A Reconstituted Cell-Free System for the Specific Transfer of Steroid-Receptor Complexes into Nuclear Chromatin Isolated from Rat Ventral Prostate Gland

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1. A system has been developed for the specific transfer of [^3H]dihydrotestosterone-receptor complexes into prostatic chromatin *in vitro*. 2. Under optimum conditions the overall transfer of [^3H]dihydrotestosterone into purified chromatin in this reconstituted system is entirely consistent with the results obtained in whole tissue both *in vivo* and *in vitro*. 3. The transfer of [^3H]dihydrotestosterone into chromatin is tissue-specific and maximal into chromatin isolated from androgen-dependent tissues. 4. The tissue specificity is maintained at two levels: first, in the presence of specific cytoplasmic androgen-receptor proteins; secondly, by the nature and composition of the chromatin itself. 5. Evidence is presented that androgenic steroids *in vivo* may maintain the tissue-specific nature of chromatin in androgen-dependent tissues by the selective induction of nuclear protein synthesis. 6. The relevance of these findings to the mechanism of action of androgenic steroids is discussed.

When [^3H]testosterone is administered intravenously or subcutaneously to male animals it is subjected to extensive metabolic conversion (Farnsworth & Brown, 1963; Ofner, 1969) and there is a selective and extensive retention of one of the principal metabolites, 5α -dihydrotestosterone (17β -hydroxy- 5α -androstane-3-one), in the nuclei of androgen-dependent tissues such as the rat ventral prostate gland (Bruchovsky & Wilson, 1968; Mainwaring, 1969a). Qualitatively similar results were obtained with minces of whole tissue *in vitro* (Anderson & Liao, 1968). Since purified prostatic nuclei alone are unable to bind or retain dihydrotestosterone to any appreciable extent (Mainwaring, 1970; Liao & Fang, 1970), these authors have suggested that cytoplasmic androgen-binding proteins (or androgen 'receptors'), which have a high and selective binding affinity for dihydrotestosterone, are implicated in the nuclear binding of this testosterone metabolite in whole tissue. From previous attempts to stimulate the transfer of dihydrotestosterone from a complex with cytoplasmic receptor into specific sites in intact prostatic nuclei (Mainwaring, 1970; Liao & Fang, 1970), two notable findings emerged. First, the overall transfer of dihydrotestosterone was significantly lower than found in whole tissue either *in vivo* or *in vitro*; secondly, nuclei from androgen-dependent tissues retained far more soluble dihydrotestosterone-receptor complex than nuclei from other

tissues. The main purpose of the present investigations was to develop a reconstituted cell-free system in which the nuclear retention of dihydrotestosterone was reproduced with fidelity and to ascertain the nature of nuclear-associated component(s) involved in this process. The development of such a system was a necessary prerequisite to an investigation of the specific biological role of dihydrotestosterone and, in a wider context, to gain further insight into the mechanism of action of androgenic steroids.

EXPERIMENTAL

Animals

Adult male Sprague-Dawley rats (250 g) were castrated by the scrotal route under Fluothane anaesthesia. All tissues employed in this study were taken from animals 24 h after bilateral castration. When administered, testosterone phenylpropionate (0.25 ml; 10 mg/ml) was given subcutaneously in oil.

Chemicals

[1,2- ^3H]Testosterone (37.2 Ci/mmol) and [5- ^3H]dihydrotestosterone (30 Ci/mmol) were synthesized by Dr M. Wilkinson of this Institute and stored in oxygen-free benzene at 8–10°C. Weekly checks were made on the radiochemical purity of [^3H]dihydrotestosterone by using t.l.c. (Mainwaring, 1969b) and the [^3H]steroid used

was at least 99% pure. [Me - 3H]Thymidine (16.2 Ci/mmol) and L-[4,5- 3H]leucine (12 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Tris base (99.5% pure), crystalline proteins, ox heart catalase, bovine serum albumin and trypsin, Triton X-100, glucose 6-phosphate dehydrogenase (type XV), glucose 6-phosphate (sodium salt), NADP⁺ (sodium salt) and calf thymus DNA were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. *Escherichia coli* alkaline phosphatase was supplied by Miles-Seravac Laboratories, Maidenhead, Berks., U.K. Pronase (grade A) and horseradish peroxidase were purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. Macherey-Nagel cellulose (type 2200 ff) was obtained from Camlab Ltd., Cambridge, U.K., and used without further purification. (NH₄)₂SO₄ was recrystallized from 1 mM-EDTA before use. Other chemicals were of A.R. grade. Glass-distilled water was employed in making up all solutions. Cyproterone acetate (17 α -acetoxy-6-chloro-1,2 α -methylenepregna-4,6-diene-3,20-dione) was kindly given by Schering Pharmaceuticals, Burgess Hill, Sussex, U.K.

Isolation of DNA

This was conducted either by the detergent method of Kay, Simmons & Dounce (1952) or the phenol procedure of Colter, Brown & Ellem (1962). The products in both cases were finally incubated with deoxyribonuclease-free enzymes, first ribonuclease and then Pronase (Mainwaring, 1968). The DNA prepared by the phenol procedure was essentially free of protein, but that prepared by the detergent procedure contained 8–12% (w/w) of residual protein. For the preparation of 3H -labelled DNA, C57 mice bearing the BP8 ascites tumour were given an intraperitoneal injection of 50 μ Ci of [3H]thymidine. The peritoneal fluid was withdrawn 6 h later with a wide-bore hypodermic syringe, and after repeated washing of the tumour cells by low-speed sedimentation in 0.9% NaCl [3H]DNA was isolated by the phenol procedure. The final preparation had approx. 1.5×10^6 c.p.m./10 μ g of DNA. The various DNA preparations used in this study had sedimentation coefficients in the range 26–40 S when examined in the Spinco model E analytical ultracentrifuge.

Preparation of nuclei and chromatin

The isolation procedures were conducted in a cold-room at temperatures as close to 0°C as possible.

Nuclei. Tissues were weighed, minced finely with scissors and homogenized in 0.25 M-sucrose containing 3 mM-CaCl₂ (10 ml/g of tissue) in a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle at a clearance of 0.20–0.24 mm. The homogenate was filtered through 100-gauge stainless-steel filters and nuclei were purified as described by Mainwaring (1969a). The overall recovery of nuclei, based on DNA analyses, was 60–70%, and the samples were essentially pure as judged by morphological and biochemical criteria (Widnell & Tata, 1964).

Chromatin. Nuclei were washed twice by resuspension in 0.25 M-sucrose containing 0.2% of Triton X-100 (Hymer & Kuff, 1964) and collected by centrifugation at 700 g for

10 min. The preparation was performed as described in detail by Mainwaring, Mangan & Peterken (1971). The final preparations of chromatin were gently resuspended in water in all-glass TenBroeck homogenizers so that the DNA content of the solution was 0.4–0.5 mg/ml. Chromatin could be stored for 4 days at 0°C without significant changes in its binding properties. Chemical analysis of chromatin in terms of DNA, RNA, histone and non-histone protein content was performed by the methods of Marushige & Bonner (1966). RNA was removed from chromatin by digestion with electrophoretically purified ribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) at 37°C for 2 h. Selective removal of histone and non-histone protein from chromatin was accomplished by the procedure of T. C. Spelsberg (personal communication). Histones were first removed by resuspending chromatin (4 mg of DNA) in a mixture of 2 M-NaCl in 5 M-urea and, after adjustment of the pH of the solution to pH 5.8 by using a glass electrode at ambient temperature, 0.1 vol. of 0.2 M-Na₂HPO₄-citric acid buffer, pH 6.0, was added. The suspensions were stirred for 30 min and centrifuged at 100 000 g_{av} in a swinging-bucket rotor for 15 h. The histones were recovered in the supernatant fraction. The non-histone proteins were removed from the sediment of histone-deficient chromatin by repeating the 2 M-NaCl–5 M-urea dissociation and subsequent centrifugation but at pH 8.3. At each stage the proteins released were examined by analytical polyacrylamide-gel electrophoresis under the conditions described by Panyim & Chalkley (1969) and Ornstein & Davis (1964) for the histone and non-histone proteins respectively. At pH 6.0 80% of the histones but only 15% of the non-histone proteins were dissociated from chromatin. Sedimented chromatin samples were exhaustively dialysed against water and resuspended by gentle homogenization by hand. Attempts to reconstitute chromatin were made by the stepwise dialysis procedure of Bekhor, Kung & Bonner (1969).

Labelling of receptor proteins with [3H]steroids

Soluble (cytoplasmic) receptor. Eight prostate glands (2–3 g wet wt.) were minced finely with scissors and homogenized in 8 ml of medium A (50 mM-tris-HCl buffer, pH 7.4, containing 0.1 mM-EDTA and 0.25 mM-dithiothreitol). The soluble supernatant extract obtained by centrifugation at 150 000 g_{av} for 1 h was made 10 mM with respect to [3H]dihydrotestosterone (added in 50 μ l of ethanol) and kept for 1 h in an ice bath. Labelled receptor was precipitated by the gradual addition of 0.5 vol. of saturated (NH₄)₂SO₄ (adjusted to pH 7.4 with aq. NH₃) and collected by centrifugation at 10 000 g_{av} for 10 min. The precipitate was dissolved in 5 ml of medium A, giving a solution containing 1.6×10^6 c.p.m./3 mg of protein per ml. Samples of labelled receptor could be stored frozen at –20°C for 3 days, if necessary. For labelling *in vivo*, animals were given a subcutaneous injection of 25 μ Ci of [3H]testosterone (Mainwaring, 1969a). At the appropriate time after the injection the animals were killed by a blow on the head and the excised prostate glands were rapidly placed in ice-cold medium A. Cell supernatant fractions were prepared as described above.

Nuclear receptor. This was prepared by labelling *in*

vitro. Minces of prostatic tissue (eight glands) were incubated for 30 min at 37°C in 5 ml of Eagle's basal medium containing 50 nM-[³H]steroid. Labelling was achieved with either [³H]dihydrotestosterone or [³H]-testosterone in the further presence of NADP⁺ (200 µg), glucose 6-phosphate (200 µg) and glucose 6-phosphate dehydrogenase (25 µg). After thorough cooling and rinsing in cold ³H-free Eagle's medium, nuclei were prepared in the standard manner. Purified nuclei were stirred for 30 min in 2 ml of medium A containing 0.4 M-KCl, and centrifugation of the viscous extract at 100 000 *g*_{av} for 15 min resulted in a preparation of labelled nuclear receptor containing 2.7×10^4 c.p.m./2.5 mg of protein per ml. For use in the reconstituted cell-free system, the ionic strength of the nuclear receptor preparation was rapidly and easily lowered by passage through a 2 cm × 14 cm column of Sephadex G-25 [coarse grade; Pharmacia (U.K.) Ltd., London W.5, U.K.] equilibrated with medium A. Marked losses of labelled receptor were encountered during the more prolonged conventional dialysis owing to the rapid aggregation of protein. Prostate nuclei were also radioactively labelled *in vivo* by the subcutaneous injection of [³H]testosterone; maximum labelling occurred 30 min after the injection.

Interaction of [³H]steroid-receptor complexes with chromatin and DNA

Chromatin. Samples of chromatin (0.1 ml, equivalent to 30–50 µg of DNA) were incubated with ³H-labelled receptors (0.1–0.4 ml; 2×10^3 – 10×10^3 c.p.m.) in a total volume of 0.5 ml in 1.0 ml-capacity centrifuge tubes (Microchemical Specialities Ltd., Berkeley, Calif., U.S.A.) for 1 h at 0–4°C. The samples were adjusted to concentrations of 0.10 M with respect to NaCl and 15 mM with respect to Na₂HPO₄-citric acid buffer, pH 7.0, before incubation. The tubes were centrifuged at 5000 *g*_{av} for 10 min, and the sedimented chromatin was washed twice by resuspension and sedimentation from 1 ml of medium B (15 mM-Na₂HPO₄-citric acid buffer, pH 7.0, containing 0.10 M-NaCl). The washed residue was resuspended in a total of 3 ml of medium B and collected on pre-soaked Millipore filters, 0.45 µm pore size (code no. HAWP 02500). The filters were washed with 3 ml of cold medium B, dried under an infrared lamp and their radioactivities counted in 10 ml of scintillation fluid (2.3 g of 2,5-diphenyloxazole/1 of toluene). In the initial experiments the filters were washed with toluene after the counting of radioactivity, dried once more and heated in 1.0 ml 0.5 M HClO₄ for 10 min at 90°C. DNA determinations were carried out on the acid-soluble extract. In later experiments a duplicate set of incubation mixtures was taken through the entire procedure and analysed directly for membrane-retained DNA. This latter procedure took less time and gave identical results. All counts were corrected for controls in which [³H]steroid-receptor complex was processed in the absence of chromatin. Experiments with histone-deficient chromatin were conducted similarly but with two experimental modifications. First, the insoluble residue from the incubation was collected at 15 000 *g*_{av}, applied for 20 min; secondly, the material was finally collected on Millipore filters under a controlled vacuum equivalent to only 150 mmHg. Despite these precautions the overall recovery of histone-deficient chromatin was only 60–

65%, as compared with the 90% recovery of whole chromatin.

DNA. The retention of [³H]steroid-receptor complexes by purified DNA could not be studied by the procedure employed for chromatin. Such an investigation could be made, however, if the DNA was immobilized by adsorption on cellulose, as suggested by Alberts, Amodio, Jenkins, Gutmann & Ferris (1968). Solutions of DNA (6 ml; 0.4 mg/ml) in medium C (10 mM-tris-HCl buffer, pH 7.4, containing 1 mM-EDTA) were mixed with 2 g of Macherey-Nagel cellulose and kept overnight. The paste was diluted with 30 ml of medium C and freeze-dried in as thin a film as possible in flasks of at least 500 ml capacity. Efficient freeze-drying is essential for the satisfactory preparation of DNA-cellulose. After several washes with medium C on a Buchner head, the DNA-cellulose was resuspended in medium C so that the suspension contained 250 mg of cellulose/ml. This material could be stored satisfactorily at –20°C for at least 6 weeks. The degree of retention of DNA on cellulose was assessed by solubilizing the adsorbed DNA by heating in HClO₄ (Alberts *et al.* 1968). Since the accuracy of this value was essential in comparative studies, batches of DNA-cellulose were prepared from DNA supplemented with small amounts of ³H-labelled ascites tumour DNA (1×10^7 c.p.m.) and the degree of adsorption of DNA was measured by the ³H released by hot acid. These results substantiated the colorimetric DNA analyses precisely. In general, 125–150 µg of DNA was adsorbed/250 mg of cellulose. In some experiments the DNA was denatured either by heating at 95°C for 10 min or by stirring at pH 13.0 for 10 min before adsorption on cellulose. Small columns (1 cm diam.) were packed with DNA-cellulose (125–150 µg of DNA) and samples of cytoplasmic [³H]steroid-receptor complex (1×10^4 – 4×10^4 c.p.m.) were applied in a total volume of 3 ml in medium D [20 mM-tris-HCl buffer, pH 7.4, containing 0.25 mM-dithiothreitol, 1 mM-EDTA and 10% (v/v) of glycerol]. With medium D as eluent, eight fractions (2 ml) were collected and the column was then eluted with medium D containing 0.6 M-KCl. The radioactivity eluted as an exceedingly sharp peak, generally in a single 2 ml fraction, and the amount was used to calculate the amount of [³H]steroid-receptor complex bound to DNA. As controls the corresponding amount of free [³H]dihydrotestosterone or steroid-receptor complex dissociated with 1 mM-N-bromosuccinimide (Mainwaring, 1969b) were used. Such values were only 4–5% of those obtained with the intact [³H]steroid-receptor complex. The binding was exclusively to the adsorbed DNA; cellulose columns alone retained negligible radioactivity.

Measurement of the incorporation of [³H]leucine into protein in vitro

Batches of minced tissue (approx. 1 g wet wt.) were placed in 5 ml of Krebs improved Ringer II medium (Krebs, 1950) in an atmosphere of O₂+CO₂ (95:5) in a Dubnoff metabolic shaker at 37°C. After temperature equilibration, 25 µCi of [³H]leucine was added and the incubations were continued with gentle shaking. At timed intervals batches of tissue were cooled rapidly and treated with 1.0 ml (100 µg) of puromycin. After a rinsing with 0.25 M-sucrose–3 mM-CaCl₂, the tissue was homogenized in this medium, filtered and centrifuged at 700 *g* for

10 min. The crude sediment of nuclei was washed three times by resuspension and sedimentation from 0.25 M-sucrose containing 0.2% of Triton X-100. All washes were added to the original 700g supernatant fraction. Washed nuclei and the 700g supernatant (1.0 ml) were heated with 5 ml of 5% (w/v) trichloroacetic acid at 95°C for 10 min and then thoroughly chilled in ice. About 15 min later the acid-insoluble residues were collected on Whatman GF/A glass-fibre discs and washed twice with 5 ml of cold 5% (w/v) trichloroacetic acid and twice with 5 ml of cold ethanol. The discs were dried and their radioactivities counted in toluene-based scintillation medium.

Sucrose density gradients

Linear 4 ml 5–20% (w/v) sucrose density gradients in medium A were prepared in a gradient former (model 570; Instrumentation Specialties Co., Lincoln, Nebraska, U.S.A.). Some gradients contained a uniform concentration of 0.5 M-KCl. Samples for analysis (0.25 ml) were layered over the gradients and centrifuged at 3°C for 15 h at 100 000 $g_{av.}$. At the completion of the runs, the contents of each gradient were automatically subdivided into 20 fractions by using an ISCO model 182 gradient analyser. Proteins of known sedimentation coefficient [catalase ($s_{20,w}$ 11.4S), *E. coli* phosphatase ($s_{20,w}$ 6.4S) and peroxidase ($s_{20,w}$ 3.6S)] were run as markers in parallel gradients.

Counting of radioactivity

Radioactivities of samples of ^3H -containing materials, retained on either Millipore membranes or glass-fibre discs, were counted in a toluene scintillation medium (Mainwaring *et al.* 1971) in a Nuclear-Chicago mark II scintillation spectrometer at an efficiency of 40–44%. Aqueous samples were counted in a water-miscible phosphor (Mainwaring, 1969a) at approx. 33% counting efficiency. Quenching was determined by the channels-ratio procedure.

Chemical determinations

Depending on the amount of material available, DNA was determined either by the colorimetric procedure of Burton (1956) or by the more sensitive fluorimetric method of Kissane & Robins (1958). Thymus DNA was used as reference standard. Protein was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as the standard.

RESULTS

Intracellular distribution of [^3H]androgens after the administration of [^3H]testosterone in vivo. As shown in Fig. 1, there is a rapid uptake of ^3H into the rat prostate after a single subcutaneous injection of [^3H]testosterone *in vivo*. Maximum labelling occurs first in the cell supernatant fraction and later within the nuclei. The ^3H remained firmly associated with the nuclei even when radioactivity was completely lost from other subcellular

Fig. 1. Intracellular distribution of radioactivity in rat prostate after a single subcutaneous injection of [^3H]testosterone. A total of 18 animals were each given 10.6 μCi of [^3H]testosterone at zero time. At timed intervals thereafter the prostate glands from two animals were pooled, homogenized in sucrose-EDTA and subcellular fractions were prepared by differential centrifugation (Harding & Samuels, 1961). Each point represents the total radioactivity recovered in each fraction. Protein-bound radioactivity in the cell supernatant fraction was precipitated with protamine sulphate (Mainwaring, 1969b). Negligible radioactivity was associated with the mitochondrial fraction, and these results are not included. ○, Total radioactivity; ●, cell supernatant; △, nuclei; ▲, microsomal fraction.

fractions. Although not shown in Fig. 1, detectable radioactivity in prostatic nuclei persists for 15–16 h after the administration of [^3H]testosterone. This agrees with the results of Tveter & Attramadal (1968). Over 80% of the ^3H bound to the cell supernatant and nuclear fractions was recoverable as [^3H]dihydrotestosterone. When considered with the results of Mainwaring (1970), the present results are consistent with a two-stage binding process for androgenic steroids *in vivo*, whereby [^3H]testosterone is rapidly metabolized to [^3H]dihydrotestosterone and bound to the cytoplasmic protein, then subsequently transferred to nuclear chromatin. Although the possibility of redistribution of radioactivity during homogenization cannot be explicitly eliminated in this experiment, this scheme of binding is also indicated by the results given below. Further, the employment of sucrose-EDTA as homogenizing medium (Harding & Samuels, 1961) should provide relatively clean subcellular fractions from rat prostate gland.

Fig. 2. Analysis of labelled prostatic supernatant fractions by sucrose-density-gradient centrifugation. Samples of cell supernatant fraction (0.25 ml) were layered over linear 5–20% (w/v) sucrose density gradients prepared in medium A and centrifuged at $100\,000g_{av}$ for 15 h at 3°C . The radioactivities of fractions (0.20 ml) were counted individually. The direction of centrifugation was from right to left. Sedimentation markers included *E. coli* alkaline phosphatase (6.4S) (A) and horseradish peroxidase (3.6S) (P) run in parallel. (a) Supernatant labelled by subcutaneous injection of $[^3\text{H}]$ testosterone *in vivo*: —, 2.0–5.0 min; ----, 10–15 min. (b) Supernatant labelled *in vitro*, 1 h at 0°C : —, $[^3\text{H}]$ dihydrotestosterone; ----, either $[^3\text{H}]$ cortisol or $[^3\text{H}]$ androstenedione alone, or $[^3\text{H}]$ dihydrotestosterone with a 100-fold excess of non-radioactive dihydrotestosterone or a 1000-fold excess of cyproterone acetate; -.-.-, $[^3\text{H}]$ dihydrotestosterone, precipitated at 33% saturation with $(\text{NH}_4)_2\text{SO}_4$.

Nature of the cytoplasmic binding protein. After short periods of labelling with $[^3\text{H}]$ testosterone *in vivo* the radioactivity in the cell supernatant fraction of rat prostate is associated with a protein of sedimentation coefficient of approx. 8S (Fig. 2a). At longer labelling times less 8S protein was identified and more binding to a slower-sedimenting 3.5S protein was found. When the cell supernatant was treated with $[^3\text{H}]$ dihydrotestosterone at 0°C *in vitro* both the 8S and the 3.5S androgen 'receptors' were identified (Fig. 2b). Some non-specific binding is present in the 3.5S region, however, because whereas binding of $[^3\text{H}]$ dihydrotestosterone to the 8S form of receptor is abolished completely by the addition of a large excess of non-radioactive dihydrotestosterone or the anti-androgen cyproterone acetate, the binding in the 3.5S region is not greatly diminished in competitive binding studies. Further, both $[^3\text{H}]$ cortisol and $[^3\text{H}]$ androstene-3,17-dione can bind to the 3.5S protein to a limited extent, but not to the 8S protein. Ammonium sulphate (33% saturated) selectively precipitated the $[^3\text{H}]$ dihydrotestosterone-8S receptor complex. Since this form of complex was the first to be identified by labelling whole tissue *in vivo*, it was largely employed in the reconstitution experiments described below. For practical con-

venience, however, it was isolated by fractionation of prostatic cytoplasm with ammonium sulphate after labelling *in vitro* at 0°C .

Preparation of chromatin. In the initial experiments nuclei that had been purified simply by sedimentation in 2.2M-sucrose were used directly for the preparation of chromatin. The results were unsatisfactory, for not only was the chemical composition of the chromatin variable but it also gave inconsistent binding of $[^3\text{H}]$ steroid-receptor complexes in the reconstituted cell-free systems. Further washing of nuclei with Triton X-100 before commencing the preparation of chromatin gave a marked improvement in two ways. Not only was the chemical composition of the chromatin constant (Table 1) but reproducible results were obtained in the systems for binding *in vitro*. The washing with detergent was particularly important with the accessory sexual tissues, both of which contain complex proteinaceous secretions, including very basic proteins (see Mann, 1963). Adsorption of such material on the nuclear membrane may explain the high basic protein/DNA ratios in the absence of detergent treatment. Certainly at the concentration employed, Triton X-100 would dissolve the outer nuclear membrane (Hymer & Kuff, 1964) and release such adherent contaminants. All the

Table 1. *Influence of Triton X-100 treatment on the final composition of nuclear chromatin*

Purified nuclei, with or without further treatment with detergent, were used for the preparation of chromatin. Results are the means of three determinations, with pooled tissue from eight animals per experiment. The s.d. was $\pm 5-8\%$ with Triton X-100 and $\pm 14-18\%$ without Triton X-100. Analyses are given relative to the amount of DNA as 1.0.

Tissue	With Triton X-100		Without Triton X-100	
	Histone	Non-histone	Histone	Non-histone
Prostate	0.92	0.60	1.71	0.81
Seminal vesicle	0.96	0.63	2.23	0.88
Liver	0.81	0.54	0.99	0.56
Kidney	0.76	0.43	0.81	0.46
Spleen	0.68	0.43	0.76	0.52

Fig. 3. Effect of varying the conditions of assay on the binding of steroid-receptor complex to chromatin. In all experiments [^3H]dihydrotestosterone-8S receptor complex from prostate (7000 c.p.m.) was incubated with chromatin (30–50 μg of DNA) for 40 min at 0°C . (a) Effect of ionic strength. Samples were incubated and washed with various concentrations of NaCl in 20 mM-citrate-phosphate buffer, pH 7.0, at 0°C ; $\circ$, prostate chromatin; Δ , liver chromatin; $\square$, spleen chromatin. (b) Effect of temperature. Samples of prostate chromatin were incubated for various times at 37°C or 0°C and washed in 0.15 M-NaCl solution; $\circ$, 0°C ; Δ , 37°C . (c) Effect of pH. Samples of prostatic chromatin were incubated and washed in 0.15 M-NaCl in 20 mM-citrate-phosphate buffers of various pH values.

chromatin preparations contained only small amounts of RNA (approx. 4–5% of chromatin DNA).

Optimum conditions for the binding of [^3H]steroid-receptor complexes to chromatin in vitro. Preliminary experiments were conducted to establish the optimum conditions for the binding of [^3H]dihydrotestosterone-8S receptor complex to various chromatin preparations. Particular attention was given to the effects of ionic strength, temperature and pH on the reactions. The results are summarized in Figs. 3(a), 3(b) and 3(c). From these experiments it was concluded that maximum binding occurred after 40 min at 0°C in a medium containing 0.15 M-sodium chloride at neutral pH. The binding process proceeded faster at 37°C , but dissociation of labelled complex was also apparent. Incubations were therefore conducted at 0°C .

Specificity of the transfer of [^3H]steroid-receptor

complexes in vitro. Cross-over experiments were performed on the binding of [^3H]dihydrotestosterone-receptor complexes to chromatin. The results are summarized in Fig. 4. Binding of [^3H]dihydrotestosterone to prostatic chromatin had an absolute requirement for the presence of a specific androgen 'receptor' protein (Fig. 4a). Transfer of radioactivity was accomplished with cytoplasmic preparations from androgen-dependent tissues, but not with those from spleen or kidney. Binding of the equivalent amount of free [^3H]dihydrotestosterone was minimal. The receptor protein complexes could not be replaced by rat serum at an identical concentration of protein, even though [^3H]dihydrotestosterone is completely bound by serum proteins (Mainwaring, 1969b). Further, the overall transfer of [^3H]dihydrotestosterone to specific sites in prostatic chromatin is suppressed by the presence of an excess of dihydro-

Fig. 4. Specificity of the binding of [³H]dihydrotestosterone-receptor complexes to chromatin. Labelled receptor complexes (2×10^3 – 10×10^3 c.p.m.) were added in increasing amounts to a constant amount of various chromatins (30–50 µg of DNA) under optimum conditions. For full details see the Experimental section. (a) Binding of [³H]steroid to prostatic chromatin with various fractions precipitated by (NH₄)₂SO₄ from labelled cytoplasmic fractions: ○, prostate; ●, seminal vesicle; △, kidney; ▲, spleen; □, free [³H]dihydrotestosterone. (b) Binding of prostatic [³H]dihydrotestosterone-8S receptor complex to various chromatin preparations: ○, prostate; ●, seminal vesicle; △, liver; ▲, kidney; □, spleen. (c) Binding of prostatic [³H]dihydrotestosterone-nuclear receptor complex to chromatin preparations: ○, prostate; △, liver; □, spleen.

testosterone or cyproterone acetate during the initial labelling of the prostatic cytoplasmic fraction, before fractionation with ammonium sulphate. With [³H]dihydrotestosterone-8S receptor complex from prostate as the model steroid donor, binding to chromatin from androgen-dependent tissues was very much higher than that obtained with other chromatin preparations (Fig. 4b). Significant binding to liver chromatin was found, however. An essentially similar result was obtained when [³H]dihydrotestosterone was presented to various chromatins in the form of a complex with prostatic nuclear receptor (Fig. 4c). On the basis of these experiments it was concluded that the selective retention of dihydrotestosterone in the chromatin of androgen-dependent tissues is controlled at two or more levels: first, in cytoplasm through the presence of specific androgen receptor protein; secondly, in the nature of the chromatin itself. Certainly the nuclear uptake of dihydrotestosterone could be described in terms of a two-step binding mechanism in accordance with the experiments *in vivo* described in Fig. 1. In such a process the dihydrotestosterone would be transferred to the chromatin complex with the cytoplasmic receptor. If such a theory is tenable, then it is necessary to demonstrate that the physical properties of the binding protein extracted from chromatin after incubation in the reconstituted cell-free system are similar to the physical properties of the material formed in whole tissue. Such a similarity was demonstrated (Fig. 5). The [³H]-dihydrotestosterone-nuclear protein complex formed in whole tissue has a sedimentation co-

efficient of approx. 4.5S when analysed in sucrose density gradients in the presence or absence of potassium chloride (Fig. 5a), although at low ionic strength a small amount of 8S protein was found, together with much aggregated material at the bottom of the gradient. A similar [³H]dihydrotestosterone-macromolecular complex could be formed *in vitro* with prostatic chromatin, but not with spleen chromatin (Fig. 5b). The consistent sedimentation coefficient of the complex isolated from chromatin and nuclei under various conditions of ionic strength is of considerable interest, because the freely reversible dissociation of [³H]dihydrotestosterone-8S receptor complex into a 3.5S form has been reported (Mainwaring, 1970; Baulieu & Jung, 1970). This disparity in behaviour suggests that the 8S receptor protein is modified either during binding to chromatin or during subsequent extraction.

Comparison of the extent of [³H]dihydrotestosterone binding in the reconstituted system with binding in whole tissue. For the cell-free transfer of dihydrotestosterone to be meaningful in terms of overall binding and the possible mechanism of action of androgenic steroids, the extent of transfer should be in agreement with results obtained in whole tissue. As seen in Table 2, the results from a variety of experimental procedures were in excellent agreement. Saturation of the nuclear binding sites *in vivo* required the administration of 50–60 µCi of [³H]testosterone to each animal. Maximum labelling *in vitro* was attained at a [³H]dihydrotestosterone concentration of 30 nM. If the average DNA content of each prostatic nucleus is taken as

Fig. 5. Analysis of nuclear-[^3H]dihydrotestosterone complexes by sucrose-density-gradient centrifugation. Samples under investigation were layered over linear 5–20% (w/v) sucrose density gradients prepared in medium A and centrifuged at $100000g_{av}$ for 15 h at 3°C . Sedimentation markers employed were catalase (11.4 S) (C) and peroxidase (3.6 S) (P). Some gradients contained a uniform concentration of 0.5 M-KCl. (a) Nuclear-[^3H]dihydrotestosterone complex formed in whole tissue *in vitro* and analysed in the presence (—) or in the absence (----) of 0.5 M-KCl. (b) [^3H]Dihydrotestosterone-protein complex formed on incubation of prostate chromatin with [^3H]dihydrotestosterone-8 S receptor complex *in vitro* and released on extraction of sedimented chromatin with 0.3 M-KCl. —, Whole tissue (a) as a comparison; ---, reconstituted cell-free systems in the presence of 0.5 M-KCl; ----, reconstituted cell-free systems in the absence of 0.5 M-KCl; ····, spleen chromatin in the presence of 0.5 M-KCl.

Table 2. Comparison of the binding of [^3H]dihydrotestosterone to prostatic chromatin measured by a variety of methods

Details of the individual procedures are given in the Experimental section. All experiments were conducted on pooled tissue from four animals, 24 h after bilateral castration, with saturating amounts of [^3H]steroids. Calculation for the reconstituted system was made from the maximum values shown in Fig. 4.

System	Retention of [^3H]dihydrotestosterone (pmol/g of DNA)
(1) Reconstituted cell-free system	
(a) With ^3H -labelled cytoplasmic receptor	1360
(b) With ^3H -labelled nuclear receptor	1470
(2) Whole tissue <i>in vivo</i>	1310
(3) Whole tissue <i>in vitro</i>	1210

8.0 pg (Mirsky & Ris, 1949; Bruchovsky & Wilson, 1968) and it is assumed that all the potential nuclear binding sites are unoccupied in 24h-castrated animals, then it can be calculated that approx. 6000 molecules of dihydrotestosterone are required to saturate all the potential nuclear binding sites in a single prostatic cell.

Nature of the chromatin-associated component(s) responsible for the tissue-specific acceptance of [^3H]steroid-receptor complexes. Bekhor *et al.* (1969) have suggested that chromatin-associated RNA is implicated in certain sequence-specific interactions of DNA and chromosomal proteins. In the present case, however, the small amount of chromatin-associated RNA does not play any part in main-

taining the tissue-specific nature of chromatin with respect to the binding of [^3H]steroid-receptor complexes. After complete removal of RNA by ribonuclease digestion the tissue-specific binding of receptor complexes to prostatic chromatin is not impaired. An indication that chromatin-associated proteins may be involved in the tissue specificity of receptor binding came from studies on the stimulation of nuclear protein synthesis in castrated animals after the administration of testosterone *in vivo*. As indicated in Fig. 6, androgens induce a small and transient increase in nuclear protein synthesis in the prostate gland at about the same time that protein synthesis in general is stimulated by hormonal treatment. The increase in protein

synthesis is a relatively late event when compared with the rapid response of nuclear RNA synthesis. It does occur, however, before a significant increase in DNA synthesis can be detected (Coffey, Shimazaki & Williams-Ashman, 1968). This increase in nuclear protein synthesis could not be demonstrated in kidney or spleen and suggests that certain chromatin-associated proteins may indeed be tissue-specific. In a further series of experiments a comparison was made of the binding of [³H]-dihydrotestosterone-receptor complex to chromatin, histone-deficient chromatin and DNA from several tissues (Table 3). Removal of the histones from chromatin resulted in a large increase in available binding sites for the [³H]dihydrotestosterone-receptor complex, but despite this the

tissue specificity of the chromatin was retained. Conversely, the removal of all DNA-associated proteins by extraction with phenol decreased the degree of binding to a common value, irrespective of the tissue of origin of the DNA. This result was contrary to findings obtained with DNA prepared by the detergent procedure and that still contained 8–15% of protein relative to DNA. With such preparations prostate DNA was superior to other DNA preparations with respect to the binding of receptor complexes *in vitro*. This tissue specificity was maintained even when the DNA was denatured, either by heating or incubation at alkaline pH. It is difficult to envisage how DNA-associated proteins could withstand either of these treatments without irreversible denaturation, and the results suggest

Fig. 6. Stimulation of activities in prostatic tissue after the administration of testosterone *in vivo*. Approx. 48 h after castration rats were given a single injection of 2.5 mg of testosterone subcutaneously, and at timed intervals thereafter groups of four animals were killed. The incorporation of [³H]leucine into proteins of prostatic nuclei and extranuclear components was measured *in vitro* as described in the Experimental section. Activities were compared with those of untreated controls. Values for the incorporation of [³H]uridine and the activities of microsomal fraction are taken from P. A. Wilce & W. I. P. Mainwaring (unpublished work). ●, Incorporation of [³H]uridine into nuclei; ○, incorporation of [¹⁴C]phenylalanine into protein with prostatic microsomal fraction; ▲, incorporation of [³H]leucine into nuclear protein; △, incorporation of [³H]leucine into extranuclear protein.

Table 3. Comparison of the binding of [³H]dihydrotestosterone-receptor complex to modified chromatin or DNA

The binding of ³H-labelled receptor to DNA was measured by the DNA-cellulose procedure. Full details are given in the Experimental section. DNA was prepared either by a detergent procedure or after extraction with phenol. Where indicated, DNA was denatured before adsorption on cellulose either by heating or by incubation at alkaline pH. Labelled receptor was prepared from prostate cytoplasm in all cases. The results are the means of three determinations at saturating concentrations of ³H-labelled receptor.

Binding of ³ H-labelled receptor (d.p.m./50 µg of DNA)					
Tissue	Chromatin	Histone-deficient chromatin	DNA (detergent method)		DNA (phenol method)
			Native	Denatured	
Prostate	6210	18421	12120	11271	3220
Liver	2330	6341	3210	3111	2620
Spleen	843	2696	2762	2840	2496

that the [^3H]steroid-receptor complexes are bound to the DNA of chromatin rather than to associated proteins. Nevertheless the DNA-associated proteins could play a major role in controlling the degree and tissue specificity of the binding to DNA. The difference in the binding behaviour of prostate DNA prepared by different methods may well be explained by the results of Teng, Teng & Allfrey (1970). They have shown that certain acidic tissue-specific nuclear proteins are so firmly bound to DNA that they can be released only by treatment with phenol. The proteins remaining on prostate DNA after treatment with detergent may well fall into this category.

Attempts were made to demonstrate the importance of DNA-associated proteins in maintaining the tissue specificity of chromatin by reconstituting the chromatin *in vitro* (Bekhor *et al.* 1969). Even when entirely homologous chromatin fractions were mixed together, however, only partial reconstitution was achieved, and the composition of the products did not agree well with the values given in Table 1. For this reason this aspect of the work was not pursued further.

DISCUSSION

The present observation that [^3H]steroid-receptor complexes may be bound to chromatin *in vitro* in a manner that closely reproduces that achieved in whole tissue is entirely consistent with the findings of King *et al.* (1971) on the binding of oestradiol-17 β in uterine nuclei *in vitro* and with those of Steggle, Spelsberg & O'Malley (1971) on the binding of progesterone in a reconstituted system derived from chick oviduct. The nuclear uptake of steroidal hormones is best explained by the initial formation of a specific cytoplasmic steroid-receptor complex and its subsequent transfer to specific sites within nuclear chromatin. The migration of proteins from the cytoplasm into the nucleus of cells of higher organisms is widely documented (Gurdon, 1967; Gurdon & Woodland, 1968; Merriam, 1969), and the only distinguishing feature of the process described here is that the transport of the cytoplasmic receptor protein requires the additional presence of a specific steroid, 5 α -dihydrotestosterone. It is possible that the steroid maintains the protein in a suitable configuration for transport. Since dihydrotestosterone cannot be retained in prostatic nuclei in the absence of cytoplasm (Mainwaring, 1970) and the hormone-dependent stimulation of RNA synthesis cannot be evoked in purified nuclei simply by the addition of dihydrotestosterone (Mainwaring *et al.* 1971), it seems likely that the transport of the dihydrotestosterone-receptor complex into chromatin may be fundamentally important in the mechanism of

action of androgenic steroids. Support for this view is provided by studies on the activation of plant chromatin by low-molecular-weight plant auxins (Hardin, O'Brien & Cherry, 1970; Matthyssse, 1970).

The overall transfer of dihydrotestosterone, approx. 6000 molecules of steroid/prostatic nucleus, is significantly higher than that observed in earlier studies *in vitro* (Liao & Fang, 1970; Mainwaring, 1970), yet it is in the range suggested for the nuclear binding of oestradiol-17 β in rat uterus (King *et al.* 1971). Nevertheless it is higher than the number of aldosterone molecules necessary to saturate the potential binding sites in a rat kidney nucleus (Swaneck, Chu & Edelman, 1970).

The tissue-specific binding of steroid-receptor complexes clearly requires the presence of cytoplasmic receptor proteins, but, as first reported by King, Gordon & Steggle (1970), some specificity also resides in the nature of the chromatin itself. Steggle *et al.* (1971b) have described chromatin as 'pre-programmed' to receive steroid-receptor complexes. The best interpretation of these findings is that the steroid-receptor complexes are bound to the DNA of chromatin and that the number of available sites is controlled by the non-histone chromatin-associated proteins. Several indirect lines of evidence substantiate this viewpoint. First, the androgen receptor proteins are acidic in character (Mainwaring, 1969a,b) and the binding of such proteins from both bacterial (Gilbert & Müller-Hill, 1967; Ptashne, 1967; Alberts *et al.* 1968) and mammalian (Kleinsmith, Heidema & Carroll, 1970; Teng *et al.* 1970; Salas & Green, 1971) sources to DNA is well established. Secondly, despite certain evidence to the contrary (Sluyser, 1966) and the possible exception of avian erythrocytes (Edwards & Hnilica, 1968), histones are insufficiently heterogeneous or tissue-specific to account satisfactorily for the subtle differences between various chromatins. Thirdly, the non-histones described by Teng *et al.* (1970) are sufficiently tissue-specific and heterogeneous to suggest their involvement in the maintenance of tissue-specific character. Finally, physicochemical investigations of chromatin (Clark & Felsenfeld, 1971; Itzhaki, 1971) have indicated that at least 40% of the DNA is readily accessible to large-molecular-weight molecules and the number of potential binding sites is higher than expected from previous work. The selective induction of nuclear protein synthesis in the prostate by testosterone may represent an example of the processes by which the tissue specificity of chromatin is maintained. Certainly other steroidal hormones such as oestradiol-17 β (Teng & Hamilton, 1970) and cortisol (Shelton & Allfrey, 1970) are known to regulate the synthesis of non-histone nuclear proteins in their respective target organs.

The development of the cell-free system for the transfer of dihydrotestosterone *in vitro* and the availability of the androgen-stimulated form I RNA polymerase (Chesterton & Butterfield, 1971) make an investigation into the biological role of steroid-receptor complexes a feasible proposition.

Note added in proof. Recent work by Steggles, Spelsberg, Glasser & O'Malley (1971) confirms the findings given in the present paper.

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