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COMPARATIVE UPTAKE OF ANDROSTENEDIOL, TESTOSTERONE AND DIHYDROTESTOSTERONE BY TISSUES OF THE MALE RAT*

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SUMMARY

Tritiated androstenediol (5-androstene-3β,17β-diol), testosterone and dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) were injected intravenously to castrated rats. The tissue radioactivity, expressed as the ratio organ/skeletal muscle, was highest in prostate, hypophysis, epididymides and seminal vesicles. Nuclei from these organs contained significantly more radioactivity than cytoplasm.

Equilibrium dialysis experiments with epididymides and androstenediol demonstrated a high affinity binding by caput (41%) and body (30%) while cauda did not bind.

In an intact cell system epididymal nuclei concentrated more radioactivity than cytoplasm. Isolated nuclei were unable to take up steroid. In equilibrium dialysis of nuclei the addition of cytoplasmatic protein to the outside solution increased the nuclear uptake by 22%.

The radioactivity was more firmly bound by nuclei than by cytoplasm. A nitrogen atmosphere, or the addition of 2, 4-dinitrophenol, reduced the *in vitro* nuclear incorporation of androstenediol and testosterone. This blocking effect could not be reversed by the addition of ATP or an ATP generating system to homogenates.

Preliminary evidence indicates that in an intact cell system, androstenediol was transformed to compounds with chromatographic mobilities similar to those of testosterone and dihydrotestosterone.

The role of cytoplasm, in our conditions, could be one of providing energy for the nuclear uptake or for the transformation of androstenediol to compounds suitable for binding with nuclear receptors.

INTRODUCTION

THE SELECTIVE uptake of androgens by accessory sexual organs of the rat has been shown by several authors[1–5]. Most of the reports deal with the concentration of testosterone by seminal vesicles, anterior hypophysis and prostatic tissue[6–8].

In the present paper we have studied the *in vivo* uptake and intracellular distribution of three biologically active androgens: androstenediol‡, testosterone and dihydrotestosterone‡, which are biosynthetic precursors of each other.

Some aspects of the *in vitro* binding of androstenediol by epididymal cell fractions have also been studied.

MATERIAL AND METHODS

Male albino rats weighing 150–200 g were castrated by the scrotal route 3 days prior to the experiments.

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‡Trivial names used: androstenediol: 5-androstene-3β,17β-diol; dihydrotestosterone: 17β-hydroxy-5α-androstan-3-one.

Radioactive steroids. [1-2 ^{3}H] testosterone, S.A. 50 Ci/mM, was purchased from New England Nuclear Co. and purified by thin layer chromatography. [1-2 ^{3}H] dihydrotestosterone was prepared by incubating [1-2 ^{3}H] testosterone (100 uc) with female rat liver microsomes (2·5 mg protein) for 7·5 min at 37°C in the presence of NADPH(1·5 mg), in a total volume of 2 ml of 0·067 M phosphate buffer, pH 7·4, containing 0·25 M sucrose. The same buffer was used for the isolation of microsomes by differential centrifugation. The incubation was stopped by extraction with ethyl acetate (3 × 10 ml). The pooled extracts were washed with water, evaporated to dryness and chromatographed on silica gel thin layer plates in the system chloroform : ether (4 : 1). The radioactive area behaving like authentic dihydrotestosterone was eluted and rechromatographed in the Bush A system to separate androsterone. A sample of [1-2 ^{3}H] dihydrotestosterone thus obtained was crystallized with pure carrier dihydrotestosterone and constant specific activity was obtained.

[7α ^{3}H]androstenediol was prepared by the sodium borohydride reduction of [7α ^{3}H]dehydroepiandrosterone, S.A. 12·9 Ci/mM, as previously described [9]. The radioactive steroids were stored at −15°C in a solution containing 10% methanol in benzene.

In vivo *experiments:* 4·7 μCi of the desired androgen were dissolved in 0·3 ml of isotonic saline and injected via the jugular vein under light ether anesthesia to each of a group of six animals.

One hour after the injection the animals were killed and the selected organs were quickly dissected, weighed, minced and subsequently homogenized in 0·067 M phosphate buffer, pH 7·4, containing 0·25 M sucrose, using an all glass Potter homogenizer. The homogenates were centrifuged at 700 **g** for 15 min at 4°C. The crude nuclear pellet was resuspended in homogenizing medium and recentrifuged at the same speed. The nuclei thus obtained were taken to a predetermined volume and sonically disrupted, at 4°C. Aliquots of both nuclear and cytoplasmatic fractions were withdrawn for protein determination. The remainder was extracted 3 times with 3 vol. of a mixture of ether : chloroform (3 : 1 v/v). The combined extracts were washed with water and evaporated to dryness in counting vials.

In vitro *experiments*: Epididymides were removed and alternatively sliced with a Steady–Riggs microtome or homogenized in 0·067 M phosphate buffer, pH 7·4. Slices were incubated in buffer containing [1-2 ^{3}H]androstenediol, 0·01μCi, and 20% (v/v) rat plasma, for 3 hr, at 37°C. The incubated slices were homogenized and the crude nuclear and cytoplasmatic fractions were obtained as described. These fractions were dialyzed in cellophane Visking tube (1 cm) for 6 hr at 37°C against buffer containing sufficient amounts of rat plasma to obtain equal protein concentration inside and outside the bag.

When required, 2,4 dinitrophenol (DNP) was added to the incubation mixture at a concentration of 2·5μM/ml. Some incubations were performed in glass stoppered Erlenmeyer flasks in which nitrogen was bubbled for 10 min before starting the incubation. In some instances, the extracts from slice incubations were chromatographed on thin layer silica plates in the system chloroform : ether (4 : 1) along with carrier testosterone and dihydrotestosterone. The radioactive areas corresponding to the carriers were eluted and rechromatographed on thin layer in the system chloroform : methanol (97 : 3 v/v).

The percentage binding of androstenediol by whole homogenates or sub-

cellular fractions was obtained through equilibrium dialysis, 16 hr at 37°C, against buffer containing the labelled steroid and sufficient rat plasma to equal the protein concentration inside the bag. Results were calculated according to Slaunwhite[10]. Protein was determined following the method of Lowry[11] using crystalline bovine albumin as standard. Radioactivity was measured in a Packard model 3320 liquid scintillation counter, using 10 ml of toluene based phosphor solution per vial.

RESULTS

In vivo *uptake and subcellular distribution*

The uptake of tritiated androgens by the subcellular fractions of different organs of the male rat, one hour after the intravenous injection of the compounds, is shown in Table 1. The results are expressed as the ratio of cpm per mg protein of the organ/cpm per mg protein of skeletal muscle, since this form of expression affords a better indication of the concentrating ability of the tissues[3].

Prostate, seminal vesicles, epididymides and anterior hypophysis were the organs in which the radioactivity concentrated most. In every case, the relative amount of tritium present in the nuclear fraction of these tissues was several fold higher than that of cytoplasm.

The liver contained a substantial amount of radioactivity. This retention of androgens has been shown by other authors, but the analysis of the compounds disclosed the presence of conjugated polar metabolites[12].

A striking feature was the very high nuclear prostate to muscle ratio for testosterone: 72·38. Recently Tveter[14] reported a ratio of 46 for whole tissue after the subcutaneous administration of [^{3}H]testosterone (S.A.: 46·5 Ci/mM) to castrated rats. Greer[3], Pearlman[2] and Harding[1] reported ratios ranging from 1·9 to 2·94. However, there are some technical dissimilarities which may account for the discrepancies. The specific activity of the compounds used by these authors (0·003 to 1·09 Ci/mM) was much lower than that used in the present study (50 Ci/mM), resulting in a wide difference in the actual mass of administered steroid. There were also differences in the injection technique, and intact animals

Table 1. *In vivo* uptake and subcellular distribution of radioactivity following the intravenous injection of tritiated androstenediol, testosterone and dihydrotestosterone. Results are expressed as the ratio of cpm per mg protein of the organ/cpm per mg protein in skeletal muscle

	Androstenediol		Testosterone		Dihydrotestosterone	
Organ	Nuclei	Cytopl.*	Nuclei	Cytopl.	Nuclei	Cytopl.
Adrenal	9·19	4·11	5·60	3·81	6·38	5·33
Prostate	7·99	2·04	72·38	9·19	22·29	14·68
Seminal vesicles	1·84	1·12	15·97	2·29	14·46	5·57
Levator ani	1·06	1·22	1·52	2·26	1·32	3·25
Submaxillary	3·54	0·63	7·08	2·00	9·60	6·28
Epididymis	3·75	2·06	18·20	7·77	13·13	7·38
Ant.hypophysis	13·92	2·71	18·24	2·03	21·58	6·49
Kidney	2·60	2·96	2·54	2·11	1·44	2·05
Liver	15·56	4·85	28·24	7·48	31·66	15·71
Plasma (cpm/mg protein)	8·15		8·61		3·97	

*Cytopl.: cytoplasm.

were used in some experiments. In addition, results were expressed in cpm/g of tissue instead of cpm/mg protein as in our case.

In vitro *experiments*

The percentage binding of [^{3}H]androstenediol by homogenates of the three anatomical regions of the epididymis, obtained by equilibrium dialysis, is shown in Fig. 1. Caput and body bound an average 41% and 30% respectively, while cauda did not show any binding. Abundant spermatozoa were present in these homogenates.

Isolated nuclei and cytoplasmatic fractions of epididymides gave a percentage binding of 2% and 17% respectively.

When the equilibrium dialysis of isolated nuclei was repeated, changing the source of external protein for epididymal cytoplasm, the percentage binding rose to 22% (Fig. 2).

Addition of 6 mM ATP to the outside solution of buffer and rat plasma did not result in any increase of the binding of androstenediol by nuclei. Incubation

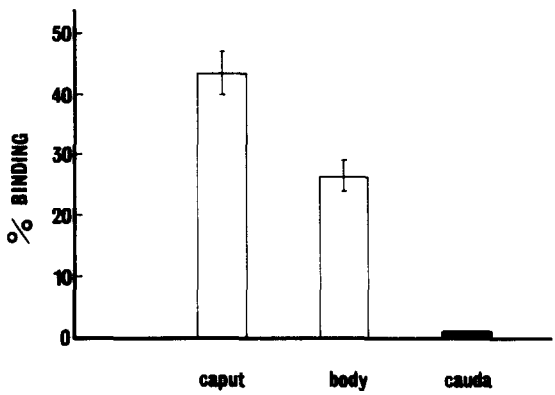

Fig. 1. Percentage binding of androstenediol by homogenates of the three anatomical regions of the epididymis.

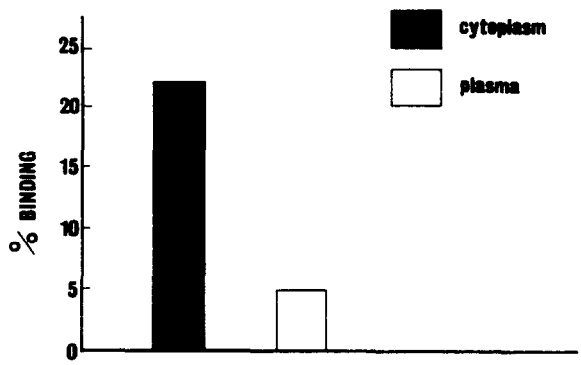

Fig. 2. Percentage binding of androstenediol by isolated epididymal nuclei obtained by equilibrium dialysis. Alternatively, epididymal cytoplasm or rat plasma were added to the dialysing fluid in sufficient amounts to equal the protein concentration inside the dialysing sack.

of slices with labelled androstenediol for 3 and 20 hr, with subsequent homogenization and fractionation, demonstrated that nuclei bound slightly more radioactivity than cytoplasm. Nuclei at 3 and 20 hr contained 915 and 465 cpm/mg protein respectively, while in cytoplasm 836 and 440 cpm/mg protein were found. The decreased amount of radioactivity present after 20 hr is thought to be due to protein denaturation during the prolonged incubation time.

The specificity of the binding was tested by subjecting the labelled subcellular fractions, obtained from the slice incubations, to dialysis against buffer and plasma for 6 hr at 37°C. Results are shown in Fig. 3. Nuclei that contained 852 cpm/mg protein after incubation, retained 645 cpm/mg protein after dialysis, which represented a loss of 24%. The specific activity of the cytoplasm was 1151 cpm/mg protein after incubation and 345 cpm/mg protein after dialysis, resulting in a loss of 70% of the bound radioactivity. The addition of 2,4-dinitrophenol to the incubations, or a nitrogen atmosphere, produced a decrease in the amount of tritium bound to the nuclei.

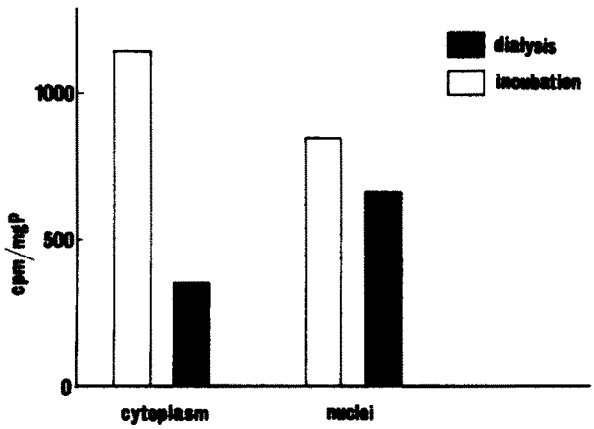

Fig. 3. Uptake and distribution of radioactivity in slices of epididymis incubated with [^{3}H]androstenediol. The affinity of the binding was tested by the retention of label after dialysis of the fractions against buffer containing rat plasma in sufficient amounts to equal the protein concentration inside the dialysing sack.

Nitrogen reduced the uptake of androstenediol by 23%, while 2,4-dinitrophenol caused a 46% decrease in nuclear radioactivity. Similar results were obtained incubating the slices with [^{3}H]testosterone. Nitrogen reduced the incorporation of tritium to the nuclei by 58% and 2,4-dinitrophenol by 44%.

Similar data were obtained from whole homogenate incubations. The inhibition of the nuclear uptake of androstenediol and testosterone by 2,4-dinitrophenol could not be reversed by the addition of 3 mM ATP or an ATP generating system to the homogenate.

DISCUSSION

The data obtained from our *in vivo* uptake experiments indicated that, with certain quantitative differences depending on the androgen injected, the organs in which radioactivity was mainly concentrated or retained were the prostate and the anterior pituitary, followed by the epididymides and seminal vesicles.

In recent years numerous reports have appeared demonstrating the quick and effective conversion of testosterone to dihydrotestosterone in target organs such as prostate[4–5, 12–13, 15–17], seminal vesicles[16, 7], epididymis [18–19] and pituitary gland[20] of several species[19].

Dihydrotestosterone was reported to be bound by the nuclear fraction of prostate[5, 12, 13, 15, 16, 21], had a direct effect on RNA synthesis in isolated prostatic nuclei[22] and promoted hyperplasia and cell division in cultured tissue [23].

On that basis it was postulated that dihydrotestosterone was the acting androgen on this tissue.

The anterior lobe of the pituitary gland concentrates or retains androgens avidly. The formation of macromolecular complexes with testosterone[24] has been demonstrated, indicating the presence of specific receptors in this tissue. It is suggested from our results that it also has a high affinity for binding with androstenediol and dihydrotestosterone.

The *in vitro* experiments performed with epididymides and [^{3}H]androstenediol indicated that this organ has a high affinity binding moiety for the steroid, since the equilibrium dialysis experiments gave a percentage binding of 41% and 30% for caput and body respectively.

It must be taken into account that rat plasma proteins, at equal concentration as the dialyzed fraction, were present in the dialyzing fluid. Therefore, the percentage binding represents the uptake of the steroid by a receptor with higher affinity than the plasma carrier proteins[25].

It is interesting that the three anatomical regions of the epididymis have a different binding affinity, which may be related to the process of spermatozoa maturation or activation known to take place in that organ[26].

The fact that the caudal portion does not have any affinity for the steroid seems to reinforce the concept that the compound is taken by the tissue and not by spermatozoa.

In a cell free medium, isolated crude nuclei were unable to take up androstenediol. When the dialysis was performed against buffer containing epididymal cytoplasmatic protein, instead of plasma, the nuclei concentrated the compound (Fig. 2), thus it became apparent that the presence of cytoplasm was necessary for nuclear uptake. This has been recently shown by Fang *et al.*[13] in their experiments with testosterone and dihydrotestosterone.

Since in the present experimental conditions, this effect could not be due to the passage of a macromolecular complex from cytoplasm to nuclei, as postulated for estradiol[27–28] and dihydrotestosterone[13], the possibility was explored that energy of some kind, provided by cytoplasm, was necessary for the uptake or transformation of androstenediol. The addition of ATP to the dialyzing buffer did not result in an increased binding of androstenediol by isolated nuclei. It was seen that a nitrogen atmosphere or the addition of DNP decreased the amount of intranuclear labelled steroid in slices and homogenates. The addition of ATP or an ATP generating system could not reverse the blocking effect of DNP added to the homogenates. The effect of DNP on the reduction of the uptake of testosterone by prostatic nuclei *in vitro* was demonstrated by Mangan [21]. A recent report by Mainwaring[5] indicated that isolated prostatic nuclei could be labelled in incubations with [^{3}H]testosterone at 22°C for 15 min. Fang *et al.*[13] demonstrated that isolated prostatic nuclei concentrated testosterone

and dihydrotestosterone but did not form the complex 3·0 S receptor-dihydrotestosterone, unless the fraction containing the cytoplasmatic binding protein was present.

It is possible that in our case the role of cytoplasm could be to transform androstenediol to another compound suitable for binding with nuclear receptors.

In an intact cell system, such as the slices incubated with androstenediol, the nuclei bound slightly more radioactivity than the cytoplasm. It seemed important to ascertain the specificity of this binding and therefore, the labelled fractions were dialysed against buffer and plasma to remove the lightly bound androgen: after dialysis the nuclei retained 76% of the tritium present at the end of the incubation period, while cytoplasm lost 70% of the radioactivity (Fig. 3). This phenomenon may be related to the shorter life of the receptor-dihydrotestosterone complex from cytoplasm than that from nuclei [13].

Unhjem *et al.* [29–30] have shown the presence of a macromolecular–testosterone complex in the 105,000 g supernatant of rat prostate. The process of binding and transport appears to be temperature dependent in a fashion similar to that described for estradiol [27–28] in uterine cells. These findings have been recently confirmed by Fang *et al.* [13].

Terms such as "nuclear binding of androstenediol" have been used in the interest of clear expression since it is possible that the three androgens used in these experiments could be rapidly metabolized within the cells of the target tissues to several compounds. Thus chromatography of extracts obtained from slices incubated with androstenediol showed areas of radioactivity corresponding to carrier testosterone and dihydrotestosterone, although no efforts were made to identify further or to quantitate these substances. It is known that androstenediol can be transformed into testosterone in several tissues [9, 31–33] and that testosterone is a precursor of dihydrotestosterone [4, 5, 12, 15–17, 20].

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