Nuclear Components Responsible for the Retention of Steroid–Receptor Complexes, especially from the Standpoint of the Specificity of Hormonal Responses

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1. By covalently linking nuclear components from hormone-sensitive cells to Sepharose 2B, it is possible to investigate the interaction between nuclear components and cytoplasmic receptor–steroid complexes by affinity chromatography. 2. Many factors are implicated in the specificity of nuclear–cytoplasmic interactions, including the nature of the nuclear components, the presence of the cytoplasmic receptor protein and the provision of the appropriate steroid ligand. 3. Two distinct sets of binding sites are present in nuclear extracts immobilized to Sepharose 2B, namely a small number of specific high-affinity sites and a larger number of non-specific low affinity-sites. 4. Considerable evidence supports the importance of the high-affinity binding sites in the manifestation of hormonal specificity in different tissues. Although the study has centred largely on androgen-responsive systems, the findings are germane to cytoplasmic–nuclear interactions in general. 5. The high-affinity or acceptor sites in nuclear extracts reside in the basic but non-histone protein fraction. 6. Hormonal specificity is seemingly maintained by both the cytoplasmic and nuclear components, and the results are discussed in the context of the mechanism of action of steroid hormones.

The current consensus of opinion (reviewed at length by King & Mainwaring, 1974) supports the view that the selective nuclear retention of steroid hormones is a characteristic feature of steroid-sensitive cells. Steroids are initially bound to specific cytoplasmic receptor proteins, and the resultant steroid–receptor complexes are then retained for a finite period of time within nuclear chromatin. Creditable attempts have been made to simulate this process in reconstituted cell-free systems with chromatin (Steggles *et al.*, 1971; Mainwaring & Peterken, 1971), intact nuclei (Fang & Liao, 1971) or soluble nuclear extracts (Tymoczko & Liao, 1971) serving as the subcellular components necessary for trapping the cytoplasmic receptor complexes. The term ‘acceptor’ was coined to describe the nuclear components engaged in the high-affinity but non-covalent attachment of the receptor complexes. These earlier studies suffered from many practical limitations, including the repeated preparation of nuclear fractions, laborious precipitation and washing procedures and experimental variability. The interpretation of certain experimental findings has also been justifiably challenged (Chamness *et al.*, 1973). These problems were adroitly circumvented in the study of Puca *et al.* (1974), in which the acceptor components were covalently

linked to Sepharose 4B, thus forming an insoluble matrix suitable for affinity chromatography. The advantages of this approach are threefold; bulk preparation of a stable acceptor matrix, extreme analytical precision and experimental flexibility.

The present study is an extension of the elegant work of Puca *et al.* (1974), particularly in seeking a plausible explanation of the specificity of hormonal responses in terms of differences in the interactions between receptor complexes and acceptor components. Hormonal specificity is expressed in two categories, termed here overt specificity and discrete specificity. Overt specificity describes the sensitivity or refractoriness of tissues to steroid hormones. Discrete specificity is used to describe the subtle differences in the responses of many tissues to the same hormone. There is currently no explanation of these extreme examples of hormonal specificity. Until cytoplasmic receptor complexes may be purified extensively, the only valid indication of tissue-specificity in receptor structure remains as differences in their binding affinity for steroid ligands. Based on this indirect criterion, such stringent specificity is limited to the glucocorticoid receptors in distinct areas of the brain (de Kloet *et al.*, 1975) and to the progesterone receptors in uteri from different species (Kontula *et al.*, 1975).

The possible involvement of acceptor components in the maintenance of hormonal specificity has not been extensively explored, and this was our principal objective.

Materials and Methods

Animals

These were sexually mature animals bred in this Institute. Bilateral adrenalectomy and castration were performed under fluothane anaesthesia, and adrenalectomized rats were maintained on 0.9% (w/v) NaCl. Prostate glands and other tissues were removed from male rats 36 h after castration because androgen receptor proteins are somewhat unstable after castration (Baulieu & Jung, 1970; Mainwaring, 1971). Adrenalectomized and ovariectomized female rats, together with ovariectomized female guinea pigs, were killed 96 h after surgery; the latter were injected with 50 µg of diethylstilboestrol intraperitoneally in 0.2 ml of 0.9% NaCl 72 h after ovariectomy to enhance the amount of uterine progesterone receptor (Milgrom *et al.*, 1970).

Chemicals

[1,2-³H]Dexamethasone (24 Ci/mmol) and [1,2,4,5,6,7(*n*)-³H]5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one; 107 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Other ³H-labelled steroids were kindly given by colleagues. As judged by t.l.c., the radiochemical purity of all ³H-labelled steroids was 98% or more. Non-radioactive steroids were supplied by Steraloids Ltd., Croydon, Surrey, U.K., and the synthetic anti-androgen, 6α-bromo-17β-methyl-4-oxa-5α-androstan-3-one, was given by Hoffman-La Roche, Basel, Switzerland. Tris base (99.5% pure), dithioerythritol, crystalline bovine serum albumin and calf thymus DNA were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Bio-Rex 70 (100–200 mesh) was supplied by V. A. Howe and Co., London W.11, U.K., and Sepharose 2B was purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. (NH₄)₂SO₄ (enzyme grade; low in heavy metals) was supplied by Fisons and Co., Loughborough, Leics., U.K.; a saturated solution was prepared and the pH adjusted to 7.4 with 1 M-NH₃. Human pregnancy serum (third trimester) was used as a source of sex steroid-binding β-globulin; serum was depleted of steroids by treatment with charcoal and stored in small portions at -20°C. Glass-distilled water was used in making up all solutions.

Preparation of subcellular fractions

These were performed at temperatures as close to 0°C as possible.

(a) Nuclei and soluble nuclear extracts. Purified

nuclei were prepared by the procedure of Mainwaring (1969a). Uterine nuclei from rats and guinea pigs were contaminated by myofibrillar elements which resisted homogenization; these were removed at the next preparative stage. Nuclei were resuspended in medium A (50 mM-Na₂HPO₄ containing 5 mM-NaHSO₃ and 2 M-NaCl) in the approximate ratio of 2 ml of medium A: 500 µg of nuclear DNA and sonicated (150 W instrument, M.S.E., Crawley, Sussex, U.K.) at 20 kHz for three periods of 10 s, with 30 s intervals to avoid heating. After vigorous stirring for 30 min, the suspension of low viscosity was centrifuged for 16 h at 100 000 *g* to sediment DNA. The clear supernatant constituted the soluble nuclear extract.

(b) *Cytoplasmic extracts.* These were prepared as described by Mainwaring & Peterken (1971) with the precautions necessary to preserve the labile receptor proteins. Extracts were made 0.5 mM with respect to ³H-labelled steroids, and labelling was allowed to proceed for 2 h at 0°C. Where indicated in the text, fractionation was performed with (NH₄)₂SO₄ as reported by Mainwaring & Peterken (1971). Provided that repeated freezing and thawing was avoided, these preparations could be stored at -20°C for up to 4 days without detectable loss of receptor-bound ³H-labelled steroid. In certain experiments, cytoplasmic fractions depleted of receptor protein were required. Cytoplasmic extracts were incubated at 50°C for 5 min and clarified by centrifugation at 5000 *g* for 5 min before labelling and (NH₄)₂SO₄ fractionation; this thermal-denaturation step inactivates the receptor proteins used in this study (King & Mainwaring, 1974). All receptor preparations were made and stored in medium B [50 mM-Tris/HCl buffer, pH 7.4, containing 0.5 mM-EDTA, 1 mM-dithiothreitol and 10% (v/v) glycerol].

Separations of nuclear-associated proteins

These were conducted throughout within the temperature range 2–4°C, with nuclear extracts, prepared in medium A, serving as starting material.

(a) *Sucrose density gradients.* Samples (0.5 ml) were layered over linear 5–20% (w/v) sucrose gradients (4.5 ml) prepared in medium A. Centrifugation was performed at 150 000 *g*_{av.} for 16 h at 3°C in the swing-out Ti 52 rotor of a Christ Omega II ultracentrifuge. Sedimentation markers, catalase (11.4S), haemoglobin (4.4S) and cytochrome *c* (1.9S), were run in a separate gradient; the metal-containing prosthetic groups were not dissociated in 2 M-NaCl.

(b) *Isoelectric focusing.* Extracts were made 3 M with respect to urea and dialysed against 1 litre of 3 M-urea overnight. Insoluble protein was removed by sedimentation at 20 000 *g*_{av.} for 15 min, and the clear supernatant (6 ml) was made 15% (w/v) with respect to sucrose and cautiously layered over a 20 ml

linear 20–60% (w/v) sucrose gradient, containing 3M-urea and 0.25% (w/v) pH-range 3–10 ampholytes [LKB (U.K.), London S.W.12, U.K.] in an apparatus made to the design of Osterman (1970). The sample was overlaid with 4ml of 3M-urea containing 0.25% pH-range 3–10 ampholytes, and after the upper (cathode) and lower electrode reservoirs were filled with 2% (v/v) ethylenediamine and 1% (v/v) H₂SO₄ respectively, an initial voltage of 100V (4mA) was applied for 30min. Focusing was continued for 24h at 320V with continuous circulation of ice-cold water through the cooling jacket. The contents of the apparatus were subdivided into 2.0ml fractions, and the pH was determined at 4°C in a Radiometer model 28 pH-meter calibrated against potassium hydrogen phthalate.

(c) *Ion-exchange chromatography.* Our procedure was based on that of Levy *et al.* (1972), with minor modifications. Nuclear extracts were made 4M with respect to urea and dialysed overnight against 1 litre of medium C (50mM-Tris/HCl buffer, pH 8.0, containing 1mM-2-mercaptoethanol, 5mM-NaHSO₃ and 4M-urea). After centrifugation of the dialysed sample at 20000g_{av} for 15 min, the clear supernatant was applied to a column (5cm×2.5cm diam.) of Bio-Rex 70, previously equilibrated with medium C. The column was eluted sequentially with medium C, medium C containing 0.40M-NaCl and finally with 10% (w/v) guanidinium chloride.

Activation of Sepharose and coupling of proteins

Our procedure was taken directly from the detailed publications of Axén *et al.* (1967) and Cuatrecasas (1970). CNBr-activated Sepharose 2B was collected on a Buchner head and washed twice with 200ml of ice-cold water and twice with 250ml of ice-cold medium A. Coupling of proteins, dissolved in medium A, was accomplished by gentle stirring overnight at 4°C. In general terms, 2g of activated Sepharose 2B was used per mg of protein in a total volume of 5ml of medium A. The matrix was collected, washed with 2M-NaCl and water (200ml each) and stirred for 2h at room temperature (20–22°C) in 100ml of 2M-glycine/NaOH buffer, pH 9.2, to block any remaining activated groups on the Sepharose 2B. After extensive washing with water, the Sepharose 2B matrix was stored in medium B containing 0.25% (w/v) NaN₃ at 2–4°C for as long as 6 weeks without deleterious change with respect to acceptor activity. Combined filtrates after the coupling stage were used to assess the extent of protein linkage.

Interactions between receptor complex and immobilized acceptor

Disposable glass Pasteur pipettes (0.5cm diam.×10cm) were plugged with cottonwool and up to 4 dozen mounted in a Perspex rack. A maximum of 500mg wet wt. of Sepharose 2B matrix was

introduced into each pipette and washed twice with 2ml of medium B. The columns were placed in a cold-room at 2–4°C, and the experiments were run in this temperature range. Cytoplasmic extract (0.05–2.5ml), labelled with ³H-labelled steroid, was applied to each matrix without any restriction on the rate of flow. By using a wash-bottle with a fine tip as dispenser, each column was washed with 7×2ml of ice-cold medium B. Elution of bound receptor complex was accomplished with 2ml of 2M-KCl and duplicate samples of eluate (0.5ml) were counted for radioactivity. The attractive feature of this technique is the facility with which large numbers of columns may be processed concomitantly. Before this routine procedure was adopted, initial experiments were performed in small chromatography columns (1.0cm diam.) containing up to 1.5g wet wt. of Sepharose 2B matrix.

Other procedures

Details of the quantitative analysis of protein, DNA and RNA may be found in Mainwaring & Peterken (1971) and Mainwaring *et al.* (1974). Aqueous samples containing ³H-labelled compounds (maximum 0.5ml) were counted at an efficiency of 50% in 10ml of water-miscible phosphor (Mainwaring & Wilce, 1972) in a Nuclear-Chicago Mark II scintillation spectrometer. The degree of quenching was determined by the channels-ratio procedure.

Results

Composition of the Sepharose 2B matrix

Most of our experiments were conducted on an affinity-chromatography matrix of Sepharose 2B containing covalently bound nuclear components of the prostate gland. Although DNA and RNA were present in the initial nuclear extracts prepared in 2M-NaCl, they were not extensively attached to Sepharose 2B (Table 1). This finding is consistent

Table 1. *Composition of the Sepharose 2B matrix containing immobilized components derived from prostate nuclear extracts*

In the standard procedure, performed on five separate occasions, nuclear extracts derived from the combined tissue from 18 prostate glands were coupled to 30g of CNBr-activated Sepharose 2B. Nuclear constituents were determined before and after coupling to assess the extent of their covalent attachment. The results are quoted as means±S.E.M.

Nuclear constituent	Input (mg)	Bound (mg)	Bound constituent (μg/g wet wt. of Sepharose 2B)
Protein	30.0±3.6	14.1±0.7	470
DNA	4.8±0.4	1.2±0.1	40
RNA	3.1±0.3	1.2±0.3	40

Fig. 1. Essential features of the interaction between immobilized nuclear components and cytoplasmic receptor complexes

Subcellular fractions were isolated from the prostate glands of rats 36h after bilateral orchidectomy. Cytoplasmic extracts (105000g_{av}, supernatant fractions) were labelled with 0.2nM-5α-[³H]dihydrotestosterone for 3h at 0°C and nuclear extracts were coupled covalently to Sepharose 2B. Labelled cytoplasmic fractions (0.5 ml) were applied to columns of Sepharose 2B (1.2 g wet wt.) and after washing in medium B, 2M-KCl was applied, where indicated by the arrows, to elute the labelled complexes retained on the columns. Variations in this approach were devised to probe the importance of various factors in the interaction between the nuclear components and the labelled receptor complex. (a) Basic system; labelled cytoplasmic receptor and Sepharose 2B matrix. (b) Labelled cytoplasmic receptor and activated Sepharose 2B, blocked with 2M-glycine buffer, pH9.2, but without coupling of nuclear components. (c) Equivalent amount of free 5α-[³H]dihydrotestosterone in medium B alone. (d) Cytoplasmic extracts alone applied to the column in the absence of 5α-[³H]dihydrotestosterone, with washing in medium B; then 5α-[³H]dihydrotestosterone applied alone, with washing in medium B. (e) As (d), but 5α-[³H]dihydrotestosterone applied before the steroid-free cytoplasmic extract. (f) Washings of free 5α-[³H]dihydrotestosterone and steroid-free cytoplasmic extracts, as in (e), combined, maintained at 0°C for 30 min and re-applied to the columns. The broken line above fractions 2 to 4 indicates that the amount of radioactivity was in excess of the scale on the ordinate.

with the work of Poonian *et al.* (1971), who established that nucleic acids must be denatured or structurally modified by limited digestion with nucleases before they may be covalently attached to agarose matrices to any appreciable extent.

Initial studies on receptor-acceptor interactions

Before undertaking an extensive study of the interaction between immobilized nuclear components and cytoplasmic receptor complexes, it was essential to delineate the role played by each component of the system. Such a series of initial experiments is summarized in Fig. 1. When labelled androgen-receptor complexes were applied to the columns containing immobilized prostatic nuclear components, a significant amount of complex was retained that could only be eluted in media of high ionic strength (Fig. 1a). Both nuclear components and

receptor protein were implicated in this process, as shown in Figs. 1(b) and 1(c). Most important of all, however, was the observation that the receptor protein and its appropriate ligand, 5α-[³H]dihydrotestosterone, must be presented concomitantly to the acceptor matrix for significant retention of ³H-labelled steroid to occur (compare Figs. 1d and 1e with Fig. 1f). This precludes any significant binding of steroid to the acceptor component in the absence of the cytoplasmic receptor protein.

More detailed studies on receptor-acceptor interactions

(a) *Specificity in the cytoplasmic receptor protein.* From the evidence presented in Table 2, the presence of the cytoplasmic receptor protein in a functional or native configuration is a mandatory feature in cytoplasmic-nuclear interactions involving the transfer of 5α-[³H]dihydrotestosterone. Cytoplasmic extracts

Table 2. *Specificity in the androgen-receptor protein in the transfer of 5 α -dihydrotestosterone to immobilized acceptor sites*

Tissues were removed from male rats 36 h after bilateral orchidectomy. Cytoplasmic extracts were prepared, diluted to a uniform concentration of 10 mg of protein/ml and labelled with 0.5 nM-5 α -[^{3} H]dihydrotestosterone for 3 h at 0°C. Prostate cytoplasmic extracts were also fractionated with (NH $_4$) $_2$ SO $_4$ and the precipitates taken up in medium B equal to the original volume of cytoplasmic extract. Receptor complex was degraded by incubation with 1 mM-*N*-ethylmaleimide and analysed after dialysis against medium B for 1 h at 0°C. Human serum, diluted to a concentration of 10 mg of protein/ml with medium B, served as the source of the sex steroid-binding β -globulin. The Sepharose 2B matrix contained immobilized prostate nuclear extracts and columns (500 mg wet wt. of matrix) were run in triplicate. Details of the washing procedure for the columns are given in the Materials and Methods section; results are quoted as mean $\pm$ S.E.M. Input of cytoplasmic extract was 0.5 ml throughout.

Source of cytoplasmic extract	Bound 5 α -[^{3} H]- dihydrotestosterone (d.p.m./500 μ g of immobilized protein)
Prostate	8280 $\pm$ 440
Prostate [0–33%–satd.-(NH $_4$) $_2$ SO $_4$ ppt.]	7440 $\pm$ 220
Prostate [33–66%–satd.-(NH $_4$) $_2$ SO $_4$ ppt.]	640 $\pm$ 80
Liver	440 $\pm$ 40
Kidney	300 $\pm$ 40
Spleen	240 $\pm$ 20
Serum	440 $\pm$ 40
None; free 5 α -[^{3} H]dihydrotestosterone	220 $\pm$ 20
Prostate+1 mM- <i>N</i> -ethylmaleimide	840 $\pm$ 20

from androgen-insensitive tissues, such as spleen, essentially devoid of androgen-receptor protein, were unable to promote the binding of 5 α -[^{3} H]dihydrotestosterone to the immobilized acceptor sites. Further, after (NH $_4$) $_2$ SO $_4$ fractionation of labelled cytoplasmic extracts of the prostate gland, transfer of 5 α -[^{3} H]dihydrotestosterone occurred only with the protein fraction precipitated at 33% saturation with respect to (NH $_4$) $_2$ SO $_4$. This precipitation step is known to yield selectively the 8S high-affinity androgen-receptor complex (Mainwaring & Peterken, 1971; Davies & Griffiths, 1974). Degraded receptor, after treatment with *N*-ethylmaleimide (Mainwaring, 1969b), is totally inactive. The most important feature of the results in Table 2, however, is the total inability of the sex steroid-binding β -globulin to simulate the effect of the androgen-receptor protein, for this plasma protein is capable of binding 5 α -dihydrotestosterone with an affinity approaching that of the receptor protein itself (Kato & Horton, 1968; Mercier-Bodard & Baulieu, 1968), but cannot transfer 5 α -dihydrotestosterone into chromatin (Mainwaring & Peterken, 1971).

(b) *Specificity in 5 α -dihydrotestosterone.* The steroid specificity of the cytoplasmic androgen-receptor protein is extremely acute (Mainwaring, 1969b; Mangan & Mainwaring, 1972), and this is reflected in the results presented in Table 3. At identical concentrations, other ^{3} H-labelled steroids could not replace 5 α -dihydrotestosterone in facilitating the association of the receptor protein with the immobilized acceptor sites. This conclusion stands despite differences in the specific radioactivities of the ^{3} H-labelled steroids. The striking dissimilarity of the non-radioactive stereoisomers of dihydrotestosterone on the transfer of 5 α -[^{3} H]dihydrotestosterone to the acceptor sites was particularly impressive. The anti-androgen, 6 α -bromo-17 β -hydroxy-17 α -methyl-4-oxa-5 α -androstan-3-one, counters the high-affinity binding of 5 α -dihydrotestosterone and negates biochemical processes implicated in the androgenic response (Mangan & Mainwaring, 1972). In keeping with these observations, the anti-androgen curtailed the association of 5 α -[^{3} H]dihydrotestosterone with the acceptor components.

(c) *Specificity in the nuclear acceptor components.* The relative ability of nuclear components from various tissues to retain the cytoplasmic androgen–

Table 3. *Specificity of 5 α -dihydrotestosterone in the interaction between the androgen-receptor complex and immobilized acceptor sites*

^{3} H-labelled steroid (0.5 nM)	Non-radioactive competitor (10 nM)	Bound ^{3} H- labelled steroid (d.p.m./500 μ g of immobilized protein)
5 α -Dihydro- testosterone	—	8980 $\pm$ 420
Testosterone	—	2160 $\pm$ 140
Dexamethasone	—	100 $\pm$ 40
Progesterone	—	200 $\pm$ 20
Androsterone	—	440 $\pm$ 60
5 α -Dihydro- testosterone	5 α -Dihydrotestosterone	1380 $\pm$ 60
	5 β -Dihydrotestosterone	8800 $\pm$ 200
	Dexamethasone	9340 $\pm$ 260
	Anti-androgen*	5040 $\pm$ 260

* 6 α -Bromo-17 β -hydroxy-17 α -methyl-4-oxa-5 α -androstan-3-one.

receptor complex was compared. As indicated in Table 4, the immobilized nuclear extracts of the prostate gland were singularly more efficient in binding the androgen-receptor complex. This result corroborates the earlier findings of Fang & Liao (1971), Tymoczko & Liao (1971) and Mainwaring & Peterken (1971) in that nuclear preparations from androgen-sensitive tissues contain appreciably more of the acceptor components than do similar preparations from androgen-insensitive tissues. The advantage of the present approach is that differences in acceptor content cannot readily be attributed to variations in the properties of chromatin during

preparation or to other limitations of experimental design.

More detailed analysis of the binding characteristics of the acceptor components

Saturation analysis provides a novel insight into the distinctive features of binding sites at concentrations of steroids within the physiological range (see review in King & Mainwaring, 1974). In these experiments partially purified receptor complex was used, since precipitation with $(\text{NH}_4)_2\text{SO}_4$ depletes the amount of free 5α - ^3H dihydrotestosterone present. Two distinct sets of acceptor sites are present in prostate nuclear extracts (Fig. 2a); one set is characterized by a low capacity but high affinity with respect to the cytoplasmic androgen-receptor complex, whereas the second was of low affinity and seemingly non-saturable under the experimental conditions used. Interactions conducted at an ionic strength known to dissociate extensively the receptor complex from intact nuclei, namely 0.5M-KCl (Mainwaring, 1969a), provided a second means of distinction between the two categories of acceptor sites. In marked contrast with the high-capacity sites, those of low capacity could not be occupied by receptor complex in media of high ionic strength (Fig. 2a). The differences between the acceptor sites were similarly demonstrable by using the method of analysis devised by Scatchard (1949); see Fig. 2(b). From these results, it may be calculated that the high-affinity sites possess an apparent K_d of 0.25 nM. These findings are quantitatively similar to the observations of Puca *et al.* (1974) on the binding characteristics of the nuclear acceptor sites for oestrogen-receptor complexes in calf uterus.

Table 4. Comparative efficiency of nuclear extracts derived from various tissues to retain the cytoplasmic androgen-receptor complex

Purified nuclei were prepared, and soluble extracts in 2M-NaCl coupled to Sepharose 2B. The amount of covalently linked protein was in the range 400–790 μg of protein/g wet wt. of agarose matrix. Cytoplasmic extracts of the prostate gland, labelled with 0.5 nM- 5α - ^3H dihydrotestosterone, were used as a source of androgen-receptor complex and samples were applied to columns containing 500 mg wet wt. of Sepharose 2B matrix, in triplicate. The results are quoted as means $\pm$ S.E.M.

Source of nuclear extract	Bound 5α - ^3H dihydrotestosterone (d.p.m./500 μg of immobilized protein)
Prostate	9200 $\pm$ 400
Liver	1300 $\pm$ 140
Kidney	2940 $\pm$ 360
Spleen	3260 $\pm$ 380
Pancreas	820 $\pm$ 60
Lung	2340 $\pm$ 140

Fig. 2. Critical appraisal of the binding properties of immobilized acceptor sites

Prostate glands were removed from male rats 36 h after castration. Nuclear extracts were immobilized on Sepharose 2B and cytoplasmic extracts, labelled with 0.5 nM- 5α - ^3H dihydrotestosterone, were precipitated at 33% saturation with respect to $(\text{NH}_4)_2\text{SO}_4$. Columns containing 500 mg wet wt. of Sepharose 2B matrix were treated with increasing amounts of labelled receptor complex. (a) Conventional saturation analysis. (b) Results plotted according to Scatchard (1949). Interactions in the absence (●) or presence (○) of 0.5M-KCl.

*Acceptor sites and the expression of hormone specificity**(a) Among tissues responsive to different hormones.*

Our next objective was to appraise critically the role played by nuclear components in the expression of hormone specificity. Four readily accessible tissues were selected, each of which demonstrates considerable hormone specificity and contains one predominant cytoplasmic receptor, specific for a distinctive class of steroid hormones. A series of cross-over experiments was undertaken between cytoplasmic receptor complexes, labelled with the appropriate ^3H -labelled steroid ligand, and immobilized nuclear acceptor components. In such comparative experiments it is imperative that every attempt should be made to attain reproducible samples of subcellular fractions from all the tissues under scrutiny; our experimental findings are presented in Table 5. Despite our precautions, the resultant receptor complexes varied considerably in the amount of associated ^3H -labelled steroid, this being conspicuously lower with the androgen- and glucocorticoid-receptor systems. In all of the selected tissues, specific receptor complexes with sedimentation coefficients in the range 7–8.5S have been described (Toft & Gorski, 1966; Mainwaring, 1969b; Milgrom *et al.*, 1970; Beato & Fiegelson, 1972). Although not reported in detail here, on analysing the labelled cytoplasmic extracts in sucrose gradients, the radioactivity recovered in the 8S region bore an excellent correlation with the radioactivity recovered after fractionation with $(\text{NH}_4)_2\text{SO}_4$. Fractionation with $(\text{NH}_4)_2\text{SO}_4$ has been advocated as a general procedure for isolating specific 8S receptor complexes (Verhoeven, 1975). Differences in the amount of ^3H -labelled steroids associated with the receptor preparations may be attributed to genuine differences

in receptor content or the extent of steroid saturation. The immobilized acceptor sites demonstrated considerable specificity in the recognition and retention of cytoplasmic receptor complexes (Table 5); in general terms, binding was consistently higher with homologous as against heterologous cytoplasm (identical compared with non-identical tissue of origin). Acceptor specificity ranged from striking lack of reciprocity exemplified by the subcellular fractions derived from prostate and liver, to the extensive mutual occupancy of acceptor sites demonstrated by progesterone- and oestradiol-receptor complexes.

A more critical evaluation of the specificity of acceptor–cytoplasmic receptor interactions is presented in Fig. 3. Selection of the subcellular fractions was based on two criteria: first, the established sensitivity of the tissues to steroids and, secondly, similar specific radioactivities in the ^3H -labelled receptor complexes, thus obviating the need for extensive correction of the experimental results. The findings on androgen–glucocorticoid interactions indicate that acceptor specificity can be absolute; the androgen–receptor complex can only occupy the high-affinity low-capacity sites in acceptor components from androgen-sensitive cells and vice versa (Figs. 3a and 3b). However, when other receptor complexes were interchanged, as for progestins and oestrogens, then partial occupation of the high-affinity acceptor sites by the heterologous receptor complex can occur (Figs. 3c and 3d).

(b) Among tissues responsive to the same hormone.

The more subtle expression of acceptor specificity was examined in a representative selection of the androgen-dependent male accessory organs (Table 6). The acceptor components from a given gland tended

Table 5. *Specificity of the interaction of cytoplasmic receptor complexes and immobilized acceptor components from different sources*

Tissues were derived from castrated animals, and subcellular fractions prepared as described in detail in the Materials and Methods section. In attempting to achieve experimental uniformity, the following procedures were adopted. First, nuclear components were covalently linked to Sepharose 2B and additional matrix, activated and subsequently blocked with glycine, was added to give a uniform amount of immobilized nuclear protein from all tissues (1.20 mg/g wet wt. of matrix). Secondly, ^3H -labelled steroids were diluted with their non-radioactive counterpart to a specific radioactivity of 24 Ci/mmol. Thirdly, cytoplasmic extracts were labelled with 0.5 nM- ^3H -labelled steroid, precipitated with $(\text{NH}_4)_2\text{SO}_4$ and analysed for protein. Thermally denatured proteins, depleted of receptor and derived from the same tissue, were added to give a protein concentration of 1.5 mg/ml. The interaction between the immobilized nuclear components and 0.5 ml of ^3H -labelled receptor complexes was performed in triplicate; results are quoted as means $\pm$ s.e.m. The extent of binding ultimately reflects the specific radioactivity of the ^3H -labelled receptor complex, and since this varied considerably (2100–12200 d.p.m./0.95 ml), disparities in the extent of the transfer of ^3H -labelled steroid are predictable when receptors from different sources are used.

Source of receptor	^3H -labelled steroid	Bound ^3H -labelled (d.p.m./g wet wt. of Sepharose 2B)			
		a. Rat prostate	b. Rat uterus	c. Rat liver	d. Guinea-pig uterus
1. Rat prostate	5 α -Dihydrotestosterone	12600 $\pm$ 400	3440 $\pm$ 280	2080 $\pm$ 200	2880 $\pm$ 160
2. Rat uterus	Oestradiol-17 β	12800 $\pm$ 460	47400 $\pm$ 1640	12600 $\pm$ 480	28800 $\pm$ 1240
3. Rat liver	Dexamethasone	6600 $\pm$ 560	9400 $\pm$ 820	22200 $\pm$ 940	10740 $\pm$ 560
4. Guinea-pig uterus	Progesterone	14800 $\pm$ 1780	44200 $\pm$ 2200	25000 $\pm$ 2400	94200 $\pm$ 4400

Fig. 3. Detailed appraisal of cytoplasmic receptor-acceptor interactions with preparations from different tissues

Partially purified cytoplasmic receptor complexes, labelled with ^3H -labelled steroids, and nuclear proteins covalently linked to Sepharose 2B, were prepared as described in the legend to Table 5. For simplicity, subcellular components are described according to the ^3H -labelled steroid principally bound in the tissue of origin. Increasing amounts of receptor complex were applied to columns containing 300mg wet wt. of Sepharose 2B, and the radioactivity eluted only in 0.5M-KCl was determined. Radioactivity in the receptor complexes (d.p.m. of ^3H -labelled steroid/0.1 ml): androgen and glucocorticoid, 2200 and 2880; oestrogen and progesterin, 1340 and 16800. In each cross-over experiment, the results are corrected to an identical concentration of ^3H -labelled steroid in the receptor complexes under comparison. In (a) and (b), androgen receptor, ●; glucocorticoid receptor, Δ; in (c) and (d), oestrogen receptor, ○; progesterin receptor, Δ. Immobilized acceptor components: (a) androgen, (b) glucocorticoid, (c) oestrogen and (d) progesterin. The difference in scale on the ordinate between the two experiments should be noted.

Table 6. Cytoplasmic receptor-acceptor interactions in preparations derived from male accessory sexual glands

Preparations were made from tissues 36h after castration. Cytoplasmic receptors were labelled with 5 nm-5 α -[^3H]dihydrotestosterone and partially purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$. The protein content of the labelled receptor preparations was adjusted to 0.4mg of protein/ml by addition, where appropriate, of heat-denatured proteins, depleted of receptor protein. The amount of covalently linked protein on Sepharose 2B was adjusted to 340 $\mu\text{g/g}$ wet wt. of matrix by addition of activated Sepharose 2B blocked with glycine. Columns containing 750mg of matrix were set up in triplicate and labelled receptor complexes were applied (0.5 ml). The results are given as means $\pm$ S.E.M. The protein content of all subcellular fractions was made with reference to the preputial gland, from which least material was available.

Source of cytoplasmic protein	Bound 5 α -[^3H]dihydrotestosterone (d.p.m./500 μg of immobilized protein)			
	a. Prostate	b. Epididymis	c. Preputial gland	d. Liver
1. Prostate	4460 $\pm$ 200	3380 $\pm$ 220	2940 $\pm$ 180	680 $\pm$ 60
2. Epididymis	1460 $\pm$ 100	2060 $\pm$ 140	1360 $\pm$ 120	420 $\pm$ 40
3. Preputial gland	540 $\pm$ 60	600 $\pm$ 60	1040 $\pm$ 80	180 $\pm$ 20
4. Liver	260 $\pm$ 20	260 $\pm$ 20	340 $\pm$ 40	300 $\pm$ 20
5. None; free [^3H]steroid	360 $\pm$ 40	340 $\pm$ 20	280 $\pm$ 40	280 $\pm$ 20

to show a preference for retaining the cytoplasmic receptor of common origin, but considerable binding of heterologous receptor was observed. The specific radioactivity of the receptor complexes in terms of associated 5 α -[³H]dihydrotestosterone differed and this was ultimately reflected in the lower extent of transfer of the ³H-labelled steroid to the acceptor site,

Table 7. Nature of the acceptor components in rat prostate nuclei

Samples of Sepharose 2B (containing 460 μ g of protein, 36 μ g of RNA and 37 μ g of DNA/g wet wt. of matrix) were incubated for 1 h at 30°C with 100 μ g of various enzymes in medium B (supplemented with 1 mM-MgCl₂ and 2 mM-CaCl₂ for trypsin and self-digested Pronase respectively). Columns were set up in triplicate and washed first with 6 ml of 6M-urea to inactivate the enzymes and then with 20 ml of medium B. The ability to retain the androgen receptor 5 α -[³H]dihydrotestosterone complex was compared; the results are given as means $\pm$ S.E.M.

Treatment	Bound 5 α -[³ H]-dihydrotestosterone (d.p.m./500 mg wet wt. of Sepharose 2B)
None	8580 $\pm$ 480
Controls; 6M-urea treatment only	8140 $\pm$ 220
Ribonuclease	7740 $\pm$ 200
Deoxyribonuclease	7680 $\pm$ 340
Trypsin	2640 $\pm$ 220
Pronase (self-digested at 37°C before use)	1060 $\pm$ 100

notably with preputial-gland receptor complex. To ensure that limited but genuine occupation of the acceptor sites had occurred under these circumstances, equivalent amounts of 5 α -[³H]dihydrotestosterone, both alone and in the presence of liver cytoplasm, were run as controls. In additional experiments with prostate and epididymis preparations, reported here only briefly, the receptor complex from one source could partially occupy the high-affinity acceptor sites from the other. Thus the differences in acceptor specificity presented in Table 6 cannot be equated with the absolute specificity demonstrated above in Figs. 3(a) and 3(b).

Nature of the acceptor components

Puca *et al.* (1974) have suggested that the acceptor components in calf uterus are proteins, and this contention is convincingly supported by our own evidence on prostate acceptor sites (Table 7). On incubation of the immobilized acceptor matrix with enzymes of restricted substrate specificity, only proteinases significantly decreased the retention of cytoplasmic androgen-receptor complex.

(a) *Ion-exchange chromatography.* The cation exchanger Bio-Rex 70 has been widely used for the fractionation of nuclear proteins (Levy *et al.*, 1972; Levy *et al.*, 1973; Richter & Sekeris, 1972), because acidic or non-histone proteins are not retained avidly, whereas the basic histones are. On the evidence summarized in Fig. 4, the high-affinity acceptor sites were concentrated in fraction III of the stepwise elution protocol, indicating the basic nature of these components. In contrast, the low-affinity

Fig. 4. Separation of prostate nuclear proteins on columns of Bio-Rex 70

Prostate nuclear extracts in medium C were applied to a column (5 cm $\times$ 2.5 cm diam.) of Bio-Rex 70, equilibrated with medium C. Non-adsorbed protein constituted fraction I, and at the points indicated by the arrows, sequential elution was performed with medium C containing 0.4M-NaCl (fraction II) and finally 10% (w/v) guanidinium chloride (fraction III). Combined peak fractions from within the bars were made 2M with respect to NaCl and dialysed for 16 h against medium A. Acceptor activity was established after covalent attachment of the proteins to Sepharose 2B and interaction with prostate cytoplasmic androgen receptor, labelled with 5 α -[³H]dihydrotestosterone. Acceptor activity (mean $\pm$ S.D. of assays conducted in triplicate) is plotted in histogram form within each protein peak; the hatched area represents the acceptor activity negated by the presence of 0.5M-KCl in the assays (see also Fig. 2).

Fig. 5. Analysis for acceptor activity in sucrose gradients containing prostate nuclear proteins

Samples of prostate nuclear extracts in 2M-NaCl (0.5 ml) were layered over 4.5 ml sucrose gradients prepared in medium A and centrifuged at $150000g_{av}$ for 16 h at 4°C. The direction of centrifugation is from right to left: sedimentation markers, catalase (C; 11.4S), haemoglobin (H; 4.6S) and cytochrome c (Cyt; 1.9S) were run in a parallel gradient. Gradient fractions (0.2 ml each) from five gradients were combined and consecutive fractions were combined in pairs, giving 13 fractions of volume 2.0 ml each. Each sample was coupled to CNBr-activated Sepharose 2B. Acceptor activity (absence of KCl), ●; acceptor activity (in the presence of 0.5M-KCl), ○; protein, ▲.

Fig. 6. Fractionation of prostate nuclear proteins by gel-exclusion chromatography

Prostate nuclear extracts in 2M-NaCl (4.0 ml) were separated on a column (35 cm × 2.3 cm diam.) of Sephadex G-75. With elution in medium A, 2.5 ml fractions were collected with automatic monitoring at a wavelength of 280 nm. Proteins in consecutive fractions were combined in pairs, coupled to Sepharose 2B and analysed for acceptor activity. E_{280} , ----; acceptor activity, ○. The exclusion limit (V_0) of the column is indicated; reference proteins included bovine serum albumin (B), ovalbumin (O), chymotrypsinogen A (Chy) and cytochrome c (Cyt). The elution volumes of these standard proteins were plotted by the methods of Porath (1963) and Reiland (1971) to estimate the molecular weight of acceptor proteins.

acceptor proteins were recovered in the non-histone or acidic protein fractions (peaks I and II; Fig. 4).

(b) *Density-gradient ultracentrifugation.* Prostate nuclear extracts in 2M-NaCl were separated in sucrose gradients and the proteins in individual fractions were covalently linked to Sepharose 2B and assayed for acceptor activity (Fig. 5). From assays conducted in the presence and absence of

0.5M-KCl, the high-affinity acceptor proteins have a sedimentation coefficient of approx. 6.5S, whereas the low-affinity acceptors were recovered in a broad region of the gradient profile corresponding to sedimentation coefficients in the range 1.5S–5.5S. Thus, although the high-affinity acceptor proteins possess an overall negative charge, they cannot be summarily equated with classical histones, because

Fig. 7. Isoelectric focusing of prostate nuclear proteins

Prostate nuclear extracts in 2M-NaCl were made 3M with respect to urea and dialysed for 24 h against 3M-urea. After removal of precipitated proteins by centrifugation at 20000g for 15 min, 6.0 ml of clear supernatant was adjusted to 15% (w/v) in sucrose and layered over a 20 ml linear 30–50% (w/v) sucrose gradient in 3M-urea. Ampholytes, pH range 3–10, were added to all solutions, including the sample, to an overall concentration of 0.25% (w/v). Isoelectric focusing was performed at 320 V for 24 h at 4°C, with the anode at the base of the apparatus. The contents of the column were subdivided into 2.0 ml fractions, and after measurement of the pH, samples were dialysed against medium A to remove urea and ampholytes. Urea-depleted samples were coupled to Sepharose 2B and assayed for acceptor activity. Acceptor activity (absence of KCl), ●; acceptor activity (0.5M-KCl), ○; protein, ▲; pH, ----.

the latter basic proteins have sedimentation coefficients of the order of only 2S (Bradbury & Crane-Robinson, 1971; Hnilica, 1972).

(c) *Gel-exclusion chromatography.* Soluble extracts of prostate nuclei in 2M-NaCl were subjected to fractionation on columns of Sephadex G-75 (Fig. 6), and proteins from combined fractions were assayed for acceptor activity. These analyses were limited by the low concentrations of protein, which raised problems in coupling to Sepharose 2B and quantitative protein analysis. Notwithstanding this, acceptor activity was essentially restricted to the proteins recovered near the exclusion limits of the column. From comparison with the elution volumes of reference proteins, it was estimated that the principal acceptor proteins have mol.wt. approx. 70000. If this assumption is indeed correct, then the high-affinity acceptors are not histones; the molecular weight of these basic proteins has been unequivocally determined to be within the range 17000–22000 (Hnilica, 1972).

(d) *Isoelectric focusing.* Although prostate nuclear proteins are soluble in 2M-NaCl, they possess a marked tendency to precipitate under conditions of low ionic strength. Isoelectric focusing, however, could be conducted in 3M-urea (Fig. 7). These experiments confirmed that the high-affinity acceptor proteins are basic (pI 8.6–9.3), whereas the low-affinity acceptor proteins are acidic (pI, broad range, 3–7). The only reservation about these experiments is the maintenance of the nuclear proteins in 3M-urea

for protracted periods; although precipitation was not evident, irreversible denaturation or the deleterious effects of the ampholytes probably accounts for the relatively low acceptor activity recovered from this procedure.

Discussion

The experimental findings reported here strongly substantiate the earlier reports by Puca *et al.* (1974, 1975a) on the nature of the high-affinity nuclear acceptor sites for receptor complexes. In particular, we conclude that the saturable acceptor is a basic protein of mol.wt. 70000 or a group of proteins with these properties. The precise classification of these nuclear proteins engenders semantic problems. In terms of sedimentation coefficient and molecular size, the acceptor proteins cannot be defined as histones, yet their basic pI runs counter to the generally acidic character of non-histone nuclear proteins. On the present evidence, it is perhaps safest to suggest that the high-affinity acceptor components are non-histone proteins of basic overall charge. It seems unlikely that the acceptors are an aggregated or polymeric form of histones. Although this possibility cannot be rigorously excluded in the present study, the similar results obtained by Puca *et al.* (1975a) using 5mm-HCl for chromatography on Sephadex G-100 tend to discount the premise of histone aggregation. However, the mol.wt. of 70000 for the prostate acceptor is difficult to

reconcile with its seemingly high sedimentation coefficient, 6.5S.

At first sight, it is also difficult to reconcile the present findings and those of Puca *et al.* (1975a) with other citations in the literature (Spelsberg *et al.*, 1971a,b, 1972) that the acceptor components are non-histone proteins and, by inference, acidic in terms of charge. These reports were based on the differential extraction of nuclear components in buffered urea-NaCl media of carefully selected pH, and the absence of basic proteins and histone was reported from an acceptor-enriched fraction, designated AP₃ (Spelsberg *et al.*, 1971a,b). However, the sensitivity of the analytical procedures used by Spelsberg and his collaborators was possibly inadequate for detecting the small amounts of basic acceptor proteins present in nuclear extracts of hormone-sensitive cells (see Puca *et al.*, 1974). Further, the absolute resolution of acidic and basic nuclear proteins on the basis of selective extraction at different pH ranges remains to be unequivocally established. For these reasons, the results obtained by using affinity chromatography by ourselves and Puca *et al.* (1974, 1975a) are not necessarily incompatible with work of Spelsberg *et al.* (1971a,b, 1972).

It is generally accepted that overt hormonal specificity is a reflexion of the steroid specificity of cytoplasmic receptor proteins (see King & Mainwaring, 1974). The present study provides additional insight into this phenomenon in that the high-affinity acceptor sites can demonstrate absolute specificity with respect to the retention of cytoplasmic receptor complexes. For example, this conclusion is consistent with the results of the cross-over experiments with subcellular fractions derived from androgen- and glucocorticoid-sensitive cells. On the other hand, oestrogens and progestins are both biologically active in the uterus, and mutual occupancy of the high-affinity acceptor sites was detected with receptor complexes containing these hormones. Androgen-sensitive tissues provide good examples of discrete hormonal specificity; certain secretory proteins are unique to the rat seminal vesicle (Tóth & Zakár, 1971) and alcohol dehydrogenase may be induced only in mouse kidney (Ohno *et al.*, 1970). Although some preference was demonstrated in androgen-receptor complexes for their homologous acceptor sites, derived from the same tissue, this was far from absolute. This renders it unlikely that cytoplasmic receptor-acceptor site interactions alone provide a plausible explanation of discrete hormonal specificity. The process of cellular differentiation clearly imparts specificity to hormonal responses by mechanisms that cannot be precisely defined at present. This important aspect of the mechanism of action of steroid hormones is the subject of a review by Mainwaring (1976), but many instances may be

cited from the current literature where the high-affinity binding of steroids to receptor and acceptor sites is not commensurate with a biological response (Munck & Brinck-Johnson, 1968; Baxter *et al.*, 1971; O'Farrell & Daniel, 1971).

It has also been implied that only the high-affinity acceptor sites are of fundamental importance, largely on the grounds that their occupation may be negated under conditions of high ionic strength. However, a significant amount of non-covalently bound steroid in the nuclei of hormone-sensitive cells resists extraction into 0.5M-KCl (King, 1967; Mainwaring, 1969a; Mester & Baulieu, 1972). Consequently, the importance of the low-affinity acceptor sites that are refractory to 0.5M-KCl should not be dismissed out of hand, but their function is difficult to evaluate.

Interactions between receptor complex and acceptor site were studied here in conditions far removed from their authentic environment in the intact cell. In particular, receptor-acceptor interactions were conducted essentially in the absence of DNA, and deoxyribonuclease treatment did not impair the acceptor activity of our affinity-chromatography matrix. Despite this, the interaction of receptor complexes with DNA immobilized on cellulose has been widely documented (Mainwaring & Mangan, 1971; King & Gordon, 1972; Yamamoto & Alberts, 1972; Yamamoto *et al.*, 1974). On currently available evidence, binding of receptor complexes to immobilized DNA or protein acceptor sites is sharply contrasted; binding to protein acceptor sites is characterized by specificity, saturability and high affinity, whereas these features are not necessarily evident in binding to DNA (Puca *et al.*, 1974; André & Rochefort, 1975). As discussed by King & Mainwaring (1974), it is generally considered that nuclear-associated proteins ultimately control the acceptor function of chromatin in either a passive or an active manner. Supporters of the passive theory (Mainwaring & Peterken, 1971; King & Gordon, 1972) suggest that DNA is the acceptor whereas nuclear proteins restrict the binding sites available on DNA; proponents of the active theory (Spelsberg *et al.*, 1971a, 1972) attribute all acceptor activity to the nuclear proteins with little, if any, involvement of DNA. At first sight, our present evidence favours the 'active' theory, but if the basic acceptor proteins described here and by Puca *et al.* (1974, 1975a) have an intrinsic propensity for DNA, then current differences of opinion about the role of DNA in acceptor activity would be largely reconciled.

Note Added in Proof (Received 5 January 1976)

Additional results by Puca, *et al.* (1975b) substantiate our present findings, especially on the

specificity of receptor-acceptor interactions. The part played by DNA in these processes is also discussed by these authors.

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