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THE INFLUENCE OF ANDROGEN RECEPTORS ON THE CONCENTRATION OF ANDROGENS IN NUCLEI OF HORMONE-RESPONSIVE CELLS

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SUMMARY

The transfer of androgens from cytoplasm to nuclei has been studied in two animal model systems, the rat prostate and the Shionogi mouse mammary carcinoma. It has been found that androgens are incorporated into the prostatic nucleus by a regulated, selective process which appears to limit the type and amount of androgen transported across the nuclear membrane. *In vivo* under optimal conditions, about 60,000 molecules of androgen penetrate the nucleus before transport stops. In the presence of an adequate concentration of testosterone or dihydrotestosterone in cytoplasm, the transfer of these androgens into the nucleus proceeds rapidly and is virtually complete within 60 min. During this time 5000, or fewer, detectable androgen receptors (mol wt. 86,000) disappear from cytoplasm and 10,000 to 20,000 receptors (mol wt. 33,000) appear in the nucleus. Because of the relatively small number of detectable cytoplasmic receptors, it is possible that several molecules of androgen are transferred into the nucleus for each molecule of receptor that either disappears from the cytoplasm or appears in the nucleus. Investigation of the intracellular transport of androgens in several lines of Shionogi carcinoma cells has also furnished evidence of a correlation between the presence of receptors in cytoplasm and the ability to transfer androgens into the nucleus. Taken together the results of these studies suggest that the intranuclear concentration of androgens is regulated in part by cytoplasmic receptors, but they leave open the possibility that the control of androgen concentration in the nucleus may be achieved by an independent process which does not rely on androgen receptors.

INTRODUCTION

Recent experimental evidence has linked the biological activity of testosterone[1] in several androgen-responsive tissues to the formation of dihydrotestosterone in the cytoplasm and 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 compounds may be determined by the concentration of dihydrotestosterone within the nucleus [6, 8], little is known about factors which regulate the assimilation and retention of dihydrotestosterone by this structure. In the following report we present the results of a study into the potential role of androgen receptors as regulators of the intranuclear concentration of testosterone and dihydrotestosterone.

EXPERIMENTAL

Animals

Male rats of the Wistar strain weighing 250 to 300 g were routinely anaesthetized with ether and castrated through a scrotal incision. In preparation for experiments *in vivo* rats were eviscerated and functionally hepatectomized 24 h after castration. Immediately following this surgery each rat was injected intravenously with radioactive steroid, and after appropriate intervals the rats in each experimental group were killed by decapitation.

Experimental tumours

The SC-115 androgen-dependent mouse mammary carcinoma and the autonomous SC-42 carcinoma were originally isolated by Minesita and Yamaguchi[9]. A number of sublines of the SC-115 dependent tumour have been developed by the research groups in Edmonton and Toronto [10]. The tumour lines termed dependent grow only in male mice whereas those termed autonomous grow at approximately equal rates in male, female, and castrated male mice of the DD/S strain. A detailed description of the properties of some of these tumours and of the methods of their propagation have been presented elsewhere [10, 11]. Tumour bearing mice were injected intravenously with 200 μCi (4·6 nmol) of [1,2-^{3}H]-testosterone, and were killed by decapitation after an interval of 30 min. The tumours, usually weighing 2–3 g, were dissected free of subcutaneous tissue and minced as described previously [12].

Homogenization of tissue

All preparative procedures were carried out at 0–4°. Prostatic tissue was rinsed in tissue culture medium and gently blotted with filter paper. After weighing, the tissue was chopped with an automatic tissue slicer, suspended in 0·01 M Tris–EDTA, ph 7·0, containing 0·05 mM EDTA, 5 mM $MgCl_2$, 0·5 mM mercaptoethanol (Tris–EDTA buffer) and 0·25 M sucrose,

[1] The trivial names used are testosterone, 17β-hydroxy-4-androsten-3-one; and dihydrotestosterone, 5α-androstan-17β-ol-3-one.

and then manually homogenized with a Dounce apparatus. Centrifugation of the homogenate at 400 *g* for 15 min in a Sorvall RC2-B Superspeed refrigerated centrifuge (SS-34 rotor, $radius_{avg}$, 5·3 cm.) yielded a supernatant fraction of cytoplasm. Cytosol was prepared from the 400 *g* supernatant through further centrifugation at 10,000 *g* for 20 min in a Sorvall RC2-B centrifuge and then at 104,000 *g* for 90 min (SW 41 rotor, $radius_{avg}$, 10·8 cm.) in a Beckman–Spinco L2-65B preparative ultracentrifuge. The 400 *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 *g* for 90 min (SW 27 rotor, $radius_{avg}$, 11·6 cm.) in a Beckman–Spinco ultracentrifuge, and the resultant nuclear pellet was resuspended in Tris–EDTA buffer containing 50 mM NaCl. Nuclei were stained with methylene blue and counted with a hemocytometer.

Essentially the same procedure was used to isolate tumour nuclei except for the following difference. The 400 *g* pellet was homogenized in an aqueous solution, pH 6·5–7·0 containing sucrose (0·44 M) and Triton N-101 (0·3% v/v); centrifugation of this homogenate through a discontinuous sucrose gradient at 53,000 *g* for 90 min yielded a purified nuclear fraction [11].

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 = $4/3\pi$ $radius^3$, the average volume was approximately 400 μm^3. The latter estimate is the same as that reported for the nuclei of other tissues of the rat [13, 14]. Similarly the average dimensions of the cell were 11 μm × 35 μm and from the relationship, volume = π $radius^2$ × 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 11×10^7 cells/g of prostate [7], 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*, a preliminary mincing of tissue was carried out with scissors and then the mince was forced through a stainless steel screen (30 mesh; 0·55 mm grid) with Teflon pestle. Tissue fragments and cells were collected in a beaker containing ice-cold tissue culture medium. The suspension was transferred to a 15 ml test tube and centrifuged at 25 *g* for 5 min in a Sorvall g.l.c.-1 centrifuge (HL-4 rotor, $radius_{avg}$, 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 volumes 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 *g* supernatant was saturated to 80% with ammonium sulphate which was added slowly over a period of 1 h while the temperature was controlled at 0°. Centrifugation of this solution at 11,000 *g* for 30 min in a Sorvall RC2-B centrifuge (HB-4 rotor, $radius_{avg}$, 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 analysis.

As mentioned earlier, nuclei were suspended in Tris–EDTA buffer containing NaCl (50 mM), sonicated, and then extracted with Tris–EDTA buffer containing NaCl (600 mM) [15]. Centrifugation of the extract (final volume, 1–2 ml) at 17,000 *g* for 20 min yielded a supernatant which was decanted and analyzed as described below.

Gel-exclusion chromatography

Chromatography of the nuclear extract and of the precipitated cytosol protein was performed on columns (0·9 cm. × 100 cm.) prepared with Sephadex G-200 or Sepharose 6B (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 per 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 cm. × 30 cm.) prepared with Sephadex G-25 (Pharmacia) and connected in 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 gradient centrifugation

Linear 5–20% gradients of sucrose in Tris–EDTA buffer (3·8 ml) containing NaCl (600 mM) were

layered with 200 μl of sample. The gradients were centrifuged at 246,000 *g* for 17 h in a Beckman–Spinco Ultracentrifuge (SW 56 rotor, radius$_{avg}$, 8·8 cm.). Following centrifugation the tubes were pierced and each gradient was collected by drops into a series of 35 counting vials. The vials were then analysed for radioactivity. Approximate sedimentation coefficients of steroid-binding components were estimated by comparison with that of bovine serum albumin [16]. Samples containing ammonium sulphate were first desalted by passage through a small column (0·5 cm. × 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 (*Kd*) of protein standards (cytochrome C, RNase I, ovalbumin, monomer and dimer forms of bovine serum albumin, aldolase, bovine gamma-globulin) was determined for each protein from the relationship $Kd = Ve - Vo/Vt - Vo$ [17] where Vo = void volume, Vt = bed volume, Ve = elution volume of the protein as determined by gel-exclusion chromatography with Sephadex G-200. In the calculation, Vg (volume of the gel) was neglected since the water regain of Sephadex G-200 is large in comparison with Vg. A plot of Kd vs. the logarithm of the Stokes radius yielded a linear relationship, which was then used to estimate the Stokes radius of steroid-binding components [17]. Stokes radius values for the standard proteins were obtained from references [17–19]. The molecular weight and frictional ratio of cytoplasmic and nuclear receptors were calculated by the use of the equations [19]: (1) $M = 6\Pi N\eta aS/1 - \bar{v}\rho$, and (2) $f/fo = a/(3\bar{v}M/4\Pi N)^{1/3}$ where M = mol wt.; f/fo = frictional ratio; a = Stokes radius; S = sedimentation coefficient; $\bar{v}$ = partial specific volume (0·725 ml/g) [16]; η = viscosity of medium at 20° (0·01005 poise); ρ = density of medium at 20° (0·9982 g/ml); and 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.*[20]. The androgen constituents of the extracts were identified by thin-layer chromatography on aluminum oxide as reported elsewhere [12].

Radioactive materials

[1,2-^{3}H]-Testosterone (5 mCi per 0·032 mg) and [1,2-^{3}H]-dihydrotestosterone (5 mCi per 0·032 mg) were purchased from New England Nuclear (Boston, Mass.). Purity was checked by thin layer, gas–liquid, and high resolution liquid chromatography, and the steroids were considered acceptable only when purity exceeded 90%. Solutions for experimental incubations and injections were prepared as follows: Radioactive steroid in ethanol–benzene solution was dried under N_2 and dissolved in a small volume of ethanol. Distilled water containing 5% (v/v) polyoxyethylene 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 per liter of toluene) for nonaqueous samples, and a solution containing 1 l. toluene, 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 ^{3}H-androgens is expressed both in terms of 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 × 10^7].

Chemicals

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

RESULTS

Effect of size of dose on the incorporation of androgens into nuclei of prostatic cells

To determine whether there is any limit to the amount of androgen that can be incorporated into the prostatic nucleus, male rats were injected intravenously with increasing doses of radioactive dihydrotestosterone and testosterone 24 h after castration and immediately after functional hepatectomy. One hour after injection the rats were killed and prostate was separated into cytoplasmic and nuclear fractions. The radioactivity incorporated into cytoplasm, as shown in Fig. 1a, increases as a linear function of dose and similar results are obtained whether dihydrotestosterone (closed circles) or testosterone (open circles) are injected. In contrast the radioactivity incorporated into nuclei as shown in Fig. 1b increases as a linear function of dose until a level of 1200 × 10^3 d.p.m./g is attained, and no more than this amount appears to be incorporated. Evidence from a series of control experiments indicates that the apparent ceiling on the amount of androgen incorporated into nuclei represents a saturation phenomenon and does not reflect a maximal rate of transport. For example, saturation is achieved within 30 min with doses of ^{3}H-androgen of 300 to 400 μCi (6·9 to 9·2 nmol) but longer periods are required for smaller doses.

The results presented in Fig. 1 also 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

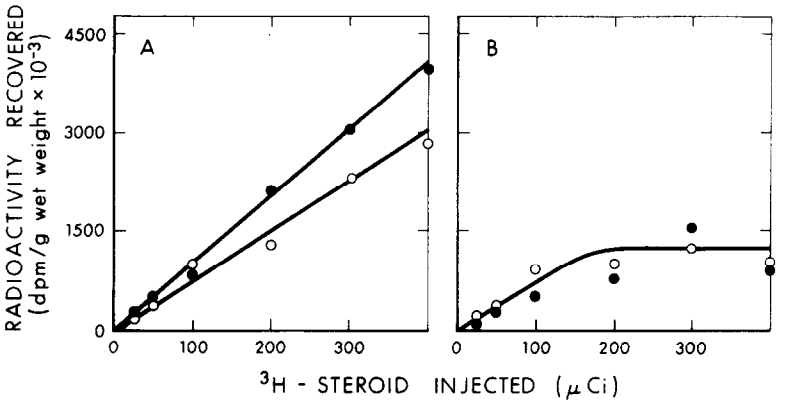

Fig. 1. *Effect of size of dose on the incorporation of androgens into nuclei of prostatic cells*
Groups of 2 to 3 male rats (250–300 g) castrated 24 h previously were functionally hepatectomized and eviscerated, and immediately afterwards were injected intravenously with doses of [1,2-^{3}H]-testosterone ranging from 25 μCi (0·57 nmol) to 400 μCi (9·1 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-^{3}H]-testosterone were identified in each fraction by thin layer and gas–liquid chromatography and the recovery of ^{3}H-dihydrotestosterone was measured. Radioactivity recovered: A, cytoplasm; B, nucleus. Radioactive androgen injected: [1,2-^{3}H]dihydrotestosterone ●——●; [1,2-^{3}H]testosterone ○——○.

compartment, the results shown in Fig. 2 are obtained.

Molar concentration of androgens in cytoplasm and nuclei

In Fig. 2a the actual concentration of total ^{3}H-androgens in nuclei (open circles) and cytoplasm (open squares) is plotted as a function of dose of [1,2-^{3}H]-testosterone injected. Whereas the final concentration of dihydrotestosterone in nuclei is in the vicinity of 250 nM, the maximal concentration in cytoplasm is 100 nM. The curves in Fig. 2b show the relative concentrations of dihydrotestosterone recovered in cytoplasm and nuclei. A 10 to 20 nM concentration of dihydrotestosterone in cytoplasm produces an intranuclear concentration of dihydrotestosterone as high as 120 nM. If dihydrotestosterone is injected into experimental animals rather than testosterone, a cytoplasmic concentration of dihydrotestosterone in the vicinity of 20 nM produces an intranuclear concentration of dihydrotestosterone in the vicinity of 250 nM (data not shown).

Since the measured concentration of dihydrotestosterone and testosterone in prostatic tissue of several species falls within the 5 to 20 nM range [3, 4, 21], it seems likely that under steady state conditions the intranuclear concentration of these androgens may be as high as 250 nM. At this concentration, the number of molecules of androgen in the nucleus is about 60,000 and therefore exceeds the number of saturable high affinity binding sites estimated at approximately 5000 [22, 23]. Consequently it is not surprising that a large amount of androgen may appear in the nucleus either weakly bound or free as has been reported before [24].

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

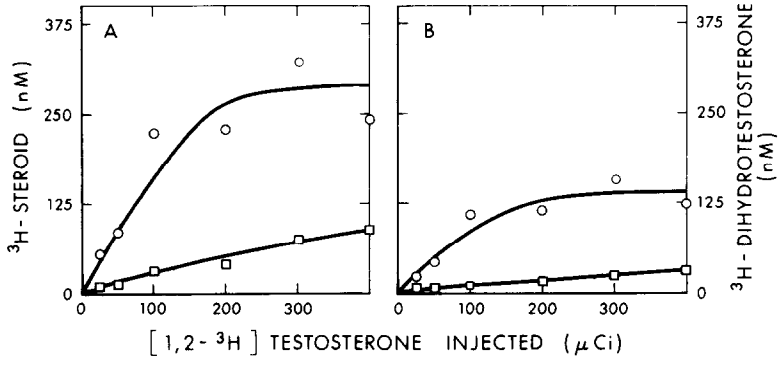

Fig. 2. *Molar concentration of androgens in cytoplasm and nuclei*
The experimental procedure was identical to that described in the legend to Fig. 1. However, the results are expressed in terms of the molar concentrations of androgens recovered in cytoplasm and nuclei, calculated as described in the "Experimental" section. Molar concentration: A, Total ^{3}H-androgens; B, ^{3}H-dihydrotestosterone. Cytoplasm, □——□; nuclei, ○——○.

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 radioactive dihydrotestosterone and testosterone into experimental animals was examined. The data in Table 1 indicates that the concentration of radioactive androgens in cytoplasm increases about thirty-fold

Table 1. Intranuclear concentration of androgens at high doses

	Concentration of ^{3}H-Androgens (Total)	
Steroid Injected	Cytoplasm	Nucleus
	nM	
[1, 2-^{3}H]testosterone	3890 ± 490	422 ± 72
[1, 2-^{3}H]dihydrotestosterone	2700 ± 130	276 ± 7

Groups of three to five rats (250 to 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-^{3}H]-testosterone and [1,2-^{3}H]-dihydrotestosterone. Sixty minutes 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 ± S.E. for each set of at least 3 experimental results.

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 maximal concentration of 250 nM previously observed. 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 transport, or to increase their release from the nucleus when the cytoplasmic concentration of androgens rises to abnormal levels.

The observation that the concentration of androgens in nuclei is ten-fold 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 by leakage of unbound steroid from the nucleus during the nuclear isolation procedure. However, when isolated nuclei themselves are incubated *in vitro* with radioactive dihydrotestosterone, the incorporation process changes to resemble passive diffusion. (N. Bruchovsky, unpublished). Not only does the intranuclear concentration of dihydrotestosterone then parallel the concentration of androgen in the 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 an effective barrier to the uncontrolled 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 radioactive dihydrotestosterone at a concentration of 750 nM. The results presented in Fig. 3a indicate that the incorporation of radioactive androgen into cytoplasm is rapid and reaches a plateau within 10 min of 350 × 10^5 d.p.m./g equivalent to a concentration of 750 nM. The incorporation of radioactive androgen into nuclei proceeds at a slower rate as shown in Fig. 3b, and reaches a plateau at 30 min of 400 × 10^3 d.p.m./g equivalent to a concentration of 60 nM. The abrupt change in this rate of incorporation and the marked discrepancy between the cytoplasmic concentration and the

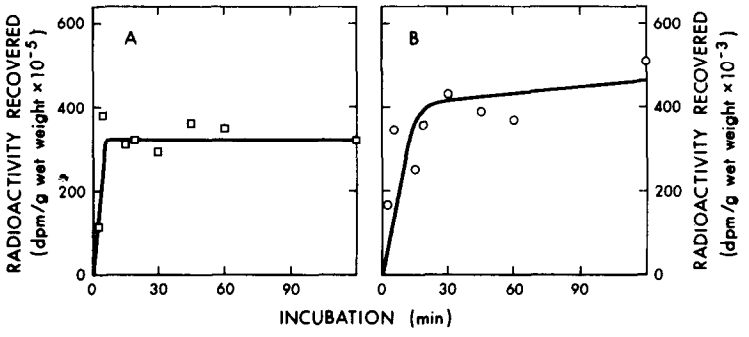

Fig. 3. In vitro *incorporation of androgens into nuclei*
Prostatic tissue of rats castrated 24 h previously was minced and incubated in tissue culture medium (2 ml) containing [1,2-^{3}H]-dihydrotesterone (750 nM) at 37° in an atmosphere of CO_2:O_2 (5:95 v/v). At the appropriate time, the incubation was terminated by the addition of 5 vol. of ice-cold tissue culture medium. 3 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.

intranuclear concentration are consistent with the idea that in whole cells the entry of androgens into the nucleus is a regulated process and that the nuclear membrane acts as a restrictive barrier to the afferent passage of androgens into the nucleus.

Time course of disappearance of androgen receptors in cytoplasm

In looking for reasons which might account for the restricted incorporation of androgens into the nucleus, we examined the possibility that the concentration of androgen receptors in either cytoplasm or nuclei might be a limiting factor. Prostate from rats castrated 24 h previously was minced and incubated at 37°C in medium containing radioactive 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 radioactive testosterone. In both experiments the disappearance of cytoplasmic androgen receptor was measured by gel-exclusion chromatography with Sephadex G-200 using either a dual-column or a single-column method. The cytoplasmic receptor (Fig. 4) which is

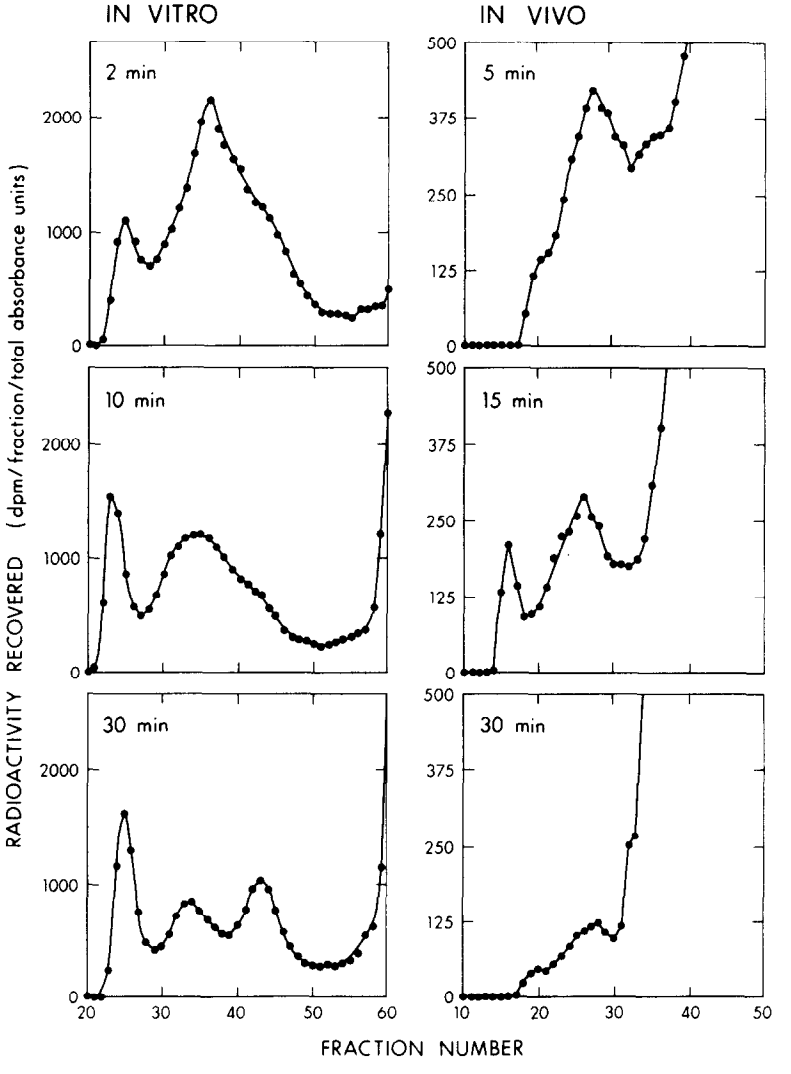

Fig. 4. *Time course of disappearance of androgen receptors in cytoplasm*
Prostatic tissue of rats castrated 24 h previously was minced and incubated in tissue culture medium (2 ml) containing [1,2-^{3}H]-dihydrotestosterone (750 nM) at 37° in an atmosphere of CO_2:O_2 (5:95 v/v) for 2, 10 and 30 min (*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-^{3}H]-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 sulphate at 80% saturation while the temperature was controlled at 0°. The precipitate was recovered by centrifugation, dissolved in Tris–EDTA buffer, and analysed by gel-exclusion chromatography with a column (0·9 cm. × 100 cm.) of Sephadex G-200 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. The radioactivity recovered in each fraction has been divided by the total number of absorbance units (absorbance measured at 280 nM) recovered from the column.

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) possesses a molecular Stokes radius of 46–48 Å, a sedimentation coefficient of 4·4 ± 0·1 Svedbergs, a molecular weight of 86,000 Daltons and a frictional ratio 1·65. In the presence of androgens this receptor rapidly disappears and is barely detectable after 30 min whether *in vitro* or *in vivo* labelling techniques are used. This period of depletion coincides approximately with the interval of 15 to 30 min which precedes the apparent shut-down of androgen transport into the nucleus. Altogether the number of cytoplasmic receptors initially present in a single prostatic cell, as determined from this data, is between 2000 and 5000.

Time course of appearance of androgen receptors in nuclei

When the nuclear fraction obtained in the previous experiments 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. 5 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 [15] and appears in the void volume; part is free and is recovered as the third of the three peaks; and part is bound to a receptor which is recovered as the second peak. The receptor possesses a molecular Stokes radius of 24 Å, a sedimentation coefficient of 3·3 ± 0·3 Svedbergs, a molecular weight of 32,700 Daltons, and a frictional ratio of 1·1. Between 2 min 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 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 about 20,000 receptor molecules and up to 60,000 androgen molecules are incorporated into the nucleus before transport stops. Because of the magnitude of these figures it is doubtful that the quantity of cytoplasmic receptor can account for the influx of androgens into the nucleus, particularly if it is assumed that androgen binding to cytoplasmic receptor followed by the translocation of androgen–receptor complex into the nucleus is a stoichiometric process. In other words, the number of androgen molecules that enter the nucleus in a regulated fashion greatly exceeds the detectable number of receptors in the cell cytoplasm. Thus if our estimate of the number of cytoplasmic receptors is a valid approximation, it appears likely that each cytoplasmic receptor promotes the transport of several androgen molecules into the nucleus, and also that some of the transported androgen molecules may themselves bind directly to chromatin.

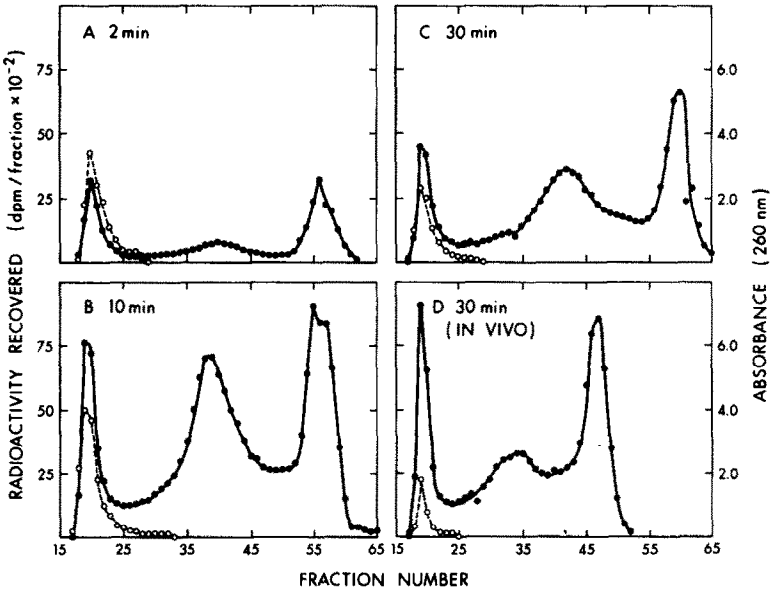

Fig. 5. *Time course of appearance of androgen receptors in nuclei*
Prostatic tissue was labelled with radioactive androgens using *in vitro* or *in vivo* labelling techniques as described in the legend to Fig. 4. The nuclear fraction was suspended in Tris–EDTA buffer containing NaCl (50 mM), sonicated, 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 per 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, O——O.

Cytoplasmic receptors and transport in Shionogi carcinoma cells

In order to study the potential action of cytoplasmic receptors in governing the intranuclear concentration of androgens, we examined the question whether any transport of androgens takes place in cells depleted of cytoplasmic receptors. For this purpose a number of variant lines of the androgen-dependent Shionogi carcinoma were surveyed in expectation that some of these might be deficient in cytoplasmic receptors and show a reduced capacity to transfer androgens into the nucleus [11]. DD/S strain mice carrying dependent and autonomous tumours of various lines were injected intravenously with 4·6 nmol (200 μCi) of radioactive testosterone; 30 min later the mice were killed and the subcutaneous tumour mass was excised. A cytoplasmic fraction was recovered from the cells and surveyed for the presence of androgen receptors. It was found that each of the dependent lines, but only two of the seven autonomous lines contained cytoplasmic receptors as detected by gel-exclusion chromatography. The receptors in such tumours are represented by two peaks of binding as shown in Fig. 6, a and b. The first peak emerges in fractions 16 to 20 which correspond to the void volume, and the second peak emerges in fractions 21 to 27. On the other hand, in tumours without receptors (Fig. 6, c and d) only a single small peak of binding is found in fractions corresponding to the void volume. We next examined the relationship between cytoplasmic receptors and the capacity of tumour cells to transport androgens into the nucleus. The results shown in Table 2 summarize the findings of studies of the autonomous lines and indicate that a high rate of transport of androgens into the nucleus appears to be correlated to the presence of cytoplasmic receptors. Thus it seems reasonable to conclude that androgen transport

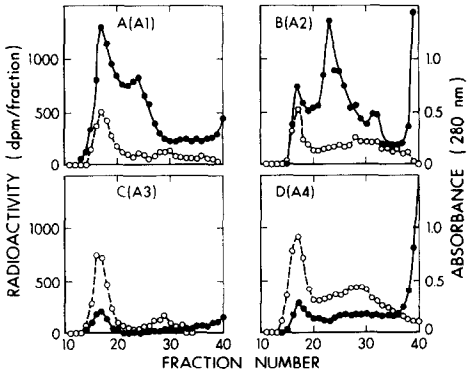

Fig. 6. *Cytoplasmic receptors in autonomous tumours*
Female mice of the DD/S strain with tumours of autonomous lines 1–7 were injected intravenously with 200 μCi (4·6 nmol) of [1,2-^{3}H]-testosterone and killed 30 min later. The protein precipitate recovered from cytosol was analysed by gel-exclusion chromatography as described in the legend to Fig. 4. Radioactivity recovered, ●——●. Absorbance at 280 nm, O——O. Analysis of binding in tumours: A and B, with receptors; C and D, without receptors.

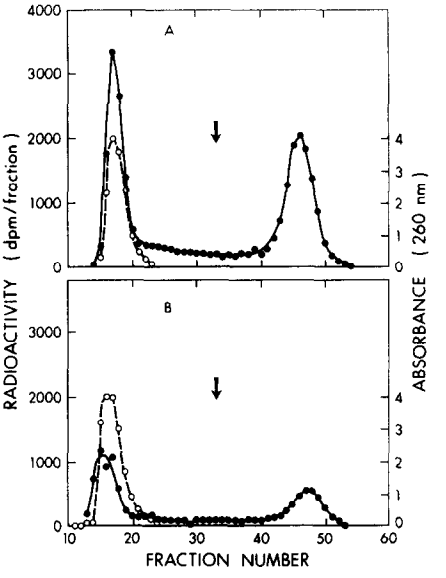

Fig. 7. *Intranuclear binding*
Male and female mice with dependent and autonomous tumours respectively were injected intravenously with 200 μCi (4·6 nmol) of [1,2-^{3}H]-testosterone and killed 30 min later. The nuclei from each tumour were extracted as described in the legend to Fig. 5, and the extract was analysed by gel-exclusion chromatography with Sephadex G-200. Fractions of 1·2–1·5 ml were collected at a flow rate of 3 ml per h. Radioactivity recovered, ●——●. Absorbance at 260 nm, O- - - -O. Panel A, dependent line 1, B, autonomous line 3. Arrow marks position of 24 Å receptor characteristic of normal target cell nuclei.

and cytoplasmic receptors are closely linked phenotypic markers of intracellular steroid–hormone action.

Androgen binding in nuclei of Shionogi carcinoma cells

An analysis of the binding of androgens in nuclei of tumour cells, whether they demonstrate high or low rates of transport, failed to reveal the presence of an intranuclear androgen receptor corresponding to the one in prostatic nuclei. Typical results are shown in Fig. 7 where the arrow marks the position of the missing receptor. The possibility that the peak of binding in the void volume represents an aggregated receptor has for the most part been ruled out by chromatographic analysis of nuclear extracts with Sepharose 6b. Although this procedure results in a significant fractionation of chromatin into its nucleic acid and protein components, the peak of binding remains associated with the void volume components (data not shown). Therefore, in order to explain the binding to excluded components, it must be assumed that aggregated receptor is very large, a possibility that seems less likely than the alternative explanations, that androgen–receptor complex is bound tightly to chromatin, or that androgens bind directly to chromatin with no requirement for receptor. The latter possibility is attractive in view of the large discrepancy between the numbers of androgen molecules and receptor molecules in nuclei of prostatic cells and in view of the usual ease with which receptor is separated from chromatin of normal target tissues such as prostate [15].

Table 2. Relationship of cytoplasmic receptors to the incorporation of ^{3}H-androgens into tumour nuclei

Tumour Line	Experiments	Radioactivity Recovered (dpm/nucleus, x10^4)	Cytoplasmic Receptors
1	3	14.1 ± 1.3	+
2	3	7.7 ± 0.6	+
3	6	2.5 ± 0.2	-
4	3	0.8 ± 0.2	-
5	3	0.7 ± 0.1	-
6	3	0.7 ± 0.1	-
7	3	0.3 ± 0.0	-

Female mice of the DD/S strain with tumours of 7 autonomous lines were injected intravenously with 200 μCi (4·6 nmoles) of [1,2-^{3}H]testosterone and the mice were killed 30 min later. Tumour nuclei were isolated and assayed for radioactivity. The results are expressed as the mean ± S.E. and the number of experiments is shown in the first column. Cytoplasmic receptors were demonstrated as described in the legend to Fig. 6.

DISCUSSION

A major objective of this work has been to assess the potential function of cytoplasmic and nuclear receptors in regulating the intranuclear concentration of androgens in two animal model systems. It has been found that androgens are incorporated into the prostatic nucleus by a regulated selective process which appears to limit the type and amount of androgens entering this structure. *In vivo* under optimal conditions about 60,000 molecules of androgen penetrate the nucleus before transport stops. In the presence of an adequate concentration of testosterone or dihydrotestosterone in cytoplasm, the transfer of these androgens into the nucleus proceeds rapidly and is virtually complete within 60 min. During this time 5000 or fewer detectable androgen receptors (mol wt. 86,000) disappear from cytoplasm and as many or more receptors (mol wt. 33,000) appear in the nucleus. Investigation of the intracellular transport of androgens in several lines of Shionogi tumour cells has also furnished evidence of a correlation between the presence of receptors in cytoplasm and the ability to transfer androgens into the nucleus. Therefore, taken together, our experimental findings are consistent with the idea that cytoplasmic receptors and androgen transport are related phenotypes, which through co-operative or independent effects may balance the intranuclear concentration of androgens. Since the number of detectable cytoplasmic receptors is relatively small it seems that several molecules of androgen are transferred into the nucleus for each molecule of receptor that either disappears from the cytoplasm or appears in the nucleus. This finding suggests that the transfer of androgens and androgen–receptor complexes may take place separately. Finally, since androgen molecules in the nucleus outnumber detectable receptor molecules it is also possible that androgens may interact directly with chromatin. This impression is supported by the apparent lack of receptor in nuclei of Shionogi tumour cells which otherwise retain the ability to transport androgens across the nuclear membrane.

In summary, although the results described in this report indicate that cytoplasmic receptors and the capacity to transport androgens into the nucleus are closely linked phenotypic markers of intracellular steroid hormone action, they leave open the possibility that the control of androgen concentration in the nucleus may be achieved by an independent process which does not rely on androgen receptors.

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