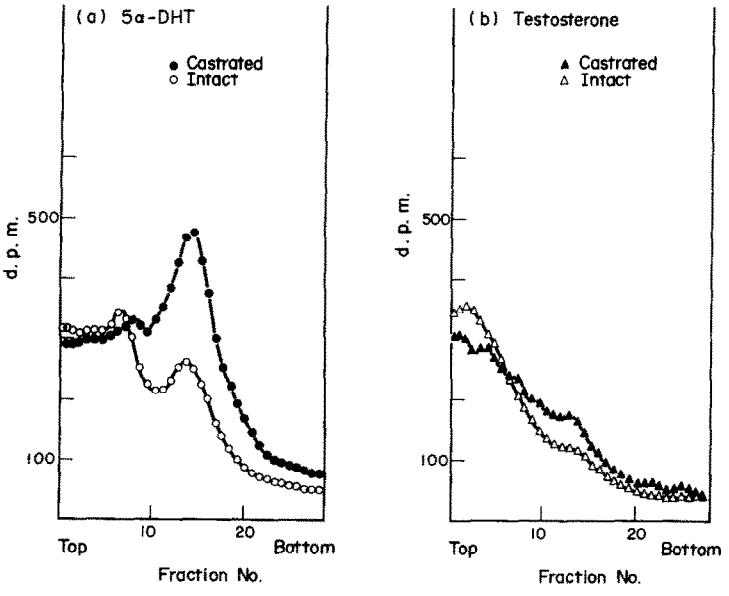

Fig. 5. Comparative effect of castration on the sedimentation pattern of (a) 5α-DHT and (b) testosterone receptors. 35 days old rats were castrated and sacrificed 15 days later. Cytosols (protein concentration 9.8 mg/ml) were incubated at 0°C for 6 h with: (a) 2×10^{-10}M [^{3}H]-5α-DHT; (b) 1×10^{-10}M [^{3}H]-testosterone. Sucrose gradient centrifugation was performed as given in the legend to Fig. 1.

Table 1. Effect of various enzymes on the macromolecular binding of testosterone, 5α-DHT and estradiol-17β in cytosol from male rats pituitaries. Results are expressed as ratio of bound radioactivity in assays to bound radioactivity in controls

	[^{3}H]-Testosterone binding (%)	[^{3}H]-5α-DHT binding (%)	[^{3}H]-Estradiol-17β binding (%)
Controls	100	100	100
Pronase	47.0	44.2	4.0
Trypsin	33.1	56.1	4.5
Chymotrypsin A	38.1	43.9	2.5
RNAse A	98.2	97.4	72.0
RNAse T_1	112.0	99.1	41.0

peaks appeared: one in the 3–4 *S* region, the other, more discrete, in the 7 *S* region. Testosterone 7 *S* peak was not easily identified whereas DHT 7 *S* peak was well demonstrated. These results were identical to those obtained in studies of prostatic cytosol. Mainwaring [16], Jung and Baulieu [17], Nozu and Tamaoki [18] found in the prostate two binding components for androgens, one very wide in the 4–5 *S* region, and another in the 8 *S* region. They established that the 8 *S* peak corresponded to specific receptor for testosterone and DHT.

Using a competition technique it was found that the 3–4 *S* peak was non-specific associations for androgens whereas the 7 *S* peak corresponded to specific receptors for testosterone and DHT. Only a small amount of radioactivity was found in the 7 *S* region. That is probably due to the instability of androgen receptors and also to the fact that, even in prepubertal rats, a part of receptor sites are occupied by endogenous androgens. Jung and Baulieu [17] expressed the same opinion referring to the prostatic 7 *S* peak for androgens. Our opinion was corroborated by the study of castrated rats cytosol. It was observed that, following castration there was an increase of the 7 *S* radioactivity in case of testosterone and specially in case of DHT. It seemed that castration emptied the receptor sites gradually so that receptors were more obvious. These results showed that, in the rat pituitary, contrary to the prostate, there was no disappearance of androgen receptors following castration. Moreover, the amount of pituitary specific receptors must be small, since as we think, they are exclusively located in gonadotrophs which represent only 10–15% of total pituitary cells [19].

Tritiated 3α-Diol was associated with a 3–4 *S* peak. It was clearly distinguishable but neither unlabelled 3α-Diol, nor 3β-Diol were competitive to binding sites. Thus it seems likely that there is no specific receptor for 3α-Diol in the male rat pituitary cytosol. This result is in good agreement with the findings of Krieg *et al.* [20] who failed to demonstrate 3α and 3β-Diol receptors in prostatic cytosol. Moreover we had recently observed that in the rat pituitary, 3α-Diol was intensely converted *in vitro* into 5α-DHT since 72% of 3α-Diol were recovered as DHT [21]. Thus the reaction 5α-DHT → 3α-Diol is reversible and it is possible that 3α-Diol acts by way of 5α-DHT. This being so, it is not surprising that specific receptors for 3α-Diol do not exist in the rat pituitary cytosol. On the other hand, we failed to establish the presence of 3α-Diol in pituitary purified nuclei. Nuclear androgens were only made up of testosterone and DHT [22–24]. Consequently if there is a two-step mechanism in the pituitary, it seems likely that only testosterone and DHT receptors would be involved in this transfer action.

Our results demonstrated that pituitary receptors for androgens were unstable. They were heat-sensitive since the hormone–receptor complex was in part destroyed by heating at 37°C. The sensitivity to heat of prostatic receptors was previously described by Jung and Baulieu [17], Bruchovsky and Wilson [25], Fang, Anderson and Liao [26] and Mainwaring [16]. In addition, Sullivan and Stroot [27] demonstrated that, in the prostate, the presence of a 8–10 *S* peak depended on low temperature (0–4°C) and that EDTA, glycerol and low ionic strength were required for its protection. The instability of androgen recep-

Table 2. Heat sensitivity of the macromolecular binding of testosterone, 5α-DHT and estradiol-17β in cytosol from male rats pituitaries. Results are expressed as ratio × 100 of bound radioactivity at 37°C to bound radioactivity at 0°C after various times

	[^{3}H]-Testosterone binding (%)	[^{3}H]-5α-DHT binding (%)	[^{3}H]-estradiol-17β binding (%)
0 min	100	100	100
15 min	63.2	76.7	38.0
30 min	54.2	84.7	32.8

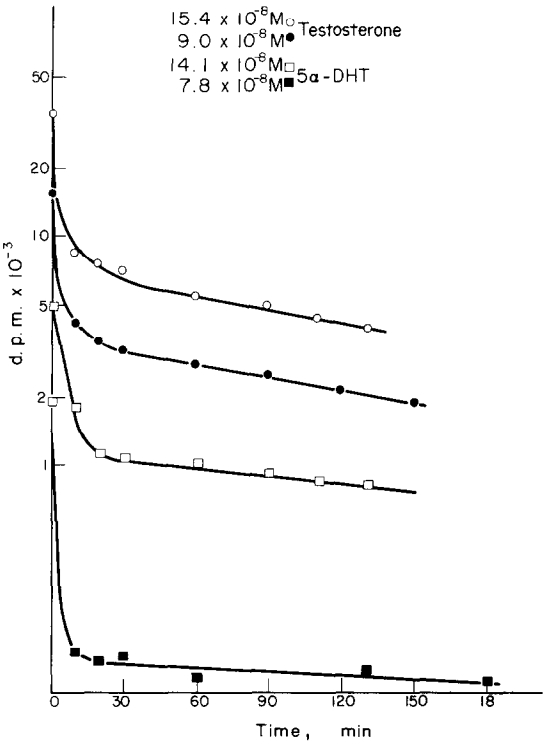

Fig. 6. Time course of the dissociation of the receptor–testosterone complex and of the receptor–DHT complex in the presence of Dextran coated charcoal: semi-log plot. Pituitary cytosol from castrated rats was labelled at equilibrium, and filtered through Sephadex G 25 column. Bound radioactivity was collected by fractions. 2 ml of a dextran coated charcoal suspension was added to 2 ml of bound radioactive fraction. Incubations were carried out at 0° for 0 to 180 min. Aliquots (300 μl) were centrifuged (5000 rev/min × 5 min) and radioactivity of the supernatant was counted. Velocity constants are calculated from the slope of the linear part of each curve.

tors gives an explanation perhaps of the fact that Korach and Muldoon failed to find such receptors in pituitary cytosol [6]. Furthermore, the use of Dextran coated charcoal corroborated the instability of androgen receptors. This technique was used previously, with favourable results to separate specific estradiol associations from non-specific ones [28]. This method was not applicable in our experimental conditions for the study of androgen binding and our conclusions have been confirmed recently by Shain and Boesel [29]. Nevertheless, using this technique, the dissociation rate constants of androgen receptors could be measured and it was shown that there were no significant differences between the constant for testosterone receptor and that for DHT receptor.

We found in the male pituitary cytosol a highly specific receptor for estradiol-17β. Our results were in agreement with those of Korach and Muldoon [6] and those of Van Beurden *et al.* [7]. Its sedimentation coefficient was 8 *S*. Only biologically active estrogens were seen to be competitive to binding sites whereas androgens and corticosterone were not. That is another difference between androgen and estrogen receptors since it was shown previously that extrogens had a competitive effect towards androgen receptor sites [2].

From our experiments involving enzyme action it could be concluded that androgen receptors were proteins. It seemed likely that estrogen receptors were also proteins, but it was observed that estrogen binding was partly decreased by RNAses.

The purpose of this work was to complete our information relating to pituitary androgen receptors. It was also an attempt to differentiate androgen receptors from estrogen receptors. This differentiation was easy: the dissociation constant (K_D) is $2.3 \cdot 10^{-9}$M for testosterone receptor, $7.8 \cdot 10^{-10}$M for DHT receptor [1, 2] and $7 \cdot 10^{-11}$M for estradiol receptor (unpublished data). The estimated amount of receptor sites is respectively: $8 \cdot 10^{-11}$M for testosterone, $1.4 \cdot 10^{-11}$M for DHT [1, 2] and $4 \cdot 10^{-10}$M for estradiol (unpublished data). When competition assays were carried out, other differences appeared: whereas androgens have no competitive action towards the binding sites for estradiol, estrogens have a competitive efficiency towards the sites for androgens [2, 3]. Similar observations were made at the prostatic level. Several authors have demonstrated a competitive effect of estrogens towards the receptor sites for androgens [26, 29–31]. Moreover androgen receptors were differentiated from estrogen receptors by their sedimentation coefficients. In addition their intracellular locations were characteristic. Indeed, from works of Sar and Stumpf [19] it appeared that [^{3}H]-testosterone was preferentially concentrated in basophils, whereas [^{3}H]-estradiol was accumulated in acidophils, chromophobs and basophils. In a recent paper, Lloyd and Karavolas corroborated these results [32]. They showed that uptake and metabolism of testosterone were more intensive in a fraction enriched in gonadotrophs than in other cellular fractions [31]. Furthermore, it is noteworthy that androgens and estrogens have contrasting effects on thymidine kinase activity [33].

Thus it is easy to differentiate androgen receptors from estrogen receptors. On the other hand, it is difficult to ascertain whether the testosterone receptor is different from the DHT receptor. Our results showed differences in number of binding sites, sensitivity to heat and sensitivity to various enzymes. It is generally admitted that cytosolic receptors for testosterone and DHT are the same because both hormones are competitive at the same binding sites. However, taking into account that the numbers of binding sites are different and that DHT receptor is more evident than that for testosterone following castration, it is possible that there are two distinct receptors. This conclusion would be in agreement with that of Rennie and Bruchovsky [34], but in the present state of our research, is premature, and the presence of two distinct receptors must be considered as a hypothetical suggestion.

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