Journal of Steroid Biochemistry. Vol. 10. pp. 401 to 410
Pergamon Press Ltd 1979. Printed in Great Britain

NUCLEAR BINDING OF 5α-DIHYDROTESTOSTERONE IN THE MALE RAT PITUITARY: EVIDENCE FOR TWO BINDING FORMS

LOUIS MERCIER, SUZANNE SAMPEREZ and PIERRE JOUAN*
Laboratoire de Neurobiologie Moléculaire, CNRS: E.R.A. 070567 Avenue du Général Leclerc, 35042 Rennes Cedex, France

(*Received* 19 *June* 1978)

SUMMARY

Purified nuclei were prepared from prepubertal male rat pituitaries incubated at 37°C in the presence of [1,2-^{3}H]-5α-DHT, and selectively extracted with saline solutions of different ionic strength and pH. This extraction process led to the identification of two nuclear 5α-DHT receptor complexes: one soluble in the nuclear sap, the other bound to chromatin. Their relative amount and their specific activity (362 fmol/mg protein and 38 fmol/mg protein, respectively), were determined after precipitation by a protamine sulphate solution. These two kinds of nuclear 5α-DHT receptor complexes were characterized using sucrose gradient centrifugation and Sephadex G-200 gel chromatography. The soluble nuclear sap 5α-DHT binding component sedimented as a 5.6S entity and was retained on Sephadex G-200 gel, whereas the complex bound to chromatin sedimented as a 4.6S entity and appeared in the void volume of the Sephadex G-200 gel column. Both are hormone specific proteins, and are distinct from the cytosol 5α-DHT complex. The physiological role of these two forms is not yet determined.

INTRODUCTION

The presence of a specific receptor for 5α-Dihydrotestosterone (5α-DHT) in the cytosol from male rat pituitary is now well established[1–5]. However, there is little information concerning the presence and properties of a 5α-DHT receptor in pituitary nuclei. Kato[4], using ultracentrifugation in linear sucrose gradients of nuclear 0.4 M KCl extracts, provided evidence for the existence of a 4–6 S receptor complex in purified nuclei from pituitaries of castrated adult male rats. Lieberburg *et al.*[6], using the same technique, after injection of tritiated hormone to male rats, found only one nuclear complex with a sedimentation coefficient of 3.6 S. Thus, the characteristics of this nuclear receptor remain to be defined.

Our work on androgen receptors in the male rat pituitary, enabled us to confirm the presence of a nuclear receptor for 5α-DHT. This paper reports its presence in nuclei in two forms: a soluble one in nucleoplasm, and another bound to chromatin.

MATERIALS AND METHODS

Steroids and chemicals

[1,2-^{3}H]-5α-dihydrotestosterone (5α-DHT) (S.A. 48 Ci/mmol) was purchased from C.E.A. (Saclay, France). Radio-inert 5α-DHT, 5α-androstane-3β, 17β-diol and 17β-oestradiol were obtained from Sigma Chemical Company. Corticosterone was from Mann Research Laboratories (New York, U.S.A.). Protamine sulphate, pancreatic ribonuclease and pronase were purchased from Koch-Light Laboratories (England), and desoxyribonuclease I from Boehringer (Mannheim, Germany). Sephadex G-200 and G-25, Dextran T 70 were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Animals

Except where otherwise stated, 36 male rats 33–35 days old, of the Wistar strain, were used in one assay. They were purchased from the "Centre d'Elevage R. Janvier" (53680 Le Genest, France). Animals were decapitated without anaesthesia, and hypophyses were quickly removed and weighed. They were kept at 0°C until incubation.

Tissue incubations

Anterior pituitaries were incubated at 37°C for 60 min, in 7.5 ml of Krebs Ringer Bicarbonate Glucose Buffer (KRBG) pH 7.4 in the presence of tritiated 5α-DHT (4×10^{-8} M).

Competitions

Competition assays were performed by adding unlabelled steroid together with the tritiated compound in the incubation medium. Several unlabelled hormones were tested: 5α-DHT (4×10^{-6} M), 5α-androstane-3β,17β-diol (2×10^{-6} M), corticosterone (4×10^{-6} M), 17β-oestradiol (4×10^{-6} M).

Preparation of subcellular fractions

All further operations were performed at 0–4°C; (a) *Purified nuclei.* After the incubation period,

* To whom requests for reprints should be addressed.

pituitaries were washed three times with ice-cold KRBG. They were then homogenized (25% w/v) in 0.32 M sucrose pH 6.5 containing 3 mM $MgCl_2$, using a Dounce homogenizer. Purified nuclei were prepared as previously described[7].

(b) *Cytosol.* Incubated pituitaries were homogenized as described above, and the homogenate was centrifuged at 600 *g* for 10 min. The supernatant was then centrifuged at 105,000 *g* for 60 min in a Beckman SW 50 or SW 65 type rotor. In some experiments, cytosol was prepared from non incubated pituitaries, in 0.05 M phosphate buffer pH 7.4. It was then incubated at 0°C for 5 h with 4×10^{-9} M tritiated 5α-DHT.

Extraction of nuclear hormone–receptor complexes with saline buffers

(a) *Direct 1 M NaCl extract.* The pellet of purified nuclei was extracted with 1 ml of 0.005 M tris-HCl buffer pH 8.0 containing 1 M NaCl; at 0°C for 18–19 h, with gentle magnetic stirring. The soluble fraction was then separated by centrifugation at 42,000 *g* for 20 min in the SS-34 Rotor of a Sorvall superspeed RC 2B. The supernatant was called direct NaCl extract.

(b) *Selective extractions*[8]. (i) *STM extract.* The pellet of purified nuclei was covered with 5 ml of 0.01 M Tris–HCl buffer pH 7.2 containing 0.14 M NaCl and 0.005 M $MgCl_2$ (STM buffer). It was submitted to gentle magnetic stirring at 0°C for 15 minutes. This extraction was repeated twice again and each time, nuclei were pelleted by centrifugation at 600 *g*. Successive supernatants were considered as STM_1, STM_2, STM_3 extracts.

(ii) *1 M NaCl extract.* Following the previous extractions, the nuclear pellet was then extracted with either 1 ml or 800 μl of 0.005 M Tris–HCl buffer pH 8.0 containing 1 M NaCl, with gentle magnetic stirring, at 0°C for 18–19 h. The soluble fraction was isolated as described above (a). The supernatant was considered as 1 M NaCl extract, and the insoluble fraction as residual pellet.

(iii) *DNA precipitation: 0.4 M NaCl extract.* DNA precipitation from the 1 M NaCl extract was accomplished by lowering the buffer molarity to 0.4 M in NaCl, by water addition[9]. Following centrifugation at 3,650 *g* for 10 min, the supernatant was considered as 0.4 M NaCl extract.

Precipitation of nuclear hormone–receptor complexes by protamine sulphate[9–10]

The hormone–receptor complex was precipitated from STM_1 extract by addition of an equal volume of a protamine sulphate solution in STM buffer (0.04 mg/ml). The protein concentration of STM_1 extract was 0.050 ± 0.007 mg/ml. (mean ± S.E.M. 9 determinations). It was precipitated from 0.4 M NaCl extract under the same conditions: respective concentrations were for protamine sulphate 0.166 mg/ml, and for proteins 0.249 ± 0.015 mg/ml. (mean ± S.E.M. 12 determinations). In both cases, the mixture was kept at 0°C for 15 min, then it was filtered through Whatman Gf/C filters (2.5 cm diameter) under slight vacuum. The precipitate was washed four times with 10 ml of buffer. Filters were then put into counting vials and radioactivity was estimated.

Effect of some enzymes

Aliquots of STM_1 extract and of 0.4 M NaCl extract were incubated at 37°C for 30 min in the presence of various enzymes at a final concentration of 50 μg/ml. Pancreatic RNAse, pronase and DNAse I were studied. For assays with DNAse I, the buffer concentration in $MgCl_2$ was brought up to 5 mM[11]. After incubation, bound radioactivity was precipitated by adding protamine sulphate solution, and it was then counted. Corrections were made with respect to controls submitted to the same incubation conditions without enzymes.

Isolation of bound radioactive fractions

Two methods were used: STM_1 extract and 1 M NaCl extract were submitted to gel filtration through Sephadex G-25 columns (28 cm × 1.5 cm) equilibrated respectively with STM buffer and 1 M NaCl buffer. In cytosol, free hormone was discarded by Dextran Coated Charcoal (DCC) treatment: Norit charcoal (0.5 g) and Dextran T 70 (0.05 g) were suspended in 100 ml of 0.01 M Tris–HCl buffer pH 7.5 containing 0.0015 M EDTA and 0.01 M Dithiothreitol. A volume of this suspension equal to the volume of cytosol was centrifuged at 3,650 *g* for 10 min. Cytosol was then added and mixed with the DCC pellet. The mixture was left at 0°C for 2–3 min and then centrifuged at 3,650 *g* for 10 min. The supernatant was undiluted cytosol devoided of free radioactive hormone.

Steroids extraction and identification

Steroids were extracted from bound fractions of STM_1 extract and 1 M NaCl extract according to the method of Folch *et al.*[12] modified by Bruchovsky *et al.*[13]. The residual pellet was suspended in 1 ml of distilled water and then submitted to the same extraction procedure. Steroids were separated by chromatography on Whatman No. 1 paper (45 cm × 2.5 cm) in the solvent system of Kochakian and Stidworthy[14]. Development at 30°C was achieved within 6–7 hours. Chromatograms were then cut into strips (2.5 cm × 1 cm), the radioactivity of which was estimated by liquid scintillation counting.

Gel chromatography on Sephadex G-200

Fractionation of the nuclear hormone–receptor complexes found in STM_1, 1 M NaCl, 0.4 M NaCl extracts and in cytosol was carried out by gel filtration through a column of Sephadex G-200 (77.5 cm × 1.5 cm) equilibrated with 1 M NaCl. Working pressure was 7.5 cm, and 2 ml fractions were collected. In each fraction proteins and radioactivity

were measured. Sometimes, the optical density of the eluates, at 260 nm and at 280 nm was estimated.

Centrifugation in linear sucrose density gradients

In all assays, sedimentation coefficients were calculated according to the method of Martin and Ames[15], using bovine serum albumin (BSA) as standard. STM_1 extracts were layered on 5–20% linear sucrose gradients prepared in STM or in 1 M NaCl buffers. 1 M NaCl extracts were layered on the same gradients prepared in 1 M NaCl buffer containing tritiated 5α-DHT (0.5×10^{-11} M)[16]. 0.4 M NaCl extracts were analyzed successively in 5–20% gradients prepared in 0.4 M NaCl, 1 M NaCl or STM buffers, and ~~always~~ containing labelled 5α-DHT (0.5×10^{-11} M).

Cytosol was first treated by DCC as previously described. Then the hormone–receptor complex was precipitated by addition of ammonium sulphate (30% w/v). After 30 min at 0°C, the pellet was recovered by centrifugation (3,650 *g*) for 10 min, and dissolved either in 0.05 M phosphate buffer or in STM buffer. It was then layered on 5–20% sucrose gradients prepared in the corresponding buffer.

Centrifugations were carried out at 4°C for 14 h either at 39,000 rev./min in a Beckman SW 50 type rotor, or at 42,500 rev./min in a SW 65 type rotor. Gradients were fractionated using an ISCO apparatus. Fractions (6 drops) were collected directly into counting vials.

Other techniques

Proteins were determined according to the method of Lowry *et al.*[17] using BSA as a standard. DNA was submitted to acid hydrolysis according to the method of Valotaire and Duval[18]. It was then estimated by spectrophotometry at 260 nm. Radioactivity was estimated by liquid scintillation counting using an Isocap 300 counter (Nuclear Chicago). Solvents were Instagel for aqueous samples, and Bray's solution[19] for glass fiber filters. Chromatogram strips were put into a scintillation counting system containing PPO (5 g), dimethyl POPOP (0.1 g), and toluene 1,000 ml. Quenching was corrected by the channel ratio method.

RESULTS

Determination of radioactivity, proteins and DNA contents in nuclear extracts

Using an extraction process which implied buffers of different ionic strength and different pH: STM buffer pH 7.2 and 1 M NaCl pH 8.0, the nuclear materials were fractionated into five fractions, of which four were soluble fractions. Their radioactivity, proteins and DNA contents are summarized in Table 1. It was noticed that only a small amount of DNA (1.4% ± 0.8%) and of proteins (12.5% ± 5.9%) was extracted with STM buffer. On the other hand, most of the radioactivity (67.8% ± 3.9%) was found in STM_1 extract.

Precipitation of hormone–receptor complexes by protamine sulphate

(a) *STM_1 extract.* Bound radioactivity was isolated by protamine sulphate precipitation as described in the materials and methods section. It was an important part of the entire STM_1 extract: 19.7% ± 1.1% (mean ± S.E.M. five determinations) and specific activity of the binding was 362 ± 22.2 fmol per mg proteins (mean ± S.E.M. five determinations).

(b) *0.4 M NaCl extract.* DNA was precipitated by lowering the NaCl concentration from 1 to 0.4 M as previously described, and bound radioactivity was isolated from the supernatant by the protamine sulphate method. Under these conditions, 85.6% ± 7.3% (mean ± S.E.M. five determinations) of the radioactivity present in 1 M NaCl extract were found in the 0.4 M NaCl extract, and the bound part represented 40.4% ± 2.1% (mean ± S.E.M. three determinations) of this amount. Its specific binding activity was

Table 1. Thirty-six anterior pituitaries from prepubertal male rats were incubated at 37°C for 60 min in the presence of [1,2-^{3}H]-5α-DHT (4×10^{-8} M). Purified nuclei were isolated and extracted with STM buffer pH 7.2 (three times) then by 1 M NaCl buffer (once); in every nuclear extract, radioactivity, proteins, and DNA were estimated. Their amounts were expressed as % ± S.E.M. of total radioactivity, proteins and DNA respectively

Extract	Proteins*	DNA†	Radioactivity Total radioactivity‡	Bound
STM_1	12.4 ± 5.9	1.3 ± 0.8	67.8 ± 3.9	14 ± 0.4§
STM_2	N.D.	N.D.	11.8 ± 2.8	N.D.
STM_3	N.D.	N.D.	4.2 ± 0.5	N.D.
1 M NaCl	54.7 ± 7.5	44.7 ± 8.3	6.3 ± 0.8	—
0.4 M NaCl	—	—	—	2.3 ± 0.2†
Residual pellet	32.7 ± 7.5	55 ± 8.2	9.9 ± 1.8	—

* Mean of nine determinations. † Mean of three determinations. ‡ Mean of six determinations. § Mean of five determinations. N.D. Not detectable.

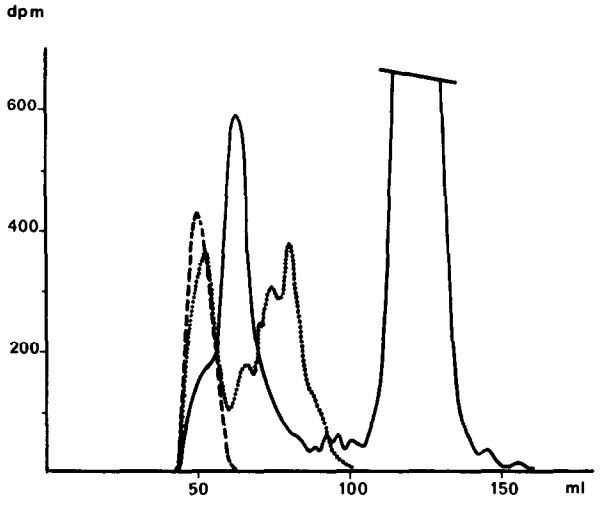

Fig. 1. Sephadex G-200 chromatography of the "direct 1 M NaCl extract". Purified nuclei were prepared from 18 pituitaries incubated at 37°C in the presence of [1,2-^{3}H]-5α-DHT and extracted overnight at 0°C with 1 ml of 1 M NaCl pH 8.0 solution. A 750 μl aliquot of the direct 1 M NaCl extract was then chromatographed on a Sephadex G-200 column (1.5 × 77.5 cm) equilibrated with 1 M NaCl solution. Results are corrected to the entire volume of the extract (1 ml). —— Radioactivity ····· Protein ------ O.D. at 260 nm.

38 ± 4.3 fmol per mg proteins (mean ± S.E.M. Three determinations).

Gel chromatography on Sephadex G-200

(a) *Direct 1 M NaCl extract.* The different 5α-DHT macromolecular bindings previously found in STM_1 and in 0.4 M NaCl extracts corresponded in fact, with two kinds of nuclear binding forms which were present in direct 1 M NaCl extract, and which were distinguishable from one another on a Sephadex G-200 column (Fig. 1). Indeed one radioactive peak was eluted together with DNA (max 50th/ml), and a second apart from DNA fraction (max 62th/ml). There was also a third peak of radioactivity (between 100th and 140th/ml) which corresponded with free hormone.

(b) *STM_1 extract.* The major part of the nuclear bound 5α-DHT was found in this extract, as previously demonstrated with protamine sulphate precipitation. During gel filtration through Sephadex G-200 column only one bound radioactive peak was eluted (max 64th ml) (Fig. 2). It seemed to correspond with the second radioactive peak (max 62th ml) of the direct 1 M NaCl extract, and also to be selectively and entirely extracted with STM buffer pH 7.2. A fraction of free hormone was also eluted from 100th to 150th ml. It was found that, when the gel was equilibrated with either STM buffer (not represented) or 1 M NaCl buffer (Fig. 2), the elution patterns were identical.

(c) *1 M NaCl extract and 0.4 M NaCl extract.* The nuclear pellet was extracted three times with STM buffer and then with 1 M NaCl pH 8.0 as described in the materials and methods section. The latter extract was then filtered through Sephadex G-200 column (Fig. 3a). Three radioactive peaks were eluted: the first one (max 56th ml) was eluted with DNA, it could represent one form of the 5α-DHT nuclear receptor, which was bound to chromatin and extractible with 1 M NaCl. The second peak (max 64th ml) had the same elution pattern as the STM_1 extract and could result from the dissociation, during gel filtration, of a part of the receptor bound to chromatin. The third peak represented free hormone (from 110th to 150th ml).

When the 0.4 M NaCl extract was analysed on Sephadex G-200 column, one radioactive peak was recovered (max 64th ml) with the same elution volume as STM_1 extract (Fig. 3b). It represented 72.4% of the hormone bound in 0.4 M NaCl extract. Free hormone was not collected in this assay.

Ultracentrifugation in 5–20% sucrose linear gradients

The analysis of nuclear extracts by sedimentation in sucrose gradients led to the identification of two nuclear binding forms for 5α-DHT.

(a) *STM_1 extract.* The sedimentation coefficient of the hormone–receptor complex was 5.6 S when gradients were prepared in STM buffer (Fig. 4). This 5.6 S form was however converted into a 4.6 S one, when gradients were prepared in 1 M NaCl.

(b) *1 M NaCl extract and 0.4 M NaCl extract.* The 1 M NaCl complex had a sedimentation coefficient of 4.6 S in a gradient containing 1 M NaCl (Fig. 5a). Unlike G-200 filtration, it was not converted into a 5.6 S form (identical to STM extract) when DNA was pelleted from the 1 M NaCl extract. The complex found in 0.4 M NaCl extract always had the same sedimentation coefficient (4.6 S), whether gradients were prepared in STM buffer or in 1 M NaCl (Fig. 5b).

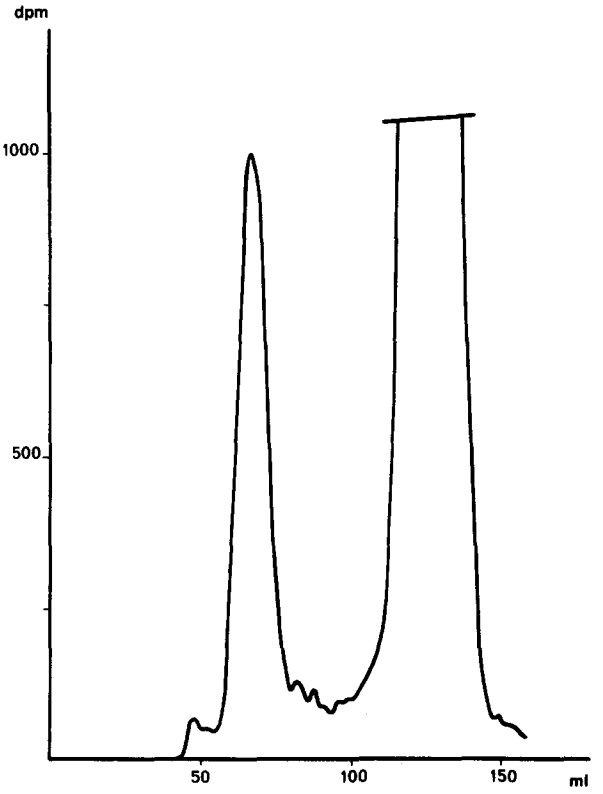

Fig. 2. Sephadex G-200 chromatography of the STM_1 extract. Purified nuclei were prepared from 36 pituitaries incubated at 37°C in the presence of [1,2-^{3}H]-5α-DHT. They were then extracted with 5 ml of STM buffer pH 7.2. A 2 ml aliquot of the STM, extract was chromatographed on a Sephadex G-200 column (1.5 × 77.5 cm) equilibrated with 1 M NaCl solution. Results are corrected to the entire volume of STM_1 extract (5 ml).

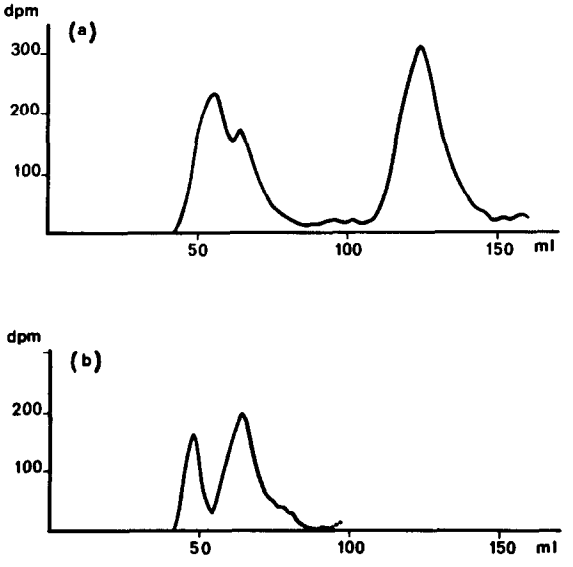

Fig. 3. Sephadex G-200 chromatography of: (a) an 800 μl aliquot of 1 M NaCl extract; (b) a 1.5 ml aliquot of 0.4 M NaCl extract. After three successive washings with STM buffer, purified nuclei obtained as outlined in the legend of Fig. 2, were then extracted overnight with 1 ml of 1 M NaCl solution pH 8.0. This 1 M NaCl extract was either directly filtered through a G-200 Sephadex column (1.5 × 77.5 cm) (a) or diluted with water to a 0.4 M NaCl concentration and then filtered (b) (for further details, see materials and methods).

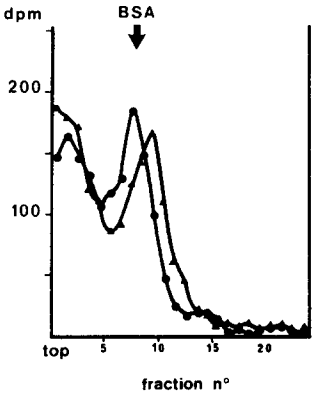

Fig. 4. Sucrose gradient analysis of the STM_1 extract. Aliquots (500 µl) were subjected to centrifugation for 14 h at 39,000 rev./min in a 5–20% linear sucrose gradient prepared with STM buffer pH 7.2 ▲——▲, or with 1 M NaCl solution pH 8.0 ●——●.

Action of enzymes

When STM_1 extract or 0.4 M NaCl extract was incubated in the presence of pronase, the binding of 5α-DHT was completely abolished. On the other hand, no decrease of binding was observed in the presence of RNAse or DNAse.

These results suggest that the nuclear binding macromolecules, which were extracted by STM buffer pH 7.2 or by 1 M NaCl pH 8.0 were proteins.

Identification of the steroid bound in nuclear extracts

In STM_1 and NaCl extracts, the steroid bound to proteins was quite exclusively 5α-DHT as demonstrated by paper chromatography: respectively 91.6% (Fig. 6a) and 80.2% (Fig. 6b) of the total chromatogram radioactivity were 5α-DHT. In the residual pellet, only a small amount of 5α-DHT was recovered (33.1%). The major radioactive part (59.9%) was found as apolar compounds at the front of the chromatogram (Fig. 6c).

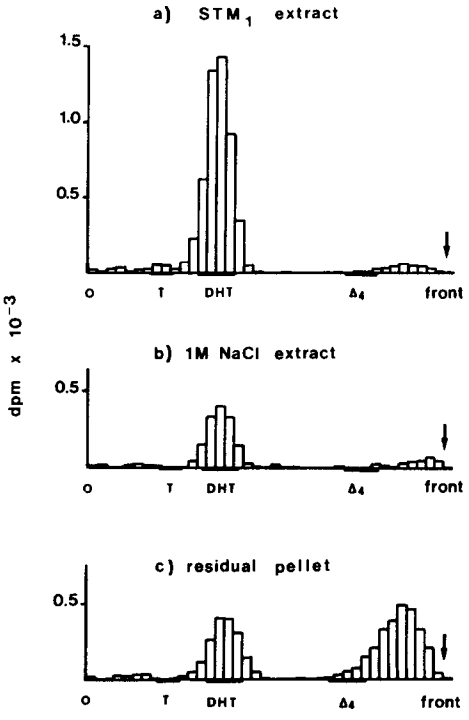

Fig. 6. Analysis by paper chromatography of chloroform-methanol extractable radioactivity from nuclear bound salt soluble fractions, and from the residual pellet (salt resistant-fraction) obtained after incubation at 37°C of male rat pituitaries in the presence of [1,2-^{3}H]-5α-DHT.

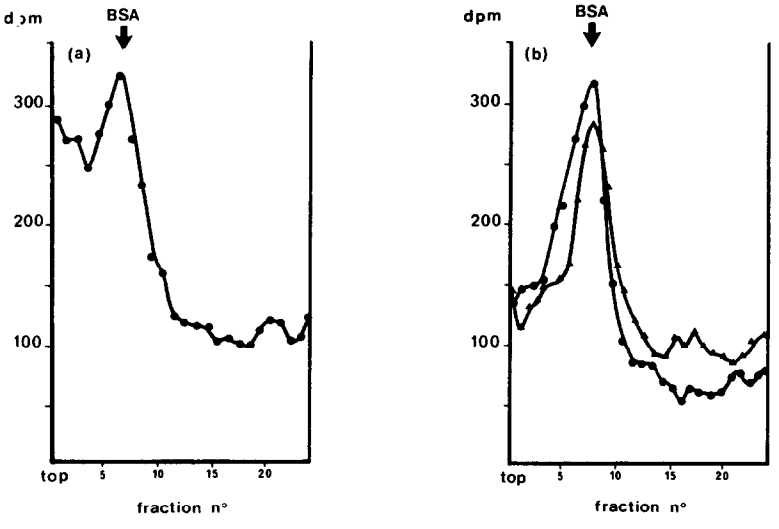

Fig. 5. Sucrose gradient analysis of (a) 1 M NaCl extract, (b) 0.4 M NaCl extract. Samples (500 µl) were subjected to centrifugation for 14 h at 39,000 rev./min. on a 5–20% linear sucrose gradients prepared with 1 M NaCl solution pH 8.0 ●——●, or with STM buffer pH 7.2 ▲——▲, both containing [1,2-^{3}H]-5α DHT (0.5×10^{-11} M).

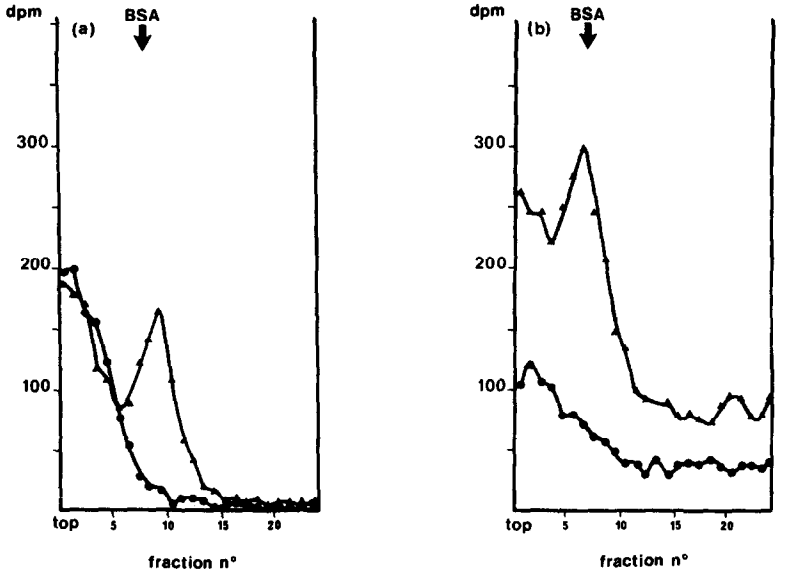

Fig. 7. Sedimentation pattern of nuclear 5α-DHT binding components obtained from pituitaries incubated in the presence of [1,2-^{3}H]-5α-DHT (4×10^{-8} M) alone ▲——▲, or in the presence of a 100 fold excess of unlabelled 5α-DHT (4×10^{-6} M) ●——●. Aliquots (500 μl) of STM_1 extract were layered on 5–20% sucrose gradients prepared with STM buffer (a). Aliquots (500 μl) of 1 M NaCl extract were layered on 5–20% sucrose gradients prepared with 1 M NaCl (b).

Binding specificity

Some competition assays were carried out by addition of unlabelled steroids together with tritiated 5α-DHT to the incubation medium. Only unlabelled 5α-DHT was competitive, since its nuclear binding was entirely abolished, as shown by sucrose gradients analysis of STM_1 extract (Fig. 7a) and 1 M NaCl extract (Fig. 7b).

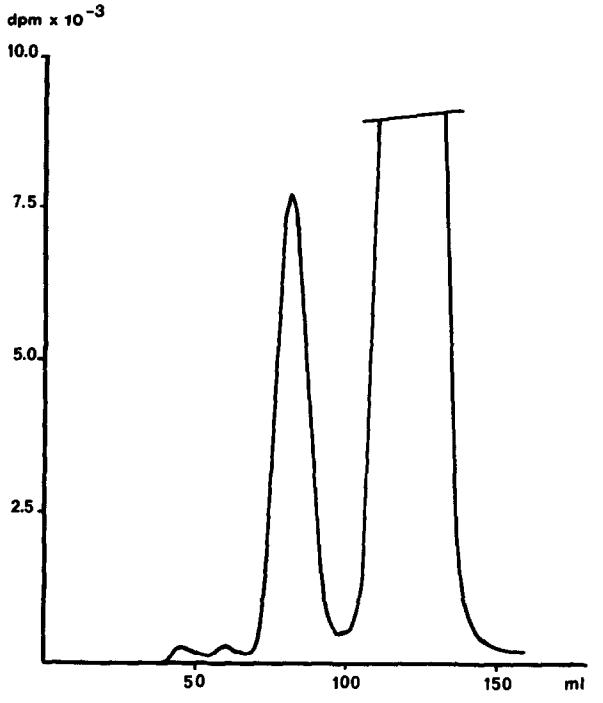

Fig. 8. Sephadex G-200 chromatography of cytosol obtained from pituitaries incubated at 37°C in the presence of [1,2-^{3}H]-5α-DHT (4×10^{-8} M). An aliquot (1 ml) of the 105,000 *g* supernatant was chromatographed on a Sephadex G-200 column (1.5 × 77.5 cm) equilibrated with 1 M NaCl solution.

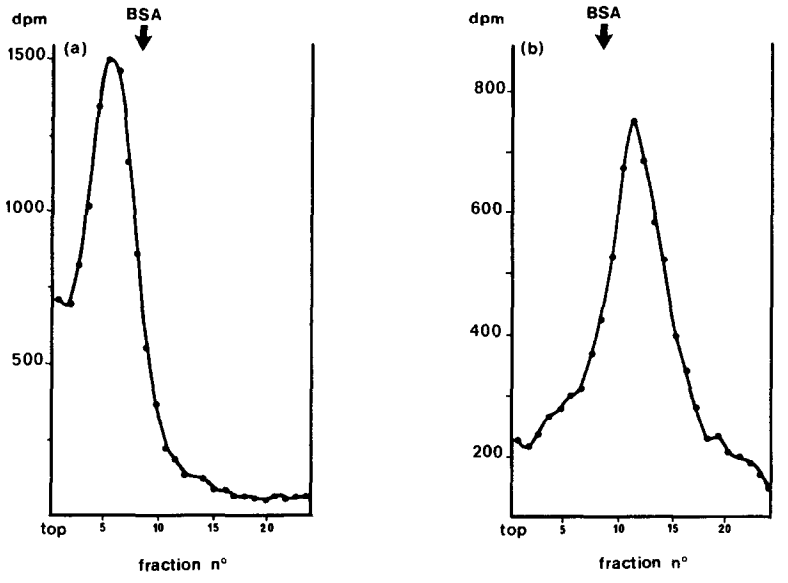

Fig. 9. Sedimentation analysis of cytosol 5α-DHT binding component. (a): cytosol was obtained from pituitaries incubated at 37°C in the presence of [1,2-^{3}H]-5α-DHT (4×10^{-8} M). (b): cytosol was directly incubated at 0°C for 5 hours with [1,2-^{3}H]-5α-DHT (4×10^{-9} M). In both cases, aliquots of the 105,000 *g* supernatant were first treated with dextran coated charcoal, then the hormone receptor complex was precipitated with ammonium sulphate (30% w/v) for 30 min. The precipitates were recovered by centrifugation at 2,000 *g*, and were dissolved either in 500 μl STM buffer (a) or in 0.05 M phosphate buffer (b) before centrifugation in sucrose gradients respectively prepared with the same buffers (STM or 0.05 M phosphate buffer).

Comparative study of cytosol

In some experiments, the cytosol binding of 5α-DHT was compared with its nuclear binding. When cytosol was prepared from pituitaries previously incubated at 37°C with tritiated hormone, and then filtered through Sephadex G-200 column, one radioactive peak was found (max 82th ml) (Fig. 8). It was different from those observed during gel filtration of STM_1 or of 1 M NaCl nuclear extracts (Figs 1, 2 and 3). In 5–20% sucrose gradients, this cytosol macromolecular complex sediments as a 3 S entity (Fig. 9a). When cytosol was directly prepared from pituitaries not exposed to hormone, and then incubated at 0°C with tritiated 5α-DHT, the complex had a sedimentation coefficient of 6.3 S (Fig. 9b). In both cases, these values differed from those found for STM_1 and NaCl 1 M complexes (Figs 4 and 5). Chromatographic analysis of steroids extracted from the 3 S complex showed that 5α-DHT itself stood for only 58% of the total chromatogram radioactivity, and that it was partially metabolized into 5α-androstanediols (39%) (Fig. 10).

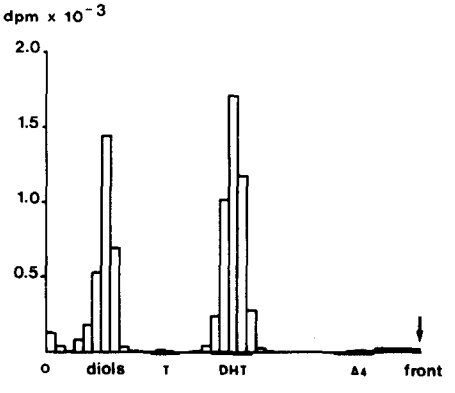

Fig. 10. Analysis by paper chromatography of chloroform–methanol extractable radioactivity from the cytosol 3S 5α-DHT–receptor complex.

DISCUSSION

We have previously shown[20] that testosterone and 5α-DHT were bound to macromolecules in purified nuclei from male rat pituitaries. In 1976, Kato[5] working on castrated male rat pituitaries incubated in the presence of tritiated 5α-DHT, found a 4–6 S receptor complex in 0.4 M KCl extract from purified nuclei. In 1977, Lieberburg *et al.*[6] confirmed the presence of a nuclear receptor complex for 5α-DHT, but found that its sedimentation coefficient was 3.6 S. Thus some differences appeared concerning the sedimentation coefficient of the pituitary nuclear receptor complex.

The present report indicates the presence of two binding forms for 5α-DHT in purified nuclei from male rat pituitary. The first one is soluble in the "nuclear sap" and can be extracted with a buffer of low

ionic strength (STM buffer), the second is bound to chromatin and is extractible with a high ionic strength solution (1 M NaCl).

General properties

These two forms of binding represent nuclear receptors for 5α-DHT. Both are hormone specific, since among the steroids tested only unlabelled 5α-DHT is able to completely abolish these two nuclear bindings. Furthermore, there is no metabolism of the bound hormone. These two kinds of receptors are proteins, since only pronase destroys the hormone–receptor complex. Moreover, they are acid proteins, since they are both precipitable by protamine sulphate.

Special properties

(a) *Intranuclear localization.* One of the two receptors is soluble in the nucleoplasm, since it can be extracted with a buffer of low ionic strength (STM pH 7.2). A similar extraction process has been used by several authors [8, 21, 22] in order to prepare purified chromatin fractions. The washing of nuclei with buffers of low ionic strength leads to the extraction of proteins from the nuclear sap, (which appear to be quite distinct from the chromosomal non histone proteins and histones, and also from cytoplasmic proteins), and ribonucleoprotein particles. The chromatin components are then extracted with 1 M or 2 M NaCl solution, leaving behind nuclear membranes and nucleolar material [8].

As an extensive washing of nuclei with STM buffer does not permit any further extraction, it seems that the second receptor is strongly bound to chromatin. The extraction of this complex together with a significant amount of DNA can be performed only with a solution of high ionic strength (1 M NaCl). It is then possible to dissociate it from DNA. By lowering the NaCl concentration from 1 M to 0.4 M, DNA is precipitated and can be pelleted by centrifugation, whereas the receptor remains in the 0.4 M NaCl supernatant.

Clark and Peck[23] have demonstrated that two kinds of oestradiol receptor are found in uterine nuclei undergoing a differential salt extraction by KCl: 80–90% of the nuclear receptor complexes are extractable from their binding sites with KCl, whereas 10–20% are non extractable. These results have been discussed by Müller *et al.*[24] who argued that discrimination between these two forms is founded only on their extractibility with KCl and that their physical properties are not taken into account. Nevertheless, these studies raise the problem of the existence of two forms of nuclear binding.

(b) *Intranuclear concentration.* 5α-DHT bound to the soluble nuclear complex represents the major fraction of the total radioactivity bound in nuclei (362 fmol/mg proteins) whereas the fraction bound to chromatin is lower (38 fmol/mg proteins). When 17β-oestradiol is studied, under the same conditions, the amount and distribution of the bound radioactivity are different: most of the bound radioactivity is recovered in the 1 M NaCl extract [25]. These results seem to be in good agreement with those of Sar and Stumpf[26]. Following oestradiol administration to male rats, they found a uniform distribution of the hormone in nuclei of all pituitary cell types. On the contrary, when testosterone was administered to animals, it was exclusively located into gonadotrophs nuclei. Gonadotrophs standing only for 10–15% of the total pituitary cells, it could be inferred that, in male rat pituitary, the number of binding sites for oestradiol is higher than that for 5α-DHT. The higher amount of oestradiol bound to chromatin could confirm this hypothesis [25].

(c) *Sedimentation coefficients.* Does the nuclear receptor exist in one or two forms? The sedimentation coefficient of STM complex is 5.6 S when gradients are prepared in STM buffer. It changes to 4.6 S when gradients are prepared in 1 M NaCl solution. On the other hand, the complex found in 1 M NaCl extract or in 0.4 M NaCl extract is 4.6 S, whether gradients are prepared in STM or in NaCl buffers. These facts suggest that the two forms of the nuclear binding are a unique 5.6 S entity, which is converted non reversibly into a 4.6 S form in a medium of high ionic strength (1 M NaCl).

The 4.6 S complex found in 1 M NaCl extract is unstable, and only observed in sucrose gradients containing tritiated 5α-DHT (0.5×10^{-11} M), whereas the 5.6 S form remains stable even when converted into a 4.6 S form by increasing the ionic strength throughout the gradient. These results are not in good agreement with the hypothesis of a unique form of nuclear receptor.

(d) *Relationship between cytosol and nuclear receptors.* The cytosol 5α-DHT receptor is 6.3 S when binding experiments are performed at 0°C in a buffer of low ionic strength (0.05 M phosphate buffer pH 7.4), whereas it is 3S at 37°C. The conversion of a 6.3S entity into a 3S entity results probably from the transformation of an inactive oligomeric form into an active one. Sedimentation coefficients and G-200 elution patterns of the active and the inactive forms are markedly different. These differences could be due to the association of the cytosol 3S receptor and another macromolecule in the nucleus, rather than to a conformational change.

The presence of phosphorylated acid proteins in 0.14 M NaCl, 0.01 M Tris–HCl (pH 7.4) extracts from mouse liver and brain nuclei has been demonstrated by McGillivray[22]. These results raise the possibility that the 5.6S nuclear receptor complex could result from association of the cytosol 3S complex and a phosphorylated acid protein. Considering the primordial role of these phosphorylated proteins in the genetic regulation of eukaryotic cells [27], it would be necessary to know whether the 5.6S receptor complex really results from association of such a protein and the 3S cytosol receptor.

(e) *Biological significance of two 5α-DHT nuclear receptor complexes.* In the present state of our work, it is difficult to explain the biological role of these two nuclear complexes. Nevertheless, it may be suggested that the 5.6S complex results from association of the cytosol 3S receptor and nuclear proteins. Its stability and also its localization in the nuclear sap suggest that it represents either the nuclear stock of 5α-DHT receptor, or a form for its transit to chromosomes and nucleoli. On the other hand, the 4.6S nuclear complex bound to chromatin may be the active form of the 5α-DHT receptor. Further studies concerning the action of these two nuclear complexes on RNA polymerases activity would make it possible to specify their respective functions. Such research is now in progress in our laboratory.

REFERENCES

1. Mercier L., Thieulant M. L., Samperez S. et Jouan P.: Sur la présence d'un récepteur spécifique de la dihydro-testostérone dans l'hypophyse antérieure du rat mâle. *C.r. hebd. Séanc. Acad. Sci. Paris* **279** (1974) 2127–2130.
2. Mercier L., Le Guellec C., Thieulant M. L., Samperez S. and Jouan P.: Androgen and estrogen receptors in the cytosol from male rat anterior hypophysis: further characteristics and differentiation between androgen and estrogen receptors. *J. steroid Biochem.* **7** (1976) 779–785.
3. Kato J. and Onouchi T.: 5α-Dihydrotestosterone "receptor" in the rat hypophysis. *Endocr. Jap.* **20** (1973) 641–644.
4. Kato J.: The role of hypothalamic and hypophyseal 5α-dihydrotestosterone, estradiol and progesterone receptors in the mechanism of feedback action. *J. steroid Biochem.* **6** (1975) 979–987.
5. Barley J., Ginsburg M., Greenstein B. D., MacLusky N. J. and Thomas P. J.: An androgen receptor in rat brain and pituitary. *Brain Res.* **100** (1975) 383–393.
6. Lieberburg I., MacLusky N. J. and McEwen B. S.: 5α-Dihydrotestosterone (DHT) receptors in rat brain and pituitary cell nuclei. *Endocrinology* **100** (1977) 598–607.
7. Jouan P., Samperez S. and Thieulant M. L.: Testosterone "receptors" in purified nuclei of rat anterior hypophysis. *J. steroid Biochem.* **4** (1973) 65–74.
8. Monahan J. J. and Hall R. H.: Preparation of chromatin from tissue culture cells—a convenient method. *Analyt. Biochem.* **65** (1975) 187–203.
9. King R. J. B., Gordon J. and Steggles A. N.: The properties of a nuclear acidic protein fraction that binds [6.7-^{3}H]-oestradiol-17β. *Biochem. J.* **114** (1969) 649–657.
10. Blondeau J. P., Corpechot C., Le Goascogne C., Baulieu E. E. and Robel P.: Androgen receptors in the rat ventral prostate and their hormonal control. *Vitams Horm.* **33** (1975) 319–345.
11. Mainwaring W. I. P.: The binding of [1,2-^{3}H]-testosterone within nuclei of the rat prostate. *J. Endocr.* **44** (1969) 323–333.
12. Folch J., Lees M., Stanley S.: Simple method for isolation and purification of total lipids from animal tissues. *J. biol. Chem.* **226** (1957) 497–509.
13. Bruchovsky N. and Wilson J.: The conversion of testosterone to 5α-androstan-17β-ol-3-one by rat prostate *in vivo* and *in vitro*. *J. biol. Chem.* **243** (1968) 2012–2021.
14. Kochakian D. and Stidworthy G.: Paper chromatography of C-19 steroids *J. biol. Chem.* **199** (1952) 607–612.
15. Martin R. G. and Ames B. N.: A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. *J. biol. Chem.* **236** (1961) 1372–1379.
16. Toft O. D. and Sherman M. R.: Receptor identification by density gradient centrifugation. *Meth. Enzymol.* **36** (1975) 156–166.
17. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
18. Valotaire Y. et Duval J.: Régulation hormonale du développement de l'adénohypophyse du rat en croissance. *Bull. Soc. Chim. Biol.* **51** (1969) 1233–1236.
19. Bray C.: A simple efficient liquid scintillation for counting aqueous solutions on a liquid scintillation counter. *Analyt. Biochem.* **1** (1960) 279–285.
20. Jouan P., Samperez S. and Thieulant M. L.: Binding and metabolism of [^{3}H]-Testosterone in the nuclei of rat pituitary *in vivo*. *J. steroid Biochem.* **4** (1973) 677–685.
21. Wang T. Y.: The isolation, properties and possible function of chromatin acidic proteins. *J. biol. Chem.* **242** (1967) 1220–1226.
22. MacGillivray A. J. and Rickwood D.: The heterogeneity of mouse chromatin non-histone proteins as evidence by two dimensional polyacrylamide-gel electrophoresis and ion-exchange chromatography. *Eur. J. Biochem.* **41** (1974) 181–190.
23. Clark J. H. and Peck E. J.: Nuclear retention of receptor–oestrogen complex and nuclear acceptor sites. *Nature* **260** (1976) 635–637.
24. Müller R. E., Traish A. M. and Wotiz H. H.: Interaction of receptor–estrogen complex (R.E) with uterine nuclei. *J. biol. Chem.* **252** (1977) 8206–8211.
25. Mercier L.: unpublished results.
26. Sar M. and Stumpf W. E.: Cellular and subcellular localization of radioactivity in the rat pituitary after injection of [1,2-^{3}H]-testosterone using dry-autoradiography. *Endocrinology* **92** (1973) 631–635.
27. Stein G. S., Stein J. S. and Kleinsmith L. J.: Chromosomal proteins and gene regulation. *Sci. Amer.* **232** (1975) 47–57.