Some Effects of Testosterone on the Rat Ventral Prostate

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1. Labelled testosterone, injected directly into the ventral prostate of castrated rats became associated, in part, with a cytoplasmic high-molecular-weight fraction, fraction 'A'. 2. The label present in fraction 'A' was found to be mainly associated with dihydrotestosterone. 3. Unlike fraction 'A' from testosterone-pelleted castrated rats, fraction 'A' obtained from untreated castrated rats, 48h or more after castration, was strongly inhibitory towards *Escherichia coli* RNA polymerase *in vitro*. 4. The inhibition of RNA polymerase by fraction 'A' from castrated rats was not changed by the addition of testosterone or dihydrotestosterone *in vitro*, but pre-heating it to 80°C resulted in a loss of its inhibitory capacity. 5. Fraction 'A' from castrated rats contained ribonuclease activity. The elution profile of ribonuclease activity from Sephadex columns indicated that this activity was responsible for the inhibitory effect on the RNA polymerase assays. 6. It is concluded that, unlike the inhibitor present in the uterus of ovariectomized rats (Talwar, Segal, Evans & Davidson, 1964), no direct connexion exists between the steroid-binding capacity of prostatic fraction 'A' and its effect on *E. coli* RNA polymerase activity *in vitro*.

Considerable interest has been focused recently on the mechanism of action of steroid hormones in mammalian target organs. Claims for the detection of specific hormone-receptor substances have been made in the case of the action of oestrogens on uterine tissue (Talwar, Segal, Evans & Davidson, 1964; Toft & Gorski, 1966) and on vaginal and breast tissue (Jungblut, Haetzel, De Sombre & Jensen, 1967). A recent finding of possible practical importance is that of Puca & Bresciani (1968), which showed that the loss of dependence of mammary tumours, growing in ovariectomized mice, on an exogenous supply of oestrogen, is correlated with the disappearance of a specific oestrogen-binding component from the high-speed supernatant fraction. The process by which a steroid hormone, after its attachment to the receptor within the target organ, exerts its effect on the cellular metabolism is still almost completely unknown. The results of Talwar *et al.* (1964), which suggest a direct connexion between the hormone-receptor complex and the subsequent effects on the metabolism of the target organ could therefore be of the greatest importance. These workers found that a fraction, that was present in

the uterus of the ovariectomized rat and that was eluted in the void volume after passage of the high-speed supernatant through Sephadex G-100, contained an oestrogen-receptor substance. This fraction was inhibitory towards *E. coli* DNA-directed RNA polymerase *in vitro*, but the inhibition could be removed by addition of 17- β -oestradiol to the assay system, or by preincubating the fraction at 80°C. De Sombre, Feldacker, Jungblut & Jensen (1966) have confirmed the presence of the inhibitory fraction in uterus, but were unable to reverse the inhibition with 17- β -oestradiol. Saporì, Biswas, Sen & Talwar (1968) have suggested that the failure to reverse the inhibition may have been due to differences in experimental technique.

The present paper deals with an examination of the rat ventral prostate for the presence of androgen-binding components, similar to the oestrogen-binding components present in the high-speed supernatant of rat uterus (Talwar *et al.* 1964). An examination of the possible relationship between androgen-binding components and RNA polymerase activity assayed *in vitro* using an *E. coli* enzyme preparation has also been made.

MATERIALS AND METHODS

Chemicals. [1,2-³H₂]Testosterone (41.8 Ci/mmol) and [8-¹⁴C]ATP (14.2 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. ATP was

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a product of the Sigma (London) Chemical Co. Ltd., London S.W.6., U.K. Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. supplied CTP, GTP, UTP and bovine serum albumin. Salmon sperm DNA was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Hyamine 10-X was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Actinomycin D was a generous gift from Merck, Sharpe and Dohme, Rahway, N.J., U.S.A. Sephadex G-100 was obtained from Pharmacia (G.B.) Ltd., London W.13, U.K. All other chemicals were from British Drug Houses Ltd., Poole, Dorset, U.K., and were of AnalaR grade.

Animals. Adult male Sprague-Dawley rats, weighing approx. 200g, were used throughout. Bilateral orchidectomy was performed via the scrotal route under anaesthesia. Testosterone pellets, weighing approximately 30mg, were placed subcutaneously in the animals' flanks, under anaesthesia, with an exploring trocar. In some experiments the ventral prostate gland was exposed by means of a ventral incision, under anaesthesia, and [^3H]testosterone was injected directly into one of the ventral lobes. The incision was closed with suture clips.

Tissue fractionation procedures. In those experiments in which homogenates were to be examined for the presence of testosterone-binding components, [$1,2\text{-}^3\text{H}_2$]testosterone ($2.0\mu\text{Ci}$) in 0.88% NaCl was injected into one ventral lobe of the prostate in a total volume of 0.05ml. After the appropriate time, the animals were killed and the prostates were removed and homogenized in 5ml of the TMK (10mm-tris-10mm-KCl-1.5mm-MgCl₂) buffer, pH 7.4, used by Talwar *et al.* (1964). After straining through nylon bolting cloth, the homogenate was centrifuged at 100000g for 90min. The clear supernatant was placed on a column (45cm $\times$ 2cm) of Sephadex G-100 prepared in the TMK buffer and fractions (5ml) were collected. Radioactivity determinations were carried out on the fractions before and after extraction with 2ml of methylene dichloride. Similar homogenizing and fractionating procedures were used to prepare fractions subsequently examined for their effect on the *E. coli* RNA polymerase system.

Analytical procedures. Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. Extinctions were measured in a Hilger and Watts Uvispek. Radioactivity determinations were carried out in either an I.D.L. liquid scintillation counter or a Beckman LS100. Radioactive samples were dissolved in Hyamine 10-X and then dioxan scintillator was added (Bray, 1960). Fractions collected from the Sephadex column were assayed in the dioxan scintillator both before and after extractions with methylene dichloride. The methylene dichloride extracts were evaporated to dryness, and the residues dissolved in toluene scintillator.

DNA-directed RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) was extracted from *E. coli* cells (obtained from the Microbiological Research Establishment, Ministry of Defence, Porton Down, Wilts., U.K.) by the method of Chamberlin & Berg (1962). The purification was carried out as far as the fraction 3 stage. This fraction (15mg of protein/ml) was stored at -70°C in the storage medium recommended by Nicholson & Peacocke (1966). Assays of RNA polymerase activity were carried out by using the assay

medium described by Chamberlin & Berg (1962). Assays were carried out in triplicate, with 0.01 ml of enzyme. All four nucleoside triphosphates were necessary for maximum activity. After 10min of incubation at 37°C , the assay tubes were chilled to 0°C and 0.5ml of cold 3.5% perchloric acid was added. The precipitate was spun down and washed three times with 3ml of 3.5% perchloric acid. The radioactive content of the precipitate was then measured. Assays carried out with increasing amounts of salmon sperm DNA as the priming agent showed a linear increase in [^{14}C]AMP incorporation up to $25\mu\text{g}$ of DNA. Incorporation varied between the different preparations of *E. coli* RNA polymerase used in this study from 0.5 to 5nmol of [^{14}C]AMP incorporated/10min per 0.01 ml of enzyme.

When the effects on RNA polymerase activity *in vitro* of fractions eluted from the Sephadex G-100 columns were to be investigated, peak tubes containing 280nm-absorbing materials were freeze-dried. When required for use, the freeze-dried powder was dissolved in the TMK buffer and dialysed against the same buffer for 18h in the cold. In other experiments, fractions (3ml) were collected and frozen immediately.

Fractions eluted from the Sephadex column were also assayed for the presence of ribonuclease, deoxyribonuclease or adenosine triphosphatase activity under the same conditions of pH and temperature as those employed in the RNA polymerase assays. The method used in the ribonuclease determinations was based on that of Mathias, Williamson, Huxley & Page (1964), except that the pH used was 7.9, the pH at which the RNA polymerase assays were carried out. Yeast RNA was purified by precipitation from 0.1M-tris buffer, pH 7.4, containing 1.5M-potassium acetate, by the addition of 2 vol. of ethanol. The purified yeast RNA was dissolved in 0.1M-tris-acetate buffer, pH 7.4, to a concentration of approximately 4mg/ml and stored at -15°C .

Assay tubes contained 1.5ml of a tenfold dilution of the stock RNA solution in 0.1M-tris-HCl buffer, pH 7.9, 0.2ml of the test solution and 0.1M-tris-HCl buffer, pH 7.9, to bring the total volume to 3ml. Assays were carried out for 90min at 37°C . Bovine pancreatic ribonuclease standards were included in every assay. Incubations were terminated by the addition of 0.1 ml of a solution of bovine serum albumin in water (1mg/ml) followed by 3.0ml of an ice-cold solution of 0.5% (w/v) La(NO₃)₃ in M-HCl-75% (v/v) ethanol. After standing for 30min in an ice bath, the precipitates were centrifuged down and the E_{260} values of the supernatants were measured.

A similar assay system was used to determine deoxyribonuclease activity present in the fractions. Adenosine triphosphatase activity was determined by incubating fractions with [^{14}C]ATP and then chromatographing the incubation medium, after addition of marker unlabelled ADP, AMP and adenosine, on Whatman no. 1 paper using isobutyric acid-conc. aq. NH₃-water (66:1:33, by vol.) as the developing solvent. The chromatograms were scanned for the presence of radioactivity in the areas of the added markers.

Steroid identification. Labelled steroids were extracted and identified by using the t.l.c. and g.l.c. technique previously reported (Neal, Belham & Williams, 1969).

RESULTS

Testosterone-binding components. Fig. 1(a) shows that 2 h after injection of labelled testosterone into the ventral prostate of 48h-castrated rats, the labelling in the 100 000g supernatant fraction of the cytoplasm was mainly associated with two regions (tubes 7–10) containing column-excluded material, i.e. mol.wt. > 100 000, referred to as fraction 'A'. A smaller peak was frequently observed following the high-molecular-weight peak, that corresponded to the haemoglobin-albumin peak. The second main peak (tubes 26–34) containing radioactivity was in the region where low-molecular-weight substances were eluted from the column. Fig. 1(b) shows that radioactivity persisted in fraction 'A' for at least 24 h after the injection of labelled testosterone. In all fractions it was found that the radioactivity could be quantitatively extracted into methylene dichloride, none being detectable in the aqueous residue. Separation and identification of the labelled compound present in fraction 'A' showed that 80–85% of the labelling was associated with dihydrotestosterone (17- β -hydroxy-5 α -androstan-3-one) and 15–20% with testosterone (17 β -hydroxy-4-androsten-3-one). When nuclei were obtained from testosterone-injected prostates, by the method of Mangan, Neal & Williams (1968), disintegrated in TMK buffer by ultrasonic treatment, and the 100 000g supernatant placed on Sephadex columns, no radioactivity was detected in the column-excluded fractions.

Effect of fraction 'A' on *E. coli* RNA polymerase in vitro. Unlabelled fraction 'A' was added to *E. coli* RNA polymerase systems, in experiments essentially comparable with those using high-molecular-weight fractions isolated from the uteri of ovariectomized rats carried out by Talwar *et al.* (1964). The results of some of these experiments are given in Table 1. Fraction 'A' isolated from castrated rats 48 h or more after castration was invariably strongly inhibitory towards the polymerase activity. Fraction 'A' from intact or testosterone-treated castrated rats was either less inhibitory than the fraction isolated from untreated castrated rats or, in some experiments, fraction 'A' from intact rats or testosterone-treated castrated rats proved to be slightly stimulatory towards the *E. coli* RNA polymerase activity. Pre-heating at 80°C for 15 min either removed the inhibitory capacity of fraction 'A' isolated from castrated rats, or actually replaced the inhibition by stimulation (Table 1). Addition of testosterone or dihydrotestosterone to the assay, however, did not prove effective in decreasing the inhibition. Additions of solutions of bovine serum albumin at protein concentrations comparable with those used in

Fig. 1. (a) Fractionation on Sephadex G-100 of the 100 000g supernatant from 6–48 h castrated rats, killed 2 h after injection of [1,2- $^3\text{H}_2$]testosterone (2.0 μCi) directly into the ventral prostate. For details of the extraction see the text. (b) Fractionation of 100 000g supernatant from castrated rats, injected 48 h after castration with [1,2- $^3\text{H}_2$]testosterone. Rats were killed 24 h after the injection. For other details see the text. ●, E_{280} ; ○, radioactivity (c.p.s.).

experiments with fraction 'A' resulted in inhibitions of less than 10% of the control activities.

Fraction 'A' from castrated rats was examined for the presence of ribonuclease, deoxyribonuclease and adenosine triphosphatase activities. Deoxyribonuclease and adenosine triphosphatase activities could not be detected. Ribonuclease activity, however, was present. Further experiments were therefore carried out in which TMK extracts, from 48h-castrated rats and 48h-castrated rats pelleted with testosterone at the time of castration, were subjected to chromatography on Sephadex G-100 columns. Fractions (3 ml) were collected and the protein and ribonuclease contents of each fraction were analysed. The effect on the *E. coli* RNA polymerase system was also determined. The results of a typical experiment are given in Fig. 2. Ribonuclease activity was abolished by heating at 80°C for 15 min.

Table 1. *Effect of fraction 'A' isolated from castrated or testosterone-treated castrated rats on E. coli RNA polymerase in vitro*

The complete system contained 10nmol of tris-HCl buffer, pH 7.9, 0.3ml of TMK buffer, 0.25 μ mol of MnCl₂, 1.0 μ mol of MgCl₂, 3.0 μ mol of β -mercaptoethanol, 0.1 μ mol each of UTP, CTP and GTP, 30nmol of [¹⁴C]ATP, 25 μ g of highly polymerized salmon sperm DNA and 0.01 ml of *E. coli* RNA polymerase in a total volume of 1.0ml. For details of extraction of fraction 'A' see the text. Incorporations are given as the means of three determinations $\pm 10\%$.

	Incorporation of [¹⁴ C]AMP (pmol/10 min)		
	Expt. (a)	Expt. (b)	Expt. (c)
Complete system	4770	635	799
Complete system + actinomycin D (10 μ g)	190		
Complete system + fraction 'A' isolated from 48 h-castrated rats (100 μ g of protein)	2610	56	383
Complete system + fraction 'A' isolated from 48 h-castrated rats (100 μ g of protein) preincubated at 80°C for 15 min	4045	750	
Complete system + fraction 'A' isolated from 48 h-castrated rats (100 μ g of protein) preincubated with 10 μ g of testosterone	2580	63	
Complete system + fraction 'A' isolated from 48 h-castrated rats (100 μ g of protein) preincubated with 10 μ g of dihydrotestosterone	2498		410
Complete system + fraction 'A' isolated from intact rats (100 μ g of protein)		570	
Complete system + fraction 'A' isolated from testosterone-treated 48 h-castrated rats (100 μ g of protein)			962

DISCUSSION

From the results of the column fractionation experiments it appears that a macromolecular component is present in the 100 000g supernatant that is capable of binding principally dihydrotestosterone, with smaller amounts of testosterone. The presence of this high-molecular-weight fraction has already been briefly reported (Belham, Neal & Williams, 1969). The complex is still readily detectable 24 h after administration of the hormone. Similar steroid binding was observed by Talwar *et al.* (1964), who found that oestradiol, administered to ovariectomized rats, becomes associated with a high-molecular-weight fraction. In the present study, no soluble binding component was observed in the nuclear sap. Previous work has shown that the label present in prostatic nuclei, after injections of radioactive testosterone *in vivo*, is associated with the chromatin fraction (Mangan *et al.* 1968). However, in the present experiments, the possibility that a labelled soluble fraction, initially present in the nucleus, was leached out during the fractionation procedures using aqueous solvent, cannot at present be excluded.

Similarly to the fraction isolated from uterus by Talwar *et al.* (1964) it was found that fraction 'A' isolated from prostates of rats 48 h or more after castration was invariably strongly inhibitory towards *E. coli* RNA polymerase *in vitro*. In contrast with the uterine fraction, addition of steroid hormone did not result in removal of the inhibitory action of the fraction isolated from prostate. However, the fact that fraction 'A'

isolated from castrated rats was always more inhibitory towards *E. coli* RNA polymerase *in vitro* than fraction 'A' isolated from testosterone-treated castrated or intact rats indicates that the degree of inhibition was linked in some way to steroid hormone-sensitive processes occurring in the ventral prostate. Similar heat-sensitivity to that reported by Talwar *et al.* (1964) was observed in the case of the inhibitory action of fraction 'A' isolated from the ventral prostate of castrated rats.

The results given in Fig. 2 indicate that the differences in effect on *E. coli* RNA polymerase *in vitro* between fraction 'A' isolated from castrated rats and that isolated from testosterone-pelleted castrated rats can be explained in terms of their relative ribonuclease contents. Prostatic extracts from castrated rats have a higher ribonuclease content than extracts from intact rats. Specific activity of the ribonuclease present in the 100 000g supernatant prepared from 72 h-castrated rats is more than ten times that of the corresponding fraction isolated from testosterone-pelleted castrated rats. It appears from Fig. 2 that the ribonuclease begins to emerge from the column in the column-excluded fraction when extracts obtained from castrated rats are chromatographed on Sephadex G-100. A close parallel exists between the ribonuclease content of the fractions and their effect on the *E. coli* RNA polymerase activity *in vitro*. Saporì *et al.* (1968) have reported that the inhibitory fractions which they isolated from the uteri of ovariectomized rats do not contain detectable ribonuclease activity. If this finding is confirmed, it would appear that the inhibitory actions towards

Fig. 2. Fractionation on Sephadex G-100 of 100000g supernatant from testosterone-pelleted (a) and untreated 48h-castrated rats (b). Fractions (3ml) were collected. The effect of 0.1ml samples on *E. coli* RNA polymerase was determined as described in the Materials and Methods section. Ribonuclease results are expressed as E_{260} after subtraction of the appropriate blanks. For all other details see the text. ●, *E. coli* RNA polymerase (% of control); ○, protein concn. (mg/ml); △, ribonuclease activity ($\Delta E_{260}/90$ min per 0.2ml of fractions).

RNA polymerase activity *in vitro* of uterine and prostatic fractions prepared from castrated rats are due to different biochemical mechanisms.

Although it is difficult to visualize that the cytoplasmic ribonuclease activity observed in fraction 'A' in this work has any particular significance in terms of steroid hormone control of nuclear RNA production [a role suggested by Talwar *et al.* (1964) for the uterine inhibitor] it nevertheless appears that the level of ribonuclease activity in the prostate is related to the hormonal status of the animal. Concerning the possible significance of this enhanced ribonuclease activity in the prostate of

castrated rats, it may be noted that Brandes & Groth (1963) found a marked decrease in the prostatic ribosomal population after orchidectomy. It could be that the increased ribonuclease levels are involved in the turnover of ribosomal RNA in the cytoplasm.

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