Radioimmunoassay measurements of nuclear dihydrotestosterone in rat prostate

Relationship to androgen receptors and androgen-regulated responses

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The concentration of dihydrotestosterone was measured by radioimmunoassay in nuclear and cytoplasmic extracts from rat ventral prostates. In the regenerating prostates of castrated rats treated with dihydrotestosterone for 4 days, the nuclear concentration of this steroid increased from approx. 70 nM to 800 nM as a linear function of the injected dose, whereas the cytoplasmic concentration remained relatively constant (70–130 nM). Isotope-exchange measurements of nuclear androgen receptors by using [³H]methyltrienolone indicated that, although the concentration of nuclear dihydrotestosterone was several-fold higher than the concentration of androgen receptors, they were logarithmically related. The recruitment of prostatic cells into the growth fraction and the stimulation of 5 α -reductase activity were more directly correlated to the nuclear concentration of androgen receptors than to the total nuclear concentration of dihydrotestosterone. Maximal restoration of a specific isoenzyme of acid phosphatase was achieved when approx. 2000 androgen receptors were present in the prostatic nuclei; higher concentrations of nuclear androgen receptors were associated with decreased amounts of this enzyme. Hence the results imply, first, that the total amount of dihydrotestosterone accumulated by nuclei is not a direct consequence of carrier-mediated transport by androgen receptors, and, secondly, that, whereas acid phosphatase may be differentially controlled by androgens in the regenerating prostate, increases in the amount of cell proliferation and 5 α -reductase activity directly parallel increases in the nuclear concentration of androgen receptors.

In the prostate, androgenic regulation of physiological functions and cellular growth is attributed largely to the formation, and subsequent binding to chromatin, of androgen–receptor complexes (King & Mainwaring, 1974). Results from experiments performed *in vitro* by several groups (Davies & Griffiths, 1973; Mainwaring & Jones, 1975; Hu *et al.*, 1975) have indicated that androgen–receptor complexes directly modulate the transcription of chromatin by RNA polymerase. Androgen receptors have also been implicated in the regulation of DNA synthesis and cell proliferation in the regenerating prostate (Coffey, 1974; Rennie *et al.*, 1975; Van Doorn *et al.*, 1976). Although it has been postulated that androgen receptors are involved in the uptake and retention of dihydrotestosterone by target-cell

nuclei (Rennie & Bruchovsky, 1973; Bruchovsky *et al.*, 1975a), a direct stoichiometric relationship has not been demonstrated. When castrated rats are injected with radioactive androgen in the ventral prostate the concentration of radioisotope measured in nuclei exceeds that measured in the cytoplasm (Bruchovsky *et al.*, 1975b). However, the concentration of androgen–receptor complexes recovered from the nuclei is insufficient to account for the nuclear influx of androgens if a mole-to-mole relationship is assumed. Furthermore, radioimmunoassay measurements of dihydrotestosterone in ventral prostates from non-castrated rats have indicated that only about 20% of the endogenous nuclear androgen is bound to androgen receptors (Bruchovsky *et al.*, 1980). Whether the large nuclear concentration of non-receptor-bound dihydrotestosterone is achieved through a receptor mechanism or

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by other processes (Lefebvre & Novosad, 1980; Barrack & Coffey, 1980) is uncertain.

The regenerating rat prostate provides an excellent model system for studying the effects of androgens on cellular growth and differentiation (Bruchovsky *et al.*, 1978). Specific responses in the ventral prostate, including the initiation of DNA synthesis and cell proliferation (Lesser & Bruchovsky, 1973), stimulation of 5 α -reductase activity (Shimazaki *et al.*, 1969) and the induction of secretory acid phosphatase (Rennie *et al.*, 1978), are elicited by different doses of dihydrotestosterone. In this context, cell proliferation is a dominant response, whereas the induction of secretory acid phosphatase, a marker of differentiation, is a subordinate one. The effects of the administration of different doses of dihydrotestosterone on cell proliferation, 5 α -reductase activity and secretory acid phosphatase activity in regenerating rat prostate were correlated with the intranuclear concentration of both dihydrotestosterone and androgen receptors to determine whether fluctuations in either of the latter parameters were directly related to specific cellular responses.

Materials and methods

Experimental animals

Male Wistar rats, weighing 275–325 g, purchased from Woodlyn Laboratories (Guelph, Ont., Canada), were castrated through a scrotal incision while the animals were under diethyl ether anaesthesia. Then, 7 days later, various doses of dihydrotestosterone (17 β -hydroxy-5 α -androstane-3-one) in 1 ml of a solution of 10% (v/v) ethanol and 10% (v/v) polyoxyethylene sorbitan monolaurate were administered daily by subcutaneous injection. After 4 days of treatment, the rats were decapitated and the ventral prostates were removed.

Preparation of subcellular fractions

All procedures were performed at 4°C. The pooled ventral prostates (1–2 g) from five to ten rats were minced with scissors, forced through a stainless-steel screen (30 mesh; 0.55 mm grid) with a Teflon pestle and then homogenized in 20–40 ml of 10 mM-Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid)/NaOH buffer (pH 7.0)/0.25 M-sucrose / 1.5 mM-CaCl₂ / 0.5 mM-2-mercaptoethanol with a Dounce homogenizer (25 and 10 strokes of the large-clearance and small-clearance pestles respectively). After samples were removed for radioimmunoassay (3 $\times$ 1 ml) and protein determinations (3 $\times$ 100 μ l), the homogenate was centrifuged at 800 g for 15 min (Sorvall GLC-1 centrifuge, HL rotor, r_{av} 12.5 cm).

The supernatant (cytoplasm), containing both intraluminal and intracellular material, was saved

for acid phosphatase analysis and the pellet was re-suspended in 50–60 ml of 10 mM-Tes/NaOH buffer (pH 7.0) / 0.88 M-sucrose / 1.5 mM-CaCl₂ / 0.5 mM-2-mercaptoethanol. Samples of the latter were taken for DNA determinations, and the remainder, in 25 ml portions, was layered over a discontinuous sucrose gradient composed of 5 ml of 1.8 M-sucrose and 5 ml of 2.2 M-sucrose, both in 10 mM-Tes/NaOH buffer (pH 7.0)/0.5 mM-CaCl₂. The sucrose gradients were centrifuged at 80000 g for 100 min (SW27 rotor, r_{av} 11.56 cm) in a Beckman L8-80 ultracentrifuge. The purified nuclei recovered in the pellet were resuspended in 10 mM-Tes/NaOH buffer (pH 7.0), and samples were taken for DNA measurements, for radioimmunoassay, and for counting in a haemocytometer after staining with aq. 1% (w/v) Methylene Blue. The nuclei were re-centrifuged through a sucrose gradient, or discarded if more than 10% of the nuclei were contaminated with cytoplasmic tags or present as clumps.

Preparation of nuclear extracts

The purified nuclei (0.5×10^8 – 1.5×10^8) were centrifuged at 800 g for 15 min, resuspended in 0.8 ml of 1 mM-Tris/HCl buffer (pH 8.0)/0.1 mM-CaCl₂ and left standing on ice for 30 min. After this, the suspension was sonicated for three 5 s pulses at a setting of 40 (yellow code) with a Biosonik III instrument (Bronwill Scientific, Rochester NY, U.S.A.) and adjusted to 1 ml with 1 mM-Tris/HCl buffer (pH 8.0)/0.1 mM-CaCl₂ containing micrococcal nuclease (Sigma Chemical Co., St. Louis, MO, U.S.A.) to a final concentration of 4 units/10⁷ nuclei. After the mixture was incubated for 30 min at 20°C, the reaction was terminated by chilling on ice and by adjusting the digest to 5 mM-Na₄EDTA. Centrifugation of the sample in a Beckman Airfuge at 100000 g for 45 min yielded a pellet, which was partially solubilized in 0.6 M-NH₄HCO₃ buffer (pH 7.5)/1.5 mM-Na₄EDTA/0.5 mM-2-mercaptoethanol. After further centrifugation at 100000 g for 20 min, the supernatant was decanted and the pellet was resuspended in 1.2 M-NH₄HCO₃. The suspension was again centrifuged at 100000 g for 20 min, and the supernatant was pooled with that obtained in the previous step, giving the nuclear extract used in the receptor assay.

Assay for nuclear receptor

One half of a sample of nuclear extract (0.5–0.7 ml) was incubated with 20 nM of the synthetic androgen [17 α -Me-³H]methyltrienolone (R1881; 17 β -hydroxy-17 α -methyl-4,9,11-oestratrien-3-one), and the other half was incubated with both radioactive methyltrienolone and 1000-fold excess (20 μ M) of unlabelled dihydrotestosterone. After incubation for 16 h at 4°C, both samples were

analysed by gel-exclusion chromatography in a dual-column apparatus consisting of a column (0.5 cm × 15 cm) of Sephadex G-25 connected in series with a column (0.5 cm × 60 cm) of Sephadex G-100 (Bruchovsky *et al.*, 1980). Samples were eluted in an upward direction with 10 mM-Tes/NaOH buffer (pH 7.0)/0.6 M-NaCl/1.5 mM-Na₄EDTA/0.5 mM-2-mercaptoethanol at a flow rate of 2–3 ml/h, and 0.5 ml fractions were assayed for radioactivity. An estimate of specific displaceable binding was calculated from the difference in the bound radioactivity of the sample without competitor and of the one with competitor.

Radioimmunoassay for dihydrotestosterone

Details of this procedure are given elsewhere (Bruchovsky *et al.*, 1980). Briefly, samples containing an external standard of [1,2-³H]dihydrotestosterone were extracted with 4 ml of ethyl acetate/hexane (1:9, v/v), and the organic phase was applied to a column (0.6 cm × 6.5 cm) of alumina (Furuyama *et al.*, 1970). After the column had been washed with 1.6 ml of ethyl acetate/hexane (1:9, v/v), dihydrotestosterone was eluted with 0.45% (v/v) ethanol/hexane (9:191, v/v) (recovery 70–80%). Samples of the purified dihydrotestosterone were dried in glass tubes and incubated with 250 µl of dilute dihydrotestosterone antiserum (DT3-154) (Endocrine Sciences, Tarzana, CA, U.S.A.) at 37°C for 1 h and then at 20°C for 2 h. The chromatographic purification of dihydrotestosterone obviated any cross-reaction problems with the antiserum. After addition of 250 µl of saturated (NH₄)₂SO₄ and removal of the precipitate by centrifugation, the supernatant was assayed for radioactivity and the androgen content was determined from a standard curve. The sensitivity of the assay was found to be 20 pg/ml, and the within-assay and between-assay coefficients of variation were 5.0% and 9.7% respectively.

Assay for 5α-reductase activity

Ventral prostates, obtained from three to six rats, were minced with scissors and homogenized in 10 mM-Tes / NaOH buffer (pH 7.0) / 0.05 mM-Na₄EDTA / 0.5 mM-2-mercaptoethanol / 5 mM-MgCl₂/50 mM-NaCl with three 7 s pulses (setting 6) of a Polytron homogenizer unit equipped with a PT 10 probe. Samples of the homogenate containing 0.3–0.6 mg of protein were incubated at 37°C for 30 min in 2 ml of an incubation mixture composed of 50 nM-[1,2-³H]testosterone and an NADPH-generating system (0.5 mM-NADP⁺, 5 mM-glucose 6-phosphate and 0.5 unit of glucose 6-phosphate dehydrogenase). The reaction was stopped by the addition of 10 ml of chloroform/methanol (2:1, v/v). Steroids were extracted, identified by t.l.c. and quantified as described by Bruchovsky &

Lieskovsky (1979). Measurement of 5α-reductase activity was based on the percentage formation of dihydrotestosterone and 3α(β)-androstenediol (5α-androstane-3α/3β,17β-diol) from testosterone.

Assay for acid phosphatase

The initial 800 g supernatant (cytoplasm) obtained during the preparation of subcellular fractions was subjected to three cycles of freezing and thawing according to the method of Harding & Samuels (1961), 80% saturated with (NH₄)₂SO₄ and then centrifuged at 17000 g for 20 min (Sorvall RC-5 centrifuge, GSA rotor, *r*_{av}, 14.6 cm). The pellet was resuspended in 50 mM-sodium acetate buffer (pH 4.8) and dialysed at 4°C for 18 h against three 3-litre changes of 50 mM-sodium acetate buffer (pH 4.8). The dialysis residue was adjusted to 33% (v/v) glycerol and then either assayed immediately or stored at –20°C.

Isoenzymes of acid phosphatase in the dialysis residues were analysed as described previously (Rennie *et al.*, 1978). Samples (50–400 µg of protein/50–150 µl) were applied to cationic disc gels, composed of a 7.5% (w/v) polyacrylamide (acrylamide/NN'-methylenebisacrylamide, 37.5:1, w/w) separating gel and a 2.5% (w/v) polyacrylamide (acrylamide/NN'-methylenebisacrylamide, 4:1, w/w) stacking gel (Williams & Reisfeld, 1964; Tenniswood *et al.*, 1976), and electrophoresed at a current of 4 mA/gel for 2.5 h at 4°C. After this, the gels were incubated for 1.5 h at 20°C with 10 ml of 50 mM-sodium acetate buffer (pH 4.8) containing α-naphthyl phosphate (1 mg/ml) and Fast Garnet GBC (1 mg/ml), rinsed several times with 7% (v/v) acetic acid, photographed and then scanned at 595 nm with a Gilford model 250 spectrophotometer (Gilford Instruments, Oberlin, OH, U.S.A.).

Other analytical procedures

Protein was measured by the Bradford (1976) method by using the Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.), with bovine γ-globulin as the reference standard. DNA determinations were done by the diphenylamine method (Burton, 1956), with calf thymus DNA as a standard.

Radioactivity was counted in a Beckman LS 7500 liquid-scintillation system with Ready-Solv EP (Beckman) scintillation 'cocktail' for aqueous samples (counting efficiency 45%) and a solution of diphenyloxazole in toluene (4 g of diphenyloxazole/l of toluene) for non-aqueous samples (counting efficiency 45%).

Chemicals

[1,2-³H]Dihydrotestosterone (50.6 Ci/mmol), [1,2-³H]testosterone (40 Ci/mol) and [17α-Me-³H]-

methyltrienolone (87 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Purity was checked by t.l.c. (Bruchovsky & Lieskovsky, 1979), and only preparations of at least 95% purity were used.

Non-radioactive steroids were supplied by Steraloids (Pawling, NY, U.S.A.), and reagents used to prepare buffers were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All aqueous solutions were made with deionized glass-distilled water.

Results

Effects of dose on the intracellular concentration of dihydrotestosterone

Although the stimulation of DNA synthesis and cell proliferation in the ventral prostates of castrated rats treated with high doses of dihydrotestosterone (400–800 µg/100g body wt.) is well documented (Coffey *et al.*, 1968; Lesser & Bruchovsky, 1973; Rennie *et al.*, 1975), the precise intracellular concentration of dihydrotestosterone necessary to obtain this and other androgen-dependent responses is not known. To determine the relationship between dose and intracellular concentration of androgen, groups of rats that had been castrated 7 days previously were given daily injections of dihydrotestosterone over a period of 4 days. After this, the amount of dihydrotestosterone present in the cytoplasm and nuclei of their prostates was measured by radioimmunoassay. The molar concentrations of dihydrotestosterone in these subcellular fractions are calculated on the basis of previous volume estimates for each cellular compartment (Bruchovsky *et al.*, 1975b) and are presented in Fig. 1. In the untreated 7-day-castrated animals (controls) both the prostate cytoplasm and nuclei have similar endogenous concentrations of dihydrotestosterone. Administration of low doses of this androgen result in an initial fall in the cytoplasmic concentration of dihydrotestosterone, whereas at a dose of 100 µg/100g body wt. the concentration of dihydrotestosterone is increased above control values. Higher doses, however, do not increase the cytoplasmic concentration of this androgen appreciably. By comparison, there is a linear relationship between the dose of dihydrotestosterone injected and the concentration attained in prostatic nuclei (correlation coefficient 1.00). At the highest dose tested (400 µg/100g body wt.) the nuclear concentration of dihydrotestosterone is approx. 40% higher than the mean endogenous concentration of this androgen in prostatic nuclei from non-castrated rats (591 ± 46 nM; mean ± S.E.M., $n = 4$). Furthermore, the pattern of nuclear assimilation of dihydrotestosterone differs in both magnitude and

Fig. 1. *Effects of dose on the cytoplasmic and nuclear concentrations of dihydrotestosterone*

Groups of five to ten rats, castrated 7 days previously, were treated daily with subcutaneous doses of dihydrotestosterone. After 4 days of treatment the animals were killed and their prostates were homogenized. Samples of the homogenates were removed, and from the remainder nuclei were isolated. Radioimmunoassay measurements for dihydrotestosterone were performed on extracts from homogenates and purified nuclei as described in the Materials and methods section. Estimates of cytoplasmic concentrations were obtained from the difference between the homogenate concentration and the nuclear concentration of dihydrotestosterone, corrected for DNA recoveries. The results are expressed as means ± S.E.M. for at least three separate determinations. O, Cytoplasmic concentration of dihydrotestosterone; ●, nuclear concentration of dihydrotestosterone.

saturability from that observed after a single injection of radioactive androgen (Bruchovsky *et al.*, 1975b; Van Doorn & Bruchovsky, 1978). In the latter studies, an approx. 250 nM concentration of androgen was the maximal intranuclear value attained under optimal conditions (a 60 min pulse of 2–3 µg of either radioactive testosterone or dihydrotestosterone). Taken together, the results imply that the process whereby prostatic nuclei accumulate dihydrotestosterone is rate-limited (i.e. 250 nM-androgen/h) but of high capacity (i.e. ≥800 nM; Fig. 1).

Relationship between androgen receptors and the nuclear concentration of dihydrotestosterone

Nuclei, isolated from the prostates of 7-day-castrated rats, treated with dihydrotestosterone, were analysed for androgen receptors by using an isotope-exchange assay with [17α -Me- 3 H]methyltrienolone. The synthetic androgen methyltrienolone was used in these experiments, since it binds to receptor sites with a higher affinity than dihydrotestosterone and is resistant to metabolism (Bonne & Raynaud, 1975, 1976). The results of gel-exclusion chromatography of a nuclear extract from rats treated with 40 μ g of dihydrotestosterone/100g body wt. are shown in Fig. 2. In agreement with previous results (Van Doorn *et al.*, 1976; Van Doorn & Bruchovsky, 1978), the amount of

displaceable binding to androgen receptor can be readily quantified by measuring the difference in radioactivity (fractions 17–30 in Fig. 2) in samples that have been incubated with radioactive androgen alone and in duplicates that have been incubated with tracer plus a 1000-fold excess of unlabelled androgen.

By using the isotope-exchange assay and gel-exclusion chromatography, estimates were made of the amount of nuclear androgen receptors in prostates from rats treated with various dosages of dihydrotestosterone, and these values were compared with results of radioimmunoassay measurements of total nuclear dihydrotestosterone. The results (Fig. 3) indicate that in regenerating prostate the nuclear concentration of androgen receptors is directly proportional to the logarithm of the concentration of dihydrotestosterone (correlation coefficient 0.92). This logarithmic relationship indicates, first, that the concentration of dihydrotestosterone retained by prostatic nuclei after daily injections of this androgen is always several-fold higher than the concentration of androgen receptors, and, secondly, that fluctuations in both the nuclear concentration of dihydrotestosterone and its recep-

Fig. 2. *Measurement of nuclear androgen receptors* A nuclear extract, prepared from the pooled prostates of eight rats (7-day-castrated) that had been treated for 4 days with a daily dosage of 40 μ g of dihydrotestosterone/100g body wt., was divided into two equal portions and incubated at 4°C for 16 h with either 20 nM-[17α -Me- 3 H]methyltrienolone (87 Ci/mmol) alone or together with a 1000-fold excess (20 μ M) of unlabelled dihydrotestosterone. After incubation the samples were analysed for binding by Sephadex G-25/G-100 dual-column chromatography. For experimental details see the text. The radioactivity recovered in 0.5 ml fractions was measured as follows: ●, after incubation with labelled steroid alone; ○, after incubation with labelled steroid and a 1000-fold excess of unlabelled dihydrotestosterone.

Fig. 3. *Relationship between nuclear androgen receptors and the nuclear concentration of dihydrotestosterone* Nuclei were isolated from the prostates of groups of five to ten 7-day-castrated rats that had been treated for 4 days with daily doses of dihydrotestosterone as shown in Fig. 1. Each point represents the mean of at least two experiments in which nuclear preparations were assayed both for nuclear androgen receptors by using the isotope-exchange assay and gel-exclusion chromatography, and for the nuclear concentration of dihydrotestosterone by using radioimmunoassay techniques. For experimental details see the text.

tor occur as related events. With regard to the first point, under the steady-state conditions present in non-castrated rats, the endogenous nuclear concentration of dihydrotestosterone (18.8 ± 1.5 pmol/mg of DNA; mean $\pm$ S.E.M., $n = 3$) is also several times greater than the comparable concentration of androgen receptor (1.0 ± 0.1 pmol/mg of DNA; mean $\pm$ S.E.M., $n = 4$).

Relationship between cell proliferation and the concentration of androgen receptors

The fraction of the cell population in the prostate that is involved in cell proliferation (growth fraction, *GF*) can be estimated by using the derivation of Cleaver (1967) as modified by Lesser & Bruchovsky (1974). The mathematical equation for the calculation of *GF* is as follows:

$$N_t/N_0 = (1 + GF)^{t/T}$$

where t is the total time of treatment (96 h), T is the cell-cycle time (20 h; Lesser & Bruchovsky, 1974), N_t is the number of cells at t and N_0 is the number of cells at $t = 0$ (7-day-castrated control). When the equation is solved for *GF* with cell number estimates based on nuclear counts and DNA recoveries after treatment with different doses of dihydrotestosterone, the curve shown in Fig. 4(a) can be constructed. From this result it is apparent that even low doses of androgen ($20 \mu\text{g}/100\text{g}$ body wt.) can effectively stimulate a degree of cell proliferation after 4 days of treatment.

When the molecules per nucleus of dihydrotestosterone and androgen receptor are plotted as a

function of growth fraction, the results shown in Fig. 4(b) are obtained. A linear relationship is observed between the mean number of receptors per nucleus and the growth fraction in the prostate (correlation coefficient 0.96). Above a threshold value of approx. 600 receptor molecules per nucleus, each increment in the number of androgen receptors is paralleled by an increase in the fraction of prostate cells involved in the growth process. By comparison, changes in the total number of dihydrotestosterone molecules per nucleus are not in phase with changes in the growth fraction. Hence the recruitment of cells into the cell-cycle may be more sensitive to androgen receptor concentration than to the nuclear concentration of dihydrotestosterone.

Effects of dose of dihydrotestosterone on 5 α -reductase activity

The activity of 5 α -reductase, the enzyme that converts testosterone into dihydrotestosterone in the ventral prostate, is decreased after castration, but can be restored to non-castrated-animal or higher values by pharmacological doses of androgens (Shimazaki *et al.*, 1969; Moore & Wilson, 1973). To determine whether the restoration of 5 α -reductase activity is related to the nuclear accumulation of either dihydrotestosterone or androgen receptor, prostatic homogenates, prepared from 7-day-castrated rats that had been treated with dihydrotestosterone, were assayed for this enzyme. The results shown in Table 1 indicate that the specific activity (pmol/h per mg of protein) of 5 α -reductase increases from the castrated-control values to

Fig. 4. *Effects of dose of dihydrotestosterone on cell proliferation and the nuclear concentration of androgen receptors* (a) Estimates of the number of nuclei, obtained from the experiments described in the legend to Fig. 1, were used to calculate the growth fraction according to the mathematical formulation given in the text. (b) The values obtained for the nuclear concentration of androgen receptors and dihydrotestosterone, shown in Fig. 3, were replotted as a function of the calculated growth fractions. For experimental details see the text. ●, Androgen receptor concentration; ○, dihydrotestosterone concentration.

non-castrated-animal values after 4 days of treatment with the lowest dose (20 µg/100 g body wt.) of dihydrotestosterone used in these experiments. Although specific activities approx. 50% higher than those measured in the prostates of non-castrated rats are observed after administration of the 40 µg /100 g body wt. dose, further increases in dosage are only marginally more effective.

When the 5α-reductase activities are expressed on a per-DNA basis (Table 1), only high doses of dihydrotestosterone (≥200 µg/100 g body wt.) effectively restore the enzyme activity to the value measured in the non-castrated controls. The amount of 5α-reductase activity in this latter group was not exceeded in the prostates of any of the treatment

Table 1. Effects of dose of dihydrotestosterone on 5α-reductase activity

Groups of three to six rats, which had been castrated 7 days previously, were given daily subcutaneous injections of various doses of dihydrotestosterone over a 4-day period. After this, the prostates were removed, homogenized and assayed for 5α-reductase activity. For experimental details see the text. The results are expressed as means ± S.E.M. for three separate determinations.

Dose (µg/100 g body wt.)	5α-Reductase activity (pmol/h)	
	(per mg of protein)	(per 10 µg of DNA)
0	45 ± 2	7 ± 1
20	106 ± 2	31 ± 2
40	166 ± 7	42 ± 6
200	161 ± 3	63 ± 1
400	205 ± 10	60 ± 5
Control (non-castrated)	106 ± 4	54 ± 4

groups. As was observed with the measurements of the growth fraction, a linear correlation is obtained (correlation coefficient 0.98) when the nuclear androgen receptor concentrations are plotted against the 5α-reductase values (results not shown).

Effects of dose of dihydrotestosterone on a specific isoenzyme of acid phosphatase

On the basis of sensitivity to inhibitors and relative mobility in cationic polyacrylamide gels, two major isoenzymes of acid phosphatase can be distinguished in extracts from rat ventral prostate (Tenniswood *et al.*, 1976; Rennie *et al.*, 1978). The secretory form of acid phosphatase is androgen-dependent, tissue-specific and faster-moving during cationic gel electrophoresis than is the lysosomal form, which is insensitive to androgens. Since secretory acid phosphatase is restored in the prostates of castrated rats treated with low doses of dihydrotestosterone, yet inhibited by the administration of high doses of androgen to these animals (Rennie *et al.*, 1978), experiments were done to re-examine this differentially controlled response in the context of the nuclear concentration of dihydrotestosterone and androgen receptors required for maximal induction.

Cytoplasmic extracts, prepared from the ventral prostates of 7-day-castrated rats that had been treated for 4 days with different doses of dihydrotestosterone, were analysed for isoenzymes of acid phosphatase by using polyacrylamide gels and specific substrate staining (Fig. 5). At doses of dihydrotestosterone of 20 and 40 µg/100 g body wt., band B, corresponding to the secretory isoenzyme, is evident; at higher doses this form of acid phosphatase is not detected. The relative amounts of this isoenzyme in each sample are compared in Table 2.

Fig. 5. Effects of dose of dihydrotestosterone on a specific isoenzyme of acid phosphatase

Samples of cytoplasmic protein (150 µg), prepared as described in the Materials and methods section, were electrophoresed on 7.5% (w/v) cationic polyacrylamide gels at a current of 4 mA/gel for 2.5 h at 4°C. Acid phosphatase isoenzymes were stained with a solution of α-naphthyl phosphate (1 mg/ml) and Fast Garnet GBC (1 mg/ml) in 50 mM-sodium acetate buffer, pH4.8. The gels were fixed in 7% (v/v) acetic acid and photographed. Results are shown for prostatic extracts from 7-day-castrated rats treated daily for 4 days with (1) vehicle alone or with (2) 20 µg, (3) 40 µg, (4) 100 µg or (5) 400 µg of dihydrotestosterone per 100 g body wt., and from (6) non-castrated control rats.

Table 2. *Effects of dose of dihydrotestosterone on a specific isoenzyme of acid phosphatase*

The band-B area of gels prepared as described in the legend to Fig. 5 was scanned at 595 nm and the results are expressed as means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. For experimental details see the text.

Dose ($\mu\text{g}/100\text{g}$ body wt.)	A_{595}
0	0.059 ± 0.018 (3)
20	0.423 ± 0.010 (7)
40	0.157 ± 0.026 (6)
100	0.124 ± 0.031 (7)
400	0.075 ± 0.013 (5)
Control (non-castrated)	0.653 ± 0.030 (3)

Gel scans at 595 nm of the appropriate regions of each gel indicate that relative to the band intensity in samples from non-castrated controls (100%) the androgen dose of $20\mu\text{g}/100\text{g}$ body wt. is the most effective for restoring the isoenzyme (65%) in the treated rats. The concentrations of dihydrotestosterone and androgen receptors measured in nuclei of animals treated with this dosage were 3.47 ± 0.42 and $0.31 \pm 0.06\text{ pmol/mg}$ of DNA (means $\pm$ S.E.M., $n = 7$ and 3 respectively). As both of these latter values increase in the regenerating prostate, there is a substantial decrease in the recovery of the band-B isoenzyme of acid phosphatase. Hence, unlike the other androgen-regulated responses examined in the regenerating prostate, the restoration of secretory acid phosphatase is not directly associated with fluctuations in the nuclear content of androgen receptors.

Discussion

Radioimmunoassay measurements of dihydrotestosterone in subcellular fractions of the regenerating prostate (Fig. 1) confirm previous impressions (Bruchovsky *et al.*, 1975b) that prostatic nuclei are able to accumulate androgens at much higher concentrations than those measured in the cytoplasm. During the 7-day period after castration, the intranuclear concentration of dihydrotestosterone is decreased by approx. 90% from 591 nM to 69 nM. After 4 days of treatment with this androgen the concentration of dihydrotestosterone attained by prostatic nuclei is increased as a linear function of the injected dose to a value exceeding that of the non-castrated controls (Fig. 1). From these results it is apparent that previous estimates of the nuclear uptake of androgens (250 nM; Bruchovsky *et al.*, 1975b) are more indicative of the maximum rate (250 nM/h) rather than the maximum capacity ($\geq 800\text{ nM}$) of nuclei to retain androgens.

Considering the large numerical differences between the concentration of dihydrotestosterone and the concentration of androgen-receptor sites in nuclei (Fig. 3), a mechanism for androgen transport in which androgen receptors are the sole carriers of dihydrotestosterone would imply that either (1) the receptors rapidly shuttle back and forth between the cytoplasm and nucleus, yielding a net surplus of androgens in the nucleus, or (2) the recovery of androgen receptors in the nucleus is only 10–20% of the actual concentration. If the first possibility were valid, then one would expect that the concentration of androgen receptors would always be evenly distributed between the cytoplasm (i.e. loading) and nucleus (i.e. unloading). However, there is no evidence to support this hypothesis; rather, it has been demonstrated (Mainwaring & Peterken, 1971; Rennie & Bruchovsky, 1973; Van Doorn & Bruchovsky, 1978) that administration of androgen results in a net increase in the concentration of androgen receptors in the nucleus that is stoichiometrically equivalent to a net decrease of androgen receptors in the cytoplasm. Although the second hypothesis cannot be completely discounted, two points argue against its acceptance: first, preparation of nuclei in the presence of a proteinase inhibitor alters the intranuclear distribution of specific dihydrotestosterone-binding sites but not their recovery (20 600 sites per nucleus; Barrack & Coffey, 1980); secondly, even with very high reported estimates for nuclear androgen receptors (26 000 sites per nucleus) the ratio of total nuclear dihydrotestosterone to receptor is too high (5:1) to be due to recovery alone (Bruchovsky *et al.*, 1980).

A more plausible explanation to account for the large discrepancy between the nuclear concentration of total and receptor-bound dihydrotestosterone is that the entry of dihydrotestosterone into the nucleus occurs largely by a non-receptor-mediated process. The presence of specific androgen-binding sites on the nuclear membrane (Lefebvre & Novosad, 1980) and nuclear matrix (Barrack & Coffey, 1980) of prostatic cells has been reported, and these appear to be potential candidates for this role. However, although androgen receptors may not directly mediate the nuclear accumulation of dihydrotestosterone, the uptakes of total dihydrotestosterone and androgen receptors by target-cell nuclei are probably closely linked events. In androgen-dependent and androgen-autonomous variants of the Shionogi mouse mammary carcinoma, nuclear uptake of androgens is only observed in tumour cells that have demonstrable cytoplasmic androgen receptors (Bruchovsky & Rennie, 1978). The results shown in Fig. 3 support this impression, in that the concentration of androgen receptors in prostatic nuclei is logarithmically related to the concentration of

nuclear dihydrotestosterone (correlation coefficient 0.92).

The pattern for androgenic regulation of the cell proliferation (Fig. 4) and 5 α -reductase activity (Table 1) in the regenerating prostate follows fluctuations in the nuclear content of androgen receptors more directly than changes in the nuclear concentration of dihydrotestosterone. The concomitant effects of the administered dose of dihydrotestosterone on both cell proliferation and androgen receptor concentration in the prostate are in good agreement with the observed temporal relationship between these observed two parameters, as reported by Van Doorn *et al.* (1976). Increases in the concentration of nuclear androgen receptors in the regenerating prostate occur as a consequence of androgenic stimulation of receptor synthesis as opposed to recycling of pre-existing androgen receptors (Van Doorn *et al.*, 1976; Van Doorn & Bruchovsky, 1978). The restoration of 5 α -reductase activity to values found in prostates from non-castrated rats is achieved in regenerating prostates after 4 days of treatment with androgen (Shimazaki *et al.*, 1969), and is dose-dependent (Table 1). As with the growth-fraction data, there is a linear correlation (correlation coefficient 0.98) between the concentration of nuclear androgen receptor and 5 α -reductase activity.

The extent to which secretory acid phosphatase is induced by androgen treatments (Fig. 5 and Table 2) is not directly proportional to the concentration of either nuclear dihydrotestosterone or its receptor. Although this enzyme has been shown to be androgen-dependent (Rennie *et al.*, 1978), the mechanism through which it is regulated may not involve androgen receptors. Alternatively, in the regenerating prostate, the appearance of secretory acid phosphatase may be controlled through the binding of androgen receptors to a finite number of specific chromatin acceptor sites (approx. 2000 per nucleus). As additional acceptor sites are filled by androgen receptors, cell proliferation becomes the dominant response and the induction of proteins such as secretory acid phosphatase is postponed until after replication (Rennie *et al.*, 1978).

Although proof of the participation of androgen receptors in the direct control of cell proliferation, 5 α -reductase activity and secretory acid phosphatase activity is contingent on a fuller understanding of the interaction of androgen receptors with chromatin acceptor sites (Davies & Griffiths, 1973; Mainwaring *et al.*, 1976; Rennie, 1979), the results obtained in the present investigation favour the hypothesis that androgen receptors, rather than non-receptor-bound nuclear androgen, modulate cell proliferation and 5 α -reductase activity. Since the association constant (K_a) for the binding of dihydrotestosterone to chromatin-bound receptor sites

is of the order of $5 \times 10^7 \text{ M}^{-1}$ (Rennie, 1979), and since the nuclear concentration of receptor sites is far lower than the concentration of dihydrotestosterone, the most obvious function of the excess nuclear dihydrotestosterone is to ensure that all the receptor sites within the nucleus are occupied with androgen.

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