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ANDROGEN RECEPTOR ASSAY IN HUMAN BENIGN AND MALIGNANT PROSTATIC TUMOUR CYTOSOL USING PROTAMINE SULPHATE PRECIPITATION

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SUMMARY

Some characteristics of a protamine sulphate precipitation assay of [^{3}H]-5α-dihydrotestosterone ([^{3}H]-DHT) receptor in human prostatic cytosol were compared with those of the assay in the rat ventral prostate. Although human prostate components may result in some underestimation of androgen receptor values, it was concluded that this simple assay is the most promising yet reported for the rapid assay of androgen receptor in small quantities of human prostatic tissue. Limited specificity studies suggested that the androgen receptor is very similar to that in the rat.

Androgen receptor assays were carried out on 84 prostatic tissue specimens from 28 patients with benign prostatic hyperplasia and 54 with prostatic carcinoma. Forty-eight of the patients had been treated with some form of hormonal manipulation. Assayable cytosol androgen receptor levels in all untreated patients were low, but in treated patients with serum testosterone levels below normal (< 300 ng/100 ml) receptor levels fell into a much wider range, the maximum concentration being equivalent to 7000 sites per cell. Binding values, expressed both on a wet weight and on a DNA basis, were more variable in tumours with extensive malignant involvement than in those with much less malignant tissue present.

Androgen receptor values were related to clinical response to homonal manipulation in 11 patients whose tumours were reported to have > 60% malignant involvement. These preliminary results suggested that low androgen receptor levels in tumour cytosols from patients whose serum testosterone values were low were related to hormonal insensitivity of the tumours.

INTRODUCTION

It is well known that sensitivity to steroid hormones is linked to the presence in the cells of the target organs of receptor proteins which are able to bind the appropriate hormone with high affinity and specificity. Receptor proteins for 5α-dihydrotestosterone*, which is considered to be the most important intracellular androgen in many target organs, have been demonstrated in the prostate glands of several species [1]. The androgen receptor concentration in the cytosol of lines of transplantable prostatic carcinomas of Copenhagen rats has been shown to be related to their androgen sensitivity [2].

The assay of androgen receptor in human prostate is complicated by the presence of sex hormone binding globulin (SHBG) in tissue preparations from the gland. This plasma protein, which has an affinity for DHT of the same order as that of androgen receptor in rat ventral prostate (RVP) ($K_a \sim 10^{-9}$ M) [3, 4], has been shown to be present in the cytosol of human hyperplastic prostate at a concentration 4–7% of that in human plasma [5, 6]. In prostatic tissue from patients treated with estrogen for prostatic carcinoma, the high affinity DHT binding not competable with cyproterone acetate (approximately equivalent to SHBG) may be as high as 20 fmol/mg [7]. Sucrose density gradient centrifugation, agar gel electrophoresis and gel chromatography have all been used with varying degrees of success in attempts to distinguish between DHT bound to intracellular androgen receptor and that bound to SHBG [6, 8–15]. In a previous publication [7] we described the use of cyproterone acetate (C.A.) competition for this purpose, as this anti-androgen competitively inhibits binding of DHT to androgen receptor to a much greater extent than to SHBG. However, this method was found to be only semi-quantitative, owing to the variability with which C.A. competed with DHT for binding sites in the sera of different patients. In a continued attempt to perfect an assay for androgen receptor in human prostate suitable for routine use on small quantities

* The abbreviations and trivial names used are: BPH; benign prostatic hyperplasia: BSA; bovine serum albumin: CA; cyproterone acetate; 17α-acetoxy-6-chloro-1α,2α-methylene-4,6-pregnadiene-3,20-dione: DCC; dextran coated charcoal: DES; diethylstilbestrol; α-α′-diethylstilbenediol: Honvol (F. W. Horner, Ltd., Montreal); diethylstilbestrol diphosphoric acid ester: TACE; cholorotrianisene; chlorotris (*p*-methoxyphenyl) ethylene: 5α-DHT; DHT; dihydrotestosterone; 17β-hydroxy-5α-androstan-3-one: PMSF; phenylmethylsulphonyl fluoride: RVP; rat ventral prostate: SHBG; sex hormone binding globulin: SN; supernatant: TE; Tris–EDTA: TEG; Tris–EDTA–glycerol: TFI; testosterone free index. TURP; transurethral prostatectomy.

of tissue, we now present an evaluation of the use of protamine sulphate for the selective precipitation of androgen receptor from human prostatic cytosol.

Protamine sulphate was used by Steggles and King [16] to precipitate estrogen receptor (ER) from rat uterus, but their method was criticised as resulting in low values compared with other methods and in precipitating large amounts of radioactivity bound with low affinity also [17]. Chamness *et al.*[18] used lower concentrations of protamine sulphate to precipitate ER from the rat uterus and found the results equivalent to those from the dextra-coated charcoal (DCC) method. Their method was used to assay androgen and estrogen receptors in human breast cancer cells by Lippman [19] who noted that human serum proteins were not precipitated. The method was validated for RVP by Blondeau *et al.*[4]. Very recently, Menon *et al.*[20] used a protamine precipitation assay in a study of androgen binding in prostatic tissue from 12 patients with benign prostatic hypertrophy. They found the method less sensitive than sucrose density gradient centrifugation owing to precipitation of low-affinity binding.

We have modified the method somewhat, and have found it possible to measure very small amounts of androgen receptor in the presence of relatively large amounts of non-receptor protein. Little low-affinity binding was precipitated at the concentration of ligand and protamine sulphate used.

The large majority of published investigations on androgen receptor assay in human prostate have dealt with benign disease only, and the amount of androgen receptor measurable has been negligible or low [6, 8–15]. No satisfactory quantitation of androgen receptors in human prostatic cancer has yet been reported. The results of the investigation to be presented here suggest that the protamine sulphate method is extremely promising for this purpose. It is simple and rapid to perform and can be carried out on as little as 150 mg tissue.

This investigation falls into three parts: first, the characteristics of the modified protamine sulphate precipitation assay of [^{3}H]-DHT receptor in RVP were examined; second, the effect on the assay of the addition of components of human prostate to known amounts of [^{3}H]-DHT receptor in RVP were investigated; and third, [^{3}H]-DHT receptor concentrations were assayed in surgical specimens of prostatic tissue from treated and untreated patients with benign prostatic hypertrophy or with prostatic carcinoma. The results of the assays were related to the endocrine status of the patients and, when possible, to the response of the patients to treatment by hormonal manipulation.

EXPERIMENTAL

Tissue

RVP was obtained from male Wistar rats weighing approximately 290 g. (Canadian Breeding Laboratories, Montreal, or Biobreeding Laboratories, Ottawa) twenty-four h after castration via the scrotal route.

Human prostatic tissue was obtained from patients undergoing surgery for benign prostatic hypertrophy (BPH) or for prostatic carcinoma. The majority of the operations performed were transurethral resections, but tissue from 5 open prostatectomies, and from multiple needle biopsies in one case, were also used. No metastatic tumour was assayed. Ten ml of venous non-heparinized blood for the estimation of free and bound serum testosterone were obtained from most patients at the time of surgery. Serum was spun off and stored at -17°C until the time of assay. Control tissue (rectus abdominis or pyramidalis muscle) was obtained from four male and two female patients undergoing urological surgery, and treated in the same way as the prostatic tissue. "Normal" prostatic tissue was taken from 3 subjects aged 29, 33 and 62 years who had died less than 16 h previously from causes unrelated to prostatic disease. This tissue was stored in liquid nitrogen and used as a source of human prostatic components other than androgen receptor. All other tissues were placed on ice as soon as possible after removal and all subsequent procedures throughout the assays were carried out at 0–4°C with pre-cooled equipment, glassware and buffer solutions. The human tissue was trimmed of damaged portions and rinsed briefly in 7 mM Tris–HCl buffer pH 7.4, containing 1.5 mM EDTA and glycerol 10% v/v (TEG buffer). Excess buffer was removed with Kimwipes, and representative portions were fixed for histological examination. The remaining tissue was either assayed immediately or refrigerated on ice overnight. This did not appear to result in loss of binding activity.

As the histology of the diseased human prostate is very variable, in addition to the histological examination of portions of the specimens received for assay, the pathology reports of the majority of the patients (63/84) were read, to ensure that the pathology of the specimen received was representative of that examined by the pathologist. In addition, the pathologists concerned assessed the approximate percentage of the tissue which was malignant in 33/56 specimens of carcinoma.

The majority of the specimens from 22 patients with BPH were described as having nodular areas of glandular and stromal hyperplasia. A few also had cystic dilatations of the glands and/or foci of acute or chronic inflammation.

The carcinoma specimens were more variable. The percentage of the tissue estimated as malignant ranged from 3–100% and was of varying degrees of differentiation. Three specimens were described as having areas of squamous metaplasia; 2 of these patients had had estrogen treatment and 1 had been orchiectomized. The prostate of one estrogen-treated patient was described as having undergone degenerative changes. Some of the specimens also contained

areas of fibromuscular and glandular hyperplasia, but only one was described as having areas of inflammation.

Chemicals

[1, 2-^{3}H]-Dihydrotestosterone (40–60 Ci/mmol) was obtained from New England Nuclear Corp.: on arrival it was diluted to 10 μCi/ml in redistilled benzene–ethanol (9:1, v/v) and stored at 4°C; an appropriate aliquot was prepared before each experiment by evaporating the solvent under nitrogen and redissolving the [^{3}H]-androgen in buffer. Cyproterone acetate (SH 714) was kindly provided by Schering Berlin. Other unlabelled steroids, TACE, DNA standard (sodium salt from salmon testes), protamine sulphate (Grade I), phenylmethylsulphonyl fluoride (PMSF) and BSA were obtained from Sigma Chemical Co., MO; Dextran T 70 from Pharmacia, Montreal; charcoal (Norit A) from Matheson, Coleman and Bell. The scintillator used was 5 g diphenyloxazole (PPO) and 0.1 g 1.4-*bis*[2-(5-phenyloxazolyl)] (POPOP) (Amersham–Searle), per liter of toluene.

For the serum testosterone assays, [1α, 2α-^{3}H]-testosterone (55 Ci/mmol) was purchased from New England Nuclear Corp. (Dorval, Quebec, Canada), checked for purity and counted as described by Bird *et al*[21]. The testosterone antibody used in the testosterone radioimmunoassay was obtained from Dr. G. Abraham (Torrance, CA, U.S.A.). All other compounds used were of reagent grade. Solvents were redistilled twice prior to use.

Assay procedures

[^{3}H]-DHT receptor assay. Prostatic tissue was homogenized (approximately 100 mg/ml TEG buffer) using a motor driven Potter–Elvejhem type homogenizer at approximately 800 rev./min in an ice-bath in 15 s bursts, with 45 s cooling intervals. If enough material was available, an aliquot of the homogenate was removed for DNA determination by the method of Dische[22]. The remainder of the homogenate was diluted 1:1 with TEG buffer and spun at 27,000 *g* for 10 m to yield a pellet and a crude supernatant (SN). For small samples the pellet was used for DNA determination. The crude SN was diluted with TEG buffer to an extent appropriate for the particular experiment, and an aliquot was removed for assay of the approximate protein concentration by the method of Layne[23]. Subsequent references to the protein concentration will refer to this crude SN from which the pure cytosol fraction was derived. The crude SN was divided into a number of aliquots (usually 2.5–3.0 ml) depending upon the number of experimental conditions to be investigated. These aliquots were transferred to polyallomer tubes and [^{3}H]-DHT was added with or without competing compounds at concentrations 1–400 times that of [^{3}H]-DHT. Blanks, using buffer instead of cytosol, were run concurrently. Cytosol was then separated from each aliquot by centrifuging at 105,000 *g* for 1 h. The supernatant cytosol was then transferred to glass tubes and allowed to stand in ice for the remainder of the 2 h incubation period. This "pre-incubation", with the addition of [^{3}H]-DHT before separation of the cytosol fraction, was carried out in order to minimize the degradation of androgen receptors, which are very labile in the absence of steroid [24]. We found that the high affinity [^{3}H]-DHT binding capacity of "pre-incubated" RVP cytosol was approximately twice that observed when [^{3}H]-DHT was incubated with separated cytosol for 2 h. For the same reason, it is important to carry out the assay with as little delay as possible after homogenization. Longer cytosol incubations were carried out in a few experiments to determine the optimum time for the assay.

Pyrex tubes (10 × 75 mm) for protamine precipitation were prepared as described by Chamness *et al*.[18]. They were etched with chromic acid, washed, and incubated for 10 min at 30°C with a solution of BSA (1 mg/ml Tris–EDTA (TE) buffer). They were then rinsed with ice-cold TE buffer. 0.5 Aliquots of incubated cytosol were transferred to the prepared tubes and protamine sulphate in an equal quantity of TE buffer was added so that the final concentration of protamine sulphate was 0.05% w/v. The tubes were vortexed and allowed to stand in ice for 10 m. They were then spun at 3000 *g* for 10 m. The supernatants were discarded and the precipitates were washed very thoroughly (at least 6 times) with Tris buffer. The washed precipitates were extracted with 1.5 ml methylene chloride overnight at 0–4°C. The solvent was quantitatively transferred to counting vials and evaporated. Scintillation fluid was added and counting was carried out in a Packard liquid spectrometer, Model 3375. High-affinity binding of [^{3}H]-DHT was calculated by subtracting low-affinity binding (in the presence of competitor) from total binding (in the absence of competitor). Binding was related to wet weight of tissue and to DNA concentration of the tissue.

"Mixture" experiments. As mentioned in the introduction, it was essential to investigate possible interference in the protamine sulphate precipitation assay by components of human prostate not present in RVP. One such contaminant is known to be SHBG, which may be responsible for as much as 20 fmol high-affinity [^{3}H]-DHT binding capacity per mg prostatic tissue from estrogen-treated patients [7]. To investigate the possible effect this and other human prostatic components might have on the [^{3}H]-DHT receptor assay in RVP, aliquots of human prostate crude SN were added to RVP SN. Diluted serum of known [^{3}H]-DHT-binding capacity was added to some aliquots of the mixture. The [^{3}H]-DHT receptor assay was carried out on the mixtures and also on each component separately, using 10 nm [^{3}H]-DHT ± 400 times unlabelled DHT as competitor. Except where mentioned otherwise, each ml of crude SN mixture contained components derived from 10 mg RVP and/or 50 mg human prostate

and/or human serum diluted to provide 900 fmol high-affinity [^{3}H]-DHT-binding capacity. The maximum concentration of protein present in any of the mixtures was 2.6 mg/ml, well within the range of linearity of the assay, (see 'Results').

Charcoal "stripping". In a few experiments with human tissue endogenous androgens were stripped from the crude SN with dextran-coated charcoal (DCC). An equal volume of TEG or TEG containing a suspension of 0.5% charcoal and 0.05% dextran was added to aliquots of crude SN of human prostate (100 mg/ml) or to diluted human serum of 1800 fmol [^{3}H]-DHT binding capacity. The tubes were shaken briefly, allowed to stand in an ice-bath for 15 min and centrifuged at 15,000 *g* for 10 min. The supernatant was removed and recentrifuged to remove the charcoal completely.

Protease inhibition. In one "mixture" experiment incubation of crude SN was carried out in the presence or absence of 1 mM PMSF in order to investigate the effect of this protease inhibitor on the assay.

[^{3}H]-DHT binding capacity of serum. Serum was diluted 1:100 or 1:200–500 (in estrogen-treated patients) with TEG buffer. Aliquots were incubated with an equal vol. of 2 nM [^{3}H]-DHT in TEG buffer for 2 h at 0°C. Unbound steroid was removed with DCC and bound [^{3}H]-DHT was extracted from the supernatant as described.

Determination of serum testosterone. Serum testosterone levels were determined by the radioimmunoassay procedure of Abraham *et al.*[25] with minor changes; these included using solvents that were not equilibrated with ethylene glycol for the Celite columns. Also 3 ml rather than 3.5 ml vols of solvents were used for elution of the columns. Levels of binding of testosterone to plasma proteins were measured by a modification [26] of the equilibrium dialysis method of Forest *et al.*[27]. The testosterone free index (TFI) was calculated [28] by multiplying the total plasma testosterone level (ng/100 ml) by the unbound fraction [%] and is expressed as ng/100 ml.

Evaluation of clinical response to treatment. Collaborating urologists were asked to provide relevant facts on the treatment and response of patients both before and after the operation from which the prostatic specimens were obtained. Whenever possible, data pertaining to objective evidence of the degree of hormonal sensitivity of the tumour were noted e.g. changes in prostatic size, serum acid phosphatase values, changes in intravenous pyelogram, changes in metastatic picture as provided by bone scans and X-rays. Changes in bone pain and the level of the patients' activity were also recorded, but evaluation of response was not based on these alone.

RESULTS

I. *Characteristics of the protamine sulphate [^{3}H]-DHT receptor assay in the 24 h castrated RVP*

Saturation of receptor sites. Aliquots of crude SN from RVP homogenates containing approximately 5.5 mg protein per ml buffer were incubated as described in 'Methods' with [^{3}H]-DHT at concentrations from 2.5–50 nM in the absence or presence of 400-fold unlabelled DHT. Saturation of high-affinity sites was found to occur at 10 nM [^{3}H]-DHT, and very little low-affinity binding was precipitated at this concentration (Fig. 1). All other experiments on RVP were therefore carried out at this concentration. In 8 experiments using pooled RVP from 8–15 animals, and using crude SN concentrations of

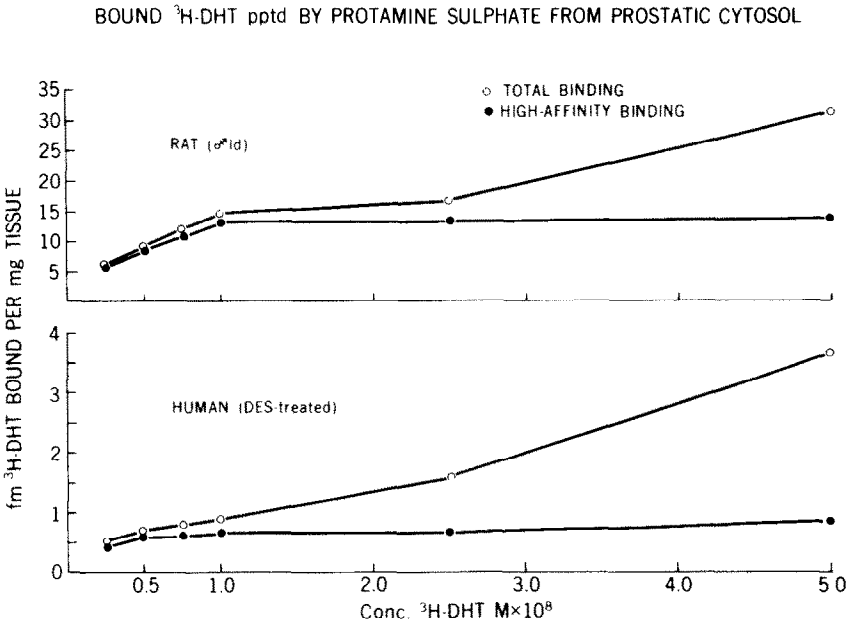

Fig. 1. Saturation curves for bound [^{3}H]-5α-dihydrotestosterone (^{3}H-DHT) precipitated by protamine sulphate from RVP cytosol (upper) and human prostate cytosol (lower). ♂ 1 day; castrated 24 h.

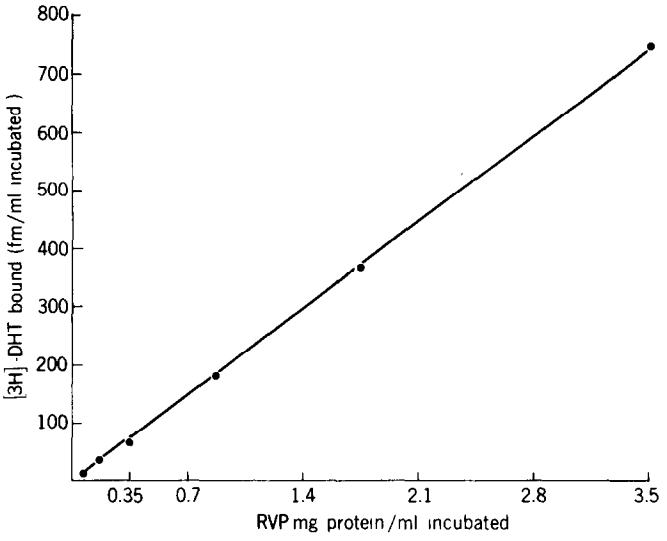

Fig. 2. Linearity and sensitivity of protamine sulphate precipitation assay in rat ventral prostate (RVP) cytosol.

approximately 3.5 mg protein per ml buffer, the mean binding capacity per mg RVP was 13.6 ± S.D. 1.6 fmol, when assayed under these conditions on different occasions. Assuming that each receptor site binds one molecule of DHT, this represents almost 12,000 high affinity cytosol sites per cell. This agrees with the estimates of other investigators, which range from 8000 [29] through 9500 [30] to 11,500 [4] sites per cell. No significant increase in high affinity binding was observed when longer incubation times (up to 6 h) were used, although there was some increase in low affinity binding.

Scatchard plot analysis would not be valid for the protamine sulphate assay when 'pre-incubation' is carried out, as the SN remaining after precipitation of [^{3}H]-DHT bound to receptor contains [^{3}H]-DHT bound to other proteins, as well as unbound [^{3}H]-DHT. In human prostate preparations these proteins would include SHBG to which [^{3}H]-DHT would be bound with high affinity.

Linearity and sensitivity of the assay. Aliquots of crude SN containing 0.07–3.5 mg protein per ml were incubated with 10 nM [^{3}H]-DHT ± 4 μM unlabelled DHT. The assay was linear over this range of tissue concentrations (Fig. 2) and high affinity binding was easily measurable in 0.5 ml aliquots at the lowest protein concentration.

Specificity of the receptor. Aliquots of crude SN containing approximately 3.5 mg protein per ml were incubated with 10 nM [^{3}H]-DHT in the absence or the presence of a number of steroids and non-steroidal synthetic estrogens at concentrations ranging from 10 nM to 4 μM. The results are presented in Fig. 3. Estradiol, cyproterone acetate and progesterone showed considerable affinity for the receptor, competing out more than 90% of the high affinity

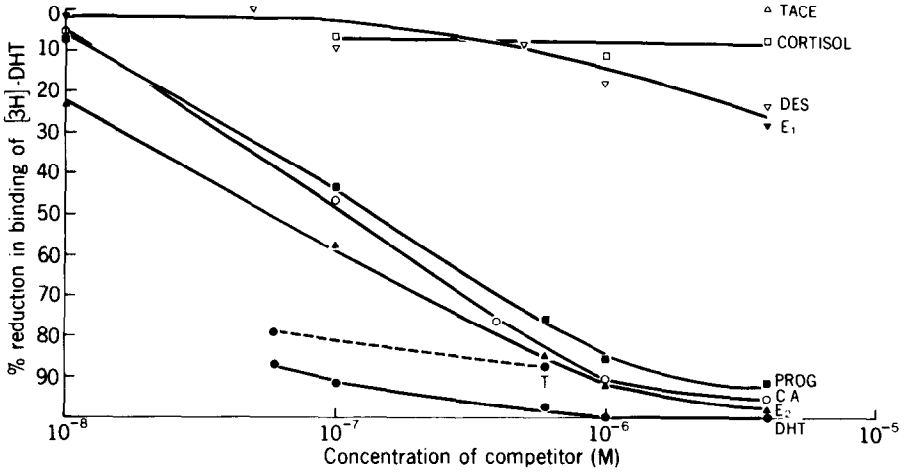

Fig. 3. Competition for high affinity binding of [^{3}H]-DHT in RVP by chlorotrianisene (TACE) △, cortisol □, diethylstilbestrol (DES) ▽, estrone (E_1) ▼, progesterone (PROG.) ■, cyproterone acetate (CA) ○, estradiol (E_2) ▲, testosterone (T) ●---●.

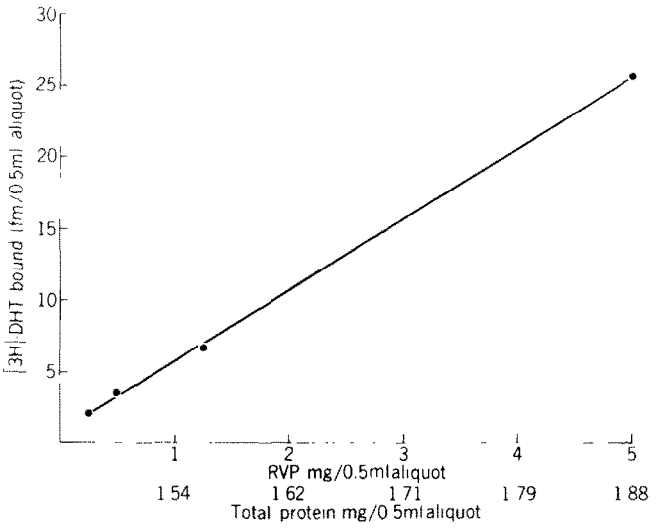

Fig. 4. Linearity and sensitivity of protamine sulphate precipitation assay in rat ventral prostate (RVP) cytosol in the presence of human serum and prostate components.

binding when used at a concentration 400 times that of [^{3}H]-DHT. DES, estrone and cortisol were poor competitors for [^{3}H]-DHT, and TACE did not compete at all, even when used at 400 times the concentration of [^{3}H]-DHT. A similar spectrum of steroid specificity of the RVP-DHT receptor has been demonstrated by Shain and Boesel[30] and Verhoeven[31], using ammonium sulphate fractionation, by ourselves[32] using a DCC assay, and by Blondeau *et al.*[4] using a protamine sulphate precipitation method, but without pre-incubation.

Using this protamine sulphate precipitation method, it is thus possible to demonstrate high affinity, saturable binding of [^{3}H]-DHT with a steroid specificity similar to that shown by other methods to be characteristic of the RVP androgen receptor. The linearity and sensitivity of the reaction are such as to suggest that it would be of use for the measurement of androgen receptors in human prostate provided that other components of the human tissue did not interfere with the assay.

II. *Characteristics of the protamine sulphate precipitation assay for RVP in the presence of components of human prostate*

Linearity and sensitivity of the assay. 0.5 ml Aliquots of crude SN derived from RVP in amounts from 0.25–5.0 mg, 25 mg human prostate and diluted human serum of 450 fmol [^{3}H]-DHT high-affinity binding capacity were assayed as previously described. It had been previously established that the human prostate used had negligible protamine sulphate-precipitable high-affinity [^{3}H]-DHT binding capacity. Linearity was observed over the entire concentration range, and high affinity binding was easily measured at the lowest concentration of RVP i.e. 2 fmol high affinity [^{3}H]-DHT binding per aliquot, assayed in the presence of 25 mg human prostate and 450 fmol SHBG. (Fig. 4).

Reproducibility. Six replicate homogenates containing 25 mg RVP and 500 mg human prostate of negligible protamine sulphate-precipitable high affinity [^{3}H]-DHT binding capacity in 5.0 ml TEG buffer were prepared. Diluted human serum was added to each crude SN so that the final components of the mixture were present in the relative concentrations described in "Methods". Four aliquots of each crude SN were assayed and the mean [^{3}H]-DHT binding capacity of the six sets of four was calculated. These results were then averaged to give a mean of 10.50 ± S.D. 1.17 fmol/mg RVP (coeff. of variation, $V = 11.1\%$).

Effect of human prostate components on RVP [^{3}H]-DHT binding capacity. The effect of adding human prostate and serum separately and together

Table 1. The effect of human prostate and serum components on the androgen binding capacity of rat ventral prostate (RVP) cytosol

Components incubated per 0.1 ml.	Protamine sulphate pptd. high affinity [^{3}H]-DHT-binding capacity (fmol)
1. RVP: 1 mg	11.34
2. Human prostate: 5 mg	0.05
3. Human serum: 90 fmol SHBG	0.6
2 + 3	0·25
1 + 3	12.10
1 + 2	9.01*
1 + 2 + 3	8.11*

* See footnote.

* Note added in proof: This experiment was carried out using human prostate obtained at autopsy. When it was repeated with prostatic tissue obtained at surgery, no reduction in [^{3}H]-DHT binding capacity of RVP was observed.

to RVP on the binding capacity of the latter is shown in Table 1. Less than 0.7% of the total high-affinity binding of the serum was precipitated by protamine sulphate. Menon *et al.*[20] also found that very little SHBG was precipitated. The human prostate used was virtually devoid of protamine sulphate precipitable high-affinity [^{3}H]-DHT binding, and these two components assayed together had a very low binding capacity. The RVP used in this experiment had a binding capacity of 11.34 fmol per mg when assayed alone; the addition of serum increased this to 12.10 fmol/mg, but the addition of human prostate, and human prostate and serum, decreased the binding capacity by 20.3 and 28.5% respectively.* This was unlikely to be due to saturation of the high-affinity binding, as the protein concentrations of all the mixtures were within the range for linearity of the assay (Fig. 4).

Further experiments were carried out using the concentrations of components in Table 1 to investigate possible reasons for the reduction in binding capacity of RVP in the presence of human prostate and serum. It was considered that the presence of endogenous DHT in the human tissues might contribute to the reduction by competing for receptor sites with the [^{3}H]-DHT added in the assay. Consequently the serum and prostate crude SN were 'stripped' of endogenous steroids before the mixtures were made up. As the results in Table 2 show, removal of endogenous steroids from the human components increased the binding capacity of the RVP in their presence somewhat, but only by a few per cent. It is of interest that the RVP component measured alone had a lower binding capacity than usually found, probably because the crude SN was kept for 20 min on ice while the other components were stripped. This emphasizes the need for speed in this assay, and suggests that the small increase in binding capacity brought about by stripping the other components was more than off-set by the delay involved.

Another factor present in human prostate which it was considered might be responsible for the reduction in binding capacity of RVP was the presence of proteolytic activity in the former. Aliquots of RVP crude SN were therefore assayed alone and in combination with human prostate and serum components in the presence and absence of PMSF (1 mM). The presence of this protease inhibitor did not affect the reduction of [^{3}H]-DHT binding capacity of RVP brought about by the presence of human prostate components, and in fact reduced the binding capacity of RVP when assayed alone by 25%.

We concluded from these results that the protamine sulphate precipitation assay for [^{3}H]-DHT receptor was sufficiently sensitive and reproducible to be used with human prostate. Human serum alone did not materially interfere with the assay, but the presence of other factors present in human prostate appeared to result in an underestimation of [^{3}H]-DHT receptor in RVP by approximately 25%.* However, these factors did not affect the linearity of the assay over a wide range of relative concentrations of androgen receptor and human prostate components (Fig. 4).

Table 2. The effect of stripping endogenous steroids from human prostate and serum components on the androgen binding capacity of rat ventral prostate (RVP) cytosol
N.D.; not determined

Components incubated per 0.1 ml.	Protamine sulphate pptd. high affinity [^{3}H]-DHT-binding capacity (fmol) Not 'Stripped'	'Stripped'
1. RVP: 1 mg	8.79	N.D.
2. Human prostate: 5 mg	0.06	0.11
3. Human serum: 90 fmol SHBG	0.18	1.06
1 + 3	N.D.	8.20
1 + 2	6.52*	7.18
1 + 2 + 3	6.40*	6.74

* See footnote on p. 294.

III. *A. Characteristics of the protamine sulphate precipitation assay for high affinity [^{3}H]-DHT binding in human prostatic tissue*

Saturation of receptor sites. The assay was carried out as described for the RVP using aliquots of crude SN (1.7 mg protein/ml) from a 45 mg/ml homogenate of a specimen from a patient under DES treatment. [^{3}H]-DHT was used at concentrations from 0.25–5 $\times$ 10^{-8} M, in the absence and presence of 400-fold unlabelled DHT. The results are presented in Fig. 1. Although the concentration of binding sites was vastly different from that in RVP, saturation of high affinity binding sites occurred at the same concentration (10 nM [^{3}H]-DHT), and very little low affinity binding was observed at this concentration. All other assays on human material were carried out at this concentration.

Specificity of the assay. Investigation of the specificity of the human prostate [^{3}H]-DHT receptor was limited by the amount of available suitable material. However specificity studies were carried out on two patients, both of whom had undergone treatment with synthetic estrogens for 7 days. The high affinity binding of [^{3}H]-DHT in the prostatic cytosols was 0.91 fmol/mg and 0.49 fmol/mg for patients 1 and 2, respectively. These limited studies (Fig. 5) suggested that the human prostate androgen receptor possesses a spectrum of specificity very similar to that in the rat. In bot. , progesterone and estradiol had considerable affinity for the receptor, but the synthetic estrogens DES and TACE were poor competitors. It is therefore unlikely that therapeutic effect of these compounds is brought about by direct competition with androgens for receptor. Cortisol was also a poor competitor, as in the rat. These competition studies will be extended as more material becomes available.

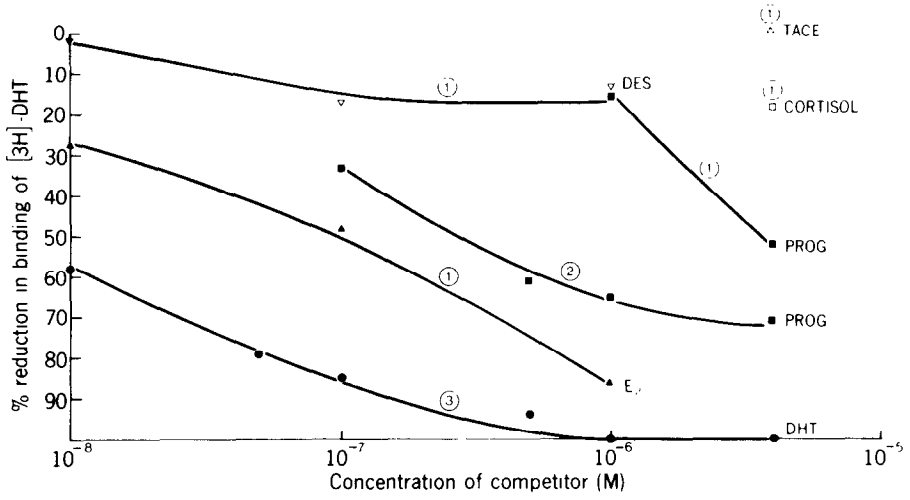

Fig. 5. Competition for high affinity binding of [^{3}H]-DHT in human prostate cytosol by chlorotrianisene (TACE) △, cortisol □, diethylstilbestrol (DES) ▽, progesterone ■, and ▲, estradiol. Investigations were carried out on 3 patients with prostatic carcinoma: 1; treated with DES, 15 mg per day for 7 days: 2; treated with TACE, 75 mg per day for 7 days: 3; treated with DES, 3 mg per day for 2 m.

III. *B. Endocrine status of the patients*

The hormonal manipulation which the 46 treated patients had undergone before we received the specimen for assay varied greatly in extent. The synthetic estrogen generally used was DES (3 mg–15 mg per day). Honvol was administered i.v. or orally (250–1000 mg per day). TACE (12.5–75 mg per day) and Estradurin (polyestradiol phosphate), were also administered. Patients had been under estrogen treatment for periods varying from 2 days to more than a year. Specimens were received from 11 patients who had been orchiectomized from 3 days to 4.5 years previously. Eight additional patients who had been orchiectomized for periods from several months to 11 years were also on estrogen treatment, sometimes for very long periods. In view of the wide variation in treatments, we considered it important to establish the endocrine status of the patients from whom prostatic specimens were received for assay.

Endocrine status of the patients was assessed by measurement of the total testosterone and TFI values in the serum, and the [^{3}H]-DHT binding capacity of the serum. The latter is considered to be equivalent to the SHBG concentration [33]. The results are shown in Table 3 subdivided according to patient treatment. The effects of treatment on endocrine status were usually, but not always, what was anticipated. The range of total serum testosterone values in untreated patients extended somewhat beyond the normal range (300–1000 ng/100 ml), but the TFI values fell within the normal range (24.6–93.6 ng/100 ml). The [^{3}H]-DHT binding capacity of the serum of one patient was unexpectedly high, possibly because of a thyroid problem [34]. The total testosterone and TFI values in all the treated patients except three were below normal, indicating adequate treatment. Two estrogen-treated patients had normal levels of total testosterone, presumably because of in-

Table 3. Serum testosterone levels and high affinity [^{3}H]-DHT binding capacity of serum (SHBG concentration) in untreated and treated patients

Treatment	Testosterone (ng/100 ml) Total (Mean and range)	Testosterone (ng/100 ml) TFI (Mean and range)	[^{3}H]-DHT binding capacity (μg/100 ml) (Mean and range)
None	455(219–1170) $n = 21$	33.9(15.8–88.9) $n = 15$	0.972(0.474–3.772) $n = 32$
Estrogen	115(12–357) $n = 18$	3.1(0.6–8.8) $n = 16$	3.712(1.56–4.972) $n = 22$
Orchiectomy	131(8–684) $n = 8$	2.3(0.6–9.8) $n = 6$	1.632(0.61–5.9) $n = 11$
Estrogen + orchiectomy	66(6–178) $n = 7$	0.8(0.1–1.8) $n = 5$	5.537(3.260–8.84) $n = 7$

Table 4. [^{3}H]-DHT binding capacity of prostatic cytosols in untreated and treated patients with benign prostatic hyperplasia (BPH) and carcinoma (ca.)

Diagnosis and treatment (No. of patients)	Protamine sulphate pptd. high affinity [^{3}H]-DHT binding (Mean and range)	
	(fmol/mg tissue)	(fmol/mg DNA)
BPH None (22)	0.21(0–0.64)	60(0–110)
Ca. None (14)	0.21(0–0.48)	57(0–120)
Non-malignant. Estrogen (6)	0.66(0.12–1.35)	210(20–250)
Ca. Estrogen (23)*	1.22(0.11–11.72)	230(20–1670)
Ca. Orchiectomy (11)	1.05(0.14–6.74)	230(40–1400)
Ca. Estrogen + Orchiectomy (8)	1.39(0.28–3.56)	240(50–660)

* 1 patient in this group did not have a serum testosterone value below normal. His prostate cytosol showed the lowest binding capacity in this group.
BPH: Benign prostatic hyperplasia

effective treatment. However, the TFI in both was low. One orchiectomized patient had an unaccountably high level of total testosterone. As expected, SHBG levels were raised in all estrogen-treated patients, except one, whose total testosterone level was also normal. The [^{3}H]-DHT binding capacity of the serum of orchiectomized patients was not higher than that in the untreated patients except in one case. Orchiectomy combined with estrogen treatment resulted in the highest [^{3}H]-DHT binding capacities and the lowest TFI values observed.

III. *C. Results of the androgen receptor assay*

Assays were carried out on crude SN containing 0.6–2.7 mg protein (from 21–75 mg tissue) per ml., according to the amount of tissue received. Replicate aliquots were assayed in the absence of excess unlabelled DHT together with corresponding aliquots in the presence of 400-fold unlabelled DHT. DNA assays were carried out on the pellet from the 27,000 *g* spin. Specimens from 2 untreated carcinoma patients were assayed before and after stripping endogenous androgens with DCC. Rectus or pyramidalis muscle was assayed from six patients, four male and two female. The mean [^{3}H]-DHT binding values were negligible ranging from 0–0.11 (mean 0.04) fmol/mg tissue. The corresponding values per mg DNA were 0–110 (mean 50) fmol/mg DNA.

The results on prostatic tissue are summarized in Table 4, and in Fig. 6, the binding values for individual patients are plotted against their serum testoster-

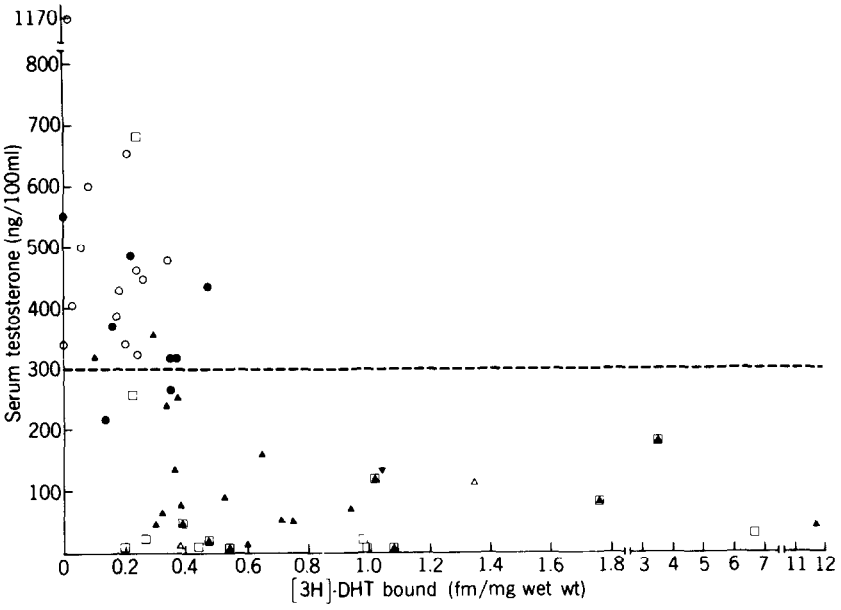

Fig. 6. High affinity binding of [^{3}H]-DHT by human prostatic cytosols plotted against serum testosterone values. ○, untreated benign prostatic hyperplasia (BPH); ●, untreated carcinoma; △, estrogen-treated non-malignant tissue; ▲, estrogen-treated carcinoma; □, orchiectomized; ■, estrogen-treated and orchiectomized. The horizontal dotted line represents the lower limit of the normal range of serum testosterone values (300 ng/100 ml).

Table 5. [^{3}H]-DHT binding capacity of prostatic cytosols in treated patients related to clinical response to hormonal manipulation

Patient	Malignant involvement in primary tumour	High affinity [^{3}H]-DHT bound (fm/mg)	High affinity [^{3}H]-DHT bound (fm/mg DNA)	Response†
A.D.	75%	0.23	90	In relapse
H.P.	65%	0.38	80	−ve
R.W.	75%	0.39	50	In relapse
H.B.	Extensive	0.60	80	+ ve
L.A.	80%	0.65	90	Brief + ve 3 m
A.L.	Extensive	0.75	420	+ ve
J. L.	70%	0.91	190	Partial +ve
E.D.	60%	0.99	210	Partial +ve 18 m
T.W.	80%	1.76	160	In relapse
J.B.	60%	3.56	660	−ve
O.O.	~100%	11.72	1670	In relapse

† − ve; negative response: + ve; positive response.

one values. It is clear that in all patients with normal serum testosterone values, whether their disease was extremely low, equivalent to approximately 250 sites per cell. Charcoal stripping of the crude SN in two cases did not alter the low values, No differences were observed between specimens from TURP and specimens from open operations. In treated patients a much wider range of values was demonstrated, and in two cases approached those found in the castrated rat. When the binding values were expressed on a DNA basis, the same pattern was obtained, but the values did not reach those observed in the rat. The maximum binding observed corresponded to about 7000 sites per cell. In treated patients whose serum testosterone levels were below 200 ng/100 ml the [^{3}H]-DHT binding values in the prostate did not appear to be related to the serum testosterone or SHBG levels, nor to the type or length of treatment. Prostatic tissue from two patients was assayed twice at intervals of several months. The values for the first were assayed six months apart, after DES treatment and orchiectomy respectively, and were both low. The initial value for the second patient, also assayed after DES treatment, was 1.56 fmol/mg tissue (240 fmol/mg DNA). Nine months later, after Estradurin treatment, the value was lower (0.46 fmol/mg tissue, 130 fmol/mg DNA). Since estradiol shows considerable competition for [^{3}H]-DHT binding by the receptor, this lower value may have been due to competitive inhibition of binding by Estradurin (polyestradiol phosphate).

As stated previously, the proportion of tissue which was estimated to be malignant varied widely between patients. The binding values in each treatment group were therefore examined in the light of the degree of malignant involvement of the tissue. In each treatment group the tumours were subdivided into those estimated as having more than 60% involvement, and those having less than 25% involvement, and the binding values in each of these subgroups were averaged. In each treatment group, higher mean binding values, and a greater spread of values was observed in the highly malignant tumours than in those with less involvement. This was not due solely to greater cellularity, as these differences were observed also when the values were expressed on a DNA basis, except in the estrogen and orchiectomized group, which contained only 3 and 2 patients in the highly and less malignant groups respectively.

III. *D. Clinical correlation*

In attempting to relate the [^{3}H]-DHT binding value in malignant prostatic tissue to the response of the patient to hormone manipulation, we have restricted ourselves to patients whose primary tumours showed extensive malignant involvement. Since untreated patients all show very low binding values, we have also been restricted to using data obtained from patients who were treated before the operation from which we received tissue. Of the patients who fulfilled these conditions, objective data on the degree of hormonal sensitivity of the tumour was obtained on eleven. The results on these are summarised in Table 5. Three tumours whith low [^{3}H]-DHT binding values (<0.4 fmol/mg, <90 fmol/mg DNA) were from patients who were in relapse at the time of surgery, or who did not respond to hormonal treatment. Five patients whose tumours bound 0.60–0.99 fmol/mg tissue (80–420 fmol/mg DNA) showed some degree of positive response to hormonal manipulation, but the 3 patients whose tumours had the highest binding values on a wet weight basis (1.76–11.72 fmol/mg), and 160–1670 fmol/mg DNA, were in relapse at the time of surgery, or showed no response to hormonal manipulation.

DISCUSSION

The results presented here confirm the validity of protamine sulphate precipitation for the assay of androgen receptor in RVP. The number of cytosol receptor sites and their specificity are in agreement with the other results using different methods [4, 30–32]. Davies and Griffiths[8] have presented evidence that the cytosol androgen receptor from RVP

and human prostate are equally efficient in their ability to stimulate RNA polymerase activity in human prostatic nuclei, thus implying that the androgen receptors in both species are extremely similar, if not identical. The competition studies presented here, although limited, support this concept.

The effect of the addition of human prostate and serum components on the assay in RVP is puzzling. Serum alone had a minimal effect, but when added to RVP together with human prostate in the experiment in Table 1, the human components appeared to decrease assayable RVP receptor by some 25%*; on the other hand, alteration of the amounts of human components relative to RVP did not result in deviation from linearity of the assay (Fig. 4). The decrease was not altered significantly by removal of endogenous androgens, nor by inhibition of proteolysis. Menon *et al.* found that androgen receptor was not completely precipitated by protamine sulphate from cytosol of benign hypertrophic prostate. Possibly there is a factor present in human, but not in rat tissue, which prevents complete precipitation.

The histological survey of the human prostates used showed that the glands diagnosed as malignant presented a wide spectrum of histological appearance, the proportion of malignant tissue present varying from 3–over 95%. Many contained foci of benign hyperplasia as well, and presumably areas with normal tissue architecture. Thus although the classification into benign and carcinomatous is necessary for clinical purposes, from a biochemical point of view (at least at the tissue, rather than at the cell, level) it is somewhat artificial, except in cases where malignant involvement of the gland is extensive. The results of the receptor assays in untreated patients, whether with BPH or carcinoma, confirm the conclusions reached in our earlier work, that very little androgen receptor protein is available for assay in the prostate glands of patients with serum testosterone values in the normal range [7]. The glands of patients treated with estrogens and/or orchiectomy, whose endogenous androgen levels are low, may have increased amounts of receptor available for assay. This may be due to the freeing of cytosol sites, as occurs in the rat after castration [29]. However, in the rat this elevation is short-lived (see later). Rosen *et al.* estimated that in BPH tissue the total binding capacity of the high affinity androgen receptor was 2.6 fmol/mg tissue, but 90% of the sites were occupied by endogenous androgen [6]. In the experiments of Bonne and Raynaud[35], endogenous androgens bound to receptor sites in the cytosol of BPH tissue were "exchanged" for radioactive methyltrienolone, a synthetic androgen. Their data suggest that their experimental material had a total androgen binding capacity of 4 fmol/mg tissue. However, there is some evidence that methyltrienolone behaves more like a progestin than an androgen in terms of ligand specificity [36], so these results may not be comparable with those of Rosen *et al.* The prostate cytosols we assayed in this investigation from treated patients with testosterone serum levels below 200 ng/ml, ranged from 0.39–1.35 fmol/mg (90–300 fmol/mg DNA) in non-malignant tissue and 0.14–11.72 fmol/mg (40–140 fmol/mg DNA) in tissue containing variable amounts of carcinoma. The preliminary correlations with response to treatment suggest that in patients with low endogenous androgens, and whose tumours have a high degree of malignant involvement, an androgen binding capacity of the prostatic cytosol of <0.4 fmol/mg may reflect androgen insensitivity of the tumour. Under the same conditions, patients whose tumours had androgen binding capacities of 0.4–1.00 fmol/mg showed at least a partial response to hormonal manipulation. Tumours with androgen binding capacity of > 1.5 fmol/mg were not sensitive to hormone treatment at the time of assay. There may be a parallel here with the situation in breast cancer *vis-a-vis* the estrogen receptor (ER). Presence of ER is a condition necessary for hormonal sensitivity, but is apparently not sufficient, as only approximately 60% of breast cancer patients whose tumours contain ER in above threshold concentrations experience tumour regression after hormonal manipulation [37].

Another possible explanation of the insensitivity of the highest binding prostatic carcinomas may be that the androgen receptor consists of more than one component. Katsumata and Goldman have demonstrated by electro-focussing that it is possible to separate multiple receptors for DHT in RVP cytosol which vary in their steroid specificity. Several of these components were observed to bind to nuclear chromatin [38]. Fang and Liao[39] also separated two components from RVP cytosol by ammonium sulphate fractionation, and observed that the protein precipitated at 35% saturation (Complex II) bound only androgens and could be transferred to the nucleus. Estradiol and progesterone showed marked competition for DHT binding to the other component (Complex I), which was not transferred to the nucleus, and was presumably non-functional regarding androgen stimulation. The strict androgen specificity of the fraction precipitated by 35% saturated ammonium sulphate has been questioned [4, 31]. However, if human prostate androgen receptor also consists of multiple components, not all of which confer androgen sensitivity, a massive increase in the latter with concomitant loss of functional androgen receptor might explain the high binding, but hormone insensitive, tumours we have observed.

Several investigators have demonstrated a loss of androgen binding capacity of RVP 2–7 days after castration [e.g. 4, 29, 31]. It might therefore have been expected that in treated patients whose endogenous androgen levels were low, the androgen binding capacity of the prostatic cytosol might have disappeared, rather than being higher than in untreated patients,

* See footnote on p. 294.

as was observed in approximately half the treated cases. This may be due to a phenomenon similar to that observed by Sullivan and Strott[40] in castrated RVP, in which androgen receptor concentration (relative to tissue wet weight and to DNA content) was restored after the initial loss, and was maintained for as long as observations were made (up to 6 weeks). Adrenalectomy and hypophysectomy did not materially affect this secondary rise, demonstrating that adrenal and pituitary hormones were not responsible.

This investigation provides no information on the effect of ageing on the human prostate, since, except for the cadaveric specimens, all the subjects were over 53 years old, and most were over 65. The cytosol from the cadaveric prostates showed negligible binding capacity, as might be expected in subjects which had undergone no hormonal manipulation. Shain and Boesel[41] have demonstrated a marked decrease in RVP androgen receptor concentration in aged rats of 2 strains as compared with that in young mature animals. This was accompanied by loss of androgen sensitivity as judged by involution after castration.

As indicated by this discussion, a number of questions are raised by this investigation. Some of them might be answered by improved assay methods, such as satisfactory exchange techniques for the measurement of total receptor sites in cytosol and nucleus regardless of the endogenous androgen levels, and by techniques for separating multiple binding components. Further investigation of the factor(s) involved in the possible underestimation of androgen receptor in the presence of human prostatic components is necessary.* A clearer view of the relationship between receptor sites and prostatic pathology might emerge from binding studies at the cytochemical level, in which presence of receptor could be related to the pathological picture. Application of these techniques to the investigation of apparently normal, as well as diseased, prostatic material from subjects at all ages might give insight into the processes accompanying hypertrophic and neoplastic change.

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* See footnote on p. 294.

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