Biochem. J. (1973) **136**, 611–622
Printed in Great Britain

Stimulation of Ribonucleic Acid Polymerase Activity *in vitro* by Prostatic Steroid–Protein Receptor Complexes

By P. DAVIES and K. GRIFFITHS
Tenovus Institute for Cancer Research, Welsh National School of Medicine, The Heath, Cardiff CF4 4XX, U.K.

(*Received* 10 *April* 1973)

A system has been developed which allows the stimulation *in vitro* of prostatic RNA polymerase by prostatic 5α-dihydrotestosterone–protein receptor complexes prepared from the tissues of castrated rats. The reconstitution *in vitro* of such a system necessitates the purification of several subcellular components. Two 5α-dihydrotestosterone–receptor complexes are located in the prostatic soluble supernatant fraction, separable by selective ammonium sulphate fractionation, and one complex can be isolated from the nuclear fraction. In the presence of all these complexes, stimulation of RNA polymerase in intact nuclei and nucleoli was observed. The complexes also increased the activity of the enzyme solubilized from whole nuclei. Greater stimulation of this system was noted in the presence of prostatic chromatin as template, as compared with that observed with calf thymus DNA or liver chromatin as template. The effects of the complexes on sub-nuclear forms of RNA polymerase, of nucleolar and extranucleolar origin, are also described. RNA polymerase solubilized from nucleoli is more susceptible to stimulation by the 5α-dihydrotestosterone–receptor complexes than is the 'nucleoplasmic' enzyme. Stimulation occurs less readily in the presence of Mn^{2+} and at high ionic strength than in the presence of Mg^{2+} and at low ionic strength. Preliminary experiments show that prostatic nucleolar RNA polymerase transcribes prostatic chromatin poorly as compared with the nucleoplasmic enzyme. The observations reported indicate an involvement of non-histone proteins associated with DNA in the process by which stimulation of enzyme activity by the 5α-dihydrotestosterone–receptor complexes is achieved. The implications of these findings in the mechanism of steroid hormone action is considered.

Reports from a number of research centres during the past few years have clearly indicated that protein receptor molecules have an essential role in transmitting the effects of steroid hormones within the cells of responsive tissues. The process involves an interaction between the steroid hormone and a specific receptor protein in the cytoplasm (Jensen *et al.*, 1968; Mainwaring, 1970; Fang & Liao, 1971; O'Malley, 1971). The complex thus formed is transferred to the nucleus, where a close association of the steroid hormone is established with certain chromosomal sites composed of DNA and nuclear acidic protein (Fang & Liao, 1971; Spelsberg *et al.*, 1971*a*; O'Malley *et al.*, 1971, 1972; Mainwaring & Peterken, 1971). The steroid–receptor complex, rather than the steroid alone, is retained within the nucleus. However, the function of such steroid–receptor complexes within the nucleus and the nature of the effects that the various components of this system may have upon gene transcription are still uncertain.

The present investigation provides some evidence that a steroid–protein receptor complex may be concerned in controlling the activity of the DNA-dependent RNA polymerase (nucleoside triphosphate–RNA nucleotidyltransferase; EC.2.7.7.6) from nuclei of rat prostatic tissue. Preliminary studies (Davies *et al.*, 1972) had shown that certain specific steroids were capable of stimulating *in vitro* the RNA polymerase in purified rat and dog prostatic nuclei. The aim of the investigation now reported was to reconstruct *in vitro*, from various purified components, a system whereby the 17β-hydroxy-5α-androstan-3-one–receptor complex formed in the prostate could stimulate the activity of the RNA polymerase.

Materials and Methods

Materials

Animals. Adult male Sprague–Dawley rats (8–12 weeks old) were acquired from Charles River Ltd., Margate, Kent, U.K. Animals were castrated by the scrotal route under ether anaesthesia. In all experiments described, ventral prostate tissue used was removed from the animals 48h after bilateral castration.

Chemicals. 17β-Hydroxy-5α-[1α,2α-$^3H_2$]androstan-3-one (specific radioactivity, 47Ci/mmol) and

[U-^{14}C]UTP (specific radioactivity, 514mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Dithiothreitol, 17β-hydroxy-5α-androstan-3-one, UTP, GTP and CTP (sodium salts) and ATP (disodium salt) were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Bovine serum albumin and *Escherichia coli* (strain K-12) ribonucleic acid polymerase [214units/mg of protein: suspension in glycerol–Tris–Cl (1:1) buffer, pH7.9] were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Tris (Aristar grade), yeast RNA, Triton X-100 and calf thymus DNA were obtained from BDH Ltd., Poole, Dorset, U.K. Aquasol is a product of the New England Nuclear Corp., and was obtained from Reading, Berks., U.K. Sephadex G-25 was obtained from Pharmacia (U.K.) Ltd., London, U.K. All other substances were of AnalaR grade and solutions used were made up in water distilled from all-glass apparatus.

Centrifuges and rotors. All high-speed centrifugation procedures were carried out in the Beckman L65 B preparative ultracentrifuge with either the SW 50 (3×5ml) swinging-bucket rotor ($r_{av.}$ = 7.3cm) or the SW 50.1 (6×5ml) swinging-bucket rotor ($r_{av.}$ = 8.35cm). Low-speed centrifugation procedures (less than 1000*g*) were carried out in the MSE Mistral 4L refrigerated centrifuge.

Methods

Preparation of subcellular fractions. All preparative procedures were carried out at temperatures between 0° and 4°C.

Nuclei. Ventral prostate glands were weighed, finely minced with scissors and homogenized in 0.25M-sucrose containing 1mM-$MgCl_2$ in a Potter–Elvehjem homogenizer with a motor-driven Teflon pestle with a clearance of 0.15–0.23mm. Homogenates were filtered through sterile gauze and the filtrate was centrifuged at 800*g* for 15min. Pellets were resuspended by gentle homogenization by hand in 2.2M-sucrose containing 1mM-$MgCl_2$ and nuclei purified by centrifugation through this medium at 50000*g* for 1h. Based on DNA determinations, 60–70% of tissue nuclei were recovered in a purified form. Satisfactory purity was assessed by biochemical and light- and electron-microscopic criteria (Widnell & Tata, 1964).

Nucleoli and nucleoplasm. Purified nuclei were washed once by centrifugation in 0.25M-sucrose and evenly suspended by homogenization by hand in 0.25M-sucrose in 5mM-Tris–HCl buffer, pH7.4. The suspensions were sonicated for 45s at 20kHz in an MSE 150W ultrasonic disintegrator (medium speed, setting 4; amplitude, 8μm). The sonicate was layered over an equal volume of 0.88M-sucrose and centrifuged at 2500*g* for 30min. The upper layers, comprising the nucleoplasm, were removed. The nucleoli were purified by repeated resuspension and re-centrifugation at 800*g* in 0.88M-sucrose (Muramatsu *et al.*, 1963). Percentage disintegration of nuclei was estimated by microscopic examination of specimens stained with Azure C (Ayres, 1948). Under the conditions described, over 80% of the nuclei were disintegrated. In most nucleolar preparations, nuclear contamination was completely absent. Approx. 15% of nuclear DNA was recovered in the nucleolar preparations. Purified nuclei and nucleoli, if not required for further preparative procedures, were resuspended in 0.25M sucrose–1mM-$MgCl_2$.

Chromatin. This was prepared from purified nuclei or nucleoli by methods similar to those described by Mainwaring *et al.* (1971) and Mainwaring & Peterken (1971). Nuclei were twice washed by resuspension in 0.25M-sucrose containing 0.2% (v/v) of Triton X-100 followed by centrifugation at 800*g* for 10min. This removes contaminating basic proteins (Hymer & Kuff, 1964) and allows for reproducible results for the chemical composition of chromatin (Mainwaring & Peterken, 1971). The main procedure consisted of two extractions with 80mM-NaCl–20mM-EDTA, titrated with citrate buffer to pH6.3, two extractions with 0.35M-NaCl and one extraction with 1.5mM-NaCl in 0.15mM-citrate buffer, pH7.0. At each successive stage, the chromatin was sedimented by centrifugation at 10000*g* for 15min. The final gel-like preparations were suspended in water to a concentration of 20–50μg of DNA/100μl by gentle homogenization by hand in an all-glass homogenizer and dialysed against distilled water. Preliminary chemical analysis of chromatin by the methods of Marushige & Bonner (1966) indicated that 85–95% of the nuclear or nucleolar DNA was recovered in the preparations. The RNA content of chromatin was less than 5% of the DNA content. Chromatin could not be prepared from nucleoplasm, by using the methods described above, with any reproducibility of chemical composition. This presumably was due to the shearing effects of the sonication procedures used to separate nuclei into nucleoli and nucleoplasm. However, chromatin was prepared from liver and spleen nuclei by the method described.

Selective removal of histone and non-histone proteins from prostatic chromatin was accomplished by methods based on those of Mainwaring & Peterken (1971) and Spelsberg *et al.* (1971*b*). Chromatin suspension (2ml) was mixed with 3ml of 2M-NaCl in 5M-urea and to the mixture was added 0.5ml of 0.2M-Na_2HPO_4–citric acid buffer, pH6.0. The suspensions were stirred for 30min and centrifuged at 100000*g* for 15h. The histones were recovered in the supernatant fraction. Non-histone (acidic) proteins were removed from the sedimented histone-

deficient chromatin by repeating the dissociation in 2M-NaCl–5M-urea, but in the presence of 10mM-Tris–HCl buffer, pH8.3, containing 5mM-NaHSO_3, followed by centrifugation at 100000*g* for 15h. The final residue remaining after this latter procedure contained 9–10% of the total protein relative to DNA. Protein-free prostatic DNA was prepared by the phenol-extraction procedure of Colter *et al.* (1962). All samples were finally resuspended in water to a final concentration of 20–50μg of DNA/100μl. Chromatin preparations were used within 24h of being suspended in water.

Labelling of receptor proteins with 17β-hydroxy-5α-[1α,2α-$^3H_2$]androstan-3-one

Cytoplasmic receptors. The method employed was based upon that of Mainwaring & Peterken (1971). Ventral prostate tissue (six glands) (2.1–2.7g wet wt.) was finely minced with scissors and homogenized in 5ml of 50mM-Tris–HCl buffer, pH7.4, containing 0.1mM-EDTA and 0.25mM-dithiothreitol (medium A). The supernatant from a preliminary centrifugation at 800*g* was re-centrifuged at 105000*g* for 1h. The resulting soluble supernatant (cytosol) fraction was made 4nM with respect to 17β-hydroxy-5α-[1α,2α-$^3H_2$]androstan-3-one ('^{3}H-labelled steroid'), added in glycerol (50μl/ml of cytosol), and retained for 1h in an ice-bath. Saturated $(NH_4)_2SO_4$ (0.5vol.), adjusted to pH7.4 with aq. NH_3 soln., was gradually added, and the resulting precipitate collected by centrifugation at 10000*g* for 10min before redissolving in 5ml of medium A. Dialysis of this solution against 2.0 litres of medium A for 2–3h removed all traces of $(NH_4)_2SO_4$ and yielded a solution containing approx 1.5×10^4c.p.m./1.26mg of protein per ml. Ammonium sulphate and free steroid could also be removed from the redissolved precipitate and from the 10000*g* supernatant (3.9×10^4c.p.m./1.23mg of protein per ml) by passage through a 2cm×14cm column of Sephadex G-25 equilibrated with medium A.

Nuclear receptor. Purified nuclei were resuspended and washed several times 0.25M-sucrose in medium A. Equal volumes of nuclear suspension (100–150μg of DNA) in medium A and labelled cytosol (adjusted with medium A so that the final concentration of ^{3}H-labelled steroid in the mixture was 0.4nM) were incubated together in a shaking water bath at 37°C for 30min. At the end of this period, the mixture was chilled in ice and centrifuged at 800*g* for 20min. The nuclear pellet was washed several times in medium A, sedimented at 800*g* and was finally resuspended in 5ml of medium A containing 0.4M-KCl. The suspension was stirred in an ice bath for 30min and the viscous extract centrifuged at 100000*g* for 30min. This resulted in a solution containing 2.6×10^4c.p.m/0.18mg of protein per ml. No radioactivity was obtained in the nuclear extract when mixtures were incubated at 0°C. For use in studies with the solubilized RNA polymerase enzyme system, the ionic strength of the nuclear extract was lowered by passage through a 2cm×14cm column of Sephadex G-25 equilibrated with medium A as outlined by Mainwaring & Peterken (1971).

Unlabelled receptor proteins for control systems were prepared by using cytosol and nuclear fractions from ventral prostate tissue of 48h castrated rats with the procedures described above, but without adding the ^{3}H-labelled steroid. The non-radioactive preparations contained the same concentration of protein as did the ^{3}H-labelled steroid–receptor complexes.

Sucrose-density-gradient centrifugation. Linear 5ml (5–20%, w/v) sucrose density gradients in a uniform concentration of medium A were prepared at least 6h before use by using a Lucite block gradient-former (Martin & Ames, 1961). Some gradients also contained a uniform concentration of 0.5M-KCl. Samples for analysis (0.4ml) were layered over the gradients and centrifuged at 3–4°C for 18h at 100000*g*. One gradient in each set was layered with bovine serum albumin ($s_{20,w}$ 4.6S) as sedimentation marker. After centrifugation, the base of each tube was pierced and two-drop fractions were collected. Radioactivity in fractions was measured in a mixture of 5ml of Aquasol and 0.5ml of water in a Nuclear–Chicago mark I liquid-scintillation spectrometer at a counting efficiency for tritium of 40%. To fractions from gradients containing bovine serum albumin, 1ml of water was added and the concentration of protein in each tube estimated spectrophotometrically.

Solubilization of RNA polymerase activity. This was accomplished by the methods of Roeder & Rutter (1970) and Mainwaring *et al.* (1971). Prostatic nuclei or nucleoli were suspended in 4ml of medium B (10mM-Tris–HCl buffer, pH7.9, containing 1.0M-sucrose, 5mM-$MgCl_2$ and 5mM-dithiothreitol), and a necessary volume of 3.0M-$(NH_4)_2SO_4$ (adjusted to pH7.9 with aq. NH_3 soln.) to bring the final concentration to 0.3M. Samples of nucleoplasm were adjusted to the same concentrations. The viscous suspensions were sonicated for four periods of 10s at 20kHz in the MSE 150W ultrasonic disintegrator (high speed, setting 4; amplitude 13–16μm) with 20s periods for cooling. After rapid dilution with 2vol. of medium C [50mM-Tris–HCl buffer, pH7.9, containing 1mM-$MgCl_2$, 0.1mM-EDTA, 0.5mM-dithiothreitol and 25% (v/v) glycerol] the samples were centrifuged at 100000*g* for 1h. To clear supernatants was added solid $(NH_4)_2SO_4$ to give 0.42g/ml and, after standing for 30min, the precipitates were sedimented by centrifugation at 100000*g* for 30min. These were then resuspended in medium C to a protein concentration of 0.6–0.8mg/ml and dialysed for 2h against this medium. Any precipitate formed

during dialysis was removed by brief centrifugation at 100000*g* and the supernatant, containing the RNA polymerase activity, was taken for further analysis. The final preparation, from prostatic nuclei from six 48h-castrated animals, contained 2-3mg of protein.

RNA polymerase prepared from whole nuclei was eluted from columns of DEAE-cellulose with a linear gradient of 0–0.8M-KCl as two discrete peaks of activity. The more rapidly eluted peak (0.3M-KCl) corresponded to activity from the nucleolus and the second peak (0.5–0.6M-KCl) to activity of extranucleolar origin. This was confirmed by the elution of enzyme prepared from nucleoli and nucleoplasm from similar columns. Both peaks contained Mg^{2+}- and Mn^{2+}-dependent activity, and preliminary experiments showed the optimum concentrations of the activating cations to be 5mM and 3mM for Mg^{2+} and Mn^{2+} respectively. More Mn^{2+}-dependent activity was observed in the nucleoplasmic peak. These results are in agreement with those of Mainwaring *et al.* (1971). The enzyme preparations during the latter stages are DNA-free, and show an absolute requirement for exogenous template. The activity is also dependent upon the presence of all four nucleoside triphosphates, and the enzyme reaction yields a ribonuclease-sensitive product.

Measurement of RNA polymerase activity. RNA polymerase activity was measured in a medium containing 60μmol of Tris–HCl buffer, pH8.1, 2.5μmol of $MgCl_2$ or 1.5μmol of $MnCl_2$, 15μmol of KCl, 200nmol of dithiothreitol, 300nmol of NaF, 300nmol each of ATP, GTP and CTP, 125pmol of [^{14}C]UTP and 20nmol of carrier UTP in a final volume of 500μl. In assays containing $MnCl_2$, the mixture also contained $(NH_4)_2SO_4$ at a final concentration of 0.4M. DNA template (10–25μg of DNA) and enzyme preparations were added in volumes of 50μl and 200μl respectively. In some experiments denatured calf-thymus DNA was used as template. Denaturation was carried out by heating DNA at 95°C for 10min, followed by rapid cooling in ice. In experiments on intact nuclei and nucleoli, DNA template and RNA polymerase were replaced by 250μl of nuclear (50–100μg of DNA) or nucleolar (20–50μg of DNA) suspension in 0.25M-sucrose–1mM-$MgCl_2$. In experiments involving *E. coli* RNA polymerase, 200μl of suspension (30μg of protein) was added to the assay. To investigate their effects on prostatic RNA polymerase activity, 17β-hydroxy-5α-androstan-3-one–receptor complexes were added to the assay system so that the final concentration of bound steroid, as determined by radioactivity measurement, was 0.25pmol/ml. Control fractions were added to the assay system in amounts, based on protein concentration, equal to the steroid–receptor complexes. Neither addition of these control fractions nor of free steroid (0.25pmol/ml) caused changes in enzymic activity. Enzyme reactions (15min at 37°C) were terminated by the addition of 2ml of 10% (w/v) trichloroacetic acid containing 1mM-$Na_4P_2O_7$. Mixtures were chilled in an ice bath and carrier yeast RNA (250μg in 250μl) was added. Washing of precipitates and preparation of acid-insoluble material for assessment of incorporated radioactivity have been previously described (Davies *et al.*, 1972). Samples were counted in a Nuclear–Chicago mark I liquid-scintillation spectrometer at 55–60% counting efficiency for ^{14}C and zero counting efficiency for ^{3}H. After correction for controls in the absence of DNA the incorporation of [^{14}C]UMP into RNA was determined in terms of d.p.m/15min per mg of DNA. For nuclear and nucleolar suspensions RNA polymerase activity was also calculated as pmol of [^{14}C]UMP incorporated/mg of DNA and for purified enzyme the activity was also calculated as pmol of [^{14}C]UMP incorporated/100μg of enzyme protein in the presence of a constant quantity of DNA template.

Chemical analyses

DNA was determined by the diphenylamine procedure of Burton (1956) as modified by Giles & Myers (1965). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results and Discussion

Characterization of 17β-hydroxy-5α-[1α,2α-$^{3}H_2$]androstan-3-one–protein receptor complexes by sucrose-density-gradient centrifugation

Centrifugation of labelled cytosol on linear 5–20% (w/v) sucrose density gradients showed two peaks of radioactivity (Fig. 1*a*). The faster-sedimenting fraction corresponded to a steroid–receptor complex of sedimentation coefficient approx. 8S, and the slower-sedimenting fraction to a complex of sedimentation coefficient of approx. 3S. The 8S form was the first receptor to be identified by labelling whole tissue *in vivo* (Mainwaring & Peterken, 1971). Preliminary experiments indicated non-specific binding in the 3S region in that other steroids, such as [^{3}H]cortisol, [^{3}H]androstenedione, [^{3}H]oestradiol and [^{3}H]testosterone, were retained by those proteins as well as 17β-hydroxy-5α-[1α,2α-$^{3}H_2$]androstan-3-one, which was the only steroid bound in the 8S region. The high capacity of the 3S region was indicated by labelling cytosol in the presence of a 10000-fold excess of non-radioactive 17β-hydroxy-5α-androstan-3-one. Whereas the low-capacity 8S peak was abolished by this treatment, the radioactivity in the 3S peak was not significantly affected (Fig. 1*a*). Ammonium sulphate selectively precipitated the 8S peak, which was recovered by dissolution

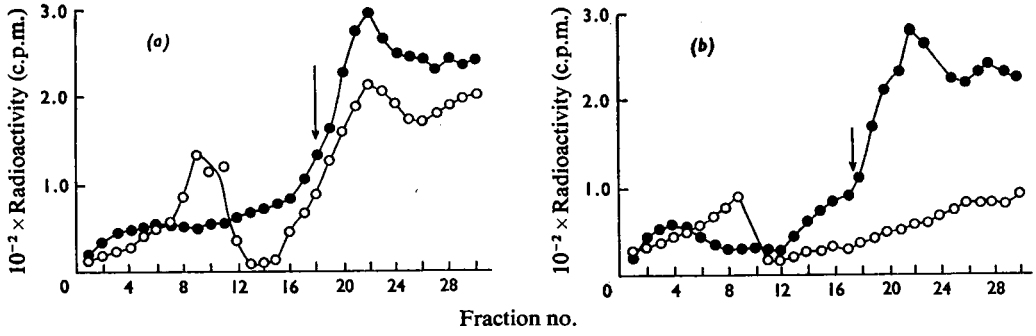

Fig. 1. *Analysis of labelled prostatic supernatant fractions by sucrose-density-gradient centrifugation*

Cell supernatant fractions (0.4ml) were layered over linear 5–20% (w/v) sucrose density gradients prepared in uniform concentration of medium A and centrifuged at 100000$g_{av.}$ for 18h at 3–4°C. The radioactivity of two-drop fractions was determined. The direction of centrifugation was from right to left. Sedimentation marker (arrows) was bovine serum albumin (4.6S). (*a*) Supernatant labelled *in vitro*, 1h at 0°C: ○, 17β-hydroxy-5α-[1α,2α-$^3H_2$]-androstan-3-one alone; ●, the ^{3}H-labelled steroid plus a 10000-fold excess of the non-radioactive steroid. (*b*) ○, Receptor precipitated by 33%-satd. $(NH_4)_2SO_4$; ●, supernatant after removal of 33%-satd.-$(NH_4)_2SO_4$ precipitate by centrifugation at 10000*g*.

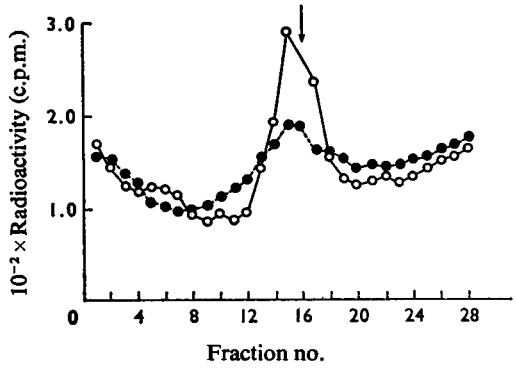

Fig. 2. *Analysis of nuclear 17β-hydroxy-5α-[1α,2α-$^3H_2$]-androstan-3-one–receptor complexes by sucrose-density-gradient centrifugation*

Samples (0.4ml) were layered over linear 5–20% (w/v) sucrose density gradients prepared in medium A, and centrifuged at 100000*g* for 18h at 3–4°C. Some gradients contained a uniform concentration of 0.5M-KCl. Sedimentation marker (arrow) was bovine serum albumin (4.6S). Nuclear ^{3}H-labelled steroid–receptor complex was formed *in vitro* by incubation of nuclei with labelled cytosol and extracted with 0.4M-KCl. ○, 0.4M-KCl-extracted receptor complex centrifuged through gradient containing 0.5M-KCl; ●, 0.4M-KCl-extracted complex after passage through Sephadex G-25, on a KCl-free gradient.

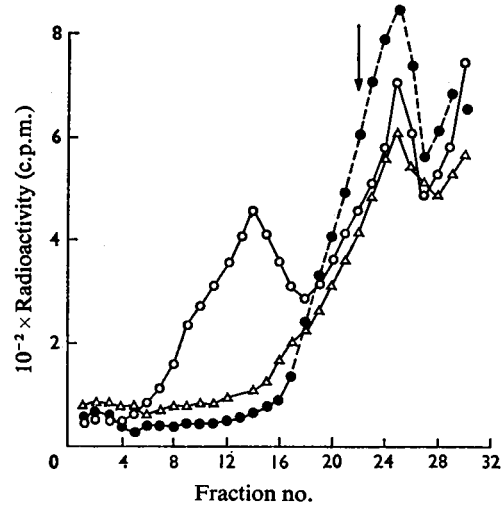

Fig. 3. *Comparison of labelling of cytosol preparations from different tissues with 17β-hydroxy-5α-[1α,2α-$^3H_2$]androstan-3-one*

High-speed supernatant fractions were prepared from ventral prostate gland, spleen and liver of castrated rats and were made 4nM with respect to 17β-[1α,2α-$^3H_2$]hydroxy-5α-androstan-3-one. Samples (0.4ml) of labelled cytosol were layered over linear 5–20% (w/v) sucrose density gradients and centrifuged at 100000*g* for 18h at 3–4°C. Centrifugation was from right to left. Sedimentation marker (arrow) was bovine serum albumin (4.6S). ○, Ventral prostate cytosol; ●, spleen cytosol; △, liver cytosol.

in medium A after re-centrifugation (Fig. 1*b*). Centrifugation of the nuclear extract on sucrose density gradients yielded one peak of radioactivity in the presence and absence of 0.5 M-KCl, corresponding to a steroid–receptor complex of $s_{20,w}$ 4.5–5S (Fig. 2).

When cytosol fractions prepared from liver and spleen and labelled with the ^{3}H-labelled steroid (4 nM) were analysed by sucrose-density-gradient centrifugation, only one peak of radioactivity was observed (Fig. 3). This corresponded to a steroid–receptor complex of sedimentation coefficient of approx 3S. No 8S region was apparent. It is evident that the low-capacity 8S region of steroid–receptor proteins is restricted to androgen target organs. Incubation of prostatic, liver or spleen nuclei with labelled cytosol from liver or spleen resulted in no incorporation of radioactivity into nuclear fractions. The 3S steroid–protein regions can be precipitated by 70% saturation with $(NH_4)_2SO_4$.

These patterns of ^{3}H-labelled steroid binding by cytoplasmic and nuclear receptor proteins are essentially similar to those reported by Mainwaring & Peterken (1971).

Transcription of chromatin by prostatic RNA polymerases

It is clear that one of the limiting factors in the development of a cell-free system which aims to reflect the effects of hormone–receptor complexes on RNA polymerase activity *in vivo* would be the rate of transcription of native chromatin that can be achieved *in vitro* by solubilized enzyme preparations. Usually, chromatin template activity has been studied by using bacterial DNA-dependent RNA polymerases with native chromatin or selectively deproteinized chromatin. Butterworth *et al.* (1971) have shown that bacterial enzymes transcribe mammalian chromatin with differing degrees of efficiency, and bind to and transcribe from sites on the chromatin DNA different from those reported for the mammalian enzyme. In view of these authors' observations on transcription of liver chromatin by liver RNA polymerases, it was considered necessary to investigate the transcriptional ability of the solubilized prostate enzyme fractions before studying the effects on the systems of the steroid–receptor complexes (Table 1). It can be seen that the nuclear enzyme transcribed prostatic nuclear chromatin with approx. 40–50% of the efficiency with which it transcribed DNA. Nucleolar RNA polymerase transcribed prostatic nuclear and nucleolar chromatin with only 15% and 19% respectively of the efficiency with which it transcribed DNA. The nucleoplasmic enzyme, however, transcribed nuclear chromatin much more efficiently, to a level of 42% of that observed with DNA, but did not transcribe nucleolar chromatin to the same extent. Transcription of chromatin by nucleolar enzyme was

Table 1. *Transcription of prostatic chromatin by various RNA polymerases compared with that of calf thymus DNA*

RNA polymerase preparations, solubilized from prostatic nuclei, nucleoli and nucleoplasm (approx. 60 μg of protein in each case) and *E. coli* RNA polymerase (30 μg of protein) were incubated in assay media containing either Mg^{2+} or Mn^{2+} and 0.4 M-$(NH_4)_2SO_4$. DNA template was 25 μg of one of the following: calf thymus DNA, prostatic nuclear chromatin or chromatin prepared from purified prostatic nucleoli. Incubations were at 37°C for 15 min. Incorporation of [^{14}C]UMP into acid-insoluble material is expressed as pmol/100 μg of enzyme protein. Incorporation is corrected for zero-time controls and control assays containing chromatin but no enzyme. Under these conditions, chromatin was devoid of RNA polymerase activity. Incorporation in the presence of chromatin as a percentage of that in the presence of purified calf-thymus DNA is shown in parentheses.

Source of enzyme	Template	[^{14}C]UMP incorporated (pmol/100 μg of protein)	
		5 mM-$MgCl_2$–0.03 M-KCl	3 mM-$MnCl_2$–0.4 M-$(NH_4)_2SO_4$
Whole nuclei	Calf thymus DNA	13.21	16.70
	Nuclear chromatin	5.69 (43)	7.19 (43)
	Nucleolar chromatin	6.77 (52)	7.97 (48)
Nucleoli	Calf thymus DNA	8.86	8.13
	Nuclear chromatin	1.39 (16)	0.94 (12)
	Nucleolar chromatin	1.71 (19)	1.23 (15)
Nucleoplasm	Calf thymus DNA	8.29	15.88
	Nuclear chromatin	3.56 (43)	6.71 (42)
	Nucleolar chromatin	0.86 (10)	0.94 (6)
E. coli	Calf thymus DNA	36.52	37.71
	Nuclear chromatin	4.83 (13)	4.42 (12)
	Nucleolar chromatin	6.72 (18)	7.12 (19)

higher in a Mg^{2+}–0.03M-KCl environment, whereas the nucleoplasmic enzyme was more effective in Mn^{2+}–0.4M-$(NH_4)_2SO_4$, but was also high in the Mg^{2+}–low salt environment. Presumably this indicates that much less of the DNA is available to the nucleolar enzyme, probably that portion restricted to the nucleolar chromatin, whereas the nucleoplasmic enzyme has access to a greater number of initiation sites. It could be worthwhile to compare transcription rates of chromatin by the two enzyme forms under various degrees of androgen-deprivation.

E. coli RNA polymerase transcribed approx. 13% of prostatic nuclear chromatin and approx. 18% of prostatic nucleolar chromatin as compared with that achieved with purified calf thymus DNA. This shows that at least one form of the prostatic enzyme is more efficient than the bacterial enzyme at transcribing prostate chromatin and is more able to gain access to those large areas of the genome now reported to be free of restricting chromosomal proteins (Clark & Felsenfeld, 1971; Itzhaki, 1971).

Stimulation of nuclear and nucleolar RNA polymerase activities

Incubation of intact nuclei and nucleoli in the standard assay system for RNA polymerase to which had been added cytoplasmic 8S or 3S steroid–receptor complexes resulted in an increased incorporation of [^{14}C]UMP into acid-insoluble material (Table 2). It is noteworthy that the so-called non-specific binding proteins in complex with 17β-hydroxy-5α-androstan-3-one stimulated enzymic activity as well as the specific binding proteins. This could suggest structural similarities between the 3S and 8S proteins, or that the 8S is an aggregated form of certain 3S subunits (Liao & Fang, 1970; Liao *et al.*, 1972). The nuclear steroid–receptor complex also produced an increase in incorporation of [^{14}C]UMP when incubated with fresh nuclei or nucleoli from 48h-castrated rats.

Stimulation of solubilized RNA polymerase activities

The various steroid–receptor complexes also increased the activity of RNA polymerase solubilized from prostatic nuclei (Table 3). Both cytoplasmic complexes and the nuclear complex stimulated the activity of the enzyme. In the presence of calf thymus DNA as template, only a slight increase in the enzyme activity was observed. The enhancement of activity was much greater, however, when calf thymus DNA was replaced by either purified prostatic nuclear or nucleolar chromatin. The stimulatory effect was not so marked with the nuclear steroid–receptor complex, and it is of interest that with enzymes from endometrium nuclei other workers have observed stimulation only with the nuclear complex (Beziat *et al.*, 1970; Hough *et al.*, 1970; Arnaud *et al.*, 1971).

Table 2. *Effect of cytoplasmic and nuclear 17β-hydroxy-5α-androstan-3-one–protein receptor complexes on RNA polymerase activity in nuclei and nucleoli*

Nuclear and nucleolar preparations were incubated for 15min at 37°C in the presence of the steroid–receptor complexes at a steroid concentration of 0.25pmol/ml, based on radioactivity, or in the presence of an equal concentration, based on protein, of 'steroid-free' receptor. RNA polymerase activity is expressed as pmol of [^{14}C]UMP incorporated/mg of DNA. Values are the means of the results of at least four experiments ±S.D. Percentage increases in activity are the average of the increases observed in each determination ±S.D.

	[^{14}C]UMP incorporated (pmol)						Increase in incorporation (%)			
	8S		3S		4.5S		8S		3S	4.5S
Complex present ...	Control	Complex	Control	Complex	Control	Complex				
Nuclei	52.41±4.88	111.86±12.46	52.75±4.19	118.41±16.10	51.88±4.33	76.67±2.69	107.0±3.69		115.2±7.29	53.5±1.73
Nucleoli	26.14±2.02	39.19±3.33	26.32±1.90	46.61±7.80	26.34±1.92	46.71±0.92	50.5±13.56		77.5±18.69	72.1±2.67

Table 3. *Effect of cytoplasmic and nuclear 17β-hydroxy-5α-androstan-3-one–protein receptor complexes on RNA polymerase solubilized from prostatic nuclei*

RNA polymerase solubilized from prostatic nuclei was incubated in the standard assay system containing the steroid–receptor complexes prepared from cytoplasm or nuclei at a final steroid concentration of 0.25 pmol/ml based on radioactivity or in the presence of an equal quantity of 'steroid-free' protein. Incorporation of [^{14}C]UMP is expressed as pmol/100 μg of protein. Values are the means of the results of at least four experiments ±S.D. Percentage increases are the average of the percentage increases observed in each determination ±S.D. The stimulation is measured in the presence of various templates (25 μg of DNA). Full details of the preparation of the various components are given in the Materials and Methods section.

DNA template	Complex present	[^{14}C]UMP incorporated (pmol)						Increase in RNA polymerase activity (%)			
		8S		3S		4.5S		8S		3S	4.5S
		Control	Complex	Control	Complex	Control	Complex				
Calf thymus DNA		12.25±0.58	14.03±0.84	12.38±0.93	13.63±1.24	12.14±0.22	15.62±0.31	14.4±1.96		10.1±3.01	28.6±2.15
Prostatic nuclear chromatin		4.69±0.57	12.33±2.07	4.79±0.85	9.18±1.78	3.97±0.34	5.57±0.43	158.2±19.90		91.9±16.02	40.7±4.69
Prostatic nucleolar chromatin		6.43±0.74	14.19±1.92	6.72±0.94	12.48±2.05	5.42±0.45	8.80±0.84	116.3±13.78		85.8±17.85	58.1±4.86
Liver chromatin		3.31±0.36	3.48±0.42	3.55±0.69	3.78±0.76	2.79±0.33	2.90±0.36	6.2±3.26		8.1±2.91	3.1±2.64

The requirement for prostatic chromatin to promote maximum stimulation by the steroid–receptor complex of enzymic activity was also demonstrated by the low stimulation observed when chromatin prepared from liver nuclei was used as template in the system. Nuclear RNA polymerase from prostate transcribed liver chromatin with approx. 25% of the efficiency with which it transcribed purified calf thymus DNA. Furthermore, if prostatic enzyme was replaced in the system by *E. coli* RNA polymerase, an increased incorporation of [^{14}C]UMP was caused by the addition of steroid–receptor complexes. These results support the view (Steggles *et al.*, 1971) that specificity of tissue-binding of steroid–receptor complexes resides in tissue chromatin.

To emphasize the tissue specificities involved in the interaction of prostatic ^{3}H-labelled steroid–receptor complexes and prostatic chromatin, investigations were carried out into the effects of the prostatic receptor complexes upon spleen RNA polymerase in the presence of chromatin from both tissues. Labelled protein fractions from spleen cytosol prepared as described for prostatic cytosol fractions were also studied to ascertain their effects upon prostatic RNA polymerase in the presence of prostatic chromatin. Since it had already been established that no radioactivity was retained in the 8S region of spleen cytosol (see Fig. 3) corresponding to the 33%-satd.-$(NH_4)_2SO_4$-precipitable protein fraction, these proteins were supplemented with the ^{3}H-labelled steroid (0.25 pmol/ml) in the enzyme assay system. The 3S region of spleen cytosol was precipitated by 70% saturation with $(NH_4)_2SO_4$. The results of these experiments are summarized in Table 4. It can be seen that no stimulation of RNA polymerase activity occurred in the presence of spleen chromatin, nor could any increase in enzyme activity be brought about by the inclusion of spleen cytosol fractions in the assay mixture. An interesting observation was that spleen RNA polymerase activity in the presence of prostatic chromatin could be stimulated by the addition of prostatic ^{3}H-labelled steroid–receptor complexes. This accentuates the importance of tissue-specific chromatin in the action of steroid–receptor complexes.

An interesting but complex aspect of the action of steroid–receptor complexes is contained in their differential effect on the RNA polymerase activities purified from subnuclear fractions (Table 5). Apparently, the degree of stimulation brought about by the complexes is dependent not only upon the DNA template provided, but also upon the intranuclear source of the enzyme and the ionic conditions employed. It has been established that, depending upon whether RNA polymerase is assayed in the presence of either Mg^{2+} or Mn^{2+} and $(NH_4)_2SO_4$, the product of the reaction is a ribosomal-type RNA or

Table 4. *Comparison of the effects of prostatic and spleen 17β-hydroxy-5α-androstan-3-one-protein receptor complexes on solubilized RNA polymerase activities*

RNA polymerase was solubilized from both prostate and spleen nuclei. Enzymes were incubated with 25μg of calf thymus DNA, or prostatic chromatin or spleen chromatin. Some incubations contained steroid–protein 'complexes' from either tissue at a final ^{3}H-labelled steroid concentration of 0.25 pmol/ml. For the 33%-satd.-$(NH_4)_2SO_4$-precipitated protein fractions from spleen cytosol, which contained no bound steroid, steroid was added at the usual concentration. RNA polymerase activity is expressed as pmol of [^{14}C]UMP incorporated/100μg of protein. Values are the means of the results of four determinations, ±S.D.

Template	[^{14}C]UMP incorporated (pmol)					
	Controls	Prostatic complexes			Spleen fractions	
	8S	4S	4.5S		33%-satd. $(NH_4)_2SO_4$	70%-satd. $(NH_4)_2SO_4$
(a) Prostatic RNA polymerase						
Calf thymus DNA	10.04±1.81	10.38±1.35	7.78±0.51	9.91±0.75	9.25±0.39	10.03±0.73
Prostatic chromatin	3.13±0.42	4.78±0.55	5.13±0.97	5.36±0.27	3.10±0.19	3.39±0.54
Spleen chromatin	1.38±0.28	1.15±0.45	1.48±0.20	1.61±0.15	1.48±0.13	1.55±0.13
(b) Spleen RNA polymerase						
Calf thymus DNA	12.31±1.13	8.86±3.07	8.24±0.93	11.40±0.39	12.52±0.25	11.30±0.61
Prostatic chromatin	3.73±0.15	5.27±0.79	4.98±0.44	4.61±0.30	3.63±0.11	3.42±0.10
Spleen chromatin	3.52±0.33	3.18±0.49	3.48±0.43	3.88±0.10	3.61±0.33	3.48±0.47

a DNA-like RNA respectively (Widnell & Tata, 1966; Hamilton *et al.*, 1968). Both Mg^{2+}- and Mn^{2+}–$(NH_4)_2SO_4$-dependent activities are present in both the nucleolar and nucleoplasmic RNA polymerases purified from rat prostate (Table 5).

It has been observed that nucleoplasmic Mn^{2+}-dependent RNA polymerase displays higher activity with denatured rather than native DNA as template. However, inclusion of denatured DNA as template in the assay mixture did not result in any significant stimulation of polymerase activity by steroid–receptor complexes.

Stimulation of nucleolar RNA polymerase by steroid–receptor complexes occurs preferentially in the presence of prostatic chromatin and 5mM-$MgCl_2$, although some stimulation does occur in the presence of 3mM-$MnCl_2$, particularly by the nuclear receptor complex. Although the nucleoplasmic enzyme transcribes prostatic chromatin more efficiently than the nucleolar enzyme, very little stimulation of this enzyme form occurred in the presence of either cation. In fact, the cytoplasmic 8S complex caused no increase in enzyme activity when Mn^{2+} was present. It would appear that the stimulation of total nuclear enzyme by these complexes is a reflexion of the nucleolar species it contains. However, it is noteworthy that the nuclear complex, with prostatic chromatin as template, stimulated the nucleoplasmic enzyme to some extent in the presence of Mn^{2+}, suggesting that stimulation of DNA-like RNA synthesis can occur *in vivo*.

Other authors have shown that hormone-stimulated RNA synthesis is of a ribosomal type, which is synthesized in the nucleolus (Liao & Lin, 1967; Jacob *et al.*, 1969; Raynaud-Jammet *et al.*, 1971; Mainwaring *et al.*, 1971). The results of our study have now shown an increase of RNA polymerase activity brought about by a steroid–receptor complex in intact nucleoli and also specifically in the purified nucleolar form. Stimulation also occurs in the presence of nucleolar chromatin. It is noteworthy that the enzyme form that transcribes native chromatin with the least efficiency is preferentially stimulated. It is tempting to relate this to a situation *in vivo*. The absence of stimulation in high-salt conditions, observed by us and others (Baulieu *et al.*, 1972), could be due to removal of some important protein factor from chromatin at high ionic strength. The stimulation caused by the nuclear complex under these conditions might suggest that the complex contains this factor. There are therefore many possibilities, which require further clarification.

Stimulation of RNA polymerase activity in the presence of altered templates

Further insight into the tissue specificity of this system *in vitro* was provided by the effects of the

Table 5. *Effect of cytoplasmic (8S) and nuclear (4.5S) 17β-hydroxy-5α-androstan-3-one–protein receptor complexes on prostatic nucleolar and nucleoplasmic RNA polymerases*

Nucleolar and nucleoplasmic RNA polymerases solubilized from the respective subnuclear fractions were incubated with various templates and the steroid–receptor complexes (0.25 pmol of 17β-hydroxy-5α-[1α,2α-$^3H_2$]androstan-3-one/ml) in assay mixtures containing either $MgCl_2$ (5 mM) and a low salt concentration (0.03 M-KCl) or $MnCl_2$ (3 mM) and at high ionic strength, 0.4 M-$(NH_4)_2SO_4$. Control assays contained equal quantities of 'steroid-free' protein. RNA polymerase activity is expressed as pmol of [^{14}C]UMP incorporated/100 μg of protein. Values are the means of the results of four or more experiments ±S.D. Percentage increases are the average of those increases observed in each determination ±S.D.

Conditions of assay	Complex present ... / Template	[^{14}C]UMP incorporated (pmol)				Increase in activity (%)	
		8S		4.5S		8S	4.5S
		Control	Complex	Control	Complex		
(a) Nucleolar enzyme (form I)							
Mg^{2+}–low salt	Calf thymus DNA	10.89±0.38	12.16±0.78	10.66±0.45	13.48±0.63	11.8±2.86	26.5±5.47
	Prostatic nuclear chromatin	2.16±0.42	4.23±0.79	1.97±0.43	2.73±0.28	96.6±6.12	39.0±1.20
	Prostatic nucleolar chromatin	3.70±0.34	8.70±0.77	2.65±0.46	3.95±0.77	143.5±10.59	50.4±9.27
	Liver chromatin	1.70±0.41	1.78±0.44	1.64±0.36	1.68±0.34	4.5±0.81	2.9±1.30
Mn^{2+}–high salt	Calf thymus DNA	9.48±0.44	9.49±0.45	9.57±1.07	10.44±1.35	0	10.6±0.62
	Prostatic nuclear chromatin	1.79±0.16	1.94±0.15	1.71±0.17	1.89±0.18	5.7±1.30	10.6±1.58
(b) Nucleoplasmic enzyme (form II)							
Mg^{2+}–low salt	Calf thymus DNA	7.67±0.46	7.82±0.52	8.05±0.29	8.68±0.32	2.0±1.89	7.8±1.49
	Prostatic nuclear chromatin	3.48±0.46	3.98±0.68	3.35±0.53	3.78±0.71	12.8±4.73	11.2±7.04
	Liver chromatin	3.95±0.31	4.28±0.53	3.61±0.38	3.62±0.36	8.0±5.68	0
Mn^{2+}–high salt	Calf thymus DNA	9.43±0.69	9.47±0.71	9.75±1.47	9.70±1.51	0	0
	Prostatic nuclear chromatin	7.22±0.41	7.26±0.45	7.83±0.10	9.09±0.39	0	16.3±4.47

Table 6. *Effect of cytoplasmic and nuclear 17β-hydroxy-5α-androstan-3-one–protein receptor complexes on nuclear RNA polymerase in the presence of selectively altered templates*

RNA polymerase solubilized from prostatic nuclei was incubated in an assay medium containing steroid–receptor complexes (0.25pmol of 17β-[1α,2α-$^3H_2$]hydroxy-5α-androstan-3-one/ml). The template for RNA synthesis varied in composition: calf-thymus DNA, liver chromatin, prostatic chromatin intact, deficient of histones, deficient of histone and acidic proteins, and prostatic DNA. Full details are given in the Materials and Methods section. The enzyme activity in the presence of the complexes or an equal quantity of steroid-deficient receptor is expressed as pmol of [^{14}C]UMP incorporated/μg of template DNA and the percentage increase in activity in the presence of steroid–receptor complex is specified in each case.

	[^{14}C]UMP incorporated (pmol)						Increase in incorporation of [^{14}C]UMP (%)		
	Control fractions								
Complex present ...									
Template	8S	3S	4.5S	8S	3S	4.5S	8S	3S	4.5S
Calf thymus DNA	1.12	1.22	1.13	1.29	1.30	1.35	15	7	20
Liver chromatin	0.28	0.26	0.30	0.30	0.28	0.33	6	8	10
Prostatic nuclear chromatin	0.46	0.56	0.42	1.17	0.93	0.82	154	66	95
Histone-deficient prostatic chromatin	0.63	0.72	0.68	1.54	1.08	1.35	144	50	99
Histone-deficient and non-histone-deficient prostatic chromatin	0.71	0.76	0.81	1.62	1.13	1.54	128	49	90
Prostatic DNA	0.89	0.75	0.97	0.97	0.86	1.15	9	15	19

cytoplasmic and nuclear steroid–receptor complexes on the nuclear enzyme in the presence of selectively altered templates (Table 6). The usual slight stimulatory effect with calf thymus DNA was noted but, as previously shown, the presence of prostatic nuclear chromatin as template produced the larger increase in enzymic activity. Removal of histone proteins from prostatic chromatin produced an increase of 17% in the rate of transcription, but did not alter the stimulatory effect. Removal of a large proportion of non-histone protein increased the rate of transcription by a further 25%, but again did not significantly alter the extent of stimulation. At this stage, 9–10% of the chromatin-associated protein remained. When protein-free, phenol-isolated prostatic DNA was introduced as template into this system *in vitro*, the template activity was increased but the percentage stimulation caused by the complexes fell to those low levels achieved with either calf thymus DNA or liver chromatin used as template.

The role of the chromatin-associated proteins is obviously of considerable interest. Steroid–receptor complexes are bound to chromatin DNA, under the control of these non-histone proteins (Mainwaring & Peterken, 1971), and they would now appear to be essential for stimulation of RNA polymerase by steroid–receptor complexes. Histones affect the template capacity, but not the stimulation of RNA polymerase activity, possibly by retaining a super-coiled structure as suggested by Shih & Bonner (1970), since they fail to produce an organ specificity for chromatin (Paul & Gilmour, 1968). This property is a feature of the non-histone proteins (Gilmour & Paul, 1970; Spelsberg & Hnilica, 1970), although the major portion of the non-histone fraction has little tissue specificity (Elgin & Bonner, 1970; MacGillivray *et al.*, 1971). These properties are limited to certain proteins which are tightly bound to DNA (Teng *et al.*, 1970) and which probably remain after chromatin dissociation in NaCl–urea at pH 8.3 (Mainwaring & Peterken, 1971). This would explain the lack of effect on the receptor complex stimulation of RNA polymerase, of removing most of the non-histone proteins from chromatin, and the major effect of the subsequent removal of the remaining small portion.

The exact mechanism of action of the steroid hormone, however, remains a problem of which chromatin-specificity is a part. It is evident that the ionic conditions for enzyme assay and the source of enzyme impose limits upon the extent of RNA polymerase stimulation, and probably further controlling factors which exist *in vivo* should be introduced into the system.

The authors are grateful to the Tenovus Organisation for their generous financial support.

References

Arnaud, M., Beziat, Y., Guilleux, J. C., Hough, A., Hough, D. & Mousseron-Canet, M. (1971) *Biochim. Biophys. Acta* **232**, 117–131

Ayres, W. W. (1948) *Cancer Res.* **8**, 352–356

Baulieu, E. E., Wira, C. R., Milgrom, E. & Raynaud-Jammet, C. (1972) in *Gene Transcription in Reproductive Tissue, 5th Karolinska Symposium* (Diczfalusy, E., ed.), pp. 396–419, Karolinska Institutet, Stockholm

Beziat, Y., Guilleux, J. C. & Mousseron-Canet, M. (1970) *C. R. Acad. Sci. Ser. D* **270**, 1620–1623

Burton, K. (1956) *Biochem. J.* **62**, 315–323

Butterworth, P. H. W., Cox, R. F. & Chesterton, C. J. (1971) *Eur. J. Biochem.* **23**, 229–241

Clark, R. J. & Felsenfeld, G. (1971) *Nature (London) New Biol.* **229**, 101–106

Colter, J. S., Brown, R. A. & Ellem, K. A. O. (1962) *Biochim. Biophys. Acta* **55**, 31–39

Davies, P., Fahmy, A. R., Pierrepoint, C. G. & Griffiths, K. (1972) *Biochem. J.* **129**, 1167–1169

Elgin, S. C. R. & Bonner, J. (1970) *Biochemistry* **9**, 4440–4447

Fang, S. & Liao, S. (1971) *J. Biol. Chem.* **246**, 16–24

Giles, K. W. & Myers, A. (1965) *Nature (London)* **206**, 93

Gilmour, R. S. & Paul, J. (1970) *FEBS Lett.* **9**, 242–244

Hamilton, T. H., Widnell, C. C. & Tata, J. R. (1968) *J. Biol. Chem.* **243**, 408–417

Hough, D., Arnaud, M. & Mousseron-Canet, M. (1970) *C. R. Acad. Sci. Ser. D* **271**, 603–606

Hymer, W. C. & Kuff, E. L. (1964) *J. Histochem. Cytochem.* **12**, 359–363

Itzhaki, R. F. (1971) *Biochem. J.* **122**, 583–592

Jacob, S. T., Sajdel, E. M. & Munro, H. N. (1969) *Eur. J. Biochem.* **7**, 449–453

Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. E. & DeSombre, E. R. (1968) *Proc. Nat. Acad. Sci. U.S.* **59**, 632–638

Liao, S. & Fang, S. (1970) In *Some Aspects of the Aetiology and Biochemistry of Prostatic Cancer, Proc. 3rd Tenovus Workshop* (Griffiths, K. & Pierrepoint, C. G., eds.), pp. 105–108, Alpha Omega Alpha Publishing Co., Caerphilly

Liao, S. & Lin, A. H. (1967) *Proc. Nat. Acad. Sci. U.S.* **57**, 379–386

Liao, S., Liang, T. & Tymoczko, J. L. (1972) *J. Steroid Biochem.* **3**, 401–408

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

MacGillivray, A. J., Carroll, D. & Paul, J. (1971) *FEBS Lett.* **13**, 204–208

Mainwaring, W. I. P. (1970) in *Some Aspects of the Aetiology and Biochemistry of Prostatic Cancer, Proc. 3rd Tenovus Workshop* (Griffiths, K. & Pierrepoint, C. G., eds.), pp. 109–114, Alpha Omega Alpha Publishing Co., Caerphilly

Mainwaring, W. I. P. & Peterken, B. M. (1971) *Biochem. J.* **125**, 285–295

Mainwaring, W. I. P., Mangan, F. R. & Peterken, B. M. (1971) *Biochem. J.* **123**, 619–628

Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379

Marushige, K. & Bonner, J. (1966) *J. Mol. Biol.* **15**, 160–174

Muramatsu, M., Smetana, K. & Busch, H. (1963) *Cancer Res.* **23**, 510–518

O'Malley, B. W. (1971) *Metabolism* **20**, 981–988

O'Malley, B. W., Toft, D. O. & Sherman, M. R. (1971) *J. Biol. Chem.* **246**, 1117–1122

O'Malley, B. W., Spelsberg, T. C., Schrader, W. T., Chytil, F. & Steggles, A. W. (1972) *Nature (London)* **235**, 141–144

Paul, J. & Gilmour, R. S. (1968) *J. Mol. Biol.* **34**, 305–316

Raynaud-Jammet, C., Bieri, F. & Baulieu, E. E. (1971) *Biochim. Biophys. Acta* **247**, 355–360

Roeder, R. G. & Rutter, W. J. (1970) *Proc. Nat. Acad. Sci. U.S.* **65**, 675–682

Shih, T. Y. & Bonner, J. (1970) *J. Mol. Biol.* **50**, 333–344

Spelsberg, T. C. & Hnilica, L. S. (1970) *Biochem. J.* **120**, 435–437

Spelsberg, T. C., Steggles, A. W. & O'Malley, B. W. (1971*a*) *J. Biol. Chem.* **246**, 4188–4197

Spelsberg, T. C., Hnilica, L. S. & Ansevin, A. T. (1971*b*) *Biochim. Biophys. Acta* **228**, 550–562

Steggles, A. W., Spelsberg, T. C. & O'Malley, B. W. (1971) *Biochem. Biophys. Res. Commun.* **43**, 20–27

Teng, G. T., Teng, G. S. & Allfrey, V. G. (1970) *Biochem. Biophys. Res. Commun.* **41**, 690–696

Widnell, C. C. & Tata, J. R. (1964) *Biochem. J.* **92**, 313–317

Widnell, C. C. & Tata, J. R. (1966) *Biochim. Biophys. Acta* **123**, 478–492