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STUDIES ON THE REGULATION OF THE CONCENTRATION OF ANDROGENS AND ANDROGEN RECEPTORS IN NUCLEI OF PROSTATIC CELLS*

NICHOLAS BRUCHOVSKY, PAUL S. RENNIE** and ADRIAN VANSON

Department of Medicine, University of Alberta, Edmonton, Alberta (Canada)

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SUMMARY

Experiments were performed to assess the effect of intracellular androgen metabolism and the availability of cytoplasmic receptors on the concentration of androgens and androgen receptors in nuclei of prostatic cells. It was found that androgens are incorporated into the nucleus by a regulated, selective process which appears to limit the type and amount of androgen transported across the nuclear membrane. The metabolic conversion of testosterone to dihydrotestosterone which takes place in cytoplasm does not reduce transport and, very likely, affects only the ratio of testosterone and dihydrotestosterone transferred into the nucleus. In vivo, when the intranuclear concentration of androgens approaches 250 nM (8 pmol per mg DNA), an apparent concentration ceiling is reached even in the presence of a downward concentration gradient that would be expected to promote further transport across the nuclear membrane. This finding strongly suggests that in vivo the nuclear membrane acts as a barrier to the passage of androgens and, therefore, mitigates against the possibility that passive diffusion is an important mechanism of afferent transport of androgens into the nucleus. The ability of the nucleus to concentrate testosterone and dihydrotestosterone was clearly demonstrated in vivo when cytoplasmic concentrations of androgens of approximately 20 nM were accompanied by intranuclear concentrations in the vicinity of 250 nM. Since the measured concentration of testosterone and dihydrotestosterone in prostate of several species fall within the 5–20 nM range, it is evident that androgen concentra-

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** Present address: Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, WC2A 3PX.

Trivial names used: testosterone, Δ^4-androsten-17β-ol-3-one; dihydrotestosterone, 5α-androstan-17β-ol-3-one; 3α(3β)-androstanediol, 5α-androstan-3α(3β),17β-diol; androsterone, 5α-androstan-3α-ol-17-one; androstanedione, 5α-androstan-3,17-dione; androstenedione, Δ^4-androsten-3,17-dione; dehydroepiandrosterone, Δ^5-androsten-3β-ol-17-one; epitestosterone, Δ^4-androsten-17α-ol-3-one; cyproterone acetate, 1,2α-methylene-6-chloro-$\Delta^{4,5}$-pregnadien-3 : 20-dione-17α-acetate; estradiol, 1,3,5(10)-estratrien-3,17β-diol.

tions in the nucleus as high as 250 nM may be typical of the physiological steady state. At the latter concentration the nucleus contains 60 000 androgen molecules: in approximate terms one third of this total is bound to a large molecular weight component of the nucleus, one third is bound to a 3.3 S receptor and one third is free or loosely bound. Since 60 000 androgen molecules and 20 000 receptor molecules appear in the nucleus before transport stops, it seems that the quantity of 4.4 S cytoplasmic receptor estimated at 174 ± 24 pmol per mg protein (equivalent to about 8000 molecules per cell) is insufficient to account for the total influx of androgens and androgen receptors into the nucleus. Thus, although these results support the view that cytoplasmic receptors and the capacity to transport androgens are closely linked phenotypic markers of intracellular steroid hormone action, they suggest that the control of androgen concentration in the nucleus is achieved in a more intricate fashion than simply through a dependence on the presumed translocation of 4.4 S androgen-receptor complex into the nucleus.

INTRODUCTION

Recent evidence suggests that the multiplication of prostatic epithelial cells is dependent on the formation of dihydrotestosterone in the cytoplasm and on the subsequent incorporation of dihydrotestosterone into the cell nucleus [1–8]. Although the magnitude of the response of target tissues to testosterone and a number of other androgenic steroids may be determined by the concentration of dihydrotestosterone within the nucleus [1, 6–8], little is known about factors which regulate the assimilation and retention of the latter compound by this structure. Nevertheless, it seems reasonable to assume that control of the intranuclear concentration of dihydrotestosterone might be achieved in at least two ways. First, since the amount of dihydrotestosterone in the nucleus is related to the amount in the cytoplasm [6], processes which change the cytoplasmic concentration may also affect the intranuclear concentration. In this regard, the concentration of dihydrotestosterone in the cytoplasm is increased by enzymes which metabolize natural androgens through one or more steps to dihydrotestosterone: conversely, its concentration is reduced by enzymes which convert dihydrotestosterone to other metabolites, chiefly 3α-androstanediol [6, 8].

The observation that the transfer of androgens into the nucleus appears to depend on the formation of androgen-receptor complexes in cytoplasm [9] suggests a second way in which the intranuclear concentration of dihydrotestosterone might be regulated. Although the cytoplasm of the prostate contains a number of androgens, only those which form androgen-receptor complexes, notably testosterone and dihydrotestosterone, are incorporated into the nucleus. Other intracellular androgens such as 3α- and 3β-androstanediol, androsterone, androstanedione, androstenedione and dehydroepiandrosterone as a rule do not enter the nucleus: in vivo, these androgens are first converted to dihydrotestosterone in the cytoplasm and the active metabolite is then transported across the nuclear membrane. If testosterone is formed, it too is transported along with dihydrotestosterone [6, 9]. Thus, since the binding of testosterone and dihydrotestosterone to cytoplasmic receptors seems to be an obligatory step in the transport process, the level of these androgens in the nucleus may depend on the number of receptors in the cytoplasm.

On the basis of the foregoing deductions, experiments were performed to determine whether the metabolism of androgens and the availability of receptors do indeed impose significant limits on the intranuclear concentration of dihydrotestosterone.

METHODS

Experimental animals

Male rats of the Wistar strain weighing 250–300 g were castrated through a scrotal incision. In preparation for experiments in vivo, rats were eviscerated and functionally hepatectomized 24 h after castration. Immediately following surgery, each rat was injected intravenously with radioactive steroid and after appropriate intervals the rats in each experimental group were killed by decapitation. For all surgical operations animals were anaesthetized with ether but no anaesthesia was used during the time course of the experiment.

Homogenization of tissues

All preparative procedures were carried out at 0–4 °C. Prostatic tissue was rinsed in tissue culture medium (CMRL 1415-ATM, Connaught Medical Research Laboratories, Toronto, Ontario) and gently blotted with filter paper. After weighing, the tissue was chopped with an automatic tissue slicer, suspended in 0.01 M Tris · HCl buffer, pH 7.0, containing 0.05 mM EDTA, 5 mM $MgCl_2$, 0.5 mM mercaptoethanol (Tris/EDTA buffer) and 0.25 M sucrose and then manually homogenized with a Dounce apparatus. Centrifugation of the homogenate at $800 \times g$ for 15 min in a Sorvall RC2-B Superspeed refrigerated centrifuge (SS-34 rotor, average radius 8.3 cm) yielded a supernatant fraction of cytoplasm. Cytosol was prepared from the $800 \times g$ supernatant through further centrifugation at $10\,000 \times g$ for 20 min in a Sorvall RC2-B centrifuge and then at $104\,000 \times g$ for 60 min (SW 41 rotor, average radius 10.8 cm) in a Beckman-Spinco L2-65B preparative ultracentrifuge. The $800 \times g$ nuclear pellet was suspended in Tris/EDTA buffer containing 1.5 mM $CaCl_2$ and 0.88 M sucrose and layered over a discontinuous sucrose gradient consisting of 5 ml of 2.2 M sucrose and 5 ml of 1.8 M sucrose in Tris/EDTA buffer with $CaCl_2$. The tubes were centrifuged at $53\,000 \times g$ for 90 min (SW 27 rotor, average radius 11.6 cm) in a Beckman-Spinco ultracentrifuge and the resultant pellet was resuspended in Tris/EDTA buffer containing 50 mM NaCl. This method normally yielded a nuclear fraction that was free of cellular debris and cytoplasmic tags. The fraction was routinely examined by light microscopy: if more than 5 % of the nuclei were contaminated with tags, the nuclei were centrifuged again through a sucrose gradient or discarded. The absence of androstanediol and other cytoplasmic androgens such as androstenedione, androstanedione and androsterone provided an additional check that the nuclei were not contaminated by cytoplasmic fragments.

Counting of nuclei

Nuclei were diluted in buffer containing methylene blue and counted with a hemocytometer. With appropriate corrections for losses based on DNA measure-

ments [1] the recovery of nuclei averaged $11 \cdot 10^7$ per g prostate, in agreement with previous results [7].

Calculation of nuclear and cytoplasmic volumes

In order to determine the molar concentration of androgens in the nucleus and cytoplasm, the dimensions of the cell and the cell nucleus were measured and their respective volumes were calculated. Sections of prostate were prepared as described previously [7] and measurements of both nuclei and whole cells were taken along two perpendicular planes with a calibrated light microscope. The average diameter of 50 prostatic nuclei was 9 μm, and from the relationship, volume = 4/3 π radius^3, the average volume was approx. 400 μm^3. The latter estimate is the same as that reported for the nuclei of other tissues of the rat [10, 11]. Similarly, the average dimensions of the cell were 11 μm $\times$ 35 μm and from the relationship, volume = π radius$^2 \times$ height, the average volume was approximately 3400 μm^3. The difference between the volume of the cell and the volume of the nucleus yields an estimate of the volume of the cytoplasm (3000 μm^3). Since there are about $11 \cdot 10^7$ cells per g of prostate, the volume of cytoplasm in 1 g of tissue is obtained and this approximation permits the calculation of the molar concentration of steroid in the cytoplasmic compartment. The molar concentration of steroid in the cell nucleus is calculated from knowledge of the radioactivity recovered per nucleus and the nuclear volume.

In vitro incubations

To prepare prostatic tissue for incubations in vitro, an initial mincing of tissue was carried out with scissors and tissue fragments were transferred to a 15-ml test-tube containing ice-cold tissue culture medium. The suspension was centrifuged at $25 \times g$ for 5 min in a Sorvall GLC-1 centrifuge (HL-4 rotor, average radius 12.5 cm). The resultant pellet was resuspended in 2 ml of tissue culture medium containing radioactive steroid. Incubation of this sample was then performed at 37 °C with gentle shaking in an atmosphere of $CO_2 : O_2$ (5 : 95, v/v). At the appropriate time, the incubation was terminated by the addition of 5 vols of ice-cold tissue culture medium. Next, three cycles of centrifugation and suspension of the tissue were performed to remove unincorporated steroid. Finally, nuclei and cytoplasm were isolated as described elsewhere in this section.

Sample preparation for assay of binding

Cytoplasmic androgen-binding protein was recovered from the cytosol fraction as follows. The final 104 000 $\times$ g supernatant was saturated to 80 % with ammonium sulfate which was added slowly over a period of 1 h while the temperature of the solution was controlled at 0 °C. Centrifugation of this solution at 11 000 $\times$ g for 30 min in a Sorvall RC2-B centrifuge (HB-4 rotor, average radius 9.7 cm) yielded a protein precipitate which was resuspended in 1–2 ml of Tris/EDTA buffer containing NaCl (600 mM) in preparation for gel-exclusion chromatography.

Purified nuclei in Tris/EDTA buffer containing NaCl (50 mM) were sonicated and then extracted with Tris/EDTA buffer containing NaCl (600 mM) in the following manner. A Biosonik III Ultrasonic System (Bronwill Scientific, Rochester, N.Y.) was fitted with a probe tip having a diameter of 5/16 inches (yellow code).

Sonication was accomplished with four 5-s intervals at an intensity setting of 50. The resulting solution was diluted with an equal volume of Tris/EDTA buffer containing 1.15 M NaCl yielding a final concentration of NaCl of 600 mM. This solution was allowed to stand for 30 min at 4 °C and then was centrifuged in a Sorvall RC2-B centrifuge at 17 000 × *g* for 30 min. The supernatant was decanted while the pellet consisting mostly of unbroken nuclei was subjected to further cycles of sonication and extraction until 95 % of the nuclear radioactivity was recovered in a soluble form. The pooled supernatants were immediately analysed by gel-exclusion chromatography and by density-gradient centrifugation.

Gel-exclusion chromatography

Chromatography of solubilized chromatin and of precipitated cytosol protein was performed on columns (0.9 × 100 cm) prepared with Sephadex G-200 (Pharmacia, Montreal, Quebec). Samples were applied to the column in a volume not exceeding 2 ml and eluted in an upward direction with Tris/EDTA buffer containing 600 mM NaCl at a flow rate of 2–3 ml/h. Fractions of 1.2–1.4 ml were collected and assayed for radioactivity. It was found that separations with the above single-column method would be improved if the sample was first passed through a column (0.9 × 30 cm) prepared with Sephadex G-25 (Pharmacia) and connected in a series with the longer column containing Sephadex G-200 (dual-column method). Sample loading and control of eluant flow were accomplished with a 3-way metering valve (Pharmacia) connected to the inlet of the pre-column of Sephadex G-25.

Sucrose density-gradient analysis

Linear 5–20 % gradients of sucrose in Tris/EDTA buffer (3.6 ml) containing 600 mM NaCl were layered with 200 μl of sample. The gradients were centrifuged at 246 000 × *g* for 17 h (SW 56 rotor, average radius 8.8 cm) in a Beckman-Spinco ultracentrifuge. Following centrifugation, the tubes were pierced and each gradient was collected by drops into a series of 35 counting vials. The vials were then analyzed for radioactivity. Bovine serum albumin was used as a reference for estimating the approximate sedimentation coefficients of steroid-binding components [12].

Samples containing ammonium sulfate were first desalted by passage through a small column (0.5 × 6 cm) of Sephadex G-25 equilibrated with Tris/EDTA buffer containing NaCl (600 mM) prior to analysis by this procedure.

Stokes radius, molecular weight and frictional ratio

The distribution coefficient (K_D) of protein standards (cytochrome *c*, RNA-ase I, ovalbumin, monomer and dimer forms of bovine serum albumin, aldolase and bovine gamma globulin) was determined for each protein by gel-exclusion chromatography with Sephadex G-200 from the relationship $K_D = (V_e - V_0)/(V_T - V_0)$ [13] where the abbreviations shown are: V_0, void volume; V_T, bed volume; V_e, elution volume of the protein. In the calculation, V_g (volume of the gel) was neglected since the water regain of Sephadex G-200 is large in comparison with V_g. A plot of K_D versus logarithm of Stokes radius yielded a linear relationship, which was then used to estimate the Stokes radius of steroid-binding components [13]. Stokes radius values for the standard proteins were obtained from refs 13–15.

The molecular weight and frictional ratio of cytoplasmic and nuclear receptors were then calculated by the use of equations [15]:

$$M = 6\pi N \eta as/(1-\bar{v}\rho) \quad (1)$$

$$f/f_0 = a/(3\bar{v}M/4\pi N)^{\frac{1}{3}} \quad (2)$$

where the abbreviations shown are: M, mol. wt; f/f_0, frictional ratio; a, Stokes radius; s, sedimentation coefficient ; $\bar{v}$, partial specific volume (0.725 ml/g) [12]; η, viscosity of medium at 20 °C (0.01005 poise); ρ, density of medium at 20 °C (0.9982 g/ml); N, Avogadro's number.

Extraction and chromatography of steroids

The extraction of steroids from aqueous solutions was accomplished by an adaptation [1, 6] of the method described by Folch et al. [16]. The androgen constituents of the extracts were indentified by thin-layer chromatography on aluminum oxide as reported elsewhere [17].

Radioactive materials

[1,2-$^3H_2$]testosterone (5 mCi/0.032 mg) and [1,2-$^3H_2$]dihydrotestosterone (5 mCi/0.032 mg) were purchased from New England Nuclear (Boston, Mass.). Purity was checked by thin-layer, gas-liquid, and high resolution liquid chromatography [18] and the steroids were considered acceptable only when the purity exeeded 90%. Solutions for experimental incubations and injections were prepared as follows. Radioactive steroid in ethanol/benzene solutions was dried under N_2 and dissolved in a small volume of ethanol. Distilled water containing 5 % (v/v) polyoxethylene sorbitan monopalmitate was then added to bring the steroid solution to the desired concentration. For in vivo administration, 250 μl of such a solution was injected into each rat.

Liquid scintillation counting

Quantitation of radioactivity was carried out using a diphenyloxazole/toluene solution (4 g of diphenyloxazole/ l toluene) for nonaqueous samples and a solution containing l l of to luene, 6 g of diphenyloxazole, 75 ml of water and 116 g of Bio-Solv (BBS-3, Beckman Instruments, Fullerton, Calif.) for aqueous samples.

The incorporation of ^3H-labelled steroids is expressed both in terms of the radioactivity recovered in cytoplasm of 1 g of prostate and in terms of the radioactivity recovered in nuclei of 1 g of prostate. The latter estimate is obtained by multiplying the radioactivity recovered per nucleus by the number of nuclei contained in 1 g of tissue ($11 \cdot 10^7$).

Other analytical procedures

Protein was measured by the method of Lowry et al. [19] and by differential ultraviolet absorption [20]. DNA was extracted from nuclei as described in an earlier report [7] and was measured using the diphenylamine procedure of Burton [21] with calf thymus DNA as standard. The results of 228 experimental determinations indicate that the concentration of DNA in prostatic nuclei is 12.7 ± 0.2 pg/ nucleus. This value is used to calculate the androgen concentration of nuclei in terms of pmol/mg DNA.

Chemicals

All steroids were purchased from Steraloids, Inc., Pawling, N.Y.

RESULTS

Effect of dose on time course of incorporation of androgens into prostate

Although the incorporation of androgens into prostate has been examined by several investigators [1, 2, 5, 6, 9], such studies have not been addressed to characterization of the maximal rates of androgen transfer across the plasma and nuclear membranes, nor to the determination of the absolute amount of androgen that can be incorporated into the nucleus under conditions which are close to physiological. Thus the question of whether there is a detectable limit to either the rate or amount of incorporation of androgens into cytoplasm and nuclei was examined in the following series of experiments. In the first of these, rats castrated 24 h previously were functionally hepatectomized and injected intravenously with 200 μCi (4.6 nmol) or 400 μCi (9.2 nmol) of [1,2-$^3H_2$]testosterone, and the incorporation of radioactivity into cytoplasm and nuclei was measured as a function of time after injection. The results presented in Fig. 1A indicate that the incorporation of radioactivity into

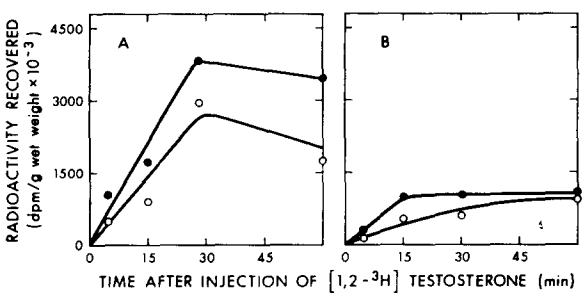

Fig. 1. Time course of incorporation of androgens into prostate. Groups of two to three male rats (250–300 g) castrated 24 h previously were functionally hepatectomized and eviscerated and immediately afterwards were injected intravenously with 200 μCi (4.6 nmol) or 400 μCi (9.2 nmol) of [1,2-$^3H_2$]testosterone. At intervals of 5–60 min the rats were killed and the cytoplasmic and nuclear fractions of prostatic tissue were prepared and assayed for radioactivity. The results are expressed in terms of the amount of radioactivity recovered in cytoplasm or nuclei from 1 g (wet weight) of prostate tissue. Radioactivity recovered: A, cytoplasm; B, nucleus. After injection of 400 μCi (●—●) and 200 μCi (○—○).

cytoplasm after a dose of 400 μCi (●) increases to a maximum at 30 min of 3800 · 10^3 dpm/g and then declines slightly. The incorporation after a dose of 200 μCi (○) is slower but also reaches a maximum at 30 min: by 60 min the level of radioactivity declines to 1700 · 10^3 dpm/g, a value which is one half the final 60 min level of 3400 · 10^3 dpm/g observed after a dose of 400 μCi. This two-fold difference in the apparent plateau levels suggests that between 30 and 60 min a form of equilibrium is established across the plasma membrane such that the concentration of androgens in cytoplasm is proportional to the dose of androgen injected into the circulatory system. Further studies of this proportional relationship are described in a later section (see Fig. 2A).

The results presented in Fig. 1B indicate that the incorporation of radioactivity into nuclei after a dose of 400 μCi (●) reaches a maximal level of approximately $1200 \cdot 10^3$ dpm/g (8 pmol/mg DNA) within 15 min: this level then remains constant for the duration of the experiment. The incorporation after a dose of 200 μCi (○) is slower, and the maximal level of $1200 \cdot 10^3$ dpm/g is attained only after an interval of 60 min. It follows from these observations that the rate of transfer of androgens into the nucleus is probably determined by the concentration of androgens in cytoplasm and also that there is an upper limit to the amount of androgens that can be incorporated.

Effect of dose on the concentration of androgens in cytoplasm and nuclei

To compare the relative concentrations of androgens in cytoplasm and nuclei under conditions that are approximately physiological, the effect of increasing doses of [1,2-$^3H_2$]testosterone on the amount of radioactive androgens incorporated into cytoplasm and nuclear fractions during an interval of 1 h was investigated. In a parallel study, [1,2-$^3H_2$]dihydrotestosterone was injected instead of [1,2-$^3H_2$]testosterone in order to determine whether androgen transfer into the nucleus is enhanced when the enzymatic conversion of testosterone to dihydrotestosterone is circumvented by the direct administration of dihydrotestosterone. The results of these experiments are shown in Fig. 2. It is evident that incorporation of both dihydrotestosterone and testosterone into cytoplasm (Fig. 2A, upper and lower curves respectively) increases as a linear function of dose: it is also evident that dihydrotestosterone is incorporated at a slightly faster rate than testosterone. In contrast the radioactivity incorporated into nuclei, as shown in Fig. 2B, increases as a linear function of dose until a level of

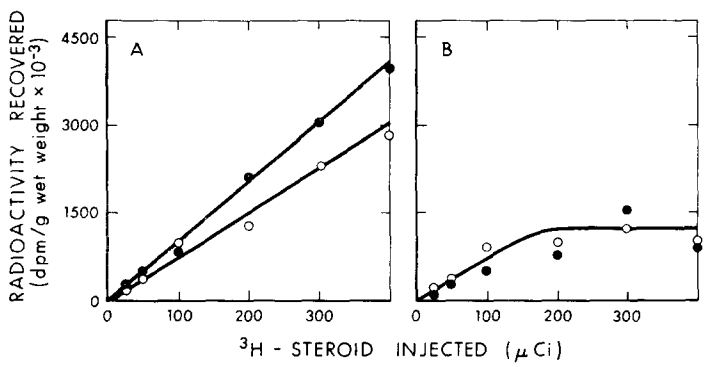

Fig. 2. Effect of dose on the concentration of androgens in cytoplasm and nuclei. Groups of two to three male rats (250–300 g) castrated 24 h previously were functionally hepatectomized and eviscerated and immediately afterwards were injected intravenously with doses of [1,2-$^3H_2$]testosterone ranging from 25 μCi (0.57 nmol) to 400 μCi (9.2 nmol). 60 min later the rats were killed and the appropriate cytoplasmic and nuclear fractions were prepared and assayed for radioactivity. In addition the metabolites of [1,2-$^3H_2$]testosterone were identified in each fraction by thin-layer and gas-liquid chromatography and the recovery of [1,2-$^3H_2$]dihydrotestosterone was measured. Radioactivity recovered: A, cytoplasm; B, nucleus. Radioactive androgen injected: [1,2-$^3H_2$]dihydrotestosterone (●—●), [1,2-$^3H_2$]testosterone (○—○).

$1200 \cdot 10^3$ dpm/g is attained: no more than this amount appears to be incorporated and the results are similar whether testosterone or dihydrotestosterone are injected. Three points merit emphasis in connection with these observations. First, the kinetics of androgen transport across the plasma and across the nuclear membranes are clearly different in that transport into the nucleus appears to be a limited process compared to transport into the cytoplasm. Second, since the incorporation of androgens into nuclei is similar after injections of either testosterone or dihydrotestosterone, it appears that the transport mechanism associated with the nuclear membrane does not discriminate between testosterone and dihydrotestosterone. In this regard, it is noteworthy that other natural androgens which are formed in the cytoplasm or enter the cytoplasm from the blood are not transferred into the nucleus at physiological concentrations [6]. Third, the metabolic conversion of testosterone to dihydrotestosterone which takes place in cytoplasm does not reduce transport, and very likely, therefore, only affects the ratio of testosterone and dihydrotestosterone transferred into the nucleus.

Molar concentration of androgens in cytoplasm and nuclei

The results presented in Fig. 2 indicate that the total amount of androgens in nuclei is exceeded by the amount in cytoplasm: this difference is particularly evident when the dose of androgen is 200 μCi (4.6 nmol) or greater. However, when the experimental data is calculated on the basis of molar concentration of androgens in cytoplasm and nuclei, thus taking into account the different volumes of each compartment, the results shown in Fig. 3 are obtained. In Fig. 3A, the actual concentration of total ^{3}H-labelled androgens in nuclei (○) and cytoplasm (□) is plotted as a function of dose of [1,2-$^3H_2$]testosterone injected. Whereas the final concentration in nuclei is in the vicinity of 250 nM, the maximal concentration in cytoplasm is 100 nM. The curves in Fig. 3B show the relative concentrations of dihydrotestosterone recovered in cytoplasm and nuclei in this experiment. A cytoplasmic concentration of dihydrotestosterone of approximately 20 nM produces an intranuclear concentration of dihydrotestosterone as high as 125 nM. Identification of the metabolites in cytoplasm and nuclei following the injection of [1,2-$^3H_2$]dihydro-

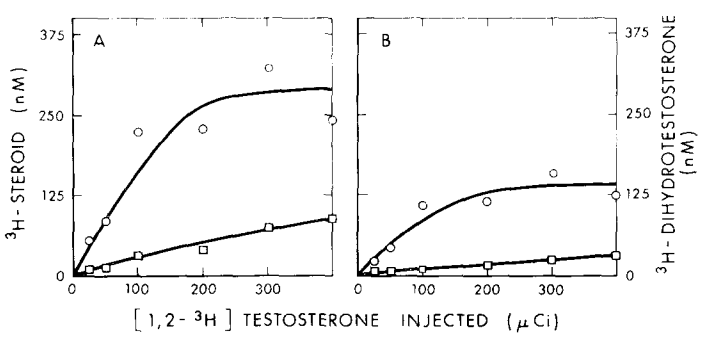

Fig. 3. Molar concentration of androgens in cytoplasm and nuclei. The experimental procedure was identical to that described in the legend to Fig. 2. However, the results are expressed in terms of the molar concentrations of androgens recovered in cytoplasm and nuclei, calculated as described in the Methods section. Molar concentration: A, total ^{3}H-labelled androgens; B, [1,2-$^3H_2$]dihydrotestosterone. Cytoplasm (□—□), nuclei (○ ○).

testosterone was not carried out: however, on the basis of previous analytical data [6] and the results presented in Fig. 2, it can be estimated that a cytoplasmic concentration of dihydrotestosterone in the vicinity of 20 nM, in the absence of testosterone, will produce an intranuclear concentration of dihydrotestosterone in the vicinity of 250 nM. Thus, in vivo, testosterone effectively competes with dihydrotestosterone in transport.

Intranuclear concentration of androgens at high doses

In the preceding experiments it was shown that there is an upper limit to the amount of androgens incorporated into nuclei which was approached when the dose of radioactive androgen injected was 4.6 nmol (200 μCi). To verify that this limit is a firm one, the effect of injecting 900 nmol (150 μCi) of [1,2-$^3H_2$]dihydrotestosterone and [1,2-$^3H_2$]testosterone into experimental animals was examined. The data in Table I indicate that the concentration of radioactive androgens in cytoplasm increases about thirtyfold over concentrations previously obtained (75–100 nM) to approximately 3000 nM. In contrast, the intranuclear concentration remains in the vicinity of 300–400 nM showing little change from the concentration of 250 nM observed in preceding experiments. Therefore, not only does the nucleus selectively concentrate dihydrotestosterone and testosterone when the cytoplasmic concentration of these steroids is below 100 nM, but it also appears to resist their incorporation, or to increase their release from the nucleus when the cytoplasmic concentration of androgens rises to abnormal levels.

TABLE I

INTRANUCLEAR CONCENTRATION OF ANDROGENS AT HIGH DOSES

Groups of three to five rats (250–300 g) castrated 24 h previously were functionally hepatectomized and eviscerated and immediately afterwards were injected intravenously with 900 nmol (150 μCi) of [1,2-$^3H_2$]testosterone and [1,2-$^3H_2$]dihydrotestosterone. 60 min later the animals were killed and the appropriate cytoplasmic and nuclear fractions were prepared and assayed for radioactivity. The results are expressed as the mean $\pm$S.E. for each set of at least three experimental results.

Steroid injected	Concentration of ^3H-labelled androgens (total) (nM)	
	Cytoplasm	Nucleus
[1,2-$^3H_2$]Testosterone	3890$\pm$490	422$\pm$72
[1,2-$^3H_2$]Dihydrotestosterone	2700$\pm$130	276$\pm$7

The observation in Table I that the concentration of androgens in nuclei can be tenfold lower than in cytoplasm suggests that, in vivo, there is little apparent transfer of androgens across the nuclear membrane by the process of passive diffusion. Clearly this result would also be explained if steroid which enters the nucleus leaks out during the nuclear isolation procedure because no unsaturated intranuclear receptor is available for binding. However, when isolated nuclei themselves are incubated in vitro with [1,2-$^3H_2$]dihydrotestosterone, the incorporation process changes to resemble passive diffusion (Bruchovsky, N., unpublished). Not only does the intranuclear concentration of dihydrotestosterone then parallel the concentration of androgen in the extranuclear medium, but also the limit on the amount

of incorporation is no longer observed. Finally, neither selectivity nor temperature dependence of transport are preserved in isolated nuclei. Together these observations indicate that the properties of the nuclear membrane are quite different in the whole cell and strongly suggest that in vivo this membrane acts as a barrier to the afferent passage of androgens into the nucleus.

In vitro incorporation of androgens into nuclei

Experiments were next performed to determine whether transport of androgens into nuclei of whole cells incubated in vitro shows any limitation similar to that observed in vivo. Prostate of rats castrated 24 h previously was minced and incubated at 37 °C in medium containing [1,2-$^3H_2$]dihydrotestosterone at a concentration of 750 nM. The results presented in Fig. 4A indicate that the incorporation of radioactive androgen into cytoplasm is rapid and reaches a plateau within 10 min of $350 \cdot 10^5$ dpm/g equivalent to a concentration of 750 nM. The incorporation of

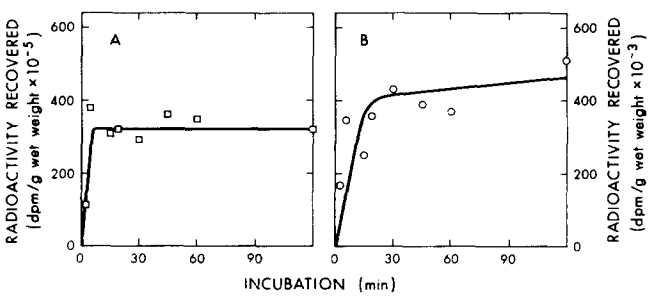

Fig. 4. In vitro incorporation of androgens into nuclei. Prostatic tissue of rats castrated 24 h previously was minced and incubated in tissue culture medium (2 ml) containing [1,2-$^3H_2$]dihydrotestosterone (750 nM) at 37 °C in an atmosphere of CO_2 : O_2 (5 : 95, v/v). At the appropriate time, the incubation was terminated by the addition of 5 vols of ice-cold tissue culture medium. Three cycles of centrifugation and suspension of the tissue were performed to remove unincorporated steroid. Cytoplasmic and nuclear fractions were then prepared and assayed for radioactivity. The results are expressed in terms of the amount of radioactivity incorporated into cytoplasm or nuclei contained in 1 g (wet weight) of tissue. The means of the results of at least 2 replicate experiments are shown. Radioactivity recovered: A, cytoplasm; B, nuclei.

radioactive androgen into nuclei also proceeds at a rapid rate as shown in Fig. 4B and reaches a plateau, within 20 min, of $400 \cdot 10^3$ dpm/g, equivalent to a concentration of 60 nM. When the preceding experiment was conducted at 4 °C instead of 37 °C, no effect was observed on the incorporation into cytoplasm while the maximal amount of radioactive androgen incorporated into nuclei was $60 \cdot 10^3$ dpm/g. The abrupt change in the rate of incorporation of androgen into nuclei, the marked discrepancy between the cytoplasmic and nuclear concentrations of androgen and the temperature dependence of incorporation are consistent with the idea that in whole cells the entry of androgens into the nucleus is a regulated process.

Time course of disappearance of androgen receptors in cytoplasm

In attempts to explain the restricted incorporation of androgens into the nucleus, experiments were performed to examine the possibility that the concentra-

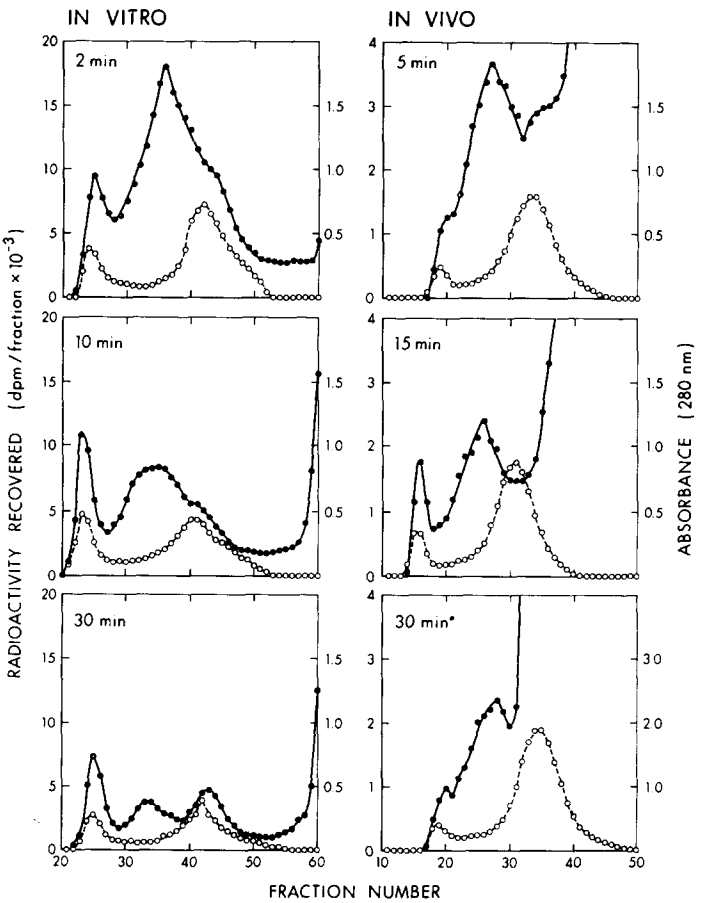

Fig. 5. Time course of disappearance of androgen receptors in cytoplasm. Prostatic tissue of rats castrated 24 h previously was minced and incubated in tissue culture medium (2 ml) containing [1,2-$^3H_2$]dihydrotestosterone (750 nM) at 37 °C in an atmosphere of CO_2 : O_2 (5 : 95, v/v) for 2, 10 and 30 min (in vitro experiment). Alternatively rats castrated 24 h previously were functionally hepatectomized and eviscerated and immediately after surgery were injected with 400 μCi (9.2 nmol) of [1,2-$^3H_2$]testosterone. At 5, 15 and 30 min after injection the rats were killed by decapitation (in vivo experiments). In both in vitro and in vivo experiments prostatic tissue was then fractionated into cytosol and nuclear samples. The cytosol fraction was precipitated with ammonium sulfate at 80 % saturation while the temperature was controlled at 0 °C. The precipitate was recovered by centrifugation, dissolved in Tris/EDTA buffer and analysed by gel-exclusion chromatography using either a dual-column method (in vitro experiments) or a single-column method (in vivo experiments) as described in the Methods section. In either case the column of Sephadex G-200 (0.9 × 100 cm) was equilibrated with Tris/EDTA buffer containing NaCl (600 mM). Fractions of 1.2–1.5 ml were collected at a flow rate of 3 ml per h. Radioactivity recovered (● — ●), absorbance at 280 nm (○ — ○).

tion of androgen receptors in either cytoplasm or nucleus might be a limiting factor. Prostate from rats castrated 24 h previously was minced and incubated at 37 °C in medium containing [1,2-$^3H_2$]dihydrotestosterone at a concentration of 750 nM. Alternatively, animals castrated 24 h previously were functionally hepatectomized and then injected intravenously with 9.2 nmol (400 μCi) of [1,2-$^3H_2$]testosterone.

In both experiments, the disappearance of cytoplasmic androgen receptor was measured by gel-exclusion chromatography using either a dual-column or a single-column method. As shown in Fig. 5, a cytoplasmic binding component is recovered in fractions 30–40 with the dual-column technique (in vitro experiments) and in fractions 20–30 with the single-column technique (in vivo experiments). Control experiments indicate that binding of radioactive dihydrotestosterone to this component is maximal under the in vitro conditions used and that it is reduced to zero in the presence of a hundred-fold excess of non-radioactive dihydrotestosterone. In addition it has been shown previously that other agents such as cyproterone acetate, epitestosterone and estradiol act in vivo as competitive inhibitors of the binding reaction but are less effective than testosterone and dihydrotestosterone [9]. The high affinity of binding is established by the stability of the complex during gel-exclusion chromatography. These observations and the physical properties of the binding component as outlined in a later section (see Fig. 9) when viewed in the light of the usual criteria for identifying receptor molecules [22–24] indicate that the use of the term "receptor" to denote the cytoplasmic binding component is justified if only for comparative purposes.

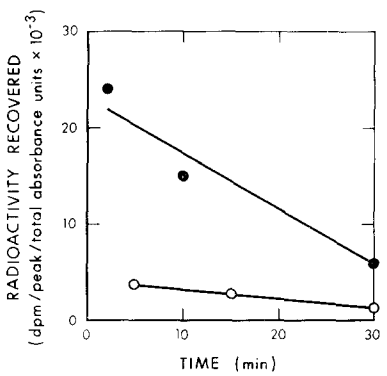

Fig. 6. Rate of disappearance of cytoplasmic receptor. The total radioactivity recovered in the receptor peak in each preparation described in Fig. 5 was estimated by doubling the sum of the radioactivity representing the most distinct and uniform side of the receptor peak. This value was then divided by the total absorbance units at 280 nm in order to correct for the different amounts of protein analysed. Receptor detected: in vitro (● – ●), in vivo (○ – ○).

In the presence of androgens the amount of radioactivity in the fractions containing receptor declines with increasing time whether in vitro or in vivo labelling techniques are used. This effect is demonstrated more clearly by the data in Fig. 6, where the estimated amount of radioactivity in the receptor peak at each time is normalized to correct for any difference in the quantity of protein applied to the analytical column. More receptor is detected by the in vitro labelling technique and its rate of disappearance (or inactivation) is noticeably faster in vitro than in vivo. In both cases, however, cytoplasmic receptor is still detected at 30 min well after the incorporation of androgens into the nucleus has stopped (Figs 1B and 4B). By extrapolating each curve in Fig. 6 to zero time, an estimate of the initial concentration of cytoplasmic receptor is obtained. With the in vitro labelling technique

this method furnishes an estimate of 174±24 fmol/mg protein (mean ±S.E. of four determinations), equivalent to about 8000 molecules/cell. Only one fifth as many receptor molecules are detected by the in vivo labelling technique. The lower amount of labelling in vivo is probably explained by the relatively slow rate of transfer of of androgens from the circulatory system into the cytoplasm: thus, at comparable times, the concentration of androgens in cytoplasm is more than tenfold lower in vivo than in vitro, and, presumably, instantaneous quantitative labelling of receptor is not possible under such conditions.

Although the receptor described above is the principal binder in cytoplasm, it is evident from the results in Fig. 5 that androgen binding also takes place to other cytoplasmic components. The radioactivity which appears in the void volume (in vitro, fractions 20-30; in vivo, fractions 10-20) probably represents binding to aggregated protein. However, a small amount of binding is sometimes observed in vitro in the fractions containing the bulk of the cytoplasmic protein (fractions 40–50). To establish whether such binding also occurs in vivo cytoplasmic protein from prostate was analysed by the dual-column method 30 min after injection of 400 μCi (9.2 nmol) of [1,2-$^3H_2$]testosterone into experimental animals. As shown in Fig. 7A, no peak

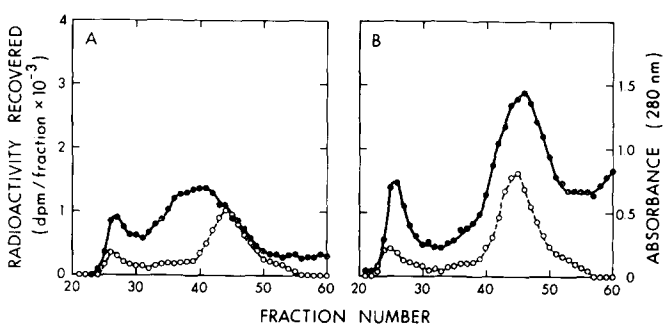

Fig. 7. Demonstration of binding to low molecular weight components in cytoplasm. (A) To establish whether binding to low molecular weight components occurs in vivo, cytoplasmic protein from prostate was analyzed by the dual-column method 30 min after the injection of 400 μCi (9.2 nmol) of [1,2-$^3H_2$]testosterone into castrated functionally hepatectomized rats. (B) Cytosol prepared from prostate of rats castrated 24 h previously was incubated in the presence of [1,2-$^3H_2$]dihydrotestosterone (20 nM) for 5 min at 20 °C. The cytosol fraction was precipitated with ammonium sulfate at 80 % saturation while the temperature was controlled at 0 °C. The precipitate was recovered by centrifugation, dissolved in Tris/EDTA buffer and analysed by gel-exclusion chromatography (dual-column method). Radioactivity recovered (● – ●), absorbance at 280 nm (○ – ○).

of radioactivity is observed in fractions 40–50 corresponding to the peak observed in vitro. However, further investigation revealed that labelling of this low molecular weight component could be achieved by incubating isolated cytosol for a short time at 4 °C or 20 °C in the presence of [1,2-$^3H_2$]dihydrotestosterone as shown in Fig. 7B. Under the same conditions, the receptor previously recovered in fractions 30–40 is poorly labelled and therefore difficult to observe. Besides demonstrating a tendency to associate with androgens only in cell-free extracts, the low molecular weight component possesses a larger if not unlimited binding capacity and it can also be differentiated on the basis of net charge distribution [22]. No competitive

inhibition of binding is observed in the presence of excess unlabelled dihydrotestosterone. Collectively, these observations indicate that the component is unlike the cytoplasmic receptor detected by in vivo labelling techniques or in vitro labelling techniques using whole cells. Moreover, the failure to observe the low molecular weight component in cytoplasm in vivo suggests that in whole cells it does not come into contact with androgens.

Time course of appearance of androgen receptors in nuclei

When the nuclear fraction recovered in each experiment described in Fig. 5 was extracted with Tris/EDTA buffer containing NaCl (600 mM) and the extract was analysed by gel-exclusion chromatography with Sephadex G-200 the results shown in Fig. 8 were obtained. Radioactive androgen is recovered in three peaks: part of the total incorporated is bound to a large molecular weight component of the nucleus presumed to be chromatin [23] and appears in the void volume: part is free and is recovered as the third of the three peaks: part is bound to a component which is recovered as the second peak. Previous studies have established that the latter represents the intranuclear form of androgen-receptor complex [9, 22, 23]. Between 2 and 30 min of incubation in vitro, there is a large increase in the size of all three peaks, with the major increase taking place in the first 10 min. Similar peaks

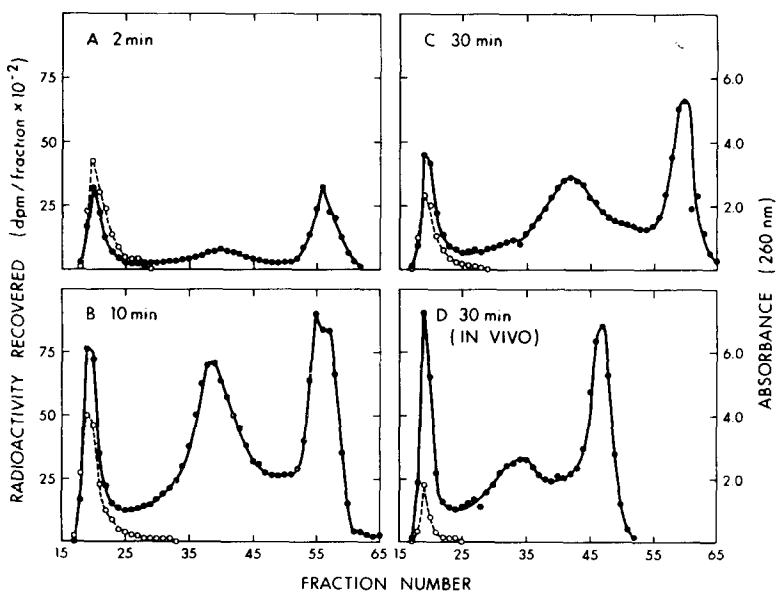

Fig. 8. Time course of appearance of androgen receptors in nuclei. Prostatic tissue was labelled with radioactive androgens using in vitro or in vivo labelling techniques as described in the legend to Fig. 5. The nuclear fraction was suspended in Tris/EDTA buffer containing NaCl (50 mM), sonicated, and then extracted with Tris/EDTA buffer containing NaCl (600 mM). Centrifugation of the extract (final volume 1–2 ml) at 17 000 × *g* for 30 min yielded a supernatant which was then analysed by gel-exclusion chromatography with Sephadex G-200. Samples were applied to the column in a volume not exceeding 2 ml and eluted in an upward direction with Tris/EDTA buffer containing NaCl (600 mM) at a flow rate of 3 ml/h. The volume of each fraction in experiments A–C was 1.1 ml, whereas that in experiment D was 1.4 ml. Radioactivity recovered (● — ●), absorbance at 260 nm (○ — ○).

are obtained when in vivo labelling techniques are used as is shown in the lower right panel of Fig. 5. Under optimal in vivo conditions (see Fig. 1) about 60 000 androgen molecules are incorporated into the nucleus before transport stops, but only about one third or 20 000 of these molecules are actually recovered in the androgen-receptor peak (Fig. 8).

Partial characterization of cytoplasmic and nuclear receptors

Measurement of the K_D of the cytoplasmic receptor detected by in vivo labelling techniques and by in vitro labelling of whole cells was accomplished by the procedure described in the Methods section and was found to be 0.258. This value corresponds to a Stokes radius of 48 Å as shown in Fig. 9 (upper arrow). The receptor possesses a sedimentation coefficient of 4.4±0.1 S (mean ±S.E. of 3 determinations) measured in 600 mM NaCl. On the basis of these parameters the apparent molecular weight of the receptor is 86 000 and its frictional ratio is 1.65.

The K_D of the intranuclear receptor was found to be 0.548 corresponding to a Stokes radius of 24 Å (Fig. 9, lower arrow). This receptor possesses a sedimentation coefficient of 3.3 ±0.3 S (mean±S.E. of three determinations) measured in 600 mM NaCl. The apparent molecular weight is 33 000 and its frictional ratio is 1.1.

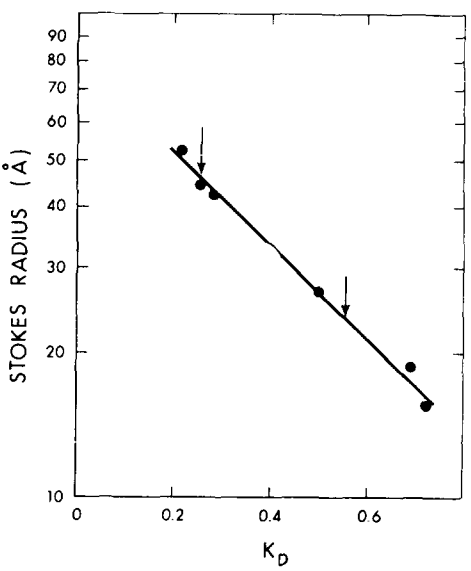

Fig. 9. Determination of Stokes radius. Distribution coefficients (K_D) of protein standards (cytochrome *c*, RNAase I, ovalbumin, monomer and dimer forms of bovine serum albumin, aldolase and bovine gamma globulin) were calculated from data obtained by gel-exclusion chromatography with Sephadex G-200 (single-column method) using the relationship $K_D = (V_e - V_0)/(V_t - V_0)$. The K_D values of the cytoplasmic and nuclear receptors are shown by the upper and lower arrows respectively.

DISCUSSION

The process whereby androgens are transferred into the nucleus of the prostatic cell contrasts to the one which promotes the entry of androgens into the cytoplasm in several respects. First, the former process is highly selective in that only testos-

terone and dihydrotestosterone enter the nucleus in significant amounts when the concentration of these androgens in cytoplasm is below 100 nM and thus within the physiological range [6]. By comparison, transport into cytoplasm lacks selectivity and a number of androgens are freely transmitted across the plasma membrane [6]. Second, the amount of androgen in the nucleus is limited to a finite maximal quantity, but there is no obvious limit to the amount that can be incorporated into cytoplasm (Fig. 2 and Table I). Third, compounds such as estradiol, cyproterone acetate, and epitestosterone which in high doses are potent competitors of androgen transport across the nuclear membrane, have no similar competitive effect on androgen transport across the plasma membrane [9]. The fourth and perhaps most differentiating feature of the nuclear membrane is its ability to concentrate testosterone and dihydrotestosterone. This action is clearly demonstrated in vivo when cytoplasmic concentrations of androgens of approximately 20 nM are accompanied by intranuclear concentrations in the vicinity of 250 nM (Fig. 3). Considered together, such evidence strongly suggests that transport of androgens into cytoplasm occurs by a process of passive diffusion while ruling out the possibility that transport of androgens into the nucleus takes place by a similar mechanism. Since the latter process is temperature dependent, selective, concentrative, sensitive to competitive inhibitors and appears to prevent excessive accumulation of androgens within the nucleus, it seems reasonable to believe that this process represents an important focal point of control of the intranuclear concentration of testosterone and dihydrotestosterone.

From the data presented in Fig. 3, certain inferences can be drawn about the potential intranuclear concentration of androgens under normal steady state conditions. For example, since the measured concentration of dihydrotestosterone and testosterone in prostatic tissue of several species falls within the 5–20 nM range [3, 4, 25], it is evident that concentrations as high as 250 nM (8 pmol/mg DNA) may indeed be typical of the physiological steady state. At the latter concentration, the number of molecules of androgen in the nucleus is about 60 000: in rough terms one third or less of the total is bound to a large molecular weight component of the nucleus presumed to be chromatin, one third is bound to receptor, and one third or more appears to be loosely bound and is recovered in a free form as has been discussed before [9]. Because 60 000 androgen molecules and 20 000 receptor molecules appear in the nucleus before transport stops, it seems that the quantity of cytoplasmic receptor (174 ± 24 fmol/mg protein or 8000 molecules/cell) is insufficient to account for the influx of androgens and androgen receptors into the nucleus if a mole to mole relationship is assumed. In considering whether this estimate of the number of cytoplasmic receptors is a valid approximation, cognizance must be given to the large body of evidence showing that the concentration of cytoplasmic receptor in analogous systems rarely exceeds a level of 200 fmol/mg protein or an equivalent value [26–34]. Thus, our estimate of the concentration of receptor is in agreement with such results.

Although our findings support the view that cytoplasmic receptors and the capacity to transport androgens into the nucleus are closely linked phenotypic markers of intracellular steroid hormone action, for the following reasons they leave open the possibility that the control of androgen concentration in the nucleus may involve the cytoplasmic receptor in a more intricate way than usually assumed. First, as already pointed out, the number of androgen molecules that enter the nucleus in a

regulated fashion greatly exceeds the detectable number of receptors in a cell cytoplasm. Second, transport of androgens into the nucleus under certain conditions (Figs 1 and 4) stops before the depletion of cytoplasmic receptor is complete (Figs 5 and 6): therefore, it seems unlikely that the apparent limit on nuclear incorporation can be explained by the lack of receptor. Third, the physical properties of the intranuclear receptor (sedimentation coefficient 3.3 S; Stokes radius 24 Å; molecular weight, 33 000; frictional ratio, 1.1) differ from those of the cytoplasmic receptor (sedimentation coefficient, 4.4 S; Stokes radius, 48 Å; molecular weight, 86 000; frictional ratio, 1.65). It has also been noted that the net charge distributions on the cytoplasmic and on the nuclear receptors are not the same [22]. Together, these observations suggest that the control of androgen concentration in the nucleus is achieved in a more subtle fashion than simply through a dependence on the translocation of 4.4 S androgen-receptor complex. However, they do not exclude the possibility that several androgen molecules are transferred with each receptor molecule, nor the possibility that the cytoplasmic receptor reduces the threshold of the barrier imposed by the nuclear membrane to the passage of androgens. These poorly understood aspects of the relationship between cytoplasmic receptors and the concentration of androgens and androgen receptors within the nucleus would perhaps be resolved by examining the transport of androgens in cells depleted of cytoplasmic receptors. Since the concentration of cytoplasmic receptor falls precipitously after castration [35–37] the use of prostate from longer-term castrated animals offers a potentially advantageous approach to the clarification of this relationship.

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