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Testosterone Action in the Rat Ventral Prostate

THE EFFECTS OF DIETHYLSTILBOESTROL AND CYPROTERONE ACETATE ON THE METABOLISM OF [^{3}H]TESTOSTERONE AND THE RETENTION OF LABELLED METABOLITES BY RAT VENTRAL PROSTATE *IN VIVO* AND *IN VITRO*

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Recent reports have indicated that the prior metabolism of testosterone by the secondary sexual tissues may be necessary for its androgenic effect. The effects of two anti-androgens, diethylstilboestrol and cyproterone acetate (17α-acetoxy-6-chloro-1,2α-methylenepregna-4,6-diene-3,20-dione) used in the chemotherapy of human prostatic carcinoma, have been examined on both the metabolism of testosterone and the retention of its metabolites by the rat ventral prostate gland. Cyproterone acetate was found to inhibit the retention of labelled metabolites of [^{3}H]-testosterone by prostatic nuclei, both *in vivo* and *in vitro*. This inhibition appeared to be competitive. In contrast with its effect on nuclear retention of metabolites of testosterone, cyproterone acetate had no significant effect on the metabolism of [^{3}H]testosterone by rat ventral prostate tissue. Diethylstilboestrol similarly had little effect on the metabolism of [^{3}H]testosterone by prostatic tissue, although it did appear partially to inhibit its initial metabolism in all the incubation systems used. Diethylstilboestrol inhibited the nuclear retention of dihydrotestosterone when both [^{3}H]testosterone and diethylstilboestrol were injected intraperitoneally *in vivo*, but had no effect on dihydrotestosterone retention when both testosterone and diethylstilboestrol were supplied directly to the prostate either *in vivo* or *in vitro*. It was concluded that if diethylstilboestrol has an anti-androgenic effect at the level of the target organ as distinct from its effect on androgen production by the testes, then it is probably due to a mechanism differing from that of cyproterone acetate.

Recent investigations (Bruchovsky & Wilson, 1968*a*,*b*; Anderson & Liao, 1968; Belham, Neal & Williams, 1969*a*) have indicated the possible involvement of dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) in the response of rat ventral prostate to the administration of testosterone. It has also been reported that the anti-androgen cyproterone acetate (17α-acetoxy-6-chloro-1,2α-methylenepregna-4,6-diene-3,20-dione) inhibits the retention of dihydrotestosterone by prostatic nuclei (Belham, Neal & Williams, 1969*b*; Fang & Liao, 1969). We have therefore carried out a study of the effects of two anti-androgens, diethylstilboestrol and cyproterone acetate, used in the chemotherapy of human prostatic carcinoma, on the metabolism of testosterone both *in vivo* and *in vitro*. The effects of these anti-androgens on the retention of labelled metabolites of testosterone by rat ventral prostatic tissue have also been examined.

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MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (250–300 g) were used throughout this study. Bilateral orchidectomy was carried out via the scrotal route under ether anaesthesia. Castrated animals were used throughout this study to increase the amount of labelling observed. Previous experiments indicated that similar patterns of results were obtained if intact animals were used. Labelled testosterone was injected, either intraperitoneally in saline (0.88% NaCl; 1 ml) or, in some experiments, directly into the ventral prostate. In the latter case the gland was exposed by means of a median ventral incision made under ether anaesthesia. A solution (0.05 ml) containing the steroid dissolved in saline was injected into each lobe of the ventral prostate, and the incision was then closed with suture clips. After the required period the animal was killed and the prostate removed. Cyproterone acetate and diethylstilboestrol were administered *in vivo* in two ways. They were injected either intraperitoneally, in suspension (5 mg/ml) twice daily, or directly into the prostate (0.1 ml) as described above. Solutions of these anti-androgens were prepared in propane-1,2-diol, and diluted to the required concentration with saline. Similar concentrations of the propane-1,2-diol were injected into the control animals. In some experiments steroid pellets

(10 mg) were implanted into the animal's flanks by using an exploring trocar.

Reagents. [1,2-^{3}H]Testosterone (41.8 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Cyproterone acetate was a gift from Schering Chemicals Ltd., Burgess Hill, Sussex., U.K. All other chemicals were AnalaR grade, from British Drug Houses Ltd., Poole, Dorset, U.K.

Chemical analyses. Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard. DNA was determined by the method of Burton (1956) as modified by Giles & Myers (1965). Calf thymus DNA was used as the standard.

Radioactivity was measured by dissolving samples in a few drops of 1 M-Hyamine solution overnight at 37°C. Dioxan scintillator (7 ml) was added (Bray, 1960) and the samples were counted for radioactivity in a Beckman LS100 liquid-scintillation counter, to a counting error of 2%. Quench corrections were applied, when necessary, by using the channels-ratio method.

Labelled metabolites of testosterone were separated, identified and their radioactive content was determined by the method described by Neal, Belham & Williams (1969).

Tissue manipulations in vitro. (*a*) Intact prostates. Ventral prostates were ligatured before removal. Saline (50 μl) containing 10 pmol of [^{3}H]testosterone was injected into each lobe of the prostate. No leakage of the fluid was observed and injections of dyes indicated that there was a good distribution of the injected fluid through the prostatic acini by using this technique. After injection the prostates were placed in 10 ml glass vials, bathed with 1.0 ml of saline and incubated at 37°C in O_2+CO_2 (95:5). Incubations were terminated by rapidly cooling the vials to 0°C. The tissue was cut finely with scissors and homogenized. Carrier steroids were added and the homogenate was deproteinized with ethanol (Neal *et al.* 1969).

(*b*) Minced prostatic tissue. These incubations were carried out in two ways. In the first method, minced prostatic tissue (100 mg fresh wt.) was incubated in bicarbonate buffer, as used by Farnsworth (1965), who used this medium for incubating slices of human prostate *in vitro*. In the present study finely minced prostatic tissue was added to 2 ml of Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs, 1950) and 10 pmol of [^{3}H]testosterone was then added; the tissue was incubated at 37°C in a shaking water bath in air. The incubations were terminated by cooling the flasks rapidly to 0°C, followed by homogenization and deproteinization with ethanol. Anti-androgens, when used, were added to the incubation flasks in 25 μl of the propanediol–saline solvent, control flasks receiving the solvent alone. In the second method, minced tissue (500 mg fresh wt.) was incubated in 10 ml of Eagles minimum essential medium (Difco Laboratories, Mich., U.S.A.) supplemented with 10% (w/v) calf serum (Difco Laboratories). Diethylstilboestrol or cyproterone acetate was added to the appropriate flasks. Immediately before incubation, [^{3}H]testosterone was added to the flasks, which were then incubated for 30 min at 37°C in O_2+CO_2 (95:5). Nuclei were subsequently prepared from the incubated tissue by using a slight modification of the technique described by Widnell & Tata (1964) for the isolation of liver nuclei (Mangan, Neal & Williams, 1968).

(*c*) Prostatic nuclear and cytoplasmic fractions. These fractions were prepared as follows. The finely minced prostatic samples were homogenized in buffer A [0.01 M-tris (pH 7.0)–50 μM-EDTA–0.5 mM-β-mercaptoethanol–0.5 mM-$MgCl_2$–0.05 M-NaCl] (Bruchovsky & Wilson, 1968*a*) and the homogenate centrifuged at 500*g* for 5 min. The supernatant from the centrifugation was termed the cytoplasmic fraction. The pellet was resuspended in 15 ml of 2.2 M-sucrose containing 1 mM-$MgCl_2$, and a purified nuclear fraction obtained by centrifuging the suspension at 48000*g* for 1 h (Mangan *et al.* 1968). Nuclear fractions were checked by microscopical examination for freedom from contamination by cytoplasmic debris. All nuclear fractions used had at least the degree of purity of 'fraction B' described by Widnell & Tata (1964). All of these operations were carried out at 1–4°C. The conditions of incubation for both nuclear and cytoplasmic fractions were essentially as described by Bruchovsky & Wilson (1968*a*). Buffer A (1.5 ml) containing subcellular fractions was supplemented with 0.4 ml of a NADPH-generating system (1 mM-glucose 6-phosphate, 1 μg of glucose 6-phosphate dehydrogenase/ml, 0.1 mM-$NADP^+$). Finally, 0.1 ml of buffer A containing [^{3}H]testosterone (40 pmol) was added and incubations were carried out at 37°C in a shaking water bath. Incubations were terminated by cooling the flasks to 0°C. Radioactive metabolites were extracted and determined. A portion was also removed for determination of protein or DNA. The effects of diethylstilboestrol and cyproterone acetate on the metabolism *in vitro* of [^{3}H]testosterone were determined by adding the anti-androgens, dissolved in propanediol–saline, to the appropriate incubation vessels. Both anti-androgens were added at a final concentration of 27 μM. Control incubations received the propanediol solvent alone. Subcellular fractions were allowed to equilibrate with the anti-androgen-containing medium for 5 min at 0°C before addition of the [^{3}H]testosterone and commencement of the incubation period.

Experiments in vivo. In those experiments in which the metabolism of [^{3}H]testosterone by the prostate *in vivo* was determined, 60 μCi, of [^{3}H]testosterone in 1.5 ml of saline was injected intraperitoneally into rats. At the end of the experimental period, the prostates were removed, rinsed in saline and homogenized in 5 ml of 0.25 M-sucrose containing 1 mM-$MgCl_2$. After being filtered through nylon bolting cloth, the homogenate was layered over 2.5 ml of 0.32 M-sucrose containing 3 mM-$MgCl_2$. This was centrifuged at 700*g* for 10 min. The supernatant was decanted and is referred to as the cytoplasmic fraction. The pellet was resuspended in 2.2 M-sucrose containing 1 mM-$MgCl_2$, and a purified nuclear pellet was obtained by centrifugation as described above. DNA, protein and radioactivity were determined as described above.

Nuclear fractions were isolated by the above technique in those experiments in which [^{3}H]testosterone and anti-androgens were injected directly into the prostate.

RESULTS

Studies in vitro

Intact ligatured prostates excised from rats castrated 24 h earlier were injected with 10 pmol of [^{3}H]testosterone and incubated *in vitro*. The

distribution of radioactivity among the metabolites was determined after various periods of incubation, and the results are given in Fig. 1 (the vertical bars, indicating the S.E.M. of three determinations, show the degree of variation encountered in these incubation studies).

Prostatic minces were incubated with [^{3}H]testosterone in Krebs–Ringer bicarbonate medium for various periods of time, and the tissue was subsequently examined for the presence of labelled metabolites. The results of one of these experiments are given in Fig. 2.

Prostatic nuclear and cytoplasmic fractions were prepared in buffer A from rats 24h after castration and were incubated *in vitro*. Each incubation vessel contained the nuclei recovered from 1g fresh wt. of tissue. Cytoplasmic fractions represented the yield from 0.5g fresh wt. of tissue. The fractions were incubated in buffer A, plus the NADPH-generating system and [^{3}H]testosterone, in a total volume of 2ml. The formation of labelled metabolites with time was followed and the results are given in Figs. 3 and 4.

Comparison of the results in Figs. 1, 2, 3 and 4 shows that the different techniques used for the incubation of prostatic tissue *in vitro* yielded dissimilar results. Although dihydrotestosterone was a major labelled metabolite produced in all incubations, labelled androstanediol (5α-androstane-3α,17β-diol) was prominent after the incubation of intact prostates or subcellular fractions, but was present only to a minor extent in the minced tissue incubated in Krebs–Ringer bicarbonate buffer.

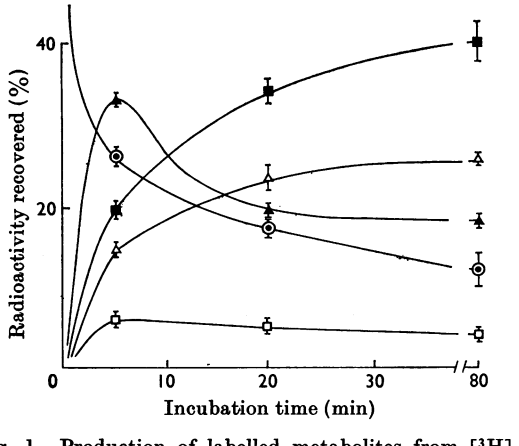


Fig. 1. Production of labelled metabolites from [^{3}H]-testosterone by intact prostate incubated *in vitro*. For experimental details see the text. Results are the means of three determinations; S.E.M. is indicated by vertical bars. Androstanediol, ■; androsterone, △; dihydrotestosterone, ▲; testosterone, ○; androstanedione, ●; androstenedione, □.

Having established the patterns of labelled metabolites produced from [^{3}H]testosterone by using the various incubation techniques *in vitro*,

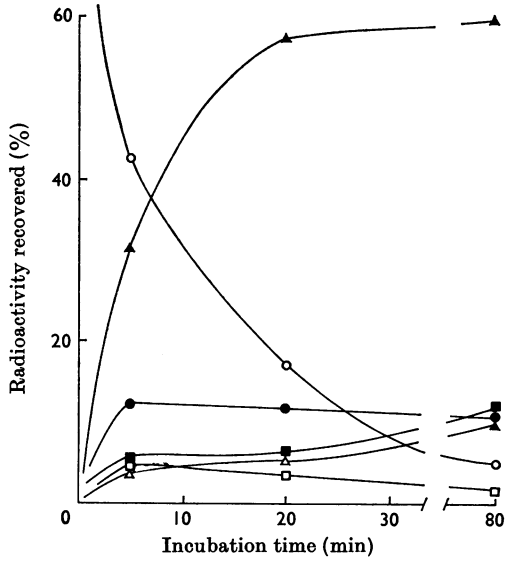


Fig. 2. Production of labelled metabolites from [^{3}H]-testosterone by minced prostatic tissue incubated in Krebs–Ringer bicarbonate buffer. Experimental details and symbols for metabolites are as given in Fig. 1.

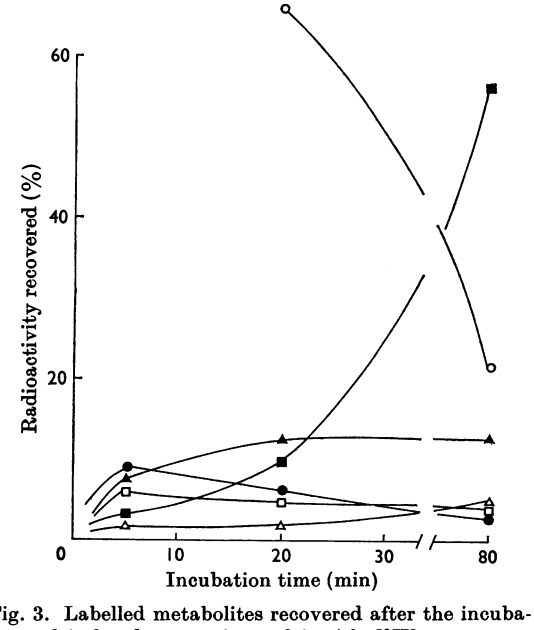


Fig. 3. Labelled metabolites recovered after the incubation of isolated prostatic nuclei with [^{3}H]testosterone *in vitro*. Experimental details and symbols for metabolites are as given in Fig. 1.

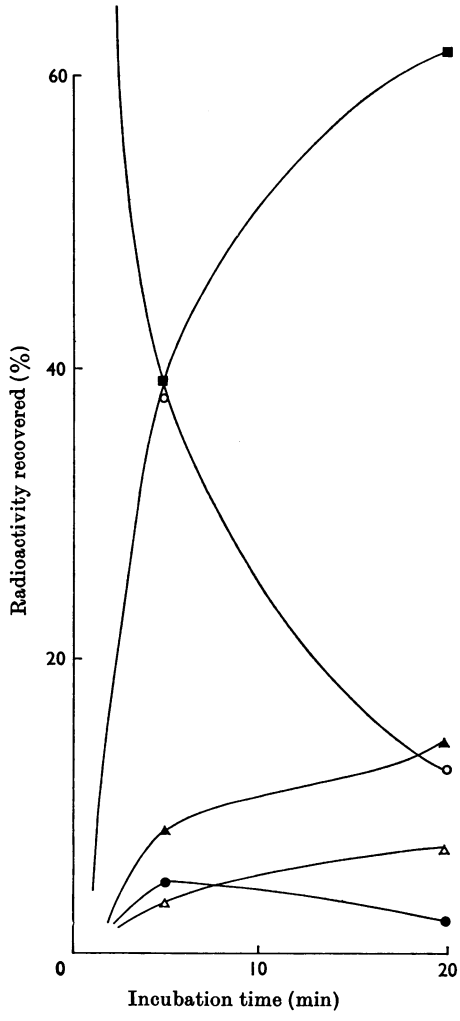


Fig. 4. Labelled metabolites recovered after the incubation of prostatic cytoplasmic fractions with [^{3}H]testosterone *in vitro*. Experimental details and symbols for metabolites are as given in Fig. 1.

we investigated the effects of the two anti-androgens, cyproterone acetate and diethylstilboestrol, on these metabolic patterns *in vitro*.

The low extents of labelling that were observed to be associated with androstanedione (5α-androstane-3,17-dione) and androstenedione (4-androstene-3,17-dione) (Figs. 1, 2, 3 and 4) were found not to be significantly altered by any of the treatments with anti-androgens employed. The effects of the anti-androgen treatments on the extents of labelling observed in testosterone, androsterone, dihydrotestosterone and androstanediol after incubations *in vitro* are indicated by the results given in Table 1. The principal findings of interest in the results given in Table 1 are as follows.

(1) In contrast with treatment with cyproterone acetate, treatment with diethylstilboestrol decreased the initial metabolism of [^{3}H]testosterone by prostatic tissue in all of the incubation systems used *in vitro*.

(2) The high yield of dihydrotestosterone and low yield of androstanediol in the incubations of minced tissue in Krebs–Ringer buffer, in contrast with the results of all other incubation systems used, is apparent. This result was unaffected by the presence of diethylstilboestrol or cyproterone acetate.

(3) Apart from the effect noted in (1), no consistent effect of cyproterone acetate or diethylstilboestrol on the metabolism of [^{3}H]testosterone was noted throughout these incubation studies *in vitro*.

The effects of the anti-androgens on the nuclear retention of labelled metabolites after incubations of prostatic tissue *in vitro* with [^{3}H]testosterone were also examined. Minced prostatic tissue was incubated in Eagle's medium by using the method of Anderson & Liao (1968), who demonstrated a selective nuclear retention of dihydrotestosterone by this technique. Ventral prostatic tissue excised from rats 24h after castration was minced and 500mg samples were incubated with 100μCi of [^{3}H]testosterone, in the presence or absence of diethylstilboestrol or cyproterone acetate. The anti-androgens were added at a final concentration of 27μM. After the incubations, the tissues were rinsed three times with 0.32M-sucrose (5ml) and the nuclei isolated in sucrose medium. Table 2 shows the total radioactivity observed in the nuclei. The labelled metabolites were isolated and their radioactive contents determined (Table 3). In contrast with the lack of effect of cyproterone acetate on the metabolism of [^{3}H]testosterone by prostatic nuclei (Table 1), the radioactivity associated with each of the metabolites retained by the nucleus had been decreased by treatment with cyproterone acetate. For the treatment with diethylstilboestrol no comparable effect to that achieved by cyproterone acetate on the nuclear retention of labelled metabolites was observed.

Studies in vivo

Distribution of radioactivity in various body tissues. An experiment was carried out to determine the distribution of radioactivity among various body organs of the rat after an injection of [^{3}H]testosterone. Rats castrated 24h before use were injected intraperitoneally with 70μCi of [^{3}H]-testosterone, and various body organs were

Table 1. *Effects of cyproterone acetate and diethylstilboestrol on the metabolism of* [^{3}H]*testosterone by prostatic tissue* in vitro

After incubations the metabolites were extracted as described in the Materials and Methods section. Replicate portions of extracts containing approx. 10000 d.p.m. were analysed by g.l.c. technique (Belham *et al.* 1969*a*). Results are means ± S.E.M. of three determinations. Incubation techniques: A, intact prostate, treated with anti-androgens *in vitro*, incubated in saline for 10 min; B, minced prostatic tissue incubated in Krebs–Ringer medium for 20 min, anti-androgens applied *in vitro*; C, as in B except that anti-androgens were administered *in vivo*; D, nuclear fraction incubated in buffer A for 40 min, anti-androgens applied *in vitro*; E, cytoplasmic fraction incubated in buffer A for 15 min, anti-androgens applied *in vitro*. Incubation times were selected on the evidence of results given in Figs. 1, 2, 3 and 4 as being consistent with extensive metabolism of [^{3}H]testosterone. For all further details of incubation systems see the Materials and Methods section.

		Radioactivity in metabolite (d.p.m.)				
Metabolite	Incubation techniques ... Treatment	A	B	C	D	E
Testosterone	Control	1055 ± 210	1620 ± 210	1210 ± 180	3445 ± 280	2150 ± 180
	Cyproterone acetate	820 ± 185	1350 ± 180	1250 ± 150	3825 ± 455	2055 ± 245
	Diethylstilboestrol	1750 ± 235	3950 ± 255	2300 ± 155	5310 ± 380	3535 ± 260
Androsterone	Control	860 ± 130	470 ± 85	530 ± 135	980 ± 125	1010 ± 155
	Cyproterone acetate	1410 ± 225	635 ± 115	425 ± 60	815 ± 95	1185 ± 125
	Diethylstilboestrol	620 ± 115	440 ± 65	570 ± 75	620 ± 105	725 ± 55
Dihydrotestosterone	Control	3710 ± 455	5650 ± 610	6310 ± 570	860 ± 135	950 ± 135
	Cyproterone acetate	3350 ± 430	5495 ± 585	6120 ± 430	1420 ± 245	1720 ± 320
	Diethylstilboestrol	2855 ± 375	3750 ± 470	4750 ± 520	1035 ± 175	685 ± 95
Androstanediol	Control	3640 ± 480	520 ± 105	850 ± 65	4260 ± 490	5625 ± 455
	Cyproterone acetate	3370 ± 450	980 ± 150	625 ± 80	3220 ± 265	4655 ± 325
	Diethylstilboestrol	4050 ± 510	750 ± 135	1105 ± 85	2320 ± 155	4325 ± 510

Table 2. *Effect of diethylstilboestrol and cyproterone acetate on the nuclear retention of radioactivity after incubation with* [^{3}H]*testosterone* in vitro

Minced prostatic tissue (500 mg) was incubated in Eagle's medium +10% (w/v) calf serum in air at 37°C for 30 min. Results are means ± S.E.M. of five determinations.

Additions to incubation medium	Radioactivity (d.p.m./10 μg of DNA)
[^{3}H]Testosterone + vehicle only	3157 ± 55
[^{3}H]Testosterone + diethylstilboestrol	3380 ± 212
[^{3}H]Testosterone + cyproterone acetate	1314 ± 6

Table 3. *Effect of diethylstilboestrol and cyproterone acetate on the retention of labelled metabolites of* [^{3}H]-*testosterone by prostatic nuclei* in vitro

Results are means ± S.E.M. for three incubated samples. Incubations were carried out by using minced prostatic tissue in Eagle's medium as described in the Materials and Methods section.

	Radioactivity (d.p.m./10 μg of DNA)		
	Control	+Diethylstilboestrol	+Cyproterone acetate
Androstanediol	130 ± 25	80 ± 20	75 ± 15
Androsterone	75 ± 20	110 ± 20	50 ± 15
Dihydrotestosterone	1650 ± 130	1725 ± 150	820 ± 115
Testosterone	870 ± 105	1375 ± 135	240 ± 35
Androstanedione	325 ± 45	350 ± 55	105 ± 20
Androstenedione	125 ± 30	110 ± 25	45 ± 15

removed 40 min later. Tissue samples (0.5 g) were homogenized in 2 ml of saline (0.5 ml of blood, removed by cardiac puncture, was deproteinized directly). The radioactive content of various lipid fractions was then determined (Table 4). These results show that only a very low proportion of the radioactivity retained by the prostate and seminal vesicles is in a hydrophilic form. The radioactivity associated with androstanediol, dihydrotestosterone and testosterone in the homogenates of the various

Table 4. *Distribution of radioactivity recovered in ethanolic extracts of various tissues after the administration of* [^{3}H]*testosterone* in vivo

Extracts in 95% (v/v) ethanol were evaporated *in vacuo*, dissolved in water and partitioned against ether. The ether extracts were subjected to t.l.c. in cyclohexane–acetone (69:31, v/v) (Belham *et al.* 1969*a*). All radioactivity counts were at least twice background rate.

	Distribution of radioactivity (%)		
Tissue	Water-soluble fraction	Ether-soluble fraction not associated with carrier steroids	Ether-soluble fraction associated with carrier steroids
Lung	44.2	25.9	29.9
Muscle	40.6	36.6	22.8
Blood	61.3	24.7	14.0
Liver	52.3	44.1	3.6
Kidney	52.2	30.7	17.1
Seminal vesicles	5.0	11.8	83.2
Prostate	2.4	6.7	90.9

Table 5. *Radioactivity recovered as androstanediol, dihydrotestosterone and testosterone after administration of* [^{3}H]*testosterone* in vivo

Four rats, castrated 24h previously, were injected intraperitoneally with 70μCi of [^{3}H]testosterone. Organs were removed 40min later; 0.5g of tissue (0.5ml of blood) was used in determinations of radioactivity of the three metabolites. All radioactivity counts were at least twice background rate.

	Radioactivity (% of total radioactivity recovered in each organ)		
Tissue	Androstanediol	Dihydrotestosterone	Testosterone
Lung	8.6	1.9	3.5
Muscle	2.1	1.5	9.4
Blood	1.8	0.5	4.9
Liver	0.8	0.4	0.1
Kidney	2.9	1.6	4.5
Seminal vesicles	6.7	56.5	4.7
Prostate	1.5	68.3	4.3

tissues was determined (Table 5). These results show that the androgen target organs, the prostate and seminal vesicles, principally contain labelled dihydrotestosterone. This is in agreement with the findings of Bruchovsky & Wilson (1968*a*). The concentration of androstanediol is generally no higher in the target than in non-target organs. This is in contrast with the large amounts of labelled androstanediol produced in some of the incubation studies *in vitro* described above.

Distribution of radioactivity in prostatic subcellular fractions. Various amounts of [^{3}H]testosterone were injected intraperitoneally into rats 48h after castration, four rats being used per group. Half of the rats in each group were killed 2h after the injections and the prostates removed. The remaining two rats in each group were killed 24h after the injections and the prostates removed. The prostates were homogenized in the sucrose medium and purified nuclear and supernatant (100000$g_{av.}$/90min) fractions were prepared. The total radioactivity present in the unfractionated homogenates was determined, and also the distribution of radioactivity between the nuclear and high-speed supernatant fraction (Table 6). The recovery of label in the prostate is clearly dependent on the dose injected at both the times examined. It also appears that the loss of radioactivity during the period between 2 and 24h after the injection is slightly greater from the nuclear than from the supernatant fraction.

The effect of treatment *in vivo* with cyproterone acetate on the distribution of radioactivity among the subcellular fractions was then determined. Rats 48h after castration were injected intraperitoneally with 10μCi of [^{3}H]testosterone. Half of the animals received the treatment *in vivo* (pelleting and twice-daily injections) with cyproterone acetate referred to in the Materials and Methods section; the other half received injections of control solution. Prostates were excised 1h after injection of the [^{3}H]testosterone, and subcellular fractions were prepared in sucrose. The radioactive contents of the subcellular fractions are given in Table 7. The

Table 6. *Uptake of radioactivity into prostate tissue after injection of* [^{3}H]*testosterone* in vivo *and the distribution of label between nuclear and high-speed-supernatant fractions*

Groups of four rats were injected intraperitoneally with various amounts of [^{3}H]testosterone 48h after castration. Two rats in each group were killed 2h later, the remaining rats being killed 24h later. Radioactivity in the total homogenate and nuclear and supernatant (100000*g*/1h) fractions was measured.

Time after injection (h)	[^{3}H]Testosterone injected (nmol)	Injected radioactivity recovered in prostate (%)	Distribution of radioactivity (% of total radioactivity present in fractions)	
			Nuclear fraction	Supernatant fraction
2	1	0.23	60	40
	0.1	0.28	65	35
	0.01	1.06	45	55
	0.001	2.49	22	78
24	1	0.05	51	49
	0.1	0.08	34	66
	0.01	0.26	22	78
	0.001	0.78	19	81

Table 7. *Effects of treatment with cyproterone acetate* in vivo *on the distribution of radioactivity among prostatic subcellular fractions after injection of* [^{3}H]*testosterone* in vivo

Two groups of three adult male Sprague–Dawley rats were castrated, and 48h later the first group received 5mg of cyproterone acetate suspended in saline by injection intraperitoneally twice daily; also 10mg pellets of cyproterone acetate were implanted subcutaneously at the time of castration. The second (control) group received aqueous vehicle and sham-implantations. Both groups were injected intraperitoneally with 10μCi of [^{3}H]testosterone in saline 1h before removal of prostates. Results are means of three determinations.

	Control rats	Rats treated with cyproterone
Supernatants (100000*g*/90min) (d.p.m./mg of protein)	338	385
Mitochondrial+microsomal pellet (d.p.m./mg of protein)	615	414
Nuclear fraction (d.p.m./mg of DNA)	19214	3722

results show that the treatment with cyproterone acetate decreased the radioactivity present in both the particulate fractions, with a particularly dramatic effect on the nuclear fraction. Similar experiments carried out with diethylstilboestrol showed that the radioactivity present in all fractions was affected by treatment *in vivo* with this anti-androgen, the radioactivity present in the supernatant and the mitochondrial–microsomal fractions being decreased by approx. 50% and that in the nuclear fraction by 65%.

Production of labelled metabolites. The rate of production of labelled metabolites of [^{3}H]testosterone *in vivo* was investigated. Groups of four rats (castrated 24h previously) were injected intraperitoneally with 60μCi of [^{3}H]testosterone. Groups were killed at 5, 40 and 80min after the injections of testosterone, the prostates removed and the radioactive metabolites present determined. The results are given in Fig. 5. Only trace amounts of radioactivity were recovered in the form of androsterone, androstanedione or androstenedione, and so the values for these steroids are not plotted. The results demonstrate the extremely rapid accumulation of labelled dihydrotestosterone by the prostate. Over 50% of the amount of the labelled dihydrotestosterone present 80min after injection of [^{3}H]testosterone was already present 5min after the injections were made.

An experiment was carried out to determine the effect of the administration *in vivo* of anti-androgens on the metabolism of [^{3}H]testosterone. Groups of four rats were castrated and treated with diethylstilboestrol, cyproterone acetate or control solutions *in vivo* for 48h as described in the Materials and Methods section. The animals were injected intraperitoneally 48h after castration with 70μCi of [^{3}H]testosterone and the prostates were excised 40min later. The prostates were homogenized in buffer A and nuclear and total cytoplasmic fractions

prepared by centrifugation as described in the Materials and Methods section. It was found in this experiment that the total radioactivity in the homogenate was decreased to 60% of the control value by the diethylstilboestrol treatment, and to 38% of the control value by treatment with cyproterone acetate. The distribution of radioactivity among the testosterone metabolites was determined, and the results are given in Tables 8 and 9. It was found during this study that homogenizing the tissue in buffer A consistently yielded higher values for labelling in the soluble fractions, and lower values for labelling in the particulate fractions, compared with those values observed when the tissue was homogenized in iso-osmotic sucrose. It is believed that this was due to solubilization of some of the particulate-bound radioactivity in the dilute buffer medium (Neal, 1970).

The effect of cyproterone acetate and diethylstilboestrol on the nuclear uptake of radioactivity *in vivo* after injections of various doses of [^{3}H]-testosterone was determined. Intraperitoneal injections of cyproterone acetate were without detectable effect on the nuclear uptake of [^{3}H]-testosterone over a period of several hours after injection of the anti-androgen, presumably owing to the low solubility of this compound. The scheme adopted therefore was to inject the [^{3}H]testosterone and the anti-androgen simultaneously directly into the prostate. Rats, castrated 72h previously, were injected with various doses of [^{3}H]testosterone

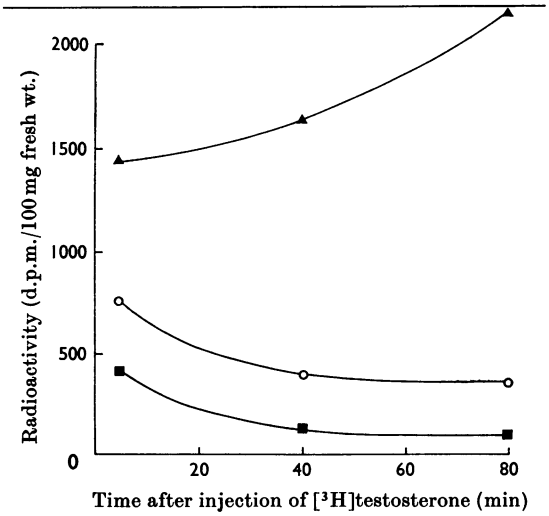


Fig. 5. Labelled metabolites present in the prostate after injections of [^{3}H]testosterone *in vivo*. Experimental details and symbols for metabolites are as given in Fig. 1.

Table 8. *Effect of diethylstilboestrol on the retention of labelled metabolites by prostatic nuclear and cytoplasmic fractions after administration of* [^{3}H]*testosterone* in vivo

Results are means for four animals ± S.E.M. For further experimental details see the text.

	Radioactivity			
	Nuclear fraction (d.p.m./100 μg of DNA)		Cytoplasmic fraction (d.p.m./mg of protein)	
	Control	+Diethylstilboestrol	Control	+Diethylstilboestrol
Androstanediol	250 ± 25	65 ± 15	126 ± 35	210 ± 10
Androsterone	135 ± 15	85 ± 5	110 ± 15	135 ± 15
Dihydrotestosterone	4520 ± 310	1220 ± 75	2100 ± 35	795 ± 15
Testosterone	745 ± 145	275 ± 35	255 ± 25	135 ± 25
Androstanedione	440 ± 75	155 ± 25	240 ± 35	225 ± 10
Androstenedione	210 ± 20	85 ± 20	75 ± 30	40 ± 5

Table 9. *Effect of cyproterone acetate on the retention of labelled metabolites by prostatic nuclear and cytoplasmic fractions after the administration of* [^{3}H]*testosterone* in vivo

Results are means for four animals ± S.E.M. For further experimental details see the text.

	Radioactivity			
	Nuclear fraction (d.p.m./100 μg of DNA)		Cytoplasmic fraction (d.p.m./mg of protein)	
	Control	Cyproterone acetate	Control	Cyproterone acetate
Androstanediol	240 ± 10	160 ± 10	75 ± 20	110 ± 25
Androsterone	105 ± 25	220 ± 35	40 ± 15	55 ± 10
Dihydrotestosterone	4570 ± 255	235 ± 25	1835 ± 30	635 ± 75
Testosterone	1245 ± 95	95 ± 20	510 ± 70	95 ± 25
Androstanedione	10 ± 15	80 ± 5	175 ± 45	110 ± 15
Androstenedione	75 ± 20	75 ± 15	60 ± 10	45 ± 10

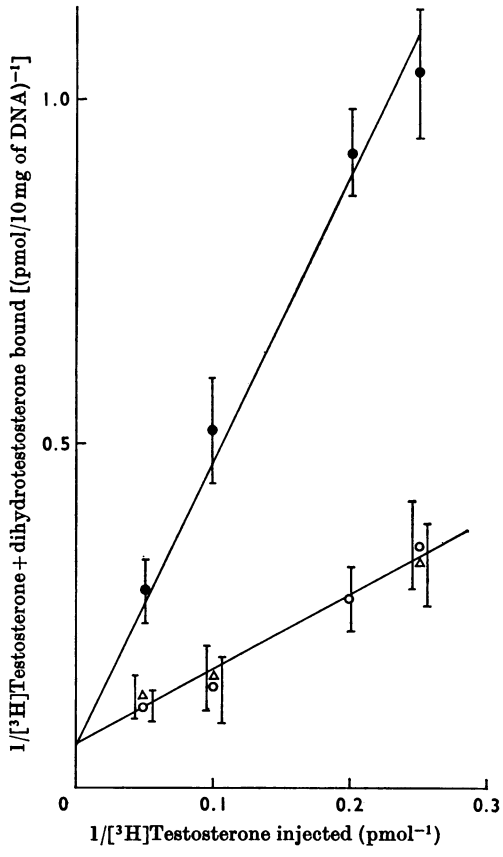


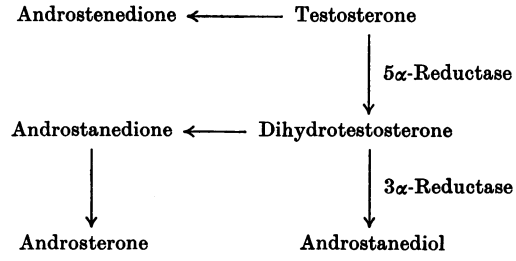
Fig. 6. Effect of cyproterone acetate and diethylstilboestrol on the retention of radioactivity by prostatic nuclei after injections of [^{3}H]testosterone directly into the prostate. ○, Testosterone; △, testosterone+diethylstilboestrol; ●, testosterone+cyproterone acetate. Vertical bars indicate S.E.M. of three determinations. For experimental details see the text.

(0.1ml) directly into the prostate, either alone or accompanied by 0.8μg of diethylstilboestrol or cyproterone acetate. The animals were killed 60min later and the nuclear fractions prepared in the sucrose–magnesium chloride medium. The effects of the anti-androgens on the extent of nuclear labelling are given in Fig. 6. It appeared that diethylstilboestrol at this concentration was without effect on the nuclear uptake of radioactivity, but that cyproterone acetate competed with [^{3}H]testosterone, or, more correctly, with dihydrotestosterone, for nuclear uptake sites. The failure to demonstrate competition between diethylstilboestrol and testosterone for nuclear sites was also observed in other experiments, in which it was found that the nuclear labelling resulting from the injection of 5nmol of [^{3}H]diethylstilboestrol directly into the prostate was not decreased by the stimultaneous injection of 50μg of unlabelled testosterone. The lack of competition between diethylstilboestrol and testosterone for nuclear sites when both compounds are supplied directly to the prostate had also been indicated by the results of the experiments *in vitro* given in Table 2.

DISCUSSION

Bruchovsky & Wilson (1968*a*) have reported that prostatic tissue incubated *in vitro* converts testosterone rapidly, principally into androstanediol, androsterone and dihydrotestosterone. Our findings are in agreement with this. These workers also reported that in their experiments *in vitro*, prostatic nuclei metabolized testosterone exclusively to dihydrotestosterone. We have found, however, in our incubations that prostatic nuclei also contain the enzyme systems necessary to produce androstanediol. It is possible that these differences in results between those of Bruchovsky & Wilson (1968*a*) and ourselves are due to differences in techniques employed. For example, the influence of different conditions of incubation *in vitro* on the products of [^{3}H]testosterone metabolism are shown by comparing the results in Figs. 1, 2, 3 and 4. In those experiments in which prostatic minces were incubated in Krebs–Ringer bicarbonate buffer, little labelled androstanediol was produced, but incubation of the intact ligatured prostates in saline or isolated nuclear or cytoplasmic fractions in buffer A resulted in a large production of this metabolite. As for the metabolic pathways involved, a slight modification of the scheme (Scheme 1) suggested by Mauvais-Jarvis & Bercovici (1968) as a result of their studies on the testicular feminization syndrome would appear to be consistent with the experimental results obtained in the present study.

The results of Belham *et al.* (1969*a*) characterizing the dihydrotestosterone as 17α-hydroxy-5α-androstan-3-one and androstanediol as 5α-androstan-3α,17β-diol is in accordance with involvement of 5α- and 3α-reductases in the above scheme. The intermediate position of dihydrotestosterone in the production of androstanediol from testosterone was also indicated by the results of our experiments (J. E. Belham & G. E. Neal, unpublished work) in which labelled dihydrotestosterone, added to incubations of prostatic nuclear or cytoplasmic fractions *in vitro*, was rapidly metabolized to form labelled androstanediol. The results given in Fig. 1 also show that when labelled testosterone was injected into intact ligatured prostates *in vitro*, label initially predominantly entered dihydrotestosterone, but that this radioactivity later decreased, whereas increased labelling was detected in androsterone and androstanediol.



Scheme 1.

The experiments carried out to determine the effects of the anti-androgens on the metabolism *in vitro* of [^{3}H]testosterone by prostatic tissue failed to reveal a consistent inhibition of the production of any metabolite to the extent that might be expected if inhibition of production of a necessary testosterone metabolite was an important factor in the anti-androgenic mechanism (Table 1). In assessing this, we can compare the magnitude of the effect of cyproterone acetate on the production of testosterone metabolites *in vitro* (Table 1) with the magnitude of its effect on the retention of those metabolites *in vitro* (Table 3). It must also be pointed out, with regard to the lack of effect of cyproterone acetate on the metabolism of testosterone by prostatic tissue, that the concentration of cyproterone acetate used in all these studies (27 μM) has been found by Hoffmann & Breuer (1968) to inhibit the production of testoterone *in vitro* by subcellular fractions of rat testis by up to 80%. One other notable point is that the marked difference in effect of cyproterone acetate on (*a*) the retention of dihydrotestosterone and (*b*) the reduction of testosterone to androstanediol via dihydrotestosterone appears to indicate that the retention of dihydrotestosterone in the nucleus cannot be by attachment to the active site of the reductase enzyme.

The results of the experiments examining the uptake of radioactivity into various body organs after the injection *in vivo* of [^{3}H]testosterone (Tables 7 and 8) emphasize that most of the label incorporated into the target organs is associated with dihydrotestosterone. Although, as we have demonstrated, both prostatic nuclear and cytoplasmic fractions are capable of the further metabolism of dihydrotestosterone to androstanediol *in vitro*, the uptake of label into androstanediol after injections of [^{3}H]testosterone *in vivo* was no higher in the target than in the non-target organs. This could possibly have been due to the conditions in intact prostatic cells being unfavourable for the extensive further reduction of dihydrotestosterone to androstanediol by the 3α-reductase, but this is not supported by the results obtained from the incubations of intact prostates *in vitro* (Fig. 1). Further experiments have also shown (G. E. Neal & J. E. Belham, unpublished work) that although large amounts of labelled androstanediol are produced by intact prostates after incubations *in vitro*, if nuclei are subsequently isolated and examined for the presence of radioactive metabolites, low values of radioactivity in androstanediol similar to those indicated in Table 3 are detected. Alternatively, it could be that the known attachment of the dihydrotestosterone to specific receptors (Parsons, Mangan & Neal, 1970; Mainwaring, 1969; Tveter & Attramadal, 1968; Fang, Anderson & Liao, 1969) *in vivo* precluded the possibility of further reduction, but in this case the action of cyproterone acetate, in blocking the specific nuclear retention of dihydrotestosterone, might have been expected to lead to overall increased contents of labelled androstanediol in the tissue *in vivo*, and this was not found to occur. The most likely explanation therefore appears to be that androstanediol formed *in vivo* is not retained in the tissue but is rapidly removed via the blood stream. The 3α-reductase, which we have also detected in the nucleus, could perhaps be concerned with the turnover of nuclear dihydrotestosterone.

As for the action of the anti-androgens in the experiments *in vivo*, the principal effect of cyproterone acetate appears to be a competitive inhibition of the specific nuclear uptake of dihydrotestosterone. A similar competition between [^{3}H]-testosterone and cyproterone in incubations *in vivo* has been reported (Stern & Eisenfeld, 1969) for the binding of label by soluble macromolecules present in the cytoplasmic fraction of seminal vesicles. In this connexion it is noteworthy that in the present study, whereas treatment *in vivo* with cyproterone acetate decreased the prostatic nuclear labelling by >80%, no corresponding decrease in radioactivity in the high-speed-supernatant fraction was observed (Table 7).

The action of the anti-androgen diethylstilboestrol on the prostate appeared to inhibit partially the 5α-reductase. Although an effect of diethylstil-

boestrol on the nuclear uptake of radioactivity after administration of [^{3}H]testosterone was observed when both diethylstilboestrol and testosterone were injected intraperitoneally, no comparable effect was observed when these hormones were supplied directly to the prostate, either *in vivo* or *in vitro*. If this anti-androgen has an effect at the level of the target organ as distinct from its known effect on androgen production by the testes, then it must be due to a mechanism differing from that of cyproterone acetate.

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