Journal of Steroid Biochemistry, 1973, Vol. 4, pp. 525–531. Pegamon Press. Printed in Great Britain

RECEPTORS FOR ANDROGENS IN RAT EPIDIDYMIS. INTERACTIONS WITH 5α-ANDROSTANE-3α,17β-DIOL*

R. S. CALANDRA† and D. A. BLAQUIER‡
Laboratorio de Esteroides, Instituto de Biologia y Medicina Experimental, Obligado 2490, Buenos Aires, Argentina

(*Received* 12 *January* 1973)

SUMMARY

The uptake of [^{3}H]-5α-androstane-3α,17β-diol* by rat epididymal tissue and the binding of this hormone to cytoplasmic and nuclear receptors were studied. One hour after its injection, the labelled steroid was found to be localized mainly in the nuclei of the epididymal cells. Radioactivity in the cytoplasmic and nuclear fractions was examined by sucrose density gradient centrifugation to detect binding to receptors. It was found that the radioactivity of the cytoplasmic fraction was bound to 8·4 *S* macromolecules, while the intranuclear radioactivity was bound to material sedimenting at 3·5 *S*.

In vitro labelling of the cytoplasmic receptor was performed by incubating minced tissue with [^{3}H]-5α-androstanediol at 0°C.

Labelling of the nuclear receptor required raising the temperature to 25°C for 30 min. The receptors could not be labelled under cell-free conditions.

The affinity of the cytoplasmic receptor for dihydrotestosterone was several times higher than for 5α-androstanediol. However, addition of dihydrotestosterone to a system containing the receptor-5α-androstanediol complex did not cause rapid dissociation of the complex. The hormone was apparently transferred from cytoplasm to nucleus bound to a receptor, since isotope dilution was not observed when non-radioactive hormone was added during the translocation step (25°C). Incubation of the tissue in hypotonic medium (50 mM Tris) caused the appearance in the nuclear extract of a second region of binding, with an approximate sedimentation coefficient of 6·0 S. [^{3}H]-5α-androstanediol was rapidly metabolized to 17β-hydroxy-5α-androstan-3-one. The latter metabolite accounted for 82 and 74·5 per cent of the radioactivity in cytoplasm and nuclei, respectively.

INTRODUCTION

THE BIOLOGICAL activity of 5α-androstane-3α,17β-diol§ has been assessed by different tests[1]. All of the reports show that this steroid possesses considerable androgenicity and in some cases is more potent than testosterone. This compound was found to be effective in maintaining spermatogenesis in hypophysectomized mice[2] and was shown to be a stimulator of the DNA polymerase activity of canine prostatic chromatin[3]. Recently, Rivarola *et al.* described the preferential metabolism of [^{14}C]-testosterone to 5α-diol by testicular tubules[4]. The rate of this conversion reached a maximum during meiosis, suggesting that this steroid might participate in the normal spermatogenic process in the rat.

*This research has been partially supported by grants from the National Research Council (Argenine), The Population Council (M72.072C) and the Universidad de Buenos Aires.

†Research Career member, Comision de Investigaciones Cientificas de la Provincia de Buenos Aires.

‡Research Career awardee, National Research Council, Argentina.

§Trivial names and abbreviations used: 5α-diol, 5α-androstanediol: 5α-androstane-3α,17β-diol: DHT, dihydrotestosterone: 17β-hydroxy-5α-androstan-3-one: BSA: bovine serum albumin, YAD: yeast alcohol dehydrogenase, CAT: beef heart catalase.

Androgens produced in the testis arrive at the epididymis in high concentration *via* the fluid secreted by the rete testis[5]. It is of interest to study the uptake and binding of 5α-androstanediol to the epididymal cytoplasmic[6] and nuclear[7] receptors.

MATERIALS AND METHODS

[1,2-^{3}H]-5α-androstane-3α,17β-diol ([^{3}H]-5α-diol), S.A. 50 Ci/mmol and [1.2-^{3}H]-dihydrotestosterone, ([^{3}H]-DHT), S.A. 44 Ci/mmol were purchased from New England Nuclear, Boston, U.S.A., and their purity checked by thin-layer chromatography.

One of the testis of adult male albino rats was excised. The rats were placed with females for 3–4 weeks by which time the epididymis were free of spermatozoa[6]. The remaining testicle was removed and experiments conducted 24 h later.

The *in vivo* studies were carried out by injecting intravenously 30 μCi of [^{3}H]-5α-diol dissolved in saline into eviscerated animals castrated 24 h before. The animals were killed one h later. The epididymides were removed, trimmed of fat and placed in ice-cold medium.

In the *in vitro* labelling experiments, 300–500 mg of minced epididymides were incubated with labelled steroids ($0{\cdot}1–1 \times 10^{-9}$M) in 5 ml of Eagle's medium or in the same vol of 50 mM Tris–HCl buffer (pH 7·4 at 0°C) containing 1·5 mM EDTA. Incubations were carried out for 2–3 h at 0°C with constant agitation. Labelling of the nuclear receptor was carried out by prolonging the incubations for an additional period of 30 min at 25°C. Alternatively, cytosol (105,000 **g** for 60 min supernatant of whole homogenate) or isolated nuclei[7] were incubated with the radioactive steroids in the same manner. After incubation the tissue was washed with excess buffer and homogenized in 1 ml of Eagle's medium in an all-plastic homogenizer. The homogenate was centrifuged at 105,000 **g** for 1 h to obtain the cytosol. Tissue labelled *in vivo* was processed in the same manner.

To obtain a purified nuclear fraction the tissues were homogenized in 1 ml of a solution of 0·6 M sucrose, 50 mM Tris–HCl (pH 7·4), 1·5 mM EDTA. The nuclei were sedimented and washed several times in the same medium, as described in a previous report[7]. After a final wash with 0·3 per cent Triton×100 the nuclear pellet was extracted with 1·0 M KCl, 50 mM Tris–HCl (pH 8·0). Cytosol receptors were investigated by density gradient ultra-centrifugation in 5–20 per cent linear sucrose gradients prepared in 500 mM Tris–HCl buffer, pH 7·4, containing 1·5 mM EDTA. Nuclear extracts were layered over linear 3–18 per cent sucrose gradients made in 50 mM Tris–HCl buffer, pH 7·4, 1·5 mM EDTA, containing 1·0 M KCl, and centrifuged in a SW 65 rotor in a Spinco L2-65B ultra-centrifuge at 0–2°C.

Bovine serum albumin (BSA), yeast alcohol dehydrogenase (YAD) and beef heart catalase (CAT) were used as sedimentation markers. The gradients were fractionated into 30 samples. Protein was determined by the method of Lowry[8]. Radioactive aqueous samples were mixed with Bray's solution and counted. The method for the extraction of steroids from incubated tissue and the identification of metabolites formed has been described previously[9].

RESULTS

In vivo *studies*

One hour after the injection of of [^{3}H]-5α-diol substantial amounts of radioac-

tivity accumulated in the epididymis. Subcellular distribution studies showed that, of the total tissue radioactivity derived from [^{3}H]-5α-diol, 56 per cent was located within the nuclei, while the remaining 44 per cent was found mainly in the non-particulate, soluble fraction of the cytoplasm (Table 1). Extraction of the intranuclear radioactivity was facilitated by using a medium which was slightly alkaline (pH 8) with a high ionic strength (1·0 M KCl). Analysis of the cytosol fraction on sucrose gradients disclosed binding of the radioactive hormone to molecules sedimenting at approximately 8·4 S (Fig. 1A). On the other hand, in-

Table 1. Uptake, subcellular distribution and metabolism of [^{3}H]-5α-androstane-3α,17β-diol by rat epididymis

Labelling	*n**	Cytosol d.p.m./mg P†	[^{3}H] d.p.m. found as DHT (%)	Nuclear d.p.m./mg P	[^{3}H] d.p.m. found as DHT (%)
in vivo‡	3	3139±325	—	3996±417	—
in vitro (0 C)¶	6	2181±192	1788 (82)	305±66	—
in vitro (0–25 C)§	6	1173±212	1120 (95·5)	1534±273	1143 (74·5)

*n**: number of epididymis used.

†Results are expressed as d.p.m. per mg of protein ± S.E.

‡Animals castrated 24 h prior to the experiment were eviscerated and injected intravenously with 30 μ Ci of [^{3}H]-5α-androstane-3α,17β-diol.

¶Epididymides from animals castrated 1 day prior to the experiment were minced and incubated in 5 ml of Eagle's medium containing 1×10^{-6} M [^{3}H]-5α-androstane-3α,17β-diol for 2 h at 0°C.

§Similar to ‡, except the incubation was prolonged for 30 min at 25°C.

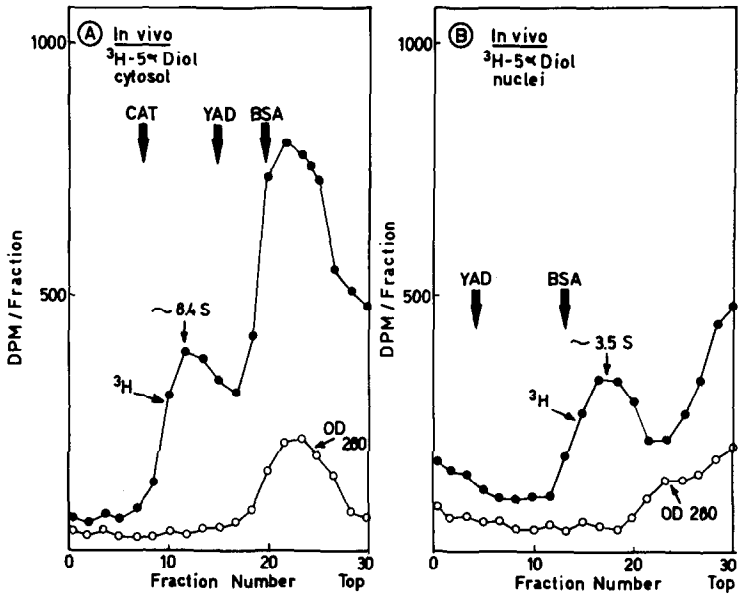

Fig. 1. *Panel* A: Sedimentation profile, in 5–20 per cent sucrose gradients, of the epididymal cytosol from rats injected 1 h previously with 30 μCi of [^{3}H]-5α-diol. Gradients were centrifuged for 14 h et 40,000 rev./min in an SW 65 rotor in a Spinco L2-65B ultracentrifuge at 0–2°C. BSA: bovine serum albumin; YAD: yeast alcohol dehydrogenase; CAT: catalase. *Panel* B: Sedimentation profile of 1·0 M KCl epididymal nuclear extracts from rats treated as in panel A. 3–18 per cent sucrose gradients containing 1·0 M KCl were centrifuged for 14 h at 60,000 rev./min in an SW 65 rotor at 0–2°C.

tranuclear radioactivity sedimented bound to material with an approximate sedimentation coefficient of 3·5 *S* (Fig. 1B).

In vitro *studies*

The cytoplasmic 8·5 *S* receptor was labelled with tritium derived from [^{3}H]-5α-diol when the minced epididymal tissue was incubated for 2 h at 0°C (Fig. 2). Further incubation at 25°C for 30 min reduced the amount of radioactive hormone bound to the receptor; however, the sedimentation coefficient remained unchanged. This receptor was not labelled when the cytosol was incubated with [^{3}H]-5α-diol.

The specificity of the receptor was assessed by competition studies in which the minced tissue was incubated with [^{3}H]-5α-diol 1046 ($0{\cdot}6 \times 10^{-9}$) in the presence of non-radioactive DHT. With equimolar amounts, DHT caused a 50 per cent reduction in the amount of [^{3}H]-5α-diol bound to the 8·4 *S* peak, while at 6×10^{-9} M DHT, no radioactivity could be detected in the receptor zone (Fig. 2). On the contrary, with [^{3}H]-DHT (1×10^{-10} M) the presence of 5α-diol (1×10^{-9} M) did not effect a significant decrease in the magnitude of the radioactive peak. No competition of DHT with [^{3}H]-5α-diol was demonstrated when the competitor was added after 2 h of incubation at 0°C.

Differences in affinity were further tested by incubating the epididymal minces with [^{3}H]-5α-diol (1×10^{-9} M = 200,000 c.p.m.). The addition of a similar amount of

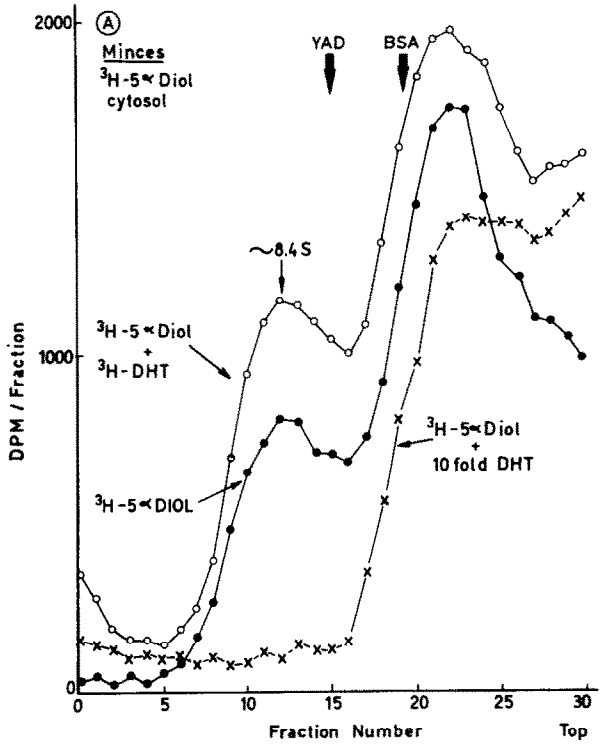

Fig. 2. *In vitro* labelling of the epididymal cytoplasmic receptor by incubation of minced tissue with [^{3}H]-5α-diol or [^{3}H]-DHT (1×10^{-9} M) for 2 h at 0°C. When indicated, DHT (1×10^{-8} M) was added at the beginning of the incubation. Centrifugation and sedimentation markers as in Fig. 1, panel A.

[^{3}H]-DHT effected an increase of 57 per cent in the amount of radioactivity bound to the receptor (Fig. 2) as compared with the [^{3}H]-5α-diol incubated controls.

Significant amounts of radioactivity entered the nucleus when the time of incubation of the minced tissue of 2 h at 0°C was extended for another 30 min at 25°C, indicating that the process of transfer of the hormone is temperature-dependent. In analysis by density gradient centrifugation, the intranuclear radioactivity was found partly bound to molecules with a sedimentation coefficient of approximately 3·5 *S*, while some remained on the top of the tube (free steroid) (Fig. 3).

This pattern of sedimentation was altered when a hypotonic medium (50 mM Tris–HCl) was used for incubation of the minced tissue. In these cases the radioactivity was found distributed in two well-defined peaks with sedimentation coefficients of 6·0 and 3·5 *S* (Fig. 3). The addition of a 100-fold excess of DHT to the medium after incubating for 2 h at 0°C and before heating at 25°C did not influence the size of the radioactive 3·5 *S* peak. The incubation of isolated nuclei with [^{3}H]-5α-diol-prelabelled cytosol obtained from the minces failed to produce labelled 3·5 *S* receptor.

The metabolism of [^{3}H]-5α-diol by epididymal tissue, under the conditions used for labelling of the receptors, was investigated. The main steroid found in the cytosol fraction of minces incubated for 2 h at 0°C was DHT, which accounted for

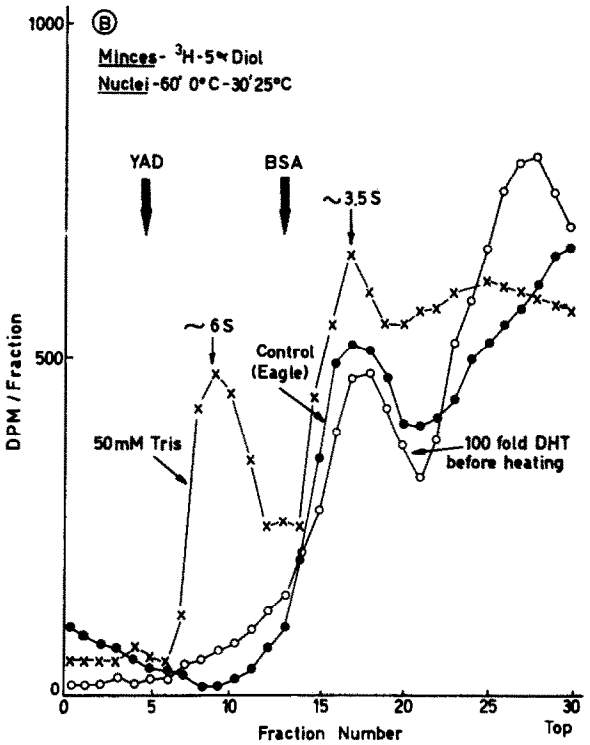

Fig. 3. *In vitro* labelling of the epididymal nuclear receptor by incubation of minced tissue with [^{3}H]-5α-diol (1×10^{-9} M) for 2 h at 0°C followed by 30 min at 25°C. Incubation media was Eagle's tissue culture medium (●—●) or 50 mM Tris-HCl pH 7·4, containing 1·5 mM EDTA (x--x). When indicated, non-radioactive DHT (1×10^{-7} M) was added after the completion of the 2 h at 0°C incubation period (0--0). Centrifugation and sedimentation markers as in Fig. 1, panel B.

about 82 per cent of the total radioactivity. When the incubation at 0°C was followed by 30 min at 25°C the proportion of DHT formed was augmented to 95·5 per cent. The intranuclear radioactivity of the latter fraction consisted of 74·5 per cent of DHT (Table 1).

DISCUSSION

The preferential intranuclear concentration of 5α-diol, both *in vivo* and *in vitro*, and the binding of this hormone to the cytoplasmic and nuclear receptors for androgens in rat epididymis has been demonstrated.

The binding appeared to take place with the same moieties previously described as cytoplasmic[6] and nuclear[7] receptors for androgens in rat epididymis. The formation of the 5α-diol-8·4 *S* complex is dependent upon cellular integrity since the cell-free fractions containing the cytoplasmic receptor which binds DHT and testosterone under the conditions described[6] failed to interact with 5α-diol. Apparently, the affinity of cytoplasmic receptor for DHT is several times higher than that for 5α-diol, as demonstrated by the competition experiments. Nevertheless, the formed 8·4 *S* receptor–5α-diol complex is not readily dissociated upon the addition of DHT.

The possibility that the transfer of hormone from the cytoplasm to the nucleus may occur as a complex bound to the receptor was investigated by the addition of a competitor steroid, after the labelling of the cytoplasmic receptor had been achieved at 0°C, but before the heating step which would allow entrance of the hormone into the nucleus. This procedure should cause a significant isotope dilution, should the hormone be detached from the binding site of the cytoplasmic receptor before being transferred to the homologous site of the nuclear receptor. However, the present finding that the amount of radioactivity of the nuclear receptor was the same as the control value suggests strongly that the transfer of the hormone from cytoplasm to nuclei might have taken place as a complex bound to a modified receptor.

The present finding that radioactivity bound to the cytoplasmic receptor was increased by 57 per cent when incubated simultaneously with [^{3}H]-5α-diol and [^{3}H]-DHT, as compared to the control sample incubated with [^{3}H]-5α-diol, most probably indicates that the binding of DHT to sites takes place with greater ease, while under the same conditions, the binding with 5α-diol is hindered.

We cannot conclude from present experimental data that different binding sites for DHT and 5α-diol exist. The analysis of [^{3}H]-5α-diol derived radioactivity in cytosol and nuclei disclosed that DHT was the major steroid species present in both fractions. The free interconversion of these two compounds in the rat prostate has been demonstrated by Bruchovsky[10], both *in vivo* and *in vitro*.

In previous publications[7, 11] we reported that labelling of the epididymal nuclear receptor on incubation of the tissue with [^{3}H]-DHT did not occur, in spite of the numerous different conditions which were studied. Nevertheless, the receptor was readily labelled by incubation with [^{3}H]-T[7], or, as in the present report, with [^{3}H]-5α-diol. These two steroids are effectively transformed to DHT by epididymal tissue under conditions employed for labelling the nuclear receptor, and DHT is the main compound bound to the receptor after incubation with [^{3}H]-T[11]. The involvement of the *in situ* conversion of the steroid bound to the 8·4 *S* receptor to form the receptor–DHT complex in the mechanism of passage of the complex to the nucleus is the subject of continuing studies. At present we are

unable to explain the appearance of two binding moieties in the nuclei of epididymal tissue incubated in hypotonic media. The existence of the two moieties should not be due to an aggregation of subunits or receptors to yield the 6·0 S form, since both the extracting solution and sucrose gradients contained 1·0 M KCl.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. S. S. Koide for revising the manuscript.
The skillful technical assistance of Mrs. Elsa Miller is gratefully acknowledged.

REFERENCES

1. Dorfman R. I.: In *Methods in Hormone Research* (Edited by R. I. Dorfman), Vol. 5, p. 235. Academic Press, New York (1966).
2. Nelson W. O. and Merckel C. E.: *Proc. Soc. exp. Biol. Med.* **38** (1938) 737.
3. Harper M. B., Pierrepoint C. G., Fahmy A. R. and Griffiths K.: *Biochem. J.* **119** (1970).
4. Rivarola M. A., Podesta E. J. and Chemes H. E.: *Endocrinology* **91** (1972) 537.
5. Voglmayr J. K., Waites G. M. and Setchell B. P.: *Nature (Lond.)* **210** (1966) 861.
6. Blaquier J. A.: *Biochem. biophys. Res. Commun.* **45** (1971) 1076.
7. Calandra R. S. and Blaquier J. A.: *Acta physiol. Latinoam.* **22** (1972) 116.
8. Lowry O., Rosebrough M., Lewis-Farr A. and Randall R.: *J. biol. Chem.* **193** (1951) 265.
9. Blaquier J. A., Cameo M. S. and Charreau E. H.: *J. steroid Biochem.* **1** (1970) 327.
10. Bruchovsky N.: *Endocrinology* **89** (1971) 1212.
11. Blaquier J. A. and Calandra R. S.: *Endocrinology* **93** (1973) (in press).