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EVIDENCE FOR ANDROGEN AND ESTROGEN RECEPTORS IN CASTRATED RAM PITUITARY CYTOSOL: INFLUENCE OF TIME AFTER CASTRATION

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SUMMARY

A comparison of testosterone, 5α-dihydrotestosterone and 17β-estradiol binding to cytosol fractions of ram anterior pituitary was conducted in animals 24 h or 2 years after castration. The three [^{3}H]-steroids were bound to macromolecules which sedimented on a sucrose density gradient at approx. 7S. Polyacrylamide gel electrophoresis and competitive binding assays with cyproterone acetate and diethylstilbestrol indicated the presence of steroid receptors as distinct from non-specific binding proteins or specific plasma proteins. Affinities of testosterone and 5α-dihydrotestosterone for cytosol macromolecules by competitive binding assays suggested the presence of androgen receptors which are clearly different from the estrogen receptor. The affinity constants for the testosterone, 5α-dihydrotestosterone and estradiol binding reactions were $3.70 \times 10^9\ M^{-1}$, $1.47 \times 10^{10}\ M^{-1}$ and $3.41 \times 10^9\ M^{-1}$ respectively in the 24 h castrated rams. These values tended to decrease for testosterone, increase for 5α-dihydrotestosterone and to remain steady for estradiol 2 years after castration. Numbers of sites were 11.75, 15.6 and 181×10^{-15} mol/mg protein for testosterone, 5α-dihydrotestosterone and estradiol respectively 24 h after castration. Number of sites decreased by 43% for testosterone and 60% for 5α-dihydrotestosterone 2 years after castration but the loss of receptor sites was 59% and 71% when the results were expressed per mg of DNA. Number of sites for estradiol did not vary after castration.

INTRODUCTION

In a previous paper, we showed that large doses of testosterone propionate were required to decrease plasma LH concentrations in long-term castrated rams [1] and observed a marked effect of the delay following castration [2]. This alteration in testosterone sensitivity could be due to a defect in the intracellular receptor machinery namely, a decrease in the number of receptor sites. While negative feed-back of androgens acts at the hypothalamic level in the ram [2], it has been shown that steroids could also modify the sensitivity of the hypophysis to LRH [3], a finding which strongly suggests the existence of androgen receptors in the ovine hypophysis as demonstrated in various other species [4–8].

Thus, in a first experiment, we examined the influence of a long delay after castration on specific androgen binding in the anterior hypophysis. Further, after observing that in long-term castrated rams estradiol benzoate is a potent inhibitor of LH release at doses 6000 times less than efficient doses of testosterone propionate (unpublished results) we conducted similar studies with estrogen receptors.

The first part of this work was devoted to characterization of androgen and estrogen receptors which have not been comprehensively studied in the ram.

MATERIALS AND METHODS

Chemicals. [1,2-^{3}H]-testosterone (S.A. 58 Ci/mmol), [1,2-^{3}H]-5α-dihydrotestosterone (S.A. 48 Ci/mmol), were obtained from the Radiochemical Centre, Amersham. [6,7-^{3}H]-17β-estradiol (S.A. 46 Ci/mmol) was purchased from C.E.A. (Gif/Yvette, France).

The purity of these compounds was checked by paper chromatography (see later). Unlabelled steroids were obtained from Sigma Chemical Company or Steraloids. Cyproterone acetate was a gift from Scher-

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Trivial names used for steroids are: Androstenedione, 4-androsten-3,17-dione; Testosterone, 17β-hydroxy-4-androsten-3-one; Epitestosterone, 17α-hydroxy-4-androstene-3-one; Dihydrotestosterone (DHT), 17β-hydroxy-5α-androstan-3-one; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; 3α-androstanediol (3α diol), 3α,17β-dihydroxy-5α-androstane; 3β-androstanediol (3β diol), 3β,17β-dihydroxy-5α-androstane; Androsterone, 3α-hydroxy-5α-androstan-17-one; Epiandrosterone, 3β-hydroxy-5α-androstan-17-one; Dehydroepiandrosterone (DHA), 3β-hydroxy-5α-androsten-17-one; Progesterone, 4-Pregnen-3,20-dione; Cortisol, 4-Pregnen-11β,17α,21-triol-3,20-dione; 17β-estradiol, 1,3,5(10)-estratrien-3,17β-diol; 17α-estradiol, 1,3,5(10)-estratrien-3,17α-diol; Estrone, 1,3,5(10)-estratrien-3-ol-17-one; Estriol, 1,3,5(10)-estratrien-3,16α,17β-triol; Diethylstilbestrol (DES), 3,4-bis(4-hydroxyphenyl)-3-hexene.

ing Company (Berlin). Dextran T 70 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), charcoal norit A from Prolabo (12 rue Pelée, 75011 Paris). Protamine sulfate, from salmon roe, was supplied by Koch Light Laboratories (Colnbrook, Bucks, England).

The sources of enzymes were: (a) protease, type VI, Sigma; (b) crystallized deoxyribonuclease DNase I, code D and lyophilized trypsin, code TL, Worthington Biochem. Co., and (c) crystallized ribonuclease RNase, purum b, Fluka (CH-9470, Buchs, Switzerland).

Animals and tissues. Adult Prealps rams, 3–5 years old were slaughtered either 24 h or 2 years after castration, performed in the former case under local anaesthesia ($n = 5$ in each group). Anterior pituitaries, removed immediately after exsanguination, were cut sagitally into two equal parts and frozen in liquid nitrogen. One half was kept frozen for measurement of DNA and the other was processed the next day for determination of cytosolic receptors. On some occasions, blood was collected at killing and allowed to clot in crushed ice. It was then centrifuged at 1500 g and 0°C for 10 min.

Preparation of cytosol. The procedure was carried out at 0–4°C. Unless stated otherwise the buffer used was 0.05 M phosphate buffer, pH 7.4. Anterior pituitary halves were thawed and weighed and the minced tissues homogenized in phosphate buffer (10%, w/v) with a Potter homogenizer. The homogenate was centrifuged at 800 *g* for 10 min and the supernatant recentrifuged at 105,000 *g*, for 1 h, in the SW-65 rotor of a Beckman L_5 ultracentrifuge. Protein concentration of the 105,000 *g* supernatant (cytosol) was determined by the method of Lowry *et al.*[9], using bovine serum albumin as standard. Cytosol protein concentration was about 7.6 mg/ml.

Protamine sulfate precipitation assay. This technique, described by Steggles and King[10] and modified by Blondeau *et al.*[11], was used for the separation of [^{3}H]-bound and free steroids in ram pituitary cytosol. Following incubation of aliquots of cytosol (0.2 ml of a preparation containing 2.4 mg protein/ml) with [^{3}H]-steroid, 0.2 ml of buffer containing 1.5 mg/ml protamine sulfate was added to each tube. The mixture was vortexed and allowed to stand at 0°C for 15 min. The precipitate was filtered on glass fiber paper (Whatman GF/C), under light vacuum and washed with 40 ml of ice-cold buffer. The filter was then transferred to a scintillation vial and counted in 10 ml of Bray's scintillator. It has been verified for ram pituitary cytosol that radioactive precipitate was maximal at the protamine sulfate concentration used (1.5 mg/mg protein).

Blank values were obtained by incubating a series of tubes containing the same concentrations of tracer in which the cytosol was replaced by 0.2 ml of buffer. These blank values were subtracted from the radioactivity of the corresponding assay tubes. Radioactivity of blank tubes using an excess of unlabelled steroid 100× that of [^{3}H]-steroid added to cytosol did not differ significantly from the phosphate blank.

Time course of steroid binding. The cytosol was diluted with buffer to the desired protein concentration (2.4 mg/ml). Aliquots of diluted cytosol (0.2 ml) were incubated at 0°C with [^{3}H]-testosterone (9.0×10^{-9} M), [^{3}H]-DHT (1.0×10^{-9} M) or [^{3}H]-estradiol (1.35×10^{-9} M) for various times, from 30 min to 24 h. Binding was measured by the protamine sulfate precipitation assay.

Absence of metabolism and purity of labelled steroids. Bound cytosol proteins (0.4 ml), labelled with [^{3}H]-testosterone (2.4×10^{-8} M), [^{3}H]-DHT (2.5×10^{-9} M) or [^{3}H]-estradiol (2.8×10^{-8} M) were extracted twice with chloroform–methanol (2:1, V/V). Extracts were dried under vacuum and redissolved in a small volume of methanol (0.2 ml). An aliquot of androgen samples was separated on Whatman No. 1 paper, in the solvent system of Kochakian and Stidworthy[12]. Estrogens were chromatographed on Whatman No. 1 paper, in the solvent system of Payne *et al.*[13]. After chromatography at +30°C, the strips of paper were cut into 2 × 1 cm segments and placed in counting vials.

Effects of protein concentration. Increasing concentrations of pituitary cytosol protein (1–12 mg/ml) were incubated at 0°C for 5 h with [^{3}H]-testosterone (9×10^{-9} M) or [^{3}H]-DHT (3.4×10^{-9}). Binding was measured by the protamine sulfate precipitation assay.

Steroid binding specificity. Aliquots of cytosol, diluted to a suitable protein concentration (2.4 mg/ml) were incubated for 4–7 h at 0°C with [^{3}H]-testosterone, [^{3}H]-DHT ($7.3–8.9 \times 10^{-10}$ M) or [^{3}H]-estradiol ($1.8–3.5 \times 10^{-10}$ M) in the presence or absence of competing unlabelled steroids. Labelled or unlabelled steroids in ethanol were diluted in buffer and added to the cytosol, to give a final ethanol concentration less than 0.4–0.8%. Competitors were added at concentrations ranging from 10^{-10} M to 10^{-6} M, i.e. 1–10,000 times that of [^{3}H]-steroid.

The percentage reduction in binding of [^{3}H]-steroid (Y) was calculated in the presence of either the same unlabelled ligand or various competitors. Data were represented by plotting the logit transformation of Y (logit $Y = 1_n Y/1 - Y$) vs the log. of the mass of competing steroid. The concentration of unlabelled steroid required to displace 50% of the bound [^{3}H]-steroid (logit $Y = 0$) was calculated for each steroid tested. The ratio of concentration of a competitor to that of the unlabelled ligand (similar to the radioligand) required to displace 50% of bound radioactivity was defined as "relative competition ratio" (RCR).

Sucrose density gradient centrifugation. Cytosols (7–8 mg/ml) and serums, diluted with buffer to the same concentrations, were incubated at 0°C in the presence of [^{3}H]-17β-estradiol (3.1×10^{-8} M), [^{3}H]-testosterone (1.4×10^{-8} M), or [^{3}H]-5α DHT ($0.4–1.8 \times 10^{-8}$ M) for 2 h. On some occasions, an

unlabelled steroid competitor at a concentration 150 times that of the radioactive steroid was added. At the end of the incubation period, the cytosol or serum was vortexed briefly with a pellet of dextran-coated charcoal (D.C.C.). Pellets were obtained from a suspension of D.C.C. (0.5% charcoal, 0.05% dextran T 70 in Tris–HCl 10 mM, EDTA 1.5 mM, dithiothreitol 1 mM buffer, pH 7.4 (TED)) equivalent in volume to that of the cytosol. The mixtures were allowed to stand for 15 min in an ice-bath and centrifuged at 3500 *g* for 10 min. (Preliminary experiments indicated that bound [^{3}H]-estradiol or [^{3}H]-androgens were not adsorbed by the Dextran-charcoal). The supernatants (300 μl) were removed and layered onto cold pre-formed sucrose gradients. These linear 5–20% sucrose density gradients were prepared in TED buffer containing 10% glycerol. The gradients were centrifuged at 200,000 *g*, for 16 h, using the SW 65 rotor of a Beckman model L_5 ultracentrifuge, at +1°C. At the end of the centrifugation, 0.2 ml fractions were collected directly into scintillation vials, using a density gradient fractionator (ISCO, Lincoln, U.S.A.). Proteins were determined at 280 nm with a U.V. analyzer (ISCO, UA-2 model). Sedimentation coefficients of binding were determined according to the method of Martin and Ames, using BSA as standard [14].

Polyacrylamide gel electrophoresis (PAGE). Pituitaries were homogenized in TEMG buffer (10 mM Tris–HCl, 1.5 mM EDTA, 2 mM mercaptoethanol, 10% glycerol), centrifuged at 1000 *g* for 10 min and the supernatant recentrifuged at 105,000 *g*, for 1 h, in the 50 Ti rotor of a Beckman L5-50 centrifuge at 0°C. 200 μl cytosol was then incubated with 2–3 nM of either [^{3}H]-testosterone, [^{3}H]-5α-dihydrotestosterone or [^{3}H]-17β-estradiol for 3 h. Controls were performed by adding a 100 fold excess of unlabelled steroid to the cytosol-[^{3}H] steroid mixture. Thereafter incubation media were transferred to a layer of dextran-charcoal solution as described above. After 15 min of contact, the mixture was centrifuged again at 5000 rev./min for 20 min, and thioglycolate 5 μM, bromophenol blue and 20% V/V glycerol were added to the supernatant. The mixture, 250 μl, was deposited onto 80 mm gels containing 7.5% acrylamide. Electrode buffer was 10 mM Tris, 77 mM glycine, pH 8.3. Electrophoresis was carried out for about 4 h at 0°C with 2 mA/tube. On some occasions, cytosol preparations were deposited onto stacking gels prepared according to Miller *et al.*[15]. Slices (2 mm) were cut with a gel fractionator (Gilson). Serums, diluted five or ten times, were processed similarly to cytosols. The quantity of proteins was 3–5 mg in 200 μl, but adjusted to be between cytosol and serum for a given run. Results were expressed as a proportion of bromophenol blue R_F.

Heat sensitivity. Cytosol first incubated as above was transferred to a water bath at +50°C for 30 min treated with charcoal-dextran and submitted to PAGE.

Enzymes effects. 100 μl cytosol diluted six times with TEMG (about 300 μg proteins) were incubated for 3 h with [^{3}H]-DHT (2.5×10^{-9} M) and then reincubated at +37°C for 30 min in the presence of various enzymes dissolved in the same buffer: protease (100 μg), trypsin, DNase I and RNase (250 μg). Incubation with DNase was carried out in the presence of 1 mM $MgCl_2$. After incubation, cytosol fractions were precipitated with protamine sulfate.

Steroid binding experiments. Aliquots of pituitary cytosol (0.2 ml) whose final protein concentration was 2.4 mg/ml were incubated at 0°C and at equilibrium (4–7 h), with varying concentrations of [^{3}H]-testosterone (1.3×10^{-10} M–3×10^{-7} M), [^{3}H]-DHT (3×10^{-11} M–1.3×10^{-7} M) or [^{3}H]-estradiol (1.3×10^{-10} M–1×10^{-7} M.) At the end of incubation, the binding was measured by protamine precipitation.

Further estimations of the association constants and receptor site concentrations were made for estradiol, testosterone and DHT simultaneously on aliquots of each cytosol from Scatchard plots [16], corrected by the Rosenthal method [17]. A least squares analysis was used to determine the linear regression coordinates of the Scatchard plot data.

DNA analysis. DNA was estimated by the method of Morimoto *et al.* [18] modified by Devinoy and Houdebine[19] using the cetyltrimethylammonium bromide (Sigma) precipitation.

Radioactivity measurement. The radioactivity of aqueous fractions or filters was counted using Bray's solution. Aliquots of sucrose gradients were counted in Instagel. The radioactivity of chromatograms was measured as previously described [20]. Polyacrylamide gel slices, pulverised by the fractionator were collected in 100 μl absolute ethanol to break down the binding of radioactive steroids to proteins [45] and left over-night at 4°C in vials containing 2 ml of toluene, PPO-POPOP scintillation medium after shaking briefly, samples were counted. Radioactivity was measured in an Isocap 300 spectrometer (Nuclear Chicago) or a Tricarb spectrometer, model 3385 (Packard). Both apparatus had a counting efficiency of 50–55%.

RESULTS

I. *Conditions of binding system*

Time course of steroid binding to pituitary cytosol. Initial studies were conducted to determine the time required for the steroid–receptor interaction to reach equilibrium at 0°C. As shown in Fig. 1, binding of [^{3}H]-estradiol was maximal after 2 h incubation and remained practically constant up to 24 h. The binding of [^{3}H]-testosterone was maximal after 3 h incubation and remained constant up to 24 h. [^{3}H]-DHT binding was maximal after 2 h incubation and tended to decrease slowly thereafter. In fact, for the three hormones we can consider that there is a plateau 2–5 h after the start of incubation.

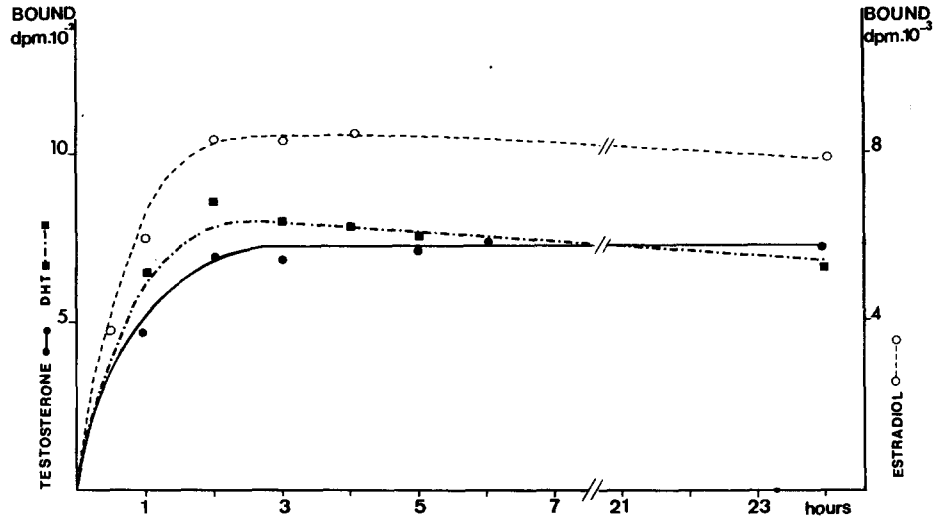

Fig. 1. Binding at equilibrium of [^{3}H]-testosterone (9.0×10^{-9} M), [^{3}H]-DHT (1.0×10^{-9} M) and [^{3}H]-17β-estradiol (1.35×10^{-9} M) to cytosol proteins from castrate ram anterior pituitary for time periods varying from 30 min to 24 h.

In all further experiments, the incubation time was 4 h for estradiol and 5–6 h for androgens except for PAGE and sucrose density gradient centrifugation where incubation time was 2–3 h.

Absence of steroid metabolism during incubation. Steroids bound at equilibrium were extracted and subjected to chromatography in a system [12] where testosterone is well separated from its main metabolites, including 5α-DHT and 3α-diol. This showed that no major testosterone or dihydrotestosterone metabolism occurred at 0°C after 5 h incubation since percentages of unchanged steroid in bound fractions were 87 and 91% respectively. Similarly when estrogen extracts were chromatographed [13] 97% of radioactivity migrated in the same way as the unlabelled estradiol.

Effect of protein cytosol concentration. Further investigation was undertaken to determine the amount of steroid binding as a function of cytosol protein concentration. As shown in Fig. 2, binding of [^{3}H]-testosterone and [^{3}H]-DHT increased linearly with increasing cytosol protein concentration, after 5 h incubation. All subsequent experiments were conducted in the lower portion of the linear curve, using 2.4 mg/ml cytosol protein concentration.

Steroid binding specificity. Amounts of steroids required to displace 50% of bound [^{3}H]-testosterone or [^{3}H]-5α-DHT are expressed as the relative competition ratios (RCR) (Table 1). Competitors of androgens can be divided into three groups:

(i) Testosterone, 5α-DHT and cyproterone acetate have similar and low RCRs, close to 1. However it may be noticed that 5α-DHT is a better competitor for [^{3}H]-testosterone binding sites than testosterone itself.

(ii) A second group of competitors presents a rela-

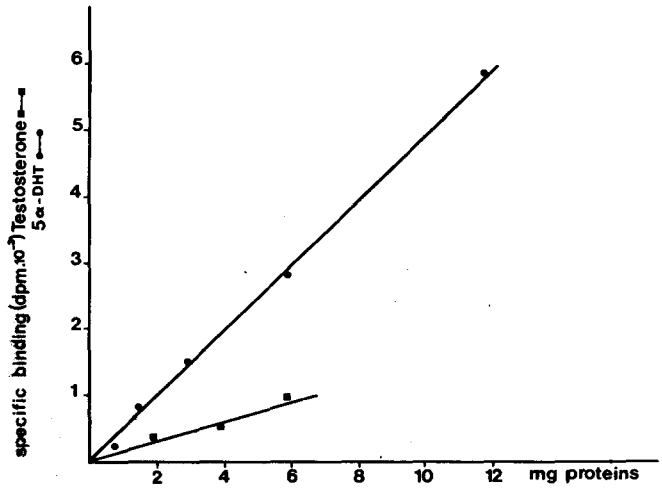

Fig. 2. Specific binding of [^{3}H]-testosterone (9×10^{-9} M) and [^{3}H]-DHT (3.4×10^{-9} M) as a function of cytosol protein concentration during a 5 h incubation.

Table 1. Testosterone and 5α-DHT binding specificity. The ratio of concentration of a competitor to that of unlabelled testosterone (left) or unlabelled 5α-DHT (right) required to displace 50% of bound radioactivity was defined as "relative competition ratio" (RCR)

Radioligand	[^{3}H]-testosterone		[^{3}H]-5α-DHT	
Time after castration	24 h	2 years	24 h	2 years
Competitor	RCR	RCR	RCR	RCR
Testosterone	1*	1†	1.7	1.3
5α-DHT	0.62	1	1‡	1§
Cyproterone acetate	1	1	2.9	3.9
Progesterone	4	5	16	22
17β-estradiol	6	9.5	17	14
3β-diol	8	13	36	38
5β-DHT	30	59	261	131
Epitestosterone	49	—	103	91
3α-diol	56	104	278	424
17α-estradiol	73	—	121	—
Androstenedione	127	222	244	369
Estrone	309	355	2000	710
DHA	1000	—	1330	1200
Cortisol	1709	2333	2470	2000

Molarity required to displace 50% of bound radioactivity: *5.5×10^{-10} M; †9×10^{-10} M; ‡1×10^{-9} M; §9.3×10^{-10} M.

tively high RCR (5 to 36) suggesting the possibility of interaction of these competitors with cytosolic androgen binding sites: it includes progesterone, 17β-estradiol and to a lesser degree 3β-androstanediol. The RCR of these competitors is less in the case of [^{3}H]-DHT than in that of [^{3}H]-testosterone.

(iii) A third group of competitors has little or no effect with an RCR greater than 40. It includes 5β-DHT, epitestosterone, 3α-androstanediol, 17α-estradiol, androstenedione, estrone. Further, DHA and cortisol have negligible potency.

In general, the RCR values are similar both in 24 h and in 2 year-castrated rams. However, RCR values in the [^{3}H]-testosterone system tend to be lower in short term than in long term castrated rams. This is also true for unlabelled testosterone.

Table 2. 17β-estradiol binding specificity. The ratio of concentration of a competitor to that of unlabelled 17β-estradiol required to displace 50% of bound [^{3}H]-17β-estradiol was defined as "relative competition ratio" (RCR)

Time after castration	24 h	2 years
Competitor	RCR	RCR
17β-estradiol	1*	1†
DES	1	1
Estrone	1.4	1.65
17α-estradiol	1.4	1.54
Estriol	2.8	5
3β-diol	31	72
5α-DHT	280	295
3α-diol	375	752
Testosterone	3600	1704
Cortisol	4000	4000
Progesterone	4000	4000

Molarity required to displace 50% of bound radioactivity: *1.05×10^{-9} M; †1.0×10^{-9} M.

In estrogen competition experiments (Table 2), two groups of competitors can be distinguished:

(i) DES, estrone, 17α-estradiol and to a lesser degree estriol, are strong competitors for 17β-estradiol binding sites. The fact that the RCR for DES is close to 1 is interesting since it suggests the absence of significant serum contamination in ram pituitary cytosol.

(ii) Other steroids studied had little or no competitive effect, particularly cortisol, testosterone and progesterone. Again RCR values are of the same magnitude in both long-term and short-term castrated animals.

Sucrose gradient sedimentation of steroid binding proteins. Figure 3 (a to c) indicates that each of the [^{3}H]-steroids (testosterone, DHT, estradiol) formed a complex with the cytosol receptors which sedimented in the 7S region of a linear sucrose gradient. When diluted serum incubated with [^{3}H]-DHT was subjected to sucrose gradient analysis (Fig. 3d) the radioactivity migrated in the 4S region and was displaced by a 150 fold excess of unlabelled DHT. These data suggest that the unlabelled DHT competed for steroid binding globulin (SBP). In cytosol experiments, the absence of a radioactive peak in the 4S region excluded contamination of cytosol by the serum binding proteins.

Polyacrylamide gel electrophoresis. When cytosol was first incubated with [^{3}H]-5α-DHT and the medium made to migrate in polyacrylamide gel without a stacking gel, one peak of radioactivity, $R_F = 0.36$ (0.32–0.39 in three experiments), was found (Fig. 4); this peak was completely abolished by the addition of an excess of unlabelled DHT before incubation.

Similarly, one peak of radioactivity was found

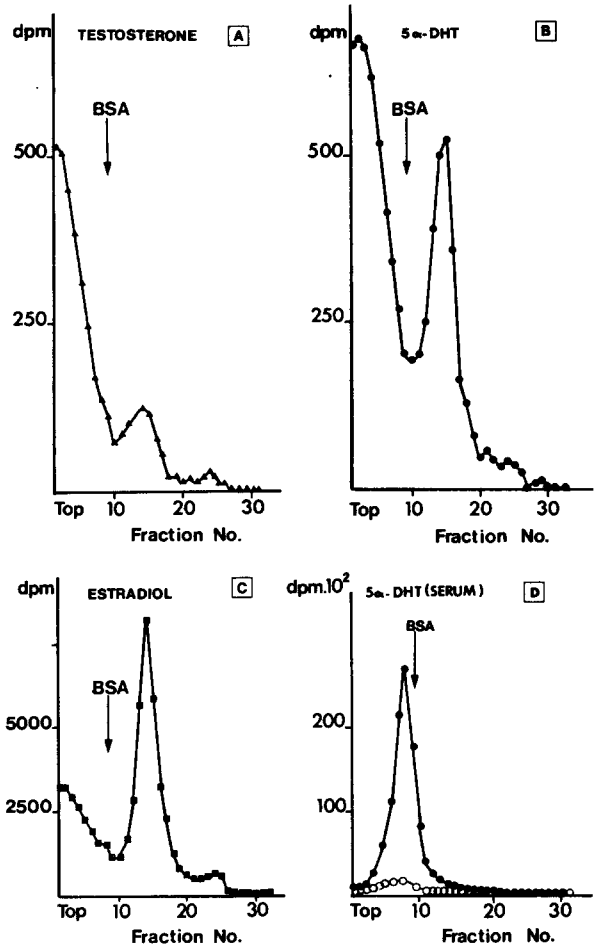

Fig. 3. Sedimentation profiles of castrate ram anterior pituitary cytosol incubated with (a) 1.4×10^{-8} M [^{3}H]-testosterone, (b) 1.8×10^{-8} M [^{3}H]-5α DHT, (c) 3.1×10^{-8} M [^{3}H]-17β estradiol and of diluted serum from castrate rams incubated with, (d) 1.8×10^{-8} M [^{3}H]-5α DHT in absence (●——●) or presence (○——○) of a 150 fold excess of unlabelled DHT.

when migration involved diluted plasma incubated with [^{3}H]-5α-DHT but the R_F was different, 0.54 (Fig. 4); 0.52 and 0.56 in two other experiments. The R_F in both cases was lower when a stacking gel was added to the separation gel, 0.29 and 0.36 for cytosol and serum respectively (Fig. 5) and the background was clearly decreased. Figure 5 also shows the influence of an excess of unlabelled DHT which in both cases abolished the peaks of radioactivity. This is in contrast to the effect of an excess of unlabelled cyproterone acetate which inhibited binding of radioactive DHT to cytosol receptors only. Experiments with [^{3}H]-testosterone gave similar results to 5α-DHT (R_F values: 0.25 and 0.27 in two experiments with a stacking gel) except that binding was two or three times less. [^{3}H]-17β-estradiol bound to cytosol but not diluted serum under the present conditions.

Heat sensitivity. No radioactive peak was apparent after PAGE when cytosol was heated before incubation with radioactive 5α-DHT (not shown).

Effects of various enzymes. Table 3 shows that binding of radioactive DHT to cytosol was strongly inhibited by proteolytic enzymes (protease, trypsin) whereas DNase and RNase were without effect.

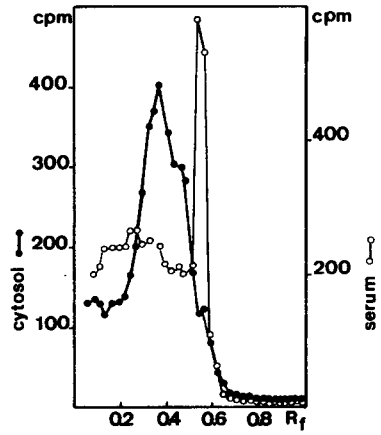

Fig. 4. Migration profiles after PAGE of [^{3}H]-DHT bound to cytosol proteins (●——●) or to 1/10 diluted serum proteins (○——○).

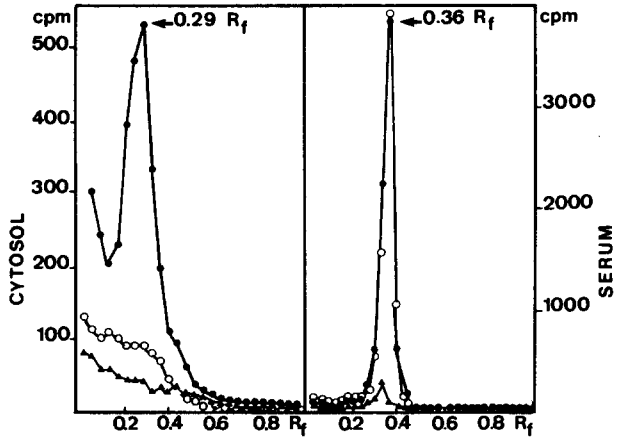

Fig. 5. Migration profiles after PAGE of [^{3}H]-DHT bound to (a) cytosol proteins or to (b) 1/10 diluted serum proteins. [^{3}H]-DHT (●——●), [^{3}H]-DHT + 100-fold excess of cyproterone acetate (○——○), [^{3}H]-DHT + 100-fold excess of DHT (▲——▲).

Saturation curve analysis. Figure 6 shows that 17β-estradiol binding sites were saturated at approx. 1.5×10^{-9} M for which bound radioactivity reached a plateau. Non-specific binding was especially weak in this case. Similarly, androgen binding sites appear to be saturated at 9×10^{-10} M of [^{3}H]-testosterone and [^{3}H]-DHT.

II. *Effect of time after castration on the affinity constants of testosterone, DHT and estradiol*

Figure 7 is a representative Scatchard plot for each of the three steroids studied. After subtraction of the blank, K_a values are given in Table 4. At 24 h after castration, K_a for testosterone and estradiol were in the same range, whereas K_a for DHT was significantly higher ($P < 0.01$).

Two years after castration, K_a for testosterone tended to decrease ($P < 0.05$) while K_a for DHT increased significantly ($P < 0.01$), thus the ratio K_a DHT/K_a testosterone increased from 4 to 11 during this time. In contrast, K_a for estradiol remained constant (3.48 vs 3.41 $\times 10^9$ M^{-1}).

Table 3. Effects of trypsin, protease, DNase and RNase on the binding of [^{3}H]-DHT to cytosolic proteins

Enzymes	Assay	% of controls
—		100
Trypsin	1	0
	2	26.3
Protease	1	0
	2	32.3
DNase		100
RNase		100

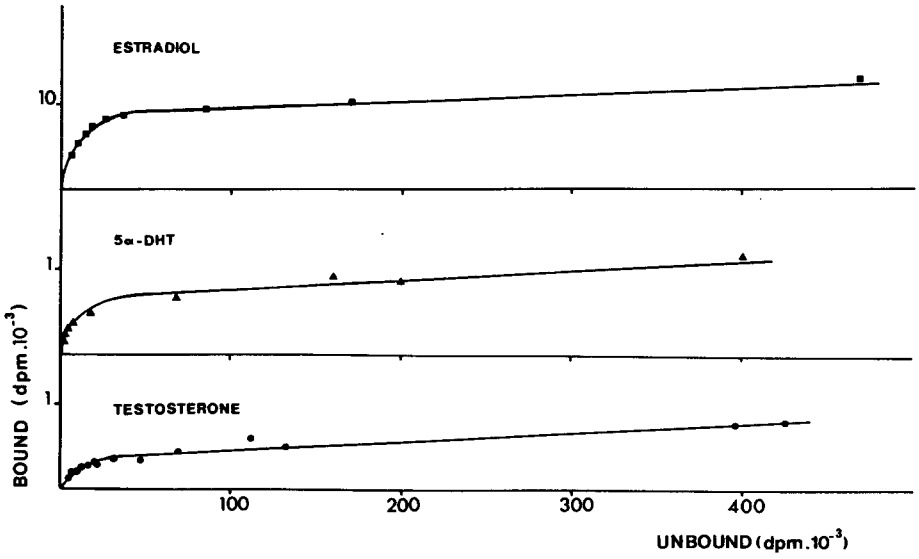

Fig. 6. Specific binding curves obtained after incubation of pituitary cytosol with [^{3}H]-17β estradiol, [^{3}H]-5α DHT and [^{3}H]-testosterone. Specific binding was defined as the difference between total binding and binding in the presence of 100-fold excess of unlabelled steroid.

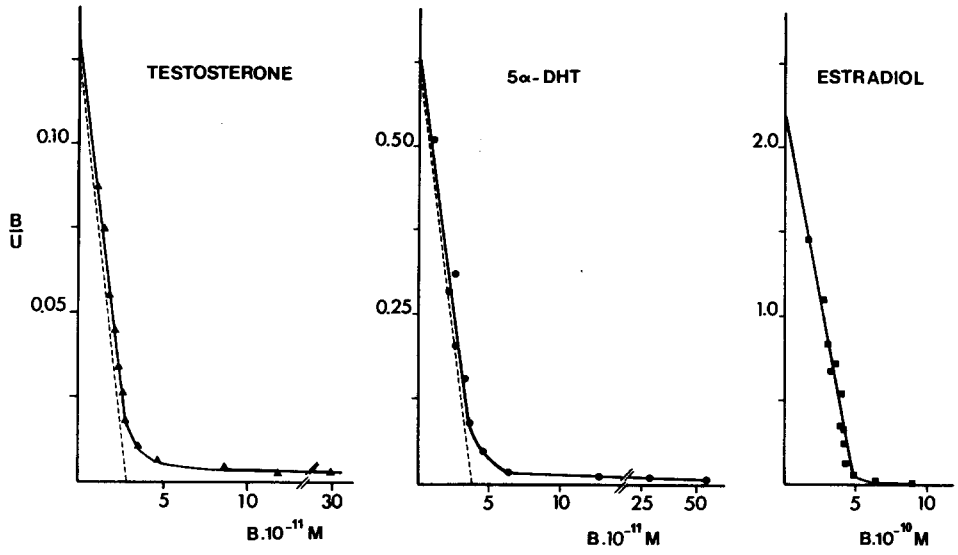

Fig. 7. Data for analysis of specific binding of: (a) [^{3}H]-testosterone. (b) [^{3}H]-5α DHT and (c) [^{3}H]-17β estradiol according to method of Scatchard[16].

III. *Effect of time after castration on the number of steroid binding sites*

Expressed as mg of proteins, the number of binding sites is similar for testosterone and DHT whatever the delay after castration (Table 4). However, in both cases a highly significant decrease is observed when long term castrated animals are compared with short term rams ($P < 0.001$). This result is still greater when results are expressed per μg of DNA and 2 years after castration, number of binding sites are only 41.4 and 29.3% of that of 24 h castrated animals for testosterone and DHT respectively.

No change or a slight decrease is shown for estradiol under the same conditions, but it appears that number of sites for estradiol is 12–33 times greater than for androgens.

DISCUSSION

The first objective of this work was to establish the presence of one or several steroid receptors in the cytosol of ram anterior pituitary. After determination of suitable working conditions (protein concentration of cytosol, equilibrium time, protamine sulfate required for precipitation) this can be considered as achieved:

(a) Macromolecules have been found to bind androgens and 17β-estradiol with a high association coefficient ($2.19 \times 10^9\ M^{-1}$ to $2.43 \times 10^{10}\ M^{-1}$).

(b) These molecules have a limited binding capacity as evidenced by saturation curves. However in the case of 5α-DHT, the plateau is short (Fig. 6).

(c) Tissue and steroid specificity. The presence of a sex binding globulin (SBP) in ram serum has been

Table 4. Number of binding sites (expressed per mg of proteins or mg of DNA) and affinity constants (K_a) for testosterone, 5α-DHT and estradiol in castrate ram pituitary cytosol (Mean ± S.E.M.)

Time after castration	No. of rams per group	24 h	2 years
K_a: $\times 10^9\ M^{-1}$			
Testosterone	4	3.70 ± 0.55	2.19 ± 0.15*
5α-DHT	5	14.70 ± 0.8	24.30 ± 2.90**
Estradiol	4	3.41 ± 0.41	3.48 ± 0.64
Number of sites fmol/mg proteins			
Testosterone	4	11.75 ± 0.65	6.70 ± 0.22***
5α-DHT	5	15.60 ± 1.30	6.31 ± 0.22***
Estradiol	4	181 ± 11.0	211 ± 28.0
Number of sites fmol/mg DNA			
Testosterone	4	172 ± 9.55	71.2 ± 2.30***
5α-DHT	5	229 ± 19.20	67.1 ± 2.30***
Estradiol	4	2661 ± 161	2244 ± 297

Difference with 24 h group significant *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

mentioned [21] and the possibility of interference in our study required a careful examination of this point. The absence of noticeable contamination of cytosolic proteins by SBP was assessed by a difference in mobility of bound radioactive steroids in serum and in cytosol submitted to sucrose gradient centrifugation or polyacrylamide gel electrophoresis. Further, cyproterone acetate, which does not inhibit DHT binding in serum [22], completely suppressed cytosolic binding. Similarly, the RCR of 1 for DES indicates that the totality of binding is due to cytosolic proteins since DES does not bind to serum.

Further, 5α-DHT binding to SBP has a K_a of one order of magnitude less than that of 5α-DHT to cytosol proteins [23], however in this latter case Scatchard analysis indicates the presence of only one major binding component.

Concerning steroid specificity two general remarks can be made:

(i) The 17β-estradiol receptor can also bind estrone, 17α-estradiol and estriol to the same extent. Strong binding of 17α-estradiol to receptor is unexpected but has been previously noted [35]. One possibility is epimerisation by hypophyseal tissue as indicated in the rat (Le Guellec, personal communication). Metabolites, 3α and 3β-diols and DHT present a higher RCR. Testosterone, cortisol and progesterone have a negligible effect. DHT which alone could compete for the same binding sites as estradiol in fact does not since estradiol binding does not vary under conditions where large changes were observed for DHT binding due to castration.

(ii) Androgen binding is similar for both testosterone and DHT and the possibility that there exists one binding entity for both is discussed later. Progesterone and 17β-estradiol compete strongly with [^{3}H]-testosterone for binding sites; however the endogenous plasma concentration of estradiol is in the picogramme range [24] and progesterone concentration is at least 100 times less than that of testosterone (Terqui, pers. comm.).

Finally, our results are in favour of two specific groups of macromolecules, one for estrogens and at least one for the androgens, testosterone and 5α-DHT.

(d) The macromolecules appear to be heat labile as shown by electrophoretic studies.

(e) It has been verified that binding was inhibited after proteolytic enzyme treatment while RNase and DNase were without effect.

The sum of the data thus obtained lead to the conclusion that steroid binding in cytosol is due to the presence of defined receptor molecules. Wise *et al.*[25] suggested that this is so in the ewe although these authors did not adequately verify the specificity of their receptor complex against serum contamination. General conclusions for the presence of cytosolic pituitary receptors are in agreement with more documented evidence in the rat [5, 7, 26] and the calf [8].

Considering the androgen receptors in ram pituitary cytosol, since the association constant, K_a, is one order of magnitude greater for 5α-DHT than for testosterone ($3.70 \times 10^9\ M^{-1}$ vs $1.47 \times 10^{10}\ M^{-1}$), this means that the binding affinity for 5α-DHT is higher than that for testosterone. Similar data have been obtained in the male rat [4, 6] and in the male calf [8].

Number of sites expressed per mg of protein are equivalent for testosterone and 5α-DHT (11.5 fmol vs 15.6 fmol), and again, these data are comparable to those measured in the rat and calf [4–6, 8].

For both androgens, migration in a sucrose gradient occurs in the 7S region, comparable to the 7–8S region found for the male rat pituitary [5, 27–29], but under these conditions, and in the electrophoretic studies, the testosterone peak is less than that of DHT. This can be related to dissociation rate constants which are less for DHT than for testosterone (the present results and 30).

Specificity experiments for steroid binding indicate that (as has also been found in the male [6, 7]), progesterone and estradiol compete relatively strongly for androgen receptors. We pointed out above that this competition presumably does not interfere *in vivo* due to the low levels of these steroids in the male. Further, the possibility that 17β-estradiol inhibited the binding of [^{3}H]-DHT to cytosol *in vitro* while it was ineffective *in vivo* has been suggested by Kato[39]. Our experiments show in particular, the remarkable similarity between [^{3}H]-testosterone and [^{3}H]-DHT in competition with various other steroids. It can be observed further that DHT competes better with [^{3}H]-testosterone than does testosterone itself.

In addition, testosterone and DHT receptors are indistinguishable by both the sucrose gradient procedure and gel electrophoresis; thus, it appears that after castration, the number of sites are similar for both hormones. The sum of these considerations favours the hypothesis that there exists only one androgen receptor with a higher affinity for DHT than testosterone. The same conclusion has been reached by various groups at both pituitary and prostate levels [8, 11, 31, 32].

Estradiol binds to ram pituitary cytosol with an association constant of $3.41 \times 10^9\ M^{-1}$, approaching that observed in rat and calf pituitary cytosol [8, 26, 33 and Thieulant—unpublished results]. The K_a for estradiol in the ewe is 4–7 times greater depending on season [34] and in addition, two slopes were shown in the Scatchard analysis, which were not observed in the ram.

The K_a value for ram pituitary cytosol is of the same order of magnitude as that measured for testosterone. The mean concentration of 17β-estradiol binding sites, 181 fmol per mg of protein is similar to that observed in the rat, 140–160 fmol, by various authors [26, 29, 35 and Thieulant—unpublished results] but about 10 times greater that observed in the ewe or the male calf.

In a sucrose gradient, the estradiol receptor migrates in the 7S region, as does the androgen receptor, a result comparable with the estrogen receptor in the ewe pituitary [25]. The 17β-estradiol receptor has an affinity for estrogen groups, but little or none for androgens, progesterone or cortisol, a result obtained by several authors using male rat pituitary cytosol [5, 26, 28, 29].

This detailed methodological study was necessary to examine under carefully defined conditions, the effects of castration on pituitary steroid receptors at the cytosolic level.

The animals which had been castrated for only 24 h were expected to have a physiological status close to that of intact animals except that the absence of circulating testicular androgens may result in a liberation of the cytosolic sites. These animals were compared with long-term castrated rams, since rams differ from rats in the long delay required before observing changes at the hypothalamo-hypophyseal level [36]. The pituitary weight in particular, does not change after castration [37], which explains why number of sites expressed per μg of DNA is not very different when based on protein concentration.

The association constant for testosterone decreases in the 2 years after castration, while that for DHT increases. For the latter steroid, the result is that in long-term castrated animals, the K_a value is significantly higher ($P < 0.001$) that in short-term animals. On the other hand, castration has no effect on the K_a for estradiol.

The number of binding sites per mg of protein decreases significantly with time after castration for both androgens (45–60%) but not for estradiol. The decrease is somewhat greater when results are expressed per μg of DNA, receptor loss being approx. 70% for DHT. The absence of changes in estradiol binding proteins in long-term castrated rams may be associated with the maintenance of a strong inhibitory effect on LH release exerted by estradiol in these animals (unpublished results).

No comparable studies have been done, even in the rat, where intact and short-term castrated animals have been compared, and where the true effect of castration is masked because the binding sites are no longer occupied by endogenous hormone [38].

The plasma concentration of 5α-DHT is low in the ram [40] but when injected *in vivo* it suppresses LH release more actively than testosterone in castrated animals, in the ram [41] as in the rat [42]. On the other hand testosterone is presumably metabolized to DHT in the pituitary as in the rat [43, 44]. Thus if the respective roles of DHT and testosterone in LH synthesis and release are still unclear both presumably involve the intervention of cytosolic androgen receptor.

Finally, if pituitary sensitivity to direct negative feed-back of androgens in long-term castrated rams is reduced, the present results indicate clearly that one component could be the partial loss of androgen receptors in the cytoplasm of the anterior pituitary.

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