Biochem. J. (1973) **134**, 113–127
Printed in Great Britain

The Use of Deoxyribonucleic Acid–Cellulose Chromatography and Isoelectric Focusing for the Characterization and Partial Purification of Steroid–Receptor Complexes

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(*Received* 4 *December* 1972)

1. Two characteristic properties of the specific high-affinity steroid-binding proteins or receptors, their ability to bind to DNA–cellulose and their relatively acidic isoelectric point, have been exploited as a means of purification. These two fundamental properties distinguish the receptors from the steroid-binding proteins in serum and the non-specific low-affinity steroid-binding proteins in hormone-responsive cells. 2. A significant degree of purification of both cytoplasmic and nuclear steroid–receptor complexes can be achieved with practical facility by these procedures. The purity of the receptor complexes is sufficient to enable studies on their possible control of metabolic processes to be investigated in the future. 3. After extensive purification the physicochemical properties of the cytoplasmic androgen–receptor complex, such as sedimentation coefficient, were unchanged. Further, the purified complex fully retained at least one of its fundamental physiological properties, namely the ability to transfer 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) into chromatin *in vitro*. 4. The methods may also be employed for studying the changes in the structure and properties of the receptor complexes that are an essential prerequisite for the transfer of cytoplasmic receptor complexes into nuclear chromatin. The temperature-dependence of the binding of androgen–receptor complexes into chromatin is essentially due to a major change in cytoplasmic receptor complex before its attachment to nuclear chromatin. 5. The resolution of these analytical procedures was sufficient to enable a critical comparison of the receptor proteins from different male accessory glands to be undertaken. From these studies, no substantial evidence in support of the tissue specificity of androgen receptors could be established; rather the receptors from different androgen-dependent glands were remarkably similar in physicochemical properties. 6. Although the methods were initially developed for the partial purification of androgen–receptor complexes, they are equally suitable for the prompt and extensive purification of oestrogen–receptor and progesterone–receptor complexes.

In recent years it has been unequivocally established that specific high-affinity steroid-binding proteins or steroid 'receptors' are present in hormone-responsive mammalian cells, and they are generally considered to play an integral part in the mechanism of action of steroid hormones. In the androgen-dependent accessory sexual glands, the tissue- and steroid-specific nuclear binding of a metabolite of testosterone, 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), occurs rapidly and certainly precedes other biochemical manifestations of the hormonal response (Mainwaring & Peterken, 1971). The implication of the high-affinity binding of dihydrotestosterone in the mechanism of action of androgens in the prostate gland is evident from studies with synthetic antiandrogenic steroids that are essentially devoid of intrinsic androgenic activity. Such antiandrogens are competitors for the dihydrotestosterone-specific binding sites in prostate nuclei (Fang & Liao, 1969; Belham & Neal, 1971; Mangan & Mainwaring, 1972) and they also antagonize well-characterized manifestations of the androgenic response, including the induction of acid phosphatase (Geller *et al.*, 1969) and the androgen-mediated stimulation of the nucleolar (form I) RNA polymerase (Mangan & Mainwaring, 1972). To gain further insight into the role of steroid–receptor complexes in the regulation of important metabolic processes, notably genetic transcription, it is evident that they must be purified extensively. This is a daunting task, since the receptors are extremely labile proteins and they are present in only small amounts in steroid-responsive cells. Further, current evidence suggests that the receptor complexes are complex subunit structures subject to major changes in form and configuration with ionic strength (Erdos, 1968; Rochefort & Baulieu, 1968; Jensen *et al.*, 1969;

Mainwaring, 1971), and although some of these forms may be stabilized by certain bivalent cations (de Sombre *et al.*, 1969) there is no evidence to suggest that such forms are equivalent to the biologically active configuration of the receptor complexes within the cell. For these diverse reasons, any potential method of purification must be mild, highly specific and not dependent on the stabilization of the receptor complex in a somewhat artificial manner. Only the partial purification of the receptor proteins for oestradiol-17β (de Sombre *et al.*, 1969; Puca *et al.*, 1971) and progesterone (O'Malley *et al.*, 1971) has been reported up to the present time, and conventional means of protein fractionation were adopted in these studies. Since the receptor complexes are ultimately bound within nuclear chromatin, it seemed possible that they may be selectively retained by DNA; certainly, deoxyribonuclease treatment of nuclei impairs their ability to retain steroid–receptor complexes (Musliner & Chader, 1971; Marver *et al.*, 1972). Prompted largely by the pioneering work of Alberts *et al.* (1968), in which binding to DNA immobilized on cellulose was successfully employed for the purification of proteins with an elective affinity for DNA, earlier studies from this laboratory demonstrated that steroid–receptor complexes were retained by columns of DNA–cellulose (Mainwaring & Mangan, 1971). The present study was undertaken in an effort to expedite the purification of receptor complexes by exploiting specific properties of these proteins, especially their binding to DNA–cellulose.

Materials and Methods

Animals and materials

Animals. All experimental animals were castrated under fluothane anaesthesia. Accessory sexual glands were removed from male Sprague–Dawley rats 24h after orchidectomy. At longer times after castration, the 8S cytoplasmic androgen receptor that features prominently in the present study is recovered in smaller amounts (Mainwaring, 1971; Baulieu & Jung, 1970). Rat uterus, used as a source of cytoplasmic oestrogen receptor, was taken from female Sprague–Dawley rats 1 week after ovariectomy. Cytoplasmic progesterone receptor was isolated from guinea-pig uterus. Animals were ovariectomized and 1 week later two injections of diethylstilboestrol (5.0μg) were given intraperitoneally in the 24h before death. This hormonal treatment increases the amounts of progesterone receptor protein (Milgrom *et al.*, 1970).

Steroid-binding proteins. Crystalline bovine serum albumin was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Human pregnancy serum (third trimester) was used as a source of testosterone-binding β-globulin. Serum was incubated with neuraminidase to ensure a uniform pI in isoelectric-focusing studies (van Baelen *et al.*, 1969), depleted of steroids by treatment with charcoal and stored in small portions at −20°C.

Chemicals. [4,5-^{3}H]Dihydrotestosterone (sp. radioactivity 28Ci/mmol) and [6,7-^{3}H]oestradiol-17β (54Ci/mmol) were synthesized by Dr. M. M. Coombs of this Institute and stored in O_2-free benzene at 8–10°C. [1,2-^{3}H]Progesterone (41Ci/mmol), [5-^{3}H]-dGTP (9.1Ci/mmol) and [4-^{14}C]testosterone (61.2mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [^{14}C] Testosterone (25μCi) was incubated with a tissue mince derived from 12 prostate glands and the components of an NADPH-generating system in 25ml of Eagle's minimal medium for 90min at 37°C (Mainwaring, 1969*a*). The radioactive steroids were extracted and separated by a combination of t.l.c. and paper chromatography to yield 2.2μCi of [^{14}C]-androstanediol (5α-androstane-3β,17β-diol). Bovine heart catalase, bovine haemoglobin, horse spleen ferritin, Tris base (99.5% pure), protamine sulphate (type II), 2-mercaptoethanol, dithiothreitol, dATP, dCTP, dGTP, TTP (sodium salts) and highly polymerized calf thymus DNA were obtained from Sigma (London) Chemical Co. Prostate DNA was isolated as described in earlier studies (Mainwaring, 1968; Mainwaring & Peterken, 1971). Macherey–Nagel cellulose (type 2200ff) was supplied by Camlabs Ltd. (Cambridge, U.K.) and used for the preparation of DNA–cellulose as described in detail by Mainwaring & Peterken (1971). On the basis of the release of deoxyribonucleotides by hot-acid digestion (Alberts *et al.*, 1968), each 1g of washed DNA–cellulose contained 400–440μg of immobilized DNA. The synthetic antiandrogen 6α-bromo-17β-hydroxy-17α-methyl-4-oxa-5α-androstan-3-one was generously given by Hoffmann–La Roche Pharmaceuticals, Basle, Switzerland. Ammonium sulphate was recrystallized twice from 5mM-EDTA and a saturated solution was adjusted to pH7.4 with aq. 2M-NH_3. Cellulose (Whatman; coarse grade) was supplied by Reeve Angel Ltd., Maidstone, Kent, U.K., and cycled successively in 0.5M-NaOH, water, 0.5M-HCl and water before use. Rabbit antibody raised against whole rat serum was supplied by Hoechst Pharmaceuticals, Brentford, London, U.K. All other chemicals were of highest available commercial grades, and glass-distilled water was employed in making up all solutions.

Fractionation procedures

Many of these were done in medium A (50mM-Tris–HCl buffer, pH7.4, containing 0.25mM-EDTA and 0.5mM-dithiothreitol); medium B additionally contained 10% (v/v) glycerol. The successful and reproducible application of these methods required their performance at temperatures within the range 0–4°C.

Sucrose density gradients. Samples (0.1ml) were analysed in linear 5–20% (w/v) sucrose gradients (4.5ml), prepared in medium B. The only departure from published methods (Mainwaring, 1969*b*; Mainwaring & Peterken, 1971) was that the gradients were formed and subsequently fractionated automatically, by using equipment supplied by Instrument Specialities Co., Lincoln, Nebr., U.S.A. (model 570 gradient former; model 182 analyser). The sedimentation markers (each 200μg) *Escherichia coli* alkaline phosphatase (6.4S) and catalase (11.4S) were run in a separate gradient.

DNA–cellulose chromatography. Complete experimental details of this procedure are given elsewhere (Mainwaring & Peterken, 1971). DNA–cellulose was prepared from native (double-stranded) DNA from prostate gland or calf thymus. Chromatography was performed on columns (1.5g) of DNA–cellulose equilibrated with medium B; adsorbed proteins were eluted with medium B containing 0.5M-KCl. In our experience, more DNA was adsorbed by freeze-drying on fine Macherey–Nagel cellulose than on coarser grades of Whatman cellulose, but the use of fine grades of cellulose led to difficulties in the operation of the columns during the separation of certain cellular extracts, notably from seminal vesicle. To improve the flow rate during chromatography, 1.5g (wet wt.) of DNA–cellulose was mixed with 0.5g of Whatman cellulose, previously equilibrated with medium B. Occasional stirring of the upper surface of the packed adsorbent with a fine glass rod enabled a good flow rate to be maintained even under the influence of gravity alone.

Isoelectric focusing. Initial attempts to purify labelled receptor complexes by isoelectric focusing in the conventional columns (110ml total volume) were not particularly successful. Under these conditions of analysis, extensive dissociation of bound ^{3}H-labelled steroid occurred, possibly owing to excessive dilution or local heating effects. Highly reproducible separations were effected in small columns (12.5ml total volume) made to the design recommended by Osterman (1970). The particular advantage of these smaller columns is the ease with which they may be efficiently cooled; additionally, up to four columns may be run concurrently from a single high-voltage power supply. Samples for analysis were desalted by passage through columns (2.2cm×25cm) of Sephadex G-25 (medium grade) equilibrated with 5% (w/v) sucrose. A linear 10–60% (w/v) sucrose gradient (10ml) containing 0.1% (v/v) narrow-range (pH5–8) ampholytes [LKB (U.K.), London S.W.12, U.K.] was formed in the apparatus and overlaid first with 2.0ml of the protein extract and finally with 0.5ml of 2.5% (w/v) sucrose. The upper electrode reservoir (cathode) contained 2% (v/v) ethylenediamine and the lower electrode reservoir contained 1% (v/v) H_2SO_4. Each sample was supplemented with internal markers before analysis: either 50μl of testosterone-binding globulin labelled with 5nM-[^{14}C]androstanediol (pI6.6; van Baelen *et al.*, 1969) or 100μg each of ferritin, catalase and haemoglobin (focuses as two bands) of pI5.0, 6.1, 7.2 and 7.6 respectively. The visibility of these coloured markers enabled the satisfactory progress of focusing to be followed readily. The apparatus was mounted in a cold-room at 4°C and thoroughly chilled by the constant circulation of ice-cold water. An initial voltage of 300V (2mA) was applied for 30min and, after adjustment to 800V, focusing was conducted overnight for 16h. The contents of the apparatus were subdivided into 0.5ml fractions, each diluted with 0.5ml of water, and the pH values were determined at 4°C in a Radiometer model 28pH-meter calibrated against potassium hydrogen phthalate.

Polyacrylamide-gel electrophoresis. This was performed in 7.5% (w/v) polyacrylamide gels at pH8.4 according to the recommendations of Ornstein (1964). As reported by other investigators (Ritzén *et al.*, 1971), preservation of receptor complexes during electrophoresis necessitated the inclusion of 10% (v/v) glycerol and 15mM-2-mercaptoethanol in the gels and electrode buffers. This concentration of 2-mercaptoethanol impeded the initial polymerization of the gels, but this was satisfactorily accomplished by incubation in a water bath at 37°C for 20min. Electrophoresis was performed at temperatures in the range 0–4°C with a maximum current of 3mA/gel. The final gels were processed in two ways: they were either stained with Coomassie Brilliant Blue R or cut into approx. 0.2mm slices, in a gel slicer made to the pattern of Maizel (1966), each of which was dispersed by overnight incubation in 0.5ml of 0.5M-KOH at 40°C and counted for radioactivity. When samples from isoelectric-focusing experiments were analysed, it was found essential to leach out the residual ampholytes before staining by placing the gels in repeated changes of cold 5% (w/v) trichloroacetic acid. Without this treatment, traces of ampholytes gave rise to staining artifacts.

Sephadex G-25 chromatography. Free and protein-bound ^{3}H-labelled steroid were separated by passage of 0.25ml of labelled extracts through columns (1cm×8cm) of Sephadex G-25 (medium grade), equilibrated with medium B. Fractions (0.25ml) were collected automatically and bound radioactivity was recovered in fraction nos. 3–6 inclusively.

Labelling and preparation of subcellular fractions. Labelling of cytoplasmic (105000$g_{av.}$ supernatant) fractions and nuclei with ^{3}H-labelled steroids was accomplished as described by Mainwaring & Peterken (1971). Cytoplasmic extracts were prepared in medium A with the precautions regarding temperature and shearing that were emphasized by Mainwaring (1969*b*). Directly after homogenization, 0.1vol. of glycerol was added. Purified nuclei were

extracted with medium B containing 0.5M-KCl to solubilize the nuclear receptor complex. The only important point to stress is that the cytoplasmic extracts may be labelled directly with ^{3}H-labelled steroids in the cold, whereas nuclei can only be labelled to any significant extent after incubation of whole tissue at 30° or 37°C. Full details of the reconstituted system for the transfer of [^{3}H]dihydrotestosterone into chromatin have been given before (Mainwaring & Peterken, 1971).

Prostate enzymes and their assay

Pooled glands (2.5g wet wt.) from eight animals were finely minced and homogenized in 10ml of 50mM-Tris–HCl buffer, pH8.0, containing 1.5mM-$MgCl_2$ and 0.5mM-dithiothreitol. After centrifugation at 105000$g_{av.}$ for 1h, the clear supernatant fraction was aspirated to within 0.5cm of the sediment of particulate material, the floating layer of lipid being carefully avoided. The fraction precipitated within the range 30–60%-satd. $(NH_4)_2SO_4$ was used as a source of alkaline phosphatase and glucose 6-phosphate dehydrogenase. Particularly sensitive fluorimetric assays of both of these enzymes have been reported (Mainwaring, 1967). For the preparation of DNA polymerase, a pH5 enzyme fraction (Mainwaring & Wilce, 1972) was dissolved in 5.0ml of medium A. Further purification was accomplished by adsorption and elution from columns (2g) of DNA–cellulose. After desalting in columns of Sephadex G-25, equilibrated with 0.1M-Tris–HCl buffer, pH8.0, containing 5mM-$MgCl_2$ and 1mM-dithiothreitol, the enzyme was diluted with an equal volume of glycerol and stored at −20°C. The assay of DNA polymerase was based essentially on that reported by Coffey *et al.* (1968). Each assay mixture (volume 0.5ml) contained 100μg of native calf thymus DNA, 50nmol each of dATP, dCTP and dTTP, 0.05μCi of [^{3}H]dGTP and 5nmol of dGTP, 0.5μmol of dithiothreitol, 1μmol of $MgCl_2$, 30μmol of Tris–HCl buffer, pH8.0, and 0.15ml of enzyme. After incubation for 1h at 37°C, the reaction was terminated by the addition of 2.0ml of ice-cold 0.5M-$HClO_4$. The acid-insoluble material was collected on Whatman GF/A glass-fibre discs, washed twice with 2.5ml of cold 0.5M-$HClO_4$, twice with 2.5ml of cold ethanol and counted for radioactivity. The incorporation of radioactivity was totally eliminated by omission of either DNA or deoxyribonucleoside 5′-triphosphates from the assay mixture or by the inclusion of 20μg of deoxyribonuclease.

Other techniques

Counting of radioactivity. The radioactivity due to ^{3}H in acid-insoluble precipitates was counted at an efficiency of 55% in a phosphor containing 2.3g of 2,5-diphenyloxazole/litre of toluene. Radioactivity due to ^{14}C and ^{3}H in aqueous samples was counted in a water-miscible phosphor (Mainwaring, 1969*a*) at efficiencies of 60 and 40% respectively in a Nuclear-Chicago mark II liquid-scintillation spectrometer. Quenching was severe in solubilized sections of polyacrylamide gels and the efficiency of the counting of ^{3}H was decreased to only 18%.

Chemical analyses. DNA was determined by the procedure of Burton (1956) with calf thymus DNA as standard. Protein was generally measured by use of the Folin–Ciocalteu reagent (Lowry *et al.*, 1951), but where greater sensitivity was demanded recourse was made to the fluorimetric procedure of Hiraoka & Glick (1963). The latter procedure is sufficiently accurate for determinations within the range 1.5–20μg of protein. Bovine serum albumin was employed as standard.

Results

Initial experiments

Apart from the extreme lability of steroid–receptor complexes, the major problems encountered in their purification are two: first, their small quantity relative to other cellular components, and, secondly, their separation from the non-specific low-affinity steroid-binding proteins present in most steroid-responsive tissues, including the prostate gland (Mainwaring, 1969*a*; Baulieu & Jung, 1970). Since there is evidence that the receptor complexes can be readily precipitated with polycationic reagents, such as protamine sulphate (Mainwaring, 1969*a*; Steggles & King, 1970), this appeared to offer a potential means of purification. On a more detailed enquiry, however, precipitation with protamine did not provide a practical method of purification; no means could be devised for releasing the receptor protein from the protamine-containing precipitates without dissociating the bound ^{3}H-labelled steroid. Alternatively, if the non-specific binding proteins represented the accumulation of plasma proteins within the prostate gland, then an attractive means for their selective removal was to treat prostate extracts with antibodies raised against whole rat serum. However, in confirmation of earlier preliminary studies (Mainwaring, 1969*b*), such antibodies did not eliminate the non-specific binding, and alternative means of purification were sought.

The binding of ^{3}H-labelled steroid–receptor complexes to DNA–cellulose was first established by Mainwaring & Mangan (1971) and later confirmed by other investigators (Clemens & Kleinsmith, 1972; Yamamoto & Alberts, 1972). A more critical appraisal of the specificity of the binding of proteins, including steroid–receptor complexes, to DNA–cellulose is summarized in Figs. 1(*a*) and 1(*b*). The

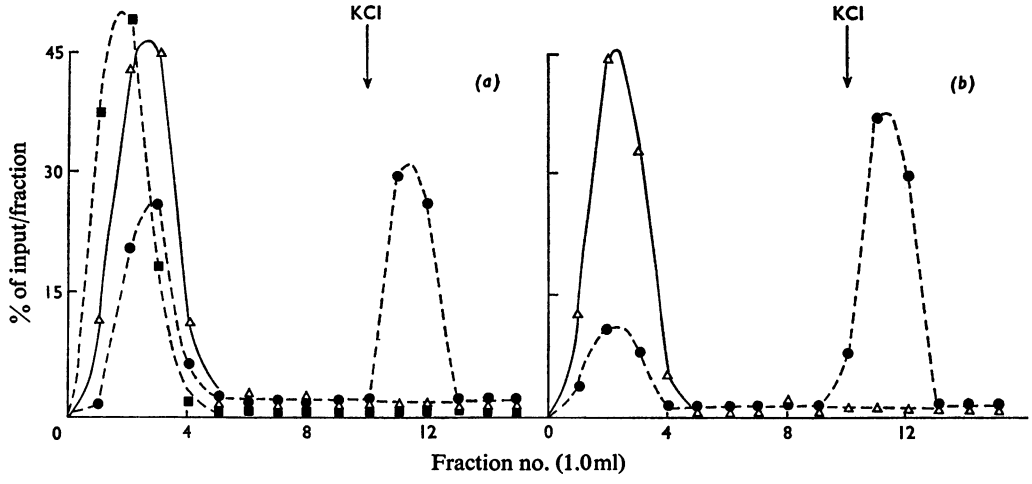


Fig. 1. *Specificity of the binding of proteins to DNA–cellulose*

Small columns (0.5g wet wt.) of DNA–cellulose were used in this study. For details of the chromatographic procedure see the Materials and Methods section. Retained proteins were eluted in a medium containing 0.5M-KCl, applied where indicated by the arrows. The % of the total input of protein, either as ^3H radioactivity in (*a*) or enzymic activity in (*b*), was determined in each fraction. (*a*) [^3H]Dihydrotestosterone–protein complexes (input 80000d.p.m.), located by scintillation spectrometry: ●, 8S androgen–receptor complex; △, complex with either testosterone-binding globulin or serum albumin; ■, free [^3H]dihydrotestosterone. (*b*) Prostate enzymes, located by specific assay of enzymic activity: ●, DNA polymerase; △, glucose 6-phosphate dehydrogenase or alkaline phosphatase.

8S [^3H]dihydrotestosterone–receptor complex from prostate cytoplasm was retained to a very significant degree (Fig. 1*a*) whereas equivalent amounts of [^3H]-dihydrotestosterone, either in the free state or in complex with either testosterone-binding globulin or bovine serum albumin, were not retained to any detectable extent. The negative result obtained with testosterone-binding globulin is particularly important because it counters the possible criticism that [^3H]dihydrotestosterone in a high-affinity complex with any protein may be indiscriminantly bound to DNA–cellulose. The dissociation constant (K_d) for the binding of dihydrotestosterone to testosterone-binding globulin is of the order of 0.2–0.6nM (Kato & Horton, 1968; Mercier-Bodard & Baulieu, 1968), which is similar to the K_d reported for its binding to androgen–receptor proteins (Ritzén *et al.*, 1971; Hansson *et al.*, 1971). Additional experiments established that the androgen–receptor protein must be in a native or functional configuration for binding to DNA and that the presence of dihydrotestosterone within the complex is similarly a mandatory requirement for binding. Low concentrations (0.5–1.0mM) of *N*-ethylmaleimide or *N*-bromosuccinimide degrade the 8S androgen–receptor complex (Mainwaring, 1969*b*) and accordingly antagonized binding to DNA. Further, when unlabelled receptor preparations were passed through columns of DNA–cellulose, followed by free [^3H]dihydrotestosterone, no radioactivity was retained by the column. The obligatory presence of adsorbed DNA was confirmed by the failure to retain radioactivity by using columns of cellulose alone, even with up to 15 times the amounts of labelled 8S receptor complex employed in Fig. 1(*a*). The acute specificity of the binding of proteins to DNA–cellulose was corroborated by additional studies on prostate enzymes (Fig. 1*b*); only those with an essential propensity for binding to DNA, such as DNA polymerase, were retained to any detectable extent.

The particular advantage of the DNA–cellulose procedure is that any non-specific binding proteins remaining after preliminary stages of purification of receptor complexes may be totally eliminated. There is some evidence that specificity exists in the source of DNA employed in the binding of receptor complexes. Clemens & Kleinsmith (1972) have claimed that DNA from bacteria does not retain receptor complexes effectively, but this was not intensively investigated in the present study. Prostate DNA was employed throughout the preliminary screening experiments,

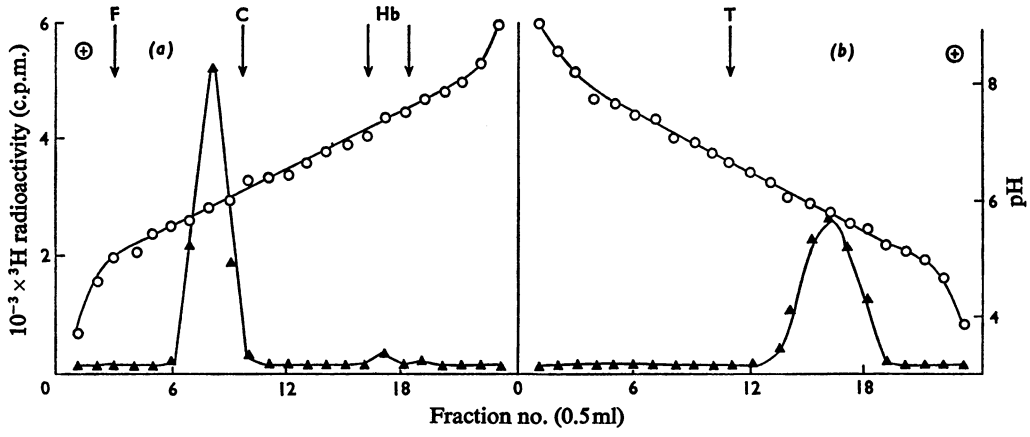


Fig. 2. *Behaviour of the 8S androgen–receptor complex during isoelectric focusing in the range pH 5–8*

The cytoplasmic fraction of rat prostate gland was labelled with 1 nM-[^{3}H]dihydrotestosterone and purified by DNA–cellulose chromatography as described in Fig. 1. Markers of known pI were added as internal markers: F, ferritin (pI 5.0); C, catalase (pI 6.1); Hb, haemoglobin (pI 7.2 and 7.6). T, Testosterone-binding β-globulin (pI 6.6), was added as a complex with [^{14}C]5α-androstanediol and located by scintillation spectrometry. (*a*) Anode to left (base of apparatus). (*b*) Anode to right. ▲, ^{3}H; ○, pH.

but commercially available DNA from other eukaryotic sources, such as calf thymus gland, is equally suitable for DNA–cellulose chromatography.

Since the androgen receptors are relatively acidic proteins (Mainwaring, 1969*a*,*b*) it was considered that isoelectric focusing offered a potential method for their purification. This technique had previously been directed towards the purification of oestrogen–receptor complexes (de Sombre *et al.*, 1969; Puca *et al.*, 1971). The results in Fig. 2 summarize preliminary studies on the 8S [^{3}H]dihydrotestosterone–receptor complex. This labelled complex had pI 5.8 when compared with two sets of internal markers, either coloured metal-containing proteins of known pI (Fig. 2*a*) or testosterone-binding globulin labelled with [^{14}C]androstanediol (Fig. 2*b*). Trial experiments established that 5α-androstanediol did not displace 5α-dihydrotestosterone from androgen receptors at these steroid concentrations. It is essential in isoelectric-focusing studies to ensure that the behaviour of the protein under investigation in the pH gradient is not influenced by the direction of migration. As shown in Fig. 2(*b*), reversal of the polarity of the electrodes did not change the pI of the 8S androgen–receptor complex; irrespective of the experimental conditions, the labelled complex was concentrated in the region of the gradient corresponding to pH 5.8. Since these gradients are fractionated from the base of the apparatus, however, some spreading of the receptor complex was found in this experiment during fractionation of the gradients (Fig. 2*b*), and subsequent analyses were always conducted with the anode as the lower electrode. At the concentrations of protein employed, no visible precipitation of protein occurred during isoelectric focusing. Provided that close attention was paid to thorough cooling, insignificant amounts of bound [^{3}H]dihydrotestosterone were dissociated during the course of the analyses.

Studies on cytoplasmic steroid–receptor complexes

Purification of the 8S dihydrotestosterone–receptor complex. The application of these methods for the purification of the 8S cytoplasmic androgen receptor from rat prostate gland is shown in Table 1. The selective precipitation of the 8S androgen–receptor complex with 30%-satd. $(NH_4)_2SO_4$ was originally devised by Mainwaring & Peterken (1971). This procedure precipitates the 8S androgen–receptor complex completely, with only small amounts of the 3.5S non-specific binding proteins. The latter are totally removed at the subsequent stage III (Table 1) involving DNA–cellulose chromatography. Although the extent of the binding of 8S receptor to DNA–cellulose was vastly superior to that reported by Clemens & Kleinsmith (1972), complete retention of radioactivity was never achieved. This cannot be explained by the use of insufficient quantities of DNA–cellulose, because doubling the amount of adsorbent did not improve the binding of receptor

Table 1. *Partial purification of the 8S cytoplasmic dihydrotestosterone–receptor complex of rat prostate gland*

Cytoplasmic (105000$g_{av.}$ supernatant) fraction was prepared from a total of 18 prostate glands, some 24h after the castration of the rats, and 18ml of cytoplasmic fraction was labelled with 5nM-[^{3}H]dihydrotestosterone for 2h at 0°C. A sample (0.5ml) was analysed in a column of Sephadex G-200 to estimate the radioactivity initially bound to the specific 8S receptor complex (see Mainwaring, 1969*b*, for details). The remainder was processed in one of two ways: either (*a*) the purification was conducted in the absence of excess of ligand ([^{3}H]dihydrotestosterone) or (*b*) 1nM-[^{3}H]dihydrotestosterone was added to all preparative media. In (*b*) samples of individual fractions from analytical procedures were processed in columns of Sephadex G-25 to measure protein-bound radioactivity (see the Materials and Methods section for details). Each purification has been repeated twice with identical results. Protein was determined by a colorimetric method, except where indicated by an asterisk (*), when the sensitive fluorimetric method of Hiraoka & Glick (1963) was used. Labelled receptor was identified by liquid-scintillation spectrometry; protein was only determined in peak fractions pooled at each stage.

		(*a*) Absence of excess of [^{3}H]dihydrotestosterone throughout			(*b*) 1nM-[^{3}H]Dihydrotestosterone present throughout		
	Stage of purification	Protein (mg)	^{3}H bound to 8S receptor (d.p.m.)	Specific radioactivity (d.p.m./mg of protein)	Protein (mg)	^{3}H bound to 8S receptor (d.p.m.)	Specific radioactivity (d.p.m./mg of protein)
I	Initial cytoplasmic fraction	364	5.5×10^7	1.52×10^5	341	5.4×10^7	1.61×10^5
II	Precipitation at 30%-satd. $(NH_4)_2SO_4$	30.4	4.9×10^7	1.62×10^6	32.2	5.4×10^7	1.68×10^6
III	DNA–cellulose chromatography; adsorbed protein fraction	3.0; 2.96*	2.4×10^7	8.02×10^6	3.1; 3.14*	4.2×10^7	1.35×10^7
IV	Desalting; Sephadex G-25	2.9; 2.84*	7.4×10^6	2.55×10^6	3.0; 3.17*	4.0×10^7	1.29×10^7
V	Isoelectric focusing; protein of pI5.8	0.105*	5.8×10^6	5.28×10^7	0.092*	3.4×10^7	3.69×10^8
Degree of purification:							
(a)	on the basis of the recovery of protein		3466			3706	
(b)	on the basis of the specific radioactivities		347			2291	

complex further. Some dissociation of dihydrotestosterone occurred during this chromatographic procedure, and, as indicated in Fig. 1(*a*), this would impair the retention of the receptor protein. When the purification was conducted in the presence of excess of [^{3}H]dihydrotestosterone (see section *b*, Table 1), the retention of receptor complex was raised considerably. Extensive dissociation of [^{3}H]dihydrotestosterone was found during the desalting stage before isoelectric focusing; the Sephadex G-25 method was adopted because losses of bound [^{3}H]dihydrotestosterone were particularly extensive during conventional dialysis. The degree of purification indicated by the specific radioactivity of the receptor complex at stage V (Table 1) is not commensurate with the approx. 3300-fold purification calculated on the overall recovery of protein. This apparent anomaly is due to dissociation of bound [^{3}H]dihydrotestosterone during the course of isolation rather than to denaturation or inactivation of the receptor protein. When the purification was performed in the presence of excess of ligand ([^{3}H]dihydrotestosterone) the specific radioactivity of the material at stage V was more in accord with the degree of purification based on the recovery of protein. It should be emphasized that the small amounts of protein present from stage III onwards precluded the reliable determination of protein in all fractions. The presence of the receptor complex was monitored throughout by determination of radioactivity, and protein was measured only in the pooled fractions representing peaks of bound radioactivity.

As judged by analysis in sucrose density gradients, the purified receptor at stage V still had a sedimentation coefficient of 8S. The maintenance of the physicochemical structure of the 8S androgen–receptor complex during its purification was confirmed by electrophoresis in polyacrylamide gels (Plate 1). The receptor complex recovered after isoelectric focusing gave a single sharp peak of bound radioactivity. Confirmation that this represented [^{3}H]dihydrotestosterone bound to the androgen-receptor protein was obtained from electrophoretic analysis of preparations derived from whole cytoplasm initially labelled in the presence of either a 200-fold excess of the antiandrogen 6α-bromo-17β-hydroxy-17α-methyl-4-oxa-5α-androstan-3-one or a 50-fold excess of non-radioactive 5α-dihydrotestosterone. 6α-Bromo-17β-hydroxy-17α-methyl-4-oxa-5α-androstan-3-one competes only for the specific high-affinity binding sites on the 8S cytoplasmic receptor protein (Mangan & Mainwaring, 1972); it does not impair binding to the non-specific low-affinity binding sites on the 3.5S proteins in prostate cytoplasm. When parallel gels were stained for protein after electrophoresis of preparations of purified receptor complex, radioactivity was associated with a protein band and only a few additional bands of slower-moving proteins were detected. This should not necessarily be taken to indicate the isolation of receptor complex of purity approaching homogeneity, since additional proteins may well be present. A more penetrating appraisal of the purity of these preparations necessitates the use of sodium dodecyl sulphate (Weber & Osborne, 1969), but this completely dissociates bound [^{3}H]dihydrotestosterone from the receptor complex (R. Irving, unpublished work).

The best available indication that the 8S androgen–receptor complex may be purified in an unchanged or active configuration was gained from its persistent ability to transfer [^{3}H]dihydrotestosterone into prostate chromatin in a reconstituted cell-free system (Fig. 3). With identical input amounts of receptor complex, in terms of bound [^{3}H]dihydrotestosterone, the purified receptor complex at stage V of the purification (Table 1) retained over 80% of the ability of the 8S complex present at purification stage II to transfer radioactivity into chromatin. The overall extent of transfer of [^{3}H]dihydrotestosterone with the purified receptor complex agreed very favourably with the results obtained in a previous study (Mainwaring & Peterken, 1971).

Tissue specificity of androgen receptors. Despite a close similarity in the androgenic response in the male

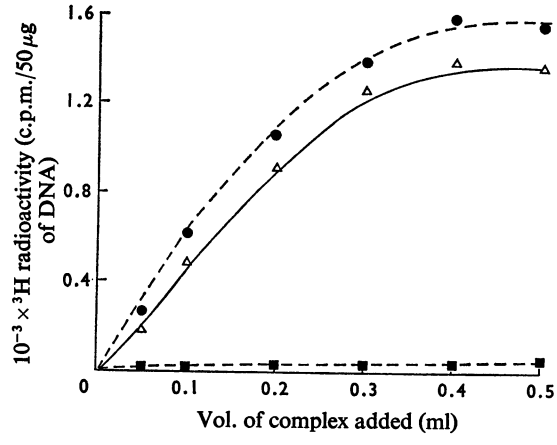
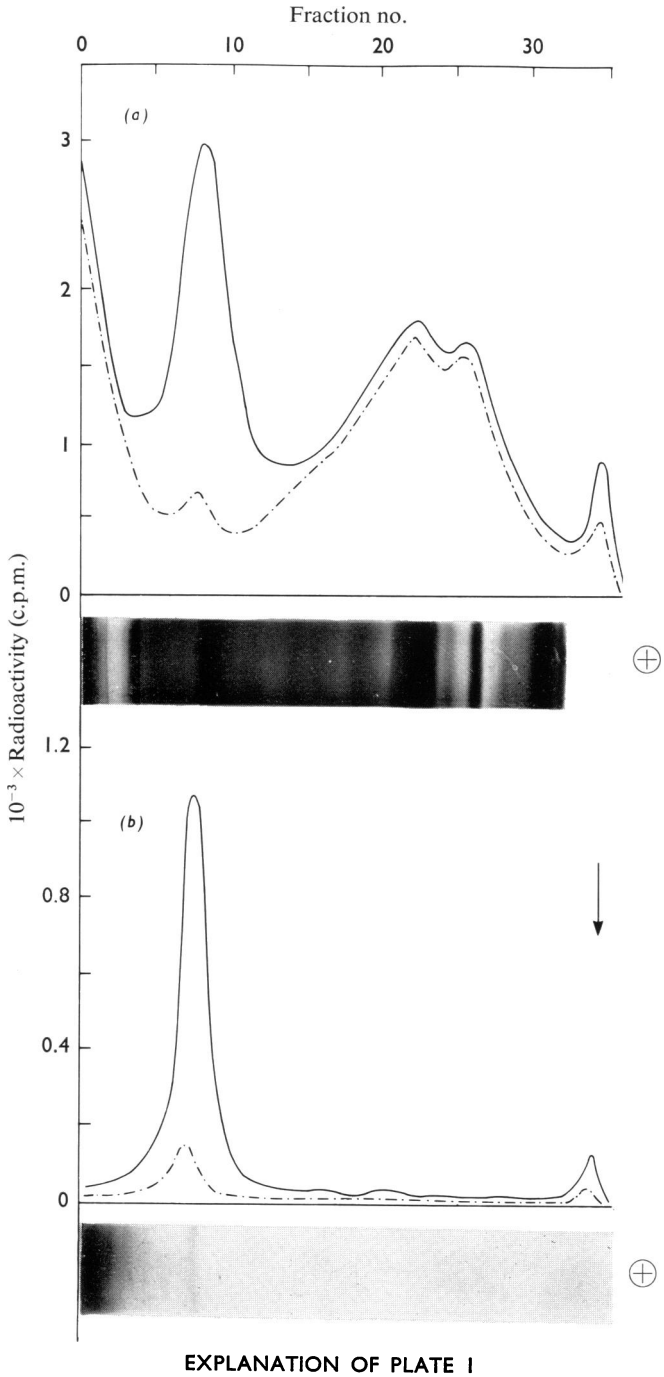


Fig. 3. *Comparison of the ability of purified 8S receptor complex to transfer [^{3}H]dihydrotestosterone into prostate chromatin*

Labelled receptor complex (2.4×10^3–24×10^3 d.p.m. of [^{3}H]dihydrotestosterone) was added in increasing amounts to unlabelled prostate chromatin (50 μg of DNA) under optimum conditions and the transfer of radioactivity into chromatin was determined. Full details are given in the Materials and Methods section. ●, Receptor at stage II of purification (Table 1); △, receptor at stage V of purification; ■, equivalent amounts of free [^{3}H]dihydrotestosterone.



EXPLANATION OF PLATE 1

Analysis of partially purified cytoplasmic 8S [^3H]dihydrotestosterone–receptor complex of rat prostate gland by polyacrylamide-gel electrophoresis

Samples of (*a*) prostate cytoplasm labelled with 5nM-[^{3}H]dihydrotestosterone (250μg of protein) or (*b*) purified receptor complex (40μg of protein; stage V; Table 1) were separated in polyacrylamide gels and either stained with Coomassie Blue or cut into 0.2mm slices and counted for radioactivity. Radioactivity in preparations labelled with [H^3]dihydrotestosterone alone (——) or in preparations labelled with [^{3}H]dihydrotestosterone in the presence of a 200-fold excess of the antiandrogen 6α-bromo-17β-hydroxy-17α-methyl-4-oxa-5α-androstan-3-one (—·—) is shown. The anode is on the right; the vertical arrow indicates the ion front.

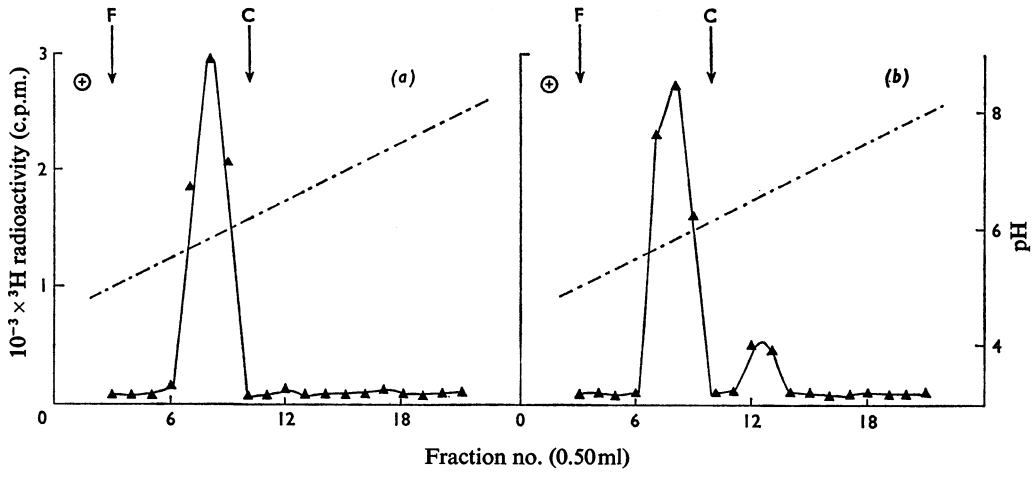


Fig. 4. *Common behaviour of cytoplasmic androgen–receptor complexes during isoelectric focusing*

Cytoplasmic (105000$g_{av.}$ supernatant) fractions of male accessory sexual glands were labelled with 1nM-[^{3}H]-dihydrotestosterone and submitted to the partial purification scheme given in Table 1. The material from stage IV of the purification (Table 1) was analysed by isoelectric focusing. The anode is to the left. Markers: F, ferritin (pI5.0); C, catalase (pI6.1). (*a*) Rat epididymis. (*b*) Guinea-pig seminal vesicle. ▲, Radioactivity; —·—, pH. Identical profiles were found with preparations from the accessory sexual glands of all species investigated.

accessory sexual glands, subtle inter- and intra-species differences exist. Some glands secrete fructose, others citric acid (Samuels *et al.*, 1962), and prolactin differentially influences the uptake of androgens into certain glands, even within the same animal (Grayhack & Lebowitz, 1967; Berswordt-Walbrabe *et al.*, 1970). One possible explanation of these differences in response is that androgen receptors differing in physicochemical and hence functional properties are present in the accessory sexual glands. Evidence favouring this viewpoint has been presented (Ritzén *et al.*, 1971), but the experimental foundation for this concept was essentially that an 8S cytoplasmic receptor complex could be detected in rat prostate gland but not rat epididymis. In contrast, other investigators have reported the presence of an 8S receptor complex in rat epididymis (Blaquier, 1971). To clarify the issue of the tissue specificity of cytoplasmic androgen receptors, a search was made for androgen receptors in the prostate gland, seminal vesicle and epididymis of the rat and guinea pig. All of these accessory sexual glands contained steroid-specific 8S androgen–receptor complexes and, without exception, these possessed a pI of 5.8 (Figs. 4*a* and 4*b*). On this evidence, a satisfactory case cannot be made in support of the tissue-specific nature of cytoplasmic androgen receptors.

Other androgen-binding proteins. Additional studies were conducted on the fundamental properties of the 3.5S non-specific steroid-binding proteins present in the majority of mammalian tissues, including the accessory sexual glands. More specifically, we investigated the physicochemical properties of the [^{3}H]-cortisol–protein and [^{3}H]dihydrotestosterone–protein complexes in rat spleen, liver and prostate gland. Considerations of sedimentation coefficient and steroid specificity apart, three additional criteria distinguish these proteins from the androgen receptors. First, they are precipitated only in the range 35–70%-satd. $(NH_4)_2SO_4$; secondly, they do not possess the ability to bind to DNA–cellulose; thirdly, they have a pI of 7.40. These properties are also shared by the α-protein distinguished in the rat prostate gland by Fang & Liao (1971), the function of which is to decrease the nuclear binding of dihydrotestosterone.

Application of the purification scheme to other steroid–receptor complexes

The purification scheme outlined for the 8S androgen–receptor complex was equally applicable to the 8S oestrogen–receptor complex from rat uterus and the 7S progesterone–receptor complex from guinea-pig uterus. The overall degree of purification was consistent with that reported for the 8S androgen–receptor complex (see Table 1), at least on the basis of the recovery of protein. Both complexes maintained a constant sedimentation coefficient throughout the

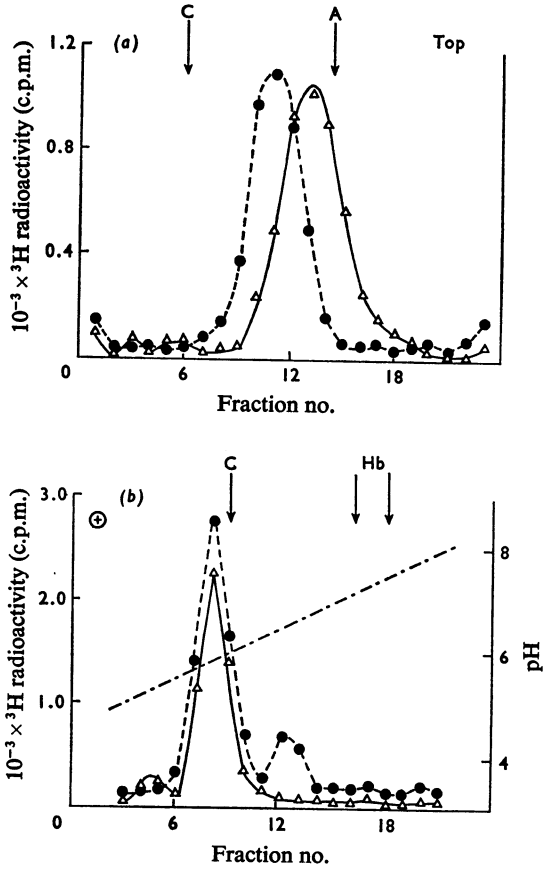


Fig. 5. *Physicochemical properties of other cytoplasmic receptor complexes after extensive purification by DNA–cellulose chromatography and isoelectric focusing*

The 8S oestrogen–receptor complex of rat uterus, labelled with [^{3}H]oestradiol-17β, and the 7S progesterone–receptor complex of guinea-pig uterus, labelled with [^{3}H]progesterone, were purified some 3000-fold by the scheme suggested in Table 1 for the 8S androgen–receptor complex. (*a*) The final material was analysed by sucrose-density-gradient centrifugation, with sedimentation from right to left; fraction size, 0.20ml. Sedimentation markers: C, catalase (11.4S), and A, alkaline phosphatase (6.4S). (*b*) Behaviour of the complexes during isoelectric focusing (stage V; see Table 1), anode to the left; fraction size, 0.50ml. Markers: C, catalase (pI6.1), and Hb, haemoglobin (pI7.2 and 7.5). For both (*a*) and (*b*): ●, [^{3}H]oestradiol; △, [^{3}H]progesterone. For (*b*): —·—, pH. For simplicity in presentation, analyses on the different receptors are presented in a single figure; the analyses were conducted separately.

purification (Fig. 5*a*) and the purified complexes had a pI of 5.8 (Fig. 5*b*). Such a pI has previously been reported for the 8S oestrogen–receptor complex by de Sombre *et al.* (1969), whereas Puca *et al.* (1971) suggest a higher pI of 6.2. These studies on the 7–8S steroid–receptor complexes suggest that they represent a class of important regulatory proteins with remarkably similar physicochemical properties.

Investigations on the temperature-dependent step in the nuclear binding of dihydrotestosterone

Studies by several research groups have established that the overall process for the nuclear binding of dihydrotestosterone in the prostate gland is a temperature-dependent process (Anderson & Liao, 1968; Fang *et al.*, 1969; Mainwaring, 1971). Purified nuclei alone have little ability to bind dihydrotestosterone, and its transfer into chromatin can only be achieved in the presence of cytoplasmic receptor protein (see Fig. 3, and also Mainwaring & Peterken, 1971). Consequently, the temperature-dependence of the nuclear binding process could be expressed at the level of the nucleus itself or in the transfer of the dihydrotestosterone–receptor complex into the nucleus. Since the first complex formed in the prostate gland after the administration of [^{3}H]testosterone *in vivo* is the cytoplasmic 8S [^{3}H]dihydrotestosterone–receptor complex (Mainwaring & Peterken, 1971), this was employed as the androgen donor in an additional series of experiments conducted on the reconstituted cell-free system (Fig. 6). Brief warming of the 8S [^{3}H]dihydrotestosterone–receptor complex before its inclusion in the reconstituted system markedly accelerated the rate but not the overall extent of the transfer of [^{3}H]dihydrotestosterone into prostate chromatin. Preincubation of chromatin at 30°C did not promote an accelerated rate of transfer of [^{3}H]-dihydrotestosterone. Warming of both chromatin or labelled cytoplasmic receptor at higher temperatures, particularly at 60°C, severely impaired the overall transfer of [^{3}H]dihydrotestosterone. If a critical distinction of the differential effect of preincubation at 30°C is made, however, there is no doubt that the temperature-dependence of the nuclear binding of dihydrotestosterone is explainable by a critical change in the 8S cytoplasmic androgen–receptor complex before its transfer into chromatin. The results of these preincubation experiments emphasize the mandatory requirement for the maintenance of low temperatures during the isolation and analysis of the 8S cytoplasmic androgen–receptor complex, as was stipulated in earlier studies (Mainwaring, 1969*b*; Baulieu & Jung, 1970).

The temperature-dependent activation of the 8S androgen–receptor complex was reflected in major changes in its physicochemical properties. Apart

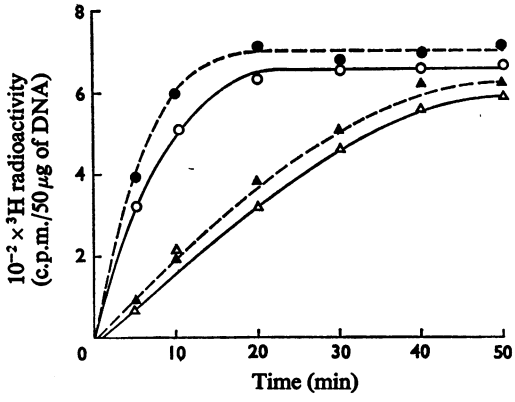


Fig. 6. *Differential effect of temperature on the 8S cytoplasmic androgen–receptor complex revealed by studies on the nuclear transfer of [^3H]dihydrotestosterone in a cell-free system*

Each point represents the radioactivity transferred from an excess of 8S [^{3}H]dihydrotestosterone–receptor complex (15000d.p.m.) into unlabelled prostate chromatin (60μg of DNA) at 4°C. For details see the Materials and Methods section. Before inclusion in the reconstituted system at 4°C, receptor complex or chromatin was incubated separately at 30°C for 20min or stored throughout at 4°C. Conditions of preincubation: ●, both receptor and chromatin to 30°C; ○, receptor only to 30°C; ▲, both receptor and chromatin kept at 4°C; △, chromatin only to 30°C.

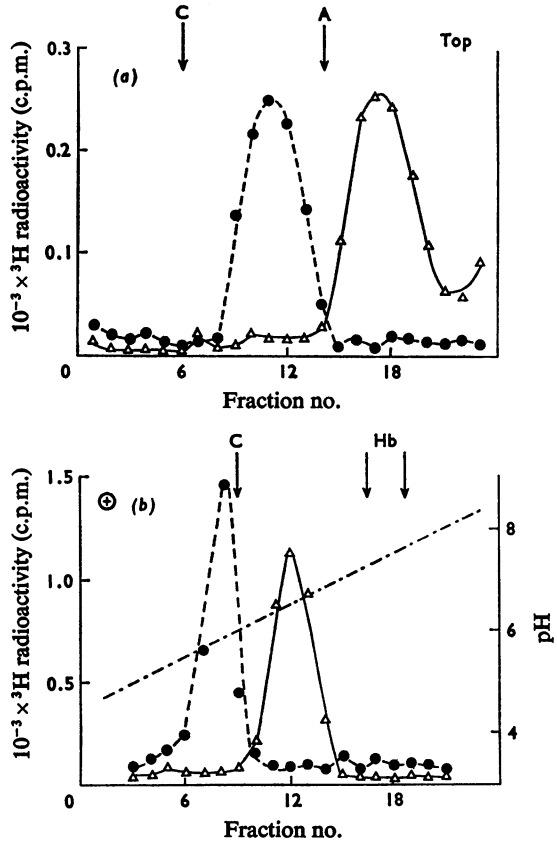


Fig. 7. *Effect of incubation at 30°C on the physicochemical properties of the 8S cytoplasmic androgen–receptor complex*

Samples of 8S androgen–receptor complex, labelled with [^{3}H]dihydrotestosterone (stage II, Table 1), were kept at 4°C (●) or warmed at 30°C for 20min (△) before analysis. (*a*) Sucrose density gradients, with the direction of centrifugation from right to left; each fraction, 0.2ml. Sedimentation markers: C, catalase (11.4S), and A, alkaline phosphatase (6.4S). (*b*) Isoelectric focusing, with direction of the anode to the left; each fraction, 0.5ml. Markers: C, catalase (pI6.1), and Hb, haemoglobin (two bands, pI7.3 and 7.6). —·—, pH. For simplicity in presentation, the results of the analyses are presented in a single figure; the analyses were conducted separately.

from a pronounced decrease in sedimentation coefficient to 4.2S (Fig. 7*a*) there was also a major change in pI, from 5.8 to 6.5 (Fig. 7*b*). At least during the course of these analytical separations, both of which were performed overnight at 0–4°C, the temperature-dependent change in the 8S androgen–receptor complex was essentially irreversible. Additional studies indicated that the activated cytoplasmic receptor complex fully retained the ability to bind to DNA–cellulose.

Studies on nuclear receptor complexes

Fundamental physicochemical properties. When prostate nuclei are labelled with [^{3}H]dihydrotestosterone *in vivo* or by incubation with labelled cytoplasmic receptor complex in a cell-free system *in vitro*, solubilization of the nuclear receptor complex is most conveniently done by extraction with 0.5M-KCl (Anderson & Liao, 1968; Mainwaring, 1969*a*). When labelled extracts were analysed in sucrose density gradients, only a single peak of bound radioactivity of sedimentation coefficient 4.2S was observed (Fig. 8*a*). In marked contrast, when KCl extracts were desalted and subjected to isoelectric focusing, a most complex profile of bound radioactivity was found (Fig. 8*b*). Since this heterogeneity was possibly attributable to the formation of complexes between the

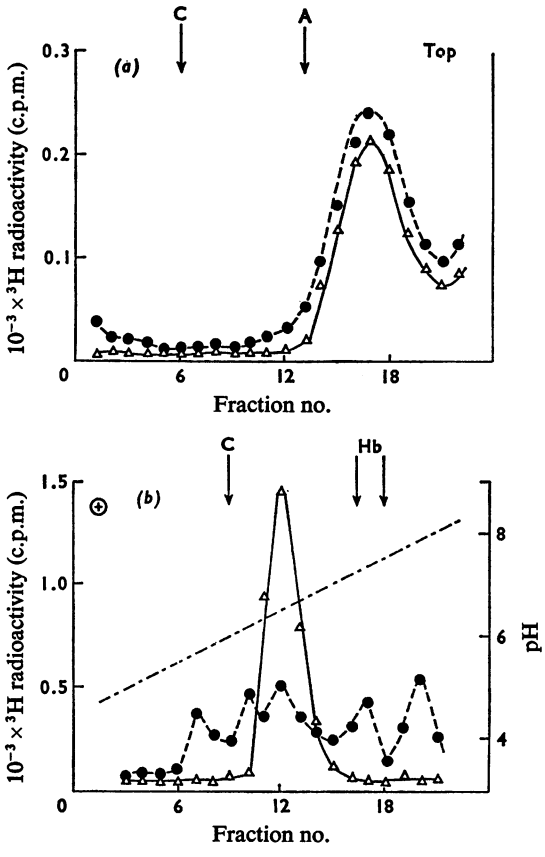


Fig. 8. *Effect of dextran sulphate on some of the physicochemical properties of the nuclear androgen–receptor complex*

Extracts of nuclear [^{3}H]dihydrotestosterone–receptor complex, prepared initially in 0.5M-KCl, were analysed for radioactivity associated with protein directly (●) or after treatment with dextran sulphate (1.5mg/ml) (△). (*a*) Sucrose gradients containing 0.5M-KCl, sedimentation from right to left; fraction size, 0.20ml. Sedimentation markers: C, catalase (11.4S), and A, alkaline phosphatase (6.4S). (*b*) Isoelectric focusing after desalting, anode to the left; fraction size, 0.50ml. Markers: C, catalase (pI6.1), and Hb, haemoglobin (two bands, pI7.3 and 7.5). —·—, pH. For simplicity in presentation, the results are presented in a single figure; the analyses were conducted separately.

relatively acidic nuclear receptor complex and solubilized histones, nuclear extracts were treated with dextran sulphate before analysis in the pH gradients. The selection of dextran sulphate for suppressing complex-formation due to cationic cellular constituents was prompted by the studies of Harris (1971). The treatment resulted in the isoelectric focusing of the nuclear receptor complex as a single sharp peak of pI6.5 (Fig. 8*b*). This determination of the pI is in excellent agreement with that of the nuclear oestrogen–receptor complex isolated from calf uterus (Puca *et al.*, 1971). This finding underlines the particular sensitivity of the isoelectric-focusing technique to artifact, yet illustrates at the same time the powerful resolution of the method. The common pI of the nuclear and activated cytoplasmic receptor complexes provides important corroboration of the general premise that nuclear receptor complexes are formed as a result of the transfer of the entire cytoplasmic receptor complex into the chromatin of steroid-responsive cells (Mainwaring & Peterken, 1971; Marver *et al.*, 1972; Baxter *et al.*, 1972).

Partial purification of the nuclear androgen–receptor complex. A method for the partial purification of the nuclear androgen–receptor complex is illustrated by the results summarized in Table 2. Desalting of the KCl-containing nuclear extracts by gel-exclusion chromatography in Sephadex G-50 provided an efficient means of separating the labelled receptor complex from nuclear constituents of much lower molecular weight, and further purification was achieved by DNA–cellulose chromatography and isoelectric focusing.

Analysis of the labelled complex by electrophoresis in polyacrylamide gels demonstrated that [^{3}H]-dihydrotestosterone was attached to a protein band, and at least two bands of contaminating protein were also present. Additional studies also indicated that the purified receptor complex was bound to chromatin when incubated under the optimum conditions described by Mainwaring & Peterken (1971).

Discussion

The properties of the androgen–receptor complexes established in the present study are generally in excellent accord with the physicochemical characteristics of the receptor complexes isolated from female accessory sexual organs (de Sombre *et al.*, 1969; Puca *et al.*, 1971; Yamamoto & Alberts, 1972). The only serious inconsistency with previously published results is the pI of 5.8 for all these 7–8S cytoplasmic receptor complexes, compared with the pI of 4.4 reported by O'Malley *et al.* (1971) for the progesterone–receptor complex of oestrogen-primed chick oviduct. This could be attributed to phylogenetic differences, since the present investigation was essentially restricted to ^{3}H-labelled steroid–receptor complexes from mammalian tissues only. In our present experience with the isoelectric focusing of labelled mammalian receptor proteins in the wide pH range

Table 2. *Partial purification of the nuclear androgen–receptor complex of rat prostate gland*

Prostate glands were removed from 16 animals 24h after castration and incubated for 30min at 37°C in 20ml of Eagle's medium containing 1nM-[^{3}H]dihydrotestosterone. Purified nuclei were isolated and the nuclear receptor complex was solubilized by extraction with 0.5M-KCl. The concentration of dextran sulphate, where used, was 1.5mg/ml. Protein was determined by the procedure of Lowry *et al.* (1951), except where indicated by an asterisk (*), when the more-sensitive fluorimetric method of Hiraoka & Glick (1963) was used. Labelled receptor was identified by liquid-scintillation spectrometry; protein was only determined in peak fractions pooled at each stage.

	Stage of purification	Protein (mg)	Bound ^{3}H (d.p.m.)	Specific radioactivity (d.p.m./mg of protein)
I	Purified nuclei	8.10	5.6×10^6	6.91×10^5
II	Soluble extract in 0.5M-KCl	6.76	5.3×10^6	7.84×10^5
III	Supernatant after treatment with dextran sulphate	6.12	3.9×10^6	6.37×10^5
IV	Desalting by Sephadex G-50 chromatography	1.54	3.5×10^6	2.27×10^6
V	DNA–cellulose chromatography	0.37; 0.370*	3.4×10^6	9.19×10^6
VI	Desalting by Sephadex G-25 chromatography	0.352*	3.2×10^6	9.09×10^6
VII	Isoelectric focusing; protein of pI6.5	0.071*	3.0×10^6	4.26×10^7

3–10, any concentration of protein-bound radioactivity below pH4.8 was accompanied by visible precipitation of protein. The otherwise remarkable similarity in the properties of the steroid–receptor complexes suggests that they are an important class of regulatory proteins differing only in the structure of the binding sites for the steroid hormones.

The marked similarity in the cytoplasmic receptor complexes from a wide range of male accessory sexual glands argues against an explanation of inter- and intra-species differences on the basis of the structure of the cytoplasmic receptor proteins, at least as judged by present methods of analysis. Differences in the androgenic response in the accessory sexual glands could be explained by the binding of the cytoplasmic receptor complexes to tissue-specific acceptor sites in chromatin, differences in the metabolism of testosterone in the androgen target organs or to the selective influence of prolactin on the uptake of testosterone into different organs (Lawrence & Landau, 1965; Grayhack & Lebowitz, 1967).

The explanation of the temperature-dependence of the nuclear binding of dihydrotestosterone in terms of the change in the structure and configuration of the cytoplasmic receptor complex before its binding to chromatin is amply supported by evidence from other steroid-sensitive systems (Baxter *et al.*, 1972; Jensen *et al.*, 1971, 1972; Marver *et al.*, 1972). Until the receptor complexes are purified to homogeneity, however, it cannot be decided whether this change is an intrinsic property of the receptor protein itself or is due to the presence of other factors in the receptor preparations. Important evidence from E. V. Jensen and his colleagues (de Sombre *et al.*, 1972) indicates that this activation or transformation of the cytoplasmic oestrogen–receptor complex can occur at low temperatures (4°C), provided that EDTA is omitted from all preparative media. Irrespective of the means of this transformation process, the activated complex can stimulate RNA synthesis in uterine nuclei *in vitro* (Mohla *et al.*, 1972). We have not yet investigated the effects of the androgen–receptor complexes on RNA synthesis in prostate nuclei, but the earlier studies by Jensen *et al.* (1971), conducted in media containing EDTA, entirely support the conclusion that the transformation of the cytoplasmic receptor complex can be effected by incubation at 30°C, as used in the present study.

The method suggested for the purification of steroid–receptor complexes is as efficient as previously recommended schemes (de Sombre *et al.*, 1969; Puca *et al.*, 1971), yet it is performed with greater practical facility. In particular, it may be eminently suitable for attempts at the purification of the receptor complexes on a larger scale. As suggested by the findings of Poonian *et al.* (1971), receptor complexes may be retained by DNA covalently linked to CNBr-activated Sepharose 4B, and this may be done in a batchwise manner, rather than in columns (W. I. P. Mainwaring, unpublished work).

Provided that certain assumptions are made, the purity of the cytoplasmic receptor complex at stage V of the purification scheme (Table 1) may be calculated. The first assumption is that only one molecule of dihydrotestosterone is bound per molecule of receptor and the second is that one molecule of 8S cytoplasmic receptor complex is converted into one molecule of nuclear receptor complex. The similarity

in the properties of the activated cytoplasmic receptor complex and the nuclear complex favours such a structural interrelationship. At saturating concentrations of dihydrotestosterone, nuclear binding of this androgenic steroid is 1.3nmol/g of DNA (Mainwaring & Peterken, 1971). With the DNA content of 2.3mg of DNA/g wet wt. of prostate gland (Coffey *et al.*, 1968) and 4.5g of starting material, as in Table 1, then maximum binding of dihydrotestosterone may be calculated to require 13.2pmol of nuclear receptor complex in this amount of tissue. Since the molecular weight of the nuclear complex is approx. 100000 (Mainwaring, 1969*a*, 1971), 1.32μg of receptor protein is required. However, the molecular weight of the 8S complex is 280000 (Mainwaring, 1969*b*), and, assuming the stoicheiometric (1:1) interconversion of the receptor complexes in the course of the nuclear binding of dihydrotestosterone, 3.74μg of 8S receptor complex is required. On this basis, our final preparation is at best 5% pure. This assessment of purity has been reported for the oestrogen–receptor complex of calf uterus (de Sombre *et al.*, 1969). A similar state of purity may be calculated for the nuclear receptor complex isolated by the scheme reported in Table 2. In both cases, however, the dissociation of [^{3}H]dihydrotestosterone during the course of purification places some doubt on the validity of the calculations. Nevertheless, the results illustrate the severity of the task of purifying these receptor complexes.

We express our gratitude to Mrs. Patricia Allen for performing the experimental castrations and to Mrs. Margaret Barker for assistance in the preparation of the manuscript.

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