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BINDING AND METABOLISM OF [^{3}H]-TESTOSTERONE IN THE NUCLEI OF RAT PITUITARY *IN VIVO*

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SUMMARY

Bound radioactivity in the nuclei of the rat anterior hypophysis reached a maximum 60 min after i.p. injection of (^{3}H)-testosterone. At this time, the level of nuclear radioactivity was 7 times greater than that of the cytoplasmic fraction. The nuclear radioactivity consisted of bound (61%) and unbound radioactivity. In the nuclei of the pituitary gland, [^{3}H]-testosterone represents 63% of the total radioactivity and dihydrotestosterone 21%.

INTRODUCTION

RECENT investigations from this laboratory indicated that testosterone and dihydrotestosterone† were bound to nuclear chromatin of rat anterior hypophysis [1]. These results were achieved by incubation of slices of hypophyseal tissue and by isolation of purified nuclei [1].

The experiments described in this paper were carried out to determine the uptake of testosterone by the anterior hypophysis and to compare it with that of cerebral tissue, muscle and serum, following the administration of (^{3}H)-testosterone *in vivo*. At the same time, the binding of testosterone to macromolecules in the nuclei of cells of rat anterior hypophysis as well as its metabolites has been studied.

MATERIAL AND METHODS

[1,2-^{3}H]-*Testosterone*, with a specific activity of 46 Ci per millimole was supplied by CEN (Belgium). It was checked for purity by paper chromatography.

The original solution (benzene–ethanol, 90:10 v/v) was evaporated to dryness under vacuum and the dry residue was dissolved in ethanol. This solution was then diluted with 0·9% NaCl solution and injected i.p. in a volume of 0·2 ml.

Biological material. Male Wistar rats, 80 days old were used. They were killed by decapitation without anaesthesia. When the uptake of injected steroids was studied, 3 rats were used for each experiment, at different time periods. Each animal received intra-peritonally 31 μCi (0·2 μg) of [1,2-^{3}H]-testosterone. They were killed 5, 30, 60, 90 and 120 min after injection.

For the study of the intranuclear binding of the hormone, 24 rats were used

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†The following abbreviations and trivial names were used: testosterone (17β-hydroxy-4-androsten-3-one); 5α-dihydrotestosterone, DHT, (17β-hydroxy-5α-androstan-3-one); androstenedione (4-androstene-3,17-dione); 5α-androstane-dione (5α-androstane-3,17-dione); androstanediol (5α-androstane-3,17β-diol); androsterone (3α-hydroxy-5α-androstan-17-one).

for each experiment. They were given the same amount of androgen as previously indicated and were killed 1 h later.

For the characterization of nuclear androgens 48 rats were needed. They were sacrificed 1 h after i.p. administration of labelled testosterone (0·2 μg).

Tissue fractionation. All procedures were performed at 4°C. In the study of the uptake of radioactivity, anterior hypophysis, hypothalamus (anterior, middle and posterior), cerebral cortex (50 mg) and muscle (diaphragm) (60 mg) were homogenized with a Potter–Elvehjem homogenizer in 2 ml of a mixture of ethanol–ether (v/v). Each homogenate was repeatedly extracted with the same mixture. The extracts were filtered through paper into scintillation vials and evaporated under vacuum. 0·2 ml of Nuclear Chicago Solubilizer (NCS) was added to each vial and after complete solubilization, 10 ml of scintillation phosphor (PPO 5 g, dimethyl POPOP 0·1 g, Toluene 1000 ml).

Radioactivity in the serum was measured by addition of 0·3 ml water and 0·2 ml of NCS to 0·1 ml of serum. After heating at 45°C until complete dissolution, 15 ml of Bray's mixture were added [2].

Sub-cellular fractionation. For sub-cellular fractionation, 24 anterior hypophysis were homogenized with an all-glass homogenizer (Dounce homogenizer. Ets. Block. 57. Strasbourg) in 0·32 M sucrose solution containing 3 mM $MgCl_2$ (25% w/v homogenate). The homogenate was diluted with an equal volume of the same buffer and the sucrose concentration was lowered to 0·25 M by addition of twice-distilled water. The homogenate was layered over a 0·32 M sucrose solution (5 ml homogenate and 3 ml 0·32 M sucrose) and centrifuged at 600 g_{av}. The 600 g_{av} supernatant was centrifuged at 39,000 g_{av} and the purified nuclei were obtained from the 600 g_{av} pellet by the procedure described previously [1]. The purified nuclear pellet was suspended in 3 ml of 1 M NaCl and submitted to magnetic stirring during 2 h in a cold room. Then, it was centrifuged at 2000 g_{av} for 20 minutes and the supernatant was analyzed by gel filtration.

Gel filtration procedure. Columns 1·5 cm × 30 cm, 16 ml void volume were prepared with Sephadex G-25 (fine). For the analysis of the 39,000 g_{av} supernatant, the gel was equilibrated with 0·05 M phosphate buffer, pH 7·4. A portion of the 39,000 g_{av} supernatant was diluted with an equal volume of 0·05 M phosphate buffer and applied to the column. Elution was carried out with the same buffer and 2 ml fractions were collected.

The bound radioactivity in the nuclei of anterior pituitary glands was detected by gel filtration on Sephadex G-25. The column (1·5 × 30 cm) was poured with gel equilibrated in 1 M NaCl. The nuclear extract in 1 M NaCl was applied to the column and elution was carried out with the same solution. Fractions of 2 ml were collected.

Gel filtrations were carried out at 7°C, and columns were tested with Dextran blue, bovine serum–albumin and [^{3}H]-testosterone. The radioactivity of the fractions obtained after gel filtration was counted using a toluene–triton mixture (PPO 5·5 g, dimethyl POPOP 0·1 g, Triton X-100 333 ml, toluene 667 ml).

Recovery and identification of steroids. After gel filtration of the 39,000 g_{av} supernatant, the fractions containing the radioactivity bound to the molecules excluded by the Sephadex G-25 (bound steroids) were extracted by the method of Folch *et al.* [3] modified by Bruchovsky and Wilson [4]. The fractions containing radioactivity retained by the column (free steroids) were extracted with ether.

Androgens in the purified nuclei of the anterior hypophysis were obtained by

direct extraction of nuclei with chloroform–methanol (2:1, v/v) in an all glass Potter homogenizer. After centrifugation, the residual pellet was extracted twice with the same solvents. The organic phases were pooled and evaporated under vacuum.

Androgens were chromatographed on Whatman No. 1 paper cut into 2 cm wide strips, in the solvent system of Kochakian and Stidworthy [5]. After a development for 4–5 h at 30°C, the strips of chromatographic paper were divided into 2 cm × 1 cm segments and these were placed in counting vials. The radioactivity of chromatograms was measured as previously described [1].

The identity of steroïds was confirmed by paper chromatography in different solvent systems. The following solvent systems were used: light petroleum: methanol: water; (100:80:20 by vol): (6), benzene, heptane, methanol, water (33·3:66·7:80:20 by vol) (7), and hexane, methanol, water; (10:9:1 v/v) (7).

Analytical procedures. The method of Lowry *et al.* [8] was used for protein determination with bovine serum albumin as standard (bovine albumin, Fr. V. National Biochemicals Corporation. Cleveland-Ohio).

RESULTS

Uptake of radioactivity by anterior hypophysis, brain tissues, muscle and serum after administration of [^{3}H]*-testosterone*

It appeared (Fig. 1) that radioactivity penetrated quickly into these tissues and that it reached a maximum level 5 min after i.p. administration. There was a rapid decrease in radioactivity between 5 and 30 min (by 80% in the anterior hypophysis, 40% in the hypothalamus, 55% in the cerebal tissue and 21% in the serum). Between 30 and 120 min after i.p. injection, radioactivity in the anterior hypophysis reached a plateau. In contrast during the same time period it decreased by 77% in the hypothalamus, by 50% in the brain and by 66% in the serum.

Uptake of androgen by hypophyseal nuclear fraction. The analysis of the 39,000 g_{av} supernatant carried out by gel filtration showed two peaks of radioactivity. The first one contained radioactivity associated with macromolecules excluded from the gel and the second corresponded to the free radioactivity.

The specific activity (d.p.m./mg protein) in the 39,000 g_{av} supernatant declined promptly from 5–120 min after i.p. injection (Fig. 2). On the other hand the specific activity of the bound fraction decreased more slowly.

The uptake of radioactivity by the pituitary nuclei differed significantly from that of the 39,000 g_{av} supernatant. The level of radioactivity extracted by 1 M NaCl rose gradually from 5–60 min after hormone administration (Fig. 2). Maximum uptake was achieved 60 min after injection at which time the specific activity of the 1 M NaCl nuclear extract was higher than that of the bound radioactivity in the 39,000 g_{av} supernatant (Fig. 2).

When the 1 M NaCl nuclear extract was submitted to gel filtration on Sephadex G-25, two peaks of radioactivity were obtained (Fig. 3). The first represented the bound radioactivity and the second, the free radioactivity. One hour after i.p. injection of [^{3}H]-testosterone the bound radioactivity represented 61% of the total radioactivity extracted by 1 M NaCl. The specific activity of the bound fraction was 460 d.p.m./mg protein and the androgen concentration was 4×10^{-15} moles/mg protein. 1 M NaCl extracted 73% of the nuclear radioactivity.

[^{3}H]*-testosterone metabolism studies.* Because of the small amount of purified nuclei obtained, it was difficult to study the identity of the bound androgen. Bound

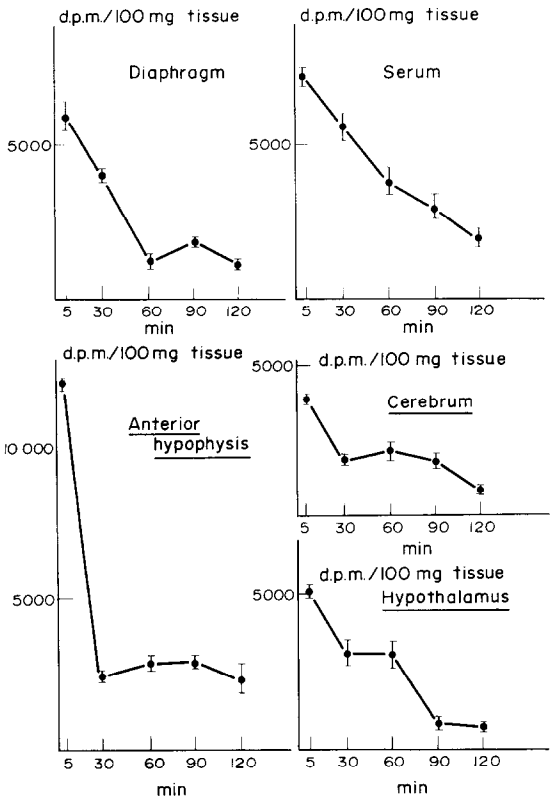

Fig. 1. [^{3}H]-testosterone uptake in anterior hypophysis, hypothalamus, cerebral cortex and muscle of diaphragm of intact male rats. Each rat was injected with [^{3}H]-testosterone (0·2 μg) and killed 5–120 min after i.p. injection. Three rats were used in each experiment. Tissular radioactivity was extracted with a mixture of chloroform–diethyl ether (1 : 1 v/v). The extract was dried in a scintillation vial, digested with N.C.S. and tritium was measured in a toluene scintillation fluid. Results are expressed as d.p.m. per 100 mg tissue. Vertical bar indicates ± S.E.M.

and unbound nuclear steroïds were therefore extracted from nuclei by a $CHCl_3$–CH_3OH mixture (2 : 1, v/v) and studied by paper chromatography in the solvent system of Kochakian and Stidworthy [5]. The results are shown in Fig. 4. The major radioactive fraction was found to be testosterone. It represented 63% of the total radioactivity recovered from the chromatogram. The amount of DHT represented 21% of the total radioactivity. Only traces of the other androgens (polar and non polar) were detected.

These results were different from those obtained after analysis of the radioactivity in 39,000 g_{av} supernatant (Fig. 4). The bound fraction of this sub-cellular fraction was mainly testosterone which represented 63% of the total radioactivity. The percentage of DHT was only 8% and polar steroïds, including androstanediols represented 10% of the total radioactivity recovered from the chromatogram. A radioactive peak appeared on the chromatogram with the same R_F as non-radioactive androsterone used as standard. It constituted 10% of radioactivity. Nevertheless, its identity has not been firmly established. The free steroïds extracted from 39,000 g_{av} supernatant were testosterone, androstenedione, DHT

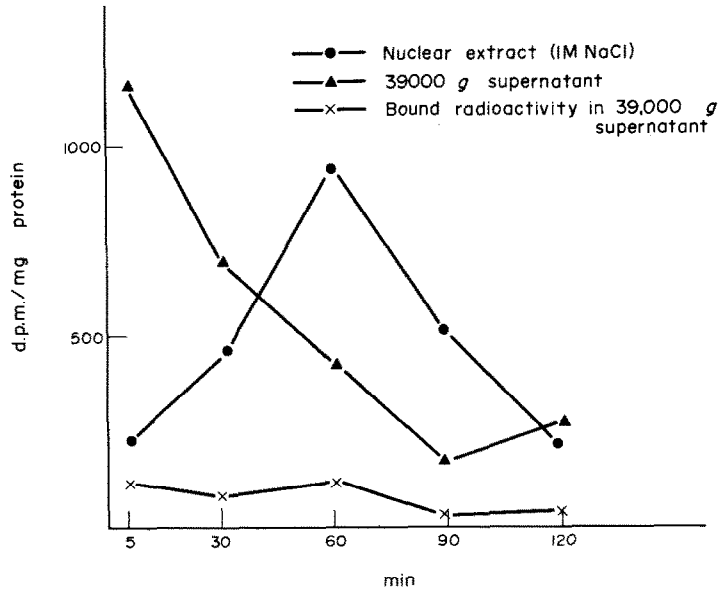

Fig. 2. Specific activity (d.p.m./mg protein) of sub-cellular fractions of the rat anterior hypophysis after i.p. injection of [^{3}H]-testosterone. —▲—▲— = Radioactivity in 39,000 g_{av} supernatant (Bound + free), —×—×— = Radioactivity bound in the 39,000 g_{av} supernatant, –●–●– = Radioactivity in 1 M NaCl nuclear extract. Each point is a mean value of three experiments with three rats each.

and androstanediol. The percentages of the radioactivity for each of these androgens were 35, 26, 7 and 8% respectively (Fig. 4). The percentages indicated in the experiments represented the means of three determinations.

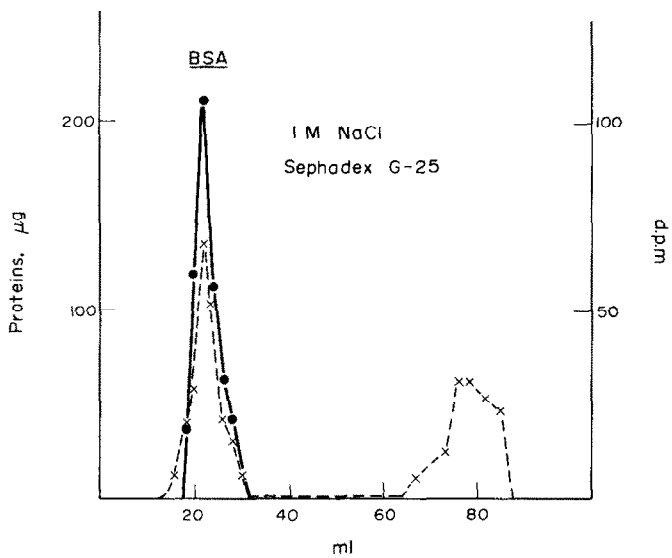

Fig. 3. Separation of nuclear radioactivity into bound and free fractions on Sephadex G-25 column. Solid line = Elution of the nuclear proteins extracted by 1 M NaCl. Dotted lines = Elution of radioactivity. B.S.A. = Elution of bovine serum-albumin.

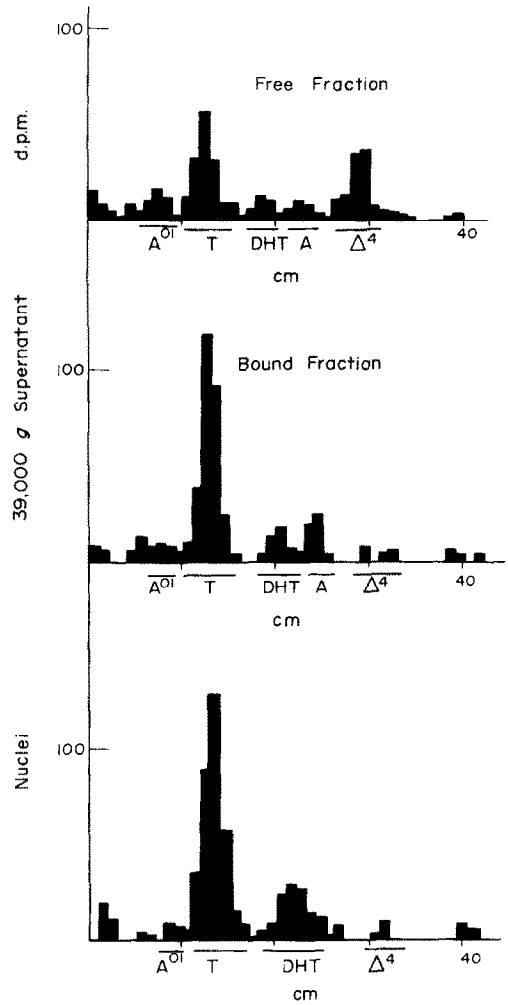

Fig. 4. Paper chromatography of radioactivity extracted from cytoplasmic and nuclear fractions of rat anterior hypophysis after i.p. injection of [^{3}H]-testosterone. Aol = 5α-androstanediol, T = Testosterone, DHT = dihydrotestosterone, Δ^4 = androstenedione, A = Androsterone (Horizontal bars indicate the region of chromatogram where standard steroïds were located).

DISCUSSION

These experiments showed a rapid uptake of radioactivity by the anterior pituitary gland of normal rats after i.p. injection of [^{3}H]-testosterone. The concentration of the radioactivity in the anterior hypophysis 5 min after i.p. injection was 2–3 times greater than in the hypothalamus, the cerebral cortex and the muscle of the diaphragm. Five min after testosterone administration, the anterior pituitary gland contained more radioactivity than serum. There was a preferential uptake of the radioactivity by this gland.

These results are similar to the findings of Roy and Laumas [10]. Using the technique of constant infusion of [^{3}H]-testosterone they have found a high uptake of this hormone in the pituitary gland of intact and castrated male rats. Also, Stern and Eisenfeld [11] have demonstrated a higher uptake of [^{3}H]-testosterone

in the pituitary gland than in the hypothalamus and the brain of castrated male rats. Similar results concerning androgen uptake were reported by Resko *et al.* [12] in the pituitary gland of castrated rats and by Whalen *et al.* [13].

The level of radioactivity decreased quickly in all the tissues studied. Nevertheless, the anterior hypophysis retained the radioactivity between 30 and 120 min after the [^{3}H]-testosterone injection. During this period, the radioactivity level was constant, showing that this gland has the ability to retain testosterone and its metabolites.

The results described here and elsewhere [9, 14] are similar to reports of accumulation of [^{3}H]-oestradiol in the anterior hypophysis and in the hypothalamus of normal or castrated female rats [15–20].

The uptake of radioactivity by the nuclei of anterior hypophysis reached a maximum by 60 min after the administration of [^{3}H]-testosterone, whereas the bound radioactivity decreases in the 39,000 g_{av} supernatant. The amount of nuclear radioactivity was 7 times greater than that of bound cytoplasmic radioactivity. This is probably related to the presence of a "receptor" in these nuclei. We have previously reported the formation of macromolecular associations *in vitro* in purified nuclear fractions of pituitary gland [1]. We are now studying the specificity of these associations.

Thus, after i.p. injection of [^{3}H]-testosterone, the nuclei of the pituitary gland takes up and retains radioactivity. Recent observations described by Sar and Stumpf, after autoradiographic studies showed a nuclear concentration of radioactivity in the male rat pituitary 1 h after [^{3}H]-testosterone injection [21, 22]. These authors observed that thyrotrophs, acidophils and chromophobes did not concentrate and retain radioactivity but that radioactivity is accumulated in certain cell nuclei which were identified as basophils. These interesting results demonstrated that [^{3}H]-testosterone is confined to gonadotrophs and suggested that these cells are target cells for androgens.

We have found that the major fraction of nuclear radioactivity (61%) was bound to macromolecules. It was surprising to detect small amounts of unbound radioactivity in the pituitary nuclei, however, the preparation of the purified nuclei requires a considerable period of time and it is possible that a slight dissociation of the bound fraction occurred during this period.

The metabolism of [^{3}H]-testosterone in the rat anterior hypophysis observed in these *in vivo* experiments confirmed our previous results obtained after incubation of slices and preparation of purified nuclei of pituitary gland [1, 14, 23]. In the cytoplasmic fractions, the main metabolites were androstanediol, dihydrotestosterone and androstenedione. Thus the presence of 3-hydroxy-dehydrogenase and 5α-reductase was again demonstrated. These findings confirm those of Jaffe [24], Massa *et al.* [25], Stern and Eisenfeld [11], Perez-Palacios *et al.* [26] and Rommerts and Van Der Molen [27].

One hour after testosterone injection, the androgens recovered in the nuclei were testosterone and dihydrotestosterone. It was found that the amount of DHT was higher *in vivo* (21%) than *in vitro* (7%). This dihydrotestosterone concentration in the nuclei, one hour after testosterone administration, raises again the problem of the 5α-reductase localization. The subcellular localization of testosterone 5α-reductase in rat ventral prostate has been investigated by Frederiksen and Wilson [28], Shimazaki *et al.* [29], Moore and Wilson [30]. It was shown that about half of the activity of the 5α-reductase was attached to the nuclear

fraction and the remaining activity was located in the microsomal fraction. On the other hand, Rommerts and Van Der Molen [27] concluded that the reductase activity, in the pituitary gland, was predominantly microsomal bound and that the reductase activity in the nuclear fraction could be explained by a microsomal contamination or a very loosely nuclear bound 5α-reductase activity. It would be important to demonstrate clearly either an intranuclear localization of the 5α-reductase activity, or a preferential concentration of DHT by the nuclei of the rat pituitary gland.

ADDENDUM

The identification of [^{3}H]-dihydrotestosterone as a conversion product of [^{3}H]-testosterone was confirmed as follows: The radio-active biological material was chromatographed in the system benzene:cyclohexane (1:1 v/v) propanediol and the zone with the same migration as authentic dihydrotestosterone was eluted. The eluate was divided into three parts. The first was subjected to a second chromatography in the system Isooctane:methanol:water (150:135:15 by vol). The second aliquot was acetylated (acetic:anhydride:pyridine 1/2 by vol) and chromatographed in the system isooctane:phenylcellosolve. The acetylated product migrated in a manner similar to that of authentic dihydrotestosterone acetate. The third aliquot was mixed with 13 mg of authentic dihydrotestosterone and crystallised successively in the following solvents: I methanol, II benzene isooctane. The data of recrystallisation are indicated in Table 1.

Table 1

	S. A. D.P.M./mg	
	Mother Liquors	Crystals
After addition of carrier		883
I Methanol	847	831
II Benezene – Isooctane	833	866

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