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IN VITRO ASSAY OF ANDROGEN BINDING BY HUMAN PROSTATE

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SUMMARY

The *in vitro* binding of 5α-dihydrotestosterone by a "cytosol" fraction of human pathological tissue was investigated, and compared with binding by the rat prostate, an androgen-responsive mouse mammary tumour, and some androgen-unresponsive control tissues. Androgen-binding levels in the human control tissues were higher than those in the rat and mouse, and were not lower than androgen-binding levels in many prostatic specimens from untreated patients. Estrogen therapy and/or orchiectomy appeared to result in higher binding levels. The steroid specificity of binding in the human prostatic tissue appeared closer to that of sex hormone binding globulin than to that of the androgen receptor in the rat prostate. It was concluded that the assay used was not sufficiently specific to distinguish androgen binding by sex hormone binding globulin from that by an intracellular prostatic androgen receptor.

INTRODUCTION

The presence of specific androgen-binding macromolecules has been demonstrated in the cytoplasm of a variety of androgen-responsive organs in several species [e.g. 1–12]. Conversely, in some pathological conditions in which androgen responsiveness has not developed or has been lost, such androgen-binding molecules are absent [2, 9, 13, 14]. Since prostatic cancer in men develops only in the presence of functioning testes [15] (although there is one report [16] of prostatic carcinoma developing 22 years after orchiectomy and irradiation for seminoma of the testis) it seems reasonable to assume that the human prostate gland also contains specific androgen-binding macromolecules, and that their concentration may indicate the degree of androgen dependence of prostatic tumours. If this is so, it may be possible to devise an *in vitro* technique which could predict the probable response of each tumour to hormonal therapy or ablation. Several authors have investigated the uptake and binding of androgen by human prostatic tissue both *in vivo* [17, 18] and *in vitro* [8, 19–23], but the material investigated has been largely normal or benign hyperplastic tissue (BPH)†.

Since the work of Bruchovsky and Wilson[24] and Fang, Anderson and Liao[5], it has been generally considered that 5α-DHT is the most potent intracellular androgen in the prostate. We have measured the binding of DHT by a cytoplasmic fraction of human carcinoma, BPH and 'normal' prostate specimens, and have compared some of the characteristics of this binding with that in two other androgen responsive tissues, the rat prostate, and an androgen-dependent mouse mammary tumour.

† The abbreviations and trivial names used are: BPH; benign prostatic hyperplasia: SHBG; sex hormone binding globulin: PL; prolactin: DNA; deoxyribonucleic acid: DES; diethylstilbestrol; α,α'-diethylstilbenediol: Honvol (F. W. Horner, Ltd., Montreal); α,α'-diethyl-4,4'-stilbenediol diphosphoric acid ester: TACE; chlorotrianisene; chlorotris (p-methoxyphenyl) ethylene: U. 28,048 (Upjohn Co. Ltd.); 19-nor-androstenediol; 5-estrene 3,17β-diol: C.A.; cyproterone acetate; 17α-acetoxy-6-chloro-1α,2α-methylene 4,6-pregnadiene-3,20-dione: DHT, dihydrotestosterone; 17β-hydroxy-5α-androstan-3-one: 3α-&-3β-androstane-diols; 5α-androstane-3α,17-diol and 5α-androstane-3β,17β-diol: estradiol; 1,3,5(10)-estratriene-3,17β-diol: estrone; 3-hydroxy-1,3,5(10)-estratriene-17-one; corticosterone; 11β,21-dihydroxy-4-pregnene-3,20-dione: progesterone; 4-pregnene-3,20-dione: testosterone; 17β-hydroxy-4-androsten-3-one.

EXPERIMENTAL

Human pathological prostatic tissue was obtained from open or transurethral prostatectomy, or occasionally by needle biopsy. The tissue was transferred to the laboratory in an iced container as soon as possible after removal from the patient. Damaged tissue was discarded and a sample of the specimen was taken for histological examination. "Normal" prostatic tissue was obtained at autopsy examination of subjects less than fifty years old, who had died several hours previously of diseases other than of prostatic origin. Human tissue was either used fresh or frozen and stored at −22°C or in liquid nitrogen.

Male Wistar rats were obtained from High Oak Ranch, Ontario. Ventral prostate and diaphragm, which was used as an androgen-unresponsive control tissue, was obtained from animals weighing approximately 250g, which had been castrated via the scrotal route, under ether anaesthesia, the previous day. Rats were killed by decapitation and the tissue was removed and immediately placed in an iced container. Tissue from several animals was pooled to obtain sufficient material for some experiments.

Mice bearing lines of the Shionogi mammary tumour were kindly provided by Dr. D. Sutherland from the Ontario Cancer Institute. Both tumour lines were derived from the Shionogi 115 androgen-responsive tumour [25], but one line had become unresponsive to androgen during passage in culture. Both lines were carried in intact male mice, and a group of mice bearing each line was castrated one day before the animals were killed by cervical dislocation and the tumours were removed. The tumours were immediately frozen and stored in liquid nitrogen.

Materials

[1, 2–^{3}H]-dihydrotestosterone (44 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]-DHT in buffer. Cyproterone acetate was provided by Schering Corp., N.J. Other non-radioactive steroids and DNA standard were obtained from Sigma Chemical Co., Miss.; Dextran T 70 from Pharmacia, Montreal, and charcoal (Norit A) from Matheson, Coleman & 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.

Tissue frozen in liquid nitrogen was pulverized in a Thermovac pulverizer cooled with liquid nitrogen. All subsequent procedures were carried out between 0 and 4°C. Fresh and previously frozen tissue was homogenized in a motor driven Potter–Elvejem type homogenizer at 790 rev./min in Tris–HCl buffer, pH 7·4, containing 1·5 mM EDTA. Homogenization was carried out in an ice-bath in 15 s bursts, with 45 s cooling intervals. The final homogenate dilution was approximately 50 mg/ml, and was centrifuged at 48,000 *g* for 1 h. When the quantity of material permitted, an aliquot of the homogenate was removed before centrifugation for the assay of DNA by the method of Dische[26].

The supernatant 'cytosol' fraction was assayed for DHT-binding activity. Replicate 1 ml aliquots were diluted 1:1 with [^{3}H-]-DHT in buffer, so that the final concentration of [^{3}H]-DHT was $0·6 \times 10^{-9}$M, either with or without non-radioactive competitor at a concentration of $2·4 \times 10^{-7}$M. Except for the specificity studies, the competitor was DHT. Blanks containing 1 ml of buffer instead of cytosol were run concurrently. Incubation was carried out at 0°C for 2 h. At the end of the incubation period, unbound hormone was removed by the addition of a suspension of 0·5% charcoal and 0·05% dextran in Tris–HCl buffer. 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. Bound DHT in the supernatant was extracted with methylene chloride which was evaporated to dryness in counting vials. Scintillator was added and radioactivity was measured in a Packard scintillation counter (Model 3375) at an efficiency of approximately 44%. Quenching was corrected for using an external standard. Specific DHT-binding was calculated by subtracting radioactivity in the presence of competitor (non-specific binding) from total binding (in the absence of competitor). Binding was expressed as femtomol [^{3}H]-DHT per mg wet weight of tissue and/or per μg DNA.

For Scatchard plot analysis [27, 28] aliquots of cytosol were incubated with several concentrations of [^{3}H]-DHT, ranging from $1·2 \times 10^{-10}$M to $8·4 \times 10^{-9}$M. Non-radioactive DHT was added as competitor in concentrations 400X that of [^{3}H]-DHT.

For specificity studies, competing steroids were added to a concentration of $2·4 \times 10^{-7}$M in aliquots of cytosol containing [^{3}H]-DHT at a concentration of $0·6 \times 10^{-9}$M. The per cent reduction in specific binding of [^{3}H]-DHT was calculated, taking the reduction caused by the presence of non-radioactive DHT as 100%.

Assessment of tumour response

A form for each patient was sent to the collaborating urologists requesting information on the treatment received prior to and after prostatectomy, and on the response of the patient to treatment, especially with respect to changes in prostatic size, renal function, serum acid phosphatase, metastatic picture and the general condition of the patient. Each clinician was asked to assess the response of the patient in light of these criteria.

RESULTS

Binding assays were carried out on four "normal" human prostates, 14 specimens of BPH, and 33 specimens of prostatic carcinoma. Two human muscle specimens (intercostal and rectus) and a prostate specimen invaded by bladder tumour were also assayed as control human tissues. Twelve of the carcinoma specimens were from patients previously untreated for prostatic disease. The remaining carcinoma patients had been treated prior to prostatectomy with estrogens (DES, TACE or Honvol) and/or had undergone orchiectomy. One of the patients with BPH had been treated before prostatectomy with DES. In the case of two carcinoma patients, assays were performed on prostatic specimens provided several months apart, before and after treatment. The DHT binding values, expressed as femtomol per mg wet weight of tissue are shown in Fig. 1. There was a range of binding values from 0–12·5 fmol/mg. In the specimens from untreated patients, the majority of specimens bound less than 2 fmol/mg. In some cases it was possible to relate the DHT-binding value to the DNA concentration of the tissue and these results are presented in Fig. 2. The pattern of distribution appears to be somewhat similar, ranging from 0–3·4 fmol per μg DNA, and less than 0·5 fmol/μg

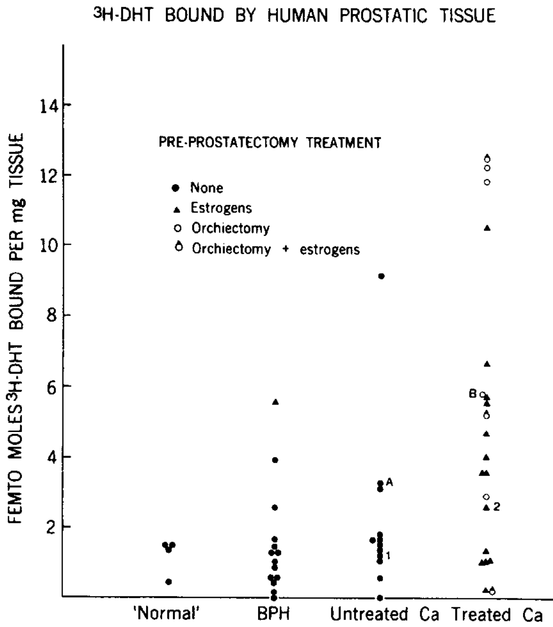

Fig. 1.

DNA in all specimens from untreated patients. Binding values in treated patients showed a wider distribution and tended to be higher than in untreated patients. The three highest binding values occurred in orchiectomized patients. In both patients from whom two specimens were obtained, the post-treatment value was somewhat higher than that before treatment (estrogen therapy and orchiectomy respectively).

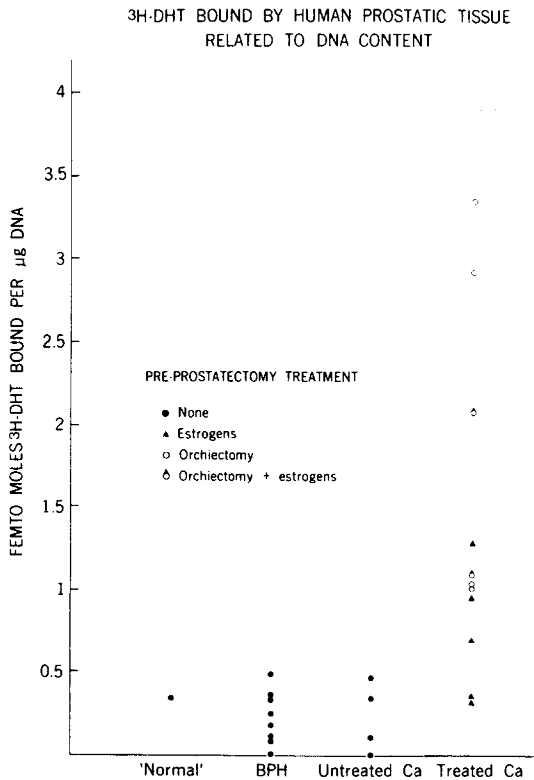

Fig. 2.

Table 1. Binding of [^{3}H]-DHT by rat and mouse androgen-responsive and -unresponsive tissues. [^{3}H]-DHT binding is expressed as femtomol/mg wet weight of tissue and as femtomol/μg DNA. All binding values are corrected for non-specific binding.

		fmoles/mg	DNA μg/mg	fmoles/μg DNA
Rat v.p. (♂ 1d.)				
230 g. b. wt.		3.7	4.4	0.8
270		3.6	4.9	0.7
275		3.1	4.3	0.7
Rat diaphragm (♂ 1d.)				
230 g. b. wt.		0.05	-	-
Mouse Shionogi mammary tumours				
Dep. 1		1.03	-	-
Dep. ♂ 1d.	- 1	1.88		
	- 4	1.34	11.1	0.1
	- 6	1.24		
Aut. 1		0	-	-
Aut. ♂ 1d.	- 1	0.11	-	-
	- 4	0.15	-	-
	- 8	0.06	6.8	0.01

In the ventral prostate of the castrated rat experiments carried out on different occasions gave almost identical results. The specific [^{3}H]-DHT binding value was approximately 3·5 fmol/mg tissue, and 0·7 fmol/μg DNA. Diaphragm from the same animals bound less than 0·1 fmol/mg tissue. The androgen-responsive Shionogi mammary tumour from an intact animal bound approximately 1·0 fmol/mg and approximately 1·5 fmol/mg in castrated animals (0·1 fmol/μg DNA). No specific [3H]-DHT binding could be detected in the autonomous Shionogi tumour in the intact animal, and that from castrated animals bound approximately 0·1 fmol/mg. The results are summarized in Table 1, and Table 2 shows [^{3}H]-DHT binding values in human control tissues; human muscle bound considerably more per unit wet weight than rat diaphragm.

Figures 3, 4 and 5 show Scatchard plots for specimens of BPH and an estrogen-treated carcinoma, rat ventral prostate, and both lines of the Shionogi mammary tumour. Plots from the human specimens indicate that a single type of molecule as regards affinity for DHT was measured and that this affinity was very high ($Kd \sim 10^{-10}$M). The concentration of binding sites was approximately twice as high in the treated carcinoma as in the BPH specimen. The Kd value in the rat ventral prostate and the androgen-responsive Shionogi tumour was $\sim 10^{-9}$M and the concentration of binding sites 8·8

Table 2. [^{3}H]-DHT binding by some androgen-unresponsive tissues

'Specific' DHT binding by control tissues

		fmoles/mg
Rat muscle	(diaphragm)	0.05
Human muscle	(intercostal) ♂	1.2
Human muscle	(rectus) ♂	1.6
Bladder tumour invading prostate		1.0

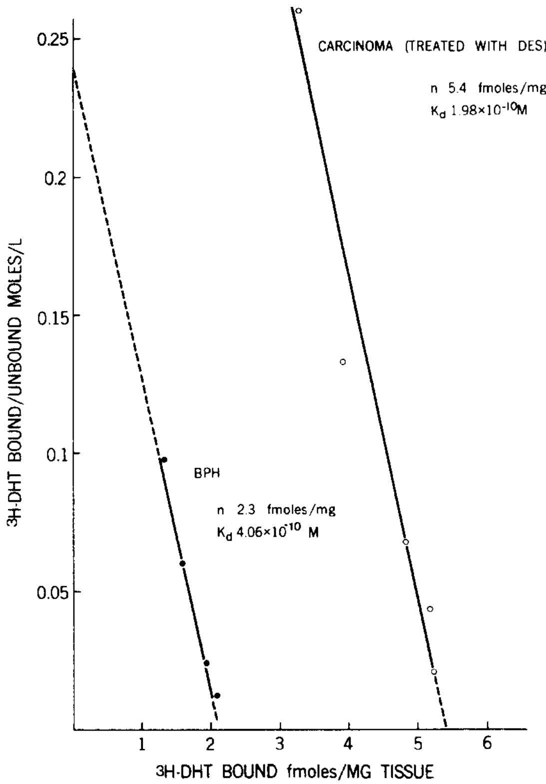

Fig. 3. Scatchard plots of $[^3H]$-DHT binding by specimens of benign prostatic hyperplasia and prostatic carcinoma from a patient treated with DES. Both plots have been corrected for non-specific binding.

and 1·9 fmol/mg respectively. The fact that the concentration was considerably higher in the rat prostate when measured by this method than when measured after incubation with $[^3H]$-DHT at a single concentration of $0{\cdot}6 \times 10^{-9}$M indicates that in high-binding specimens saturation may not have occurred at this concentration and that an underestimation of the androgen-binding capacity may have resulted in some assays. This emphasizes the importance of using the Scatchard plot method whenever possible.

Evaluation of response to therapy in the patients was difficult, as the majority had local disease only and prostatectomy relieved their symptoms. If the remaining tissue is slow-growing, assessment of response may need a long follow-up period, during which the androgen responsiveness of the tissue may change. Objective evidence of disease progression or regression was not available in many elderly patients with metastatic disease. However, Table 3 shows an attempt to relate DHT-binding values to the responsiveness of prostatic carcinomas to hormonal manipulation in some patients. Five of the twelve patients were untreated prior to prostatectomy; the remainder had undergone estrogen therapy and/or orchiectomy. In this small number of patients there was no consistent relationship between DHT-binding values as measured by this method and response to therapy.

Table 4 is a summary of experiments carried out on rat ventral prostate and human prostatic tissue to investigate the steroid specificity of the binding molecule as measured in this assay. The results indicate that binding by the rat prostate exhibits a different steroid specificity from that in the human. In

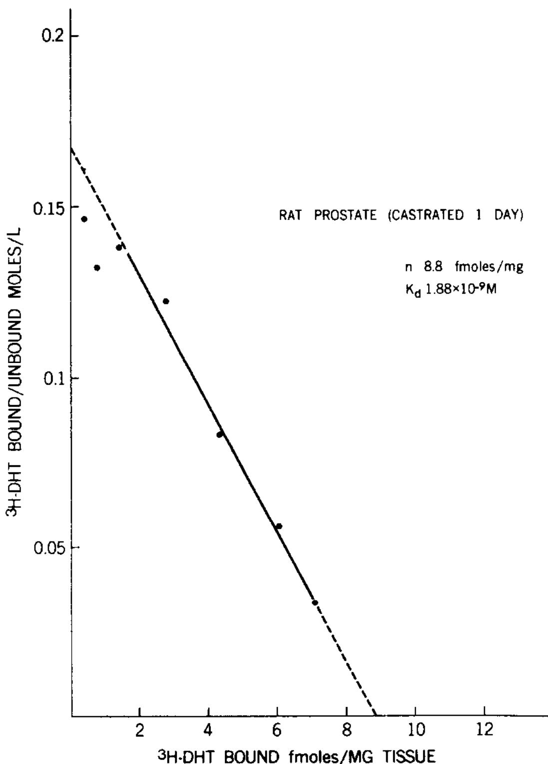

Fig. 4. Scatchard plot of $[^3H]$-DHT binding by pooled ventral prostates of rat castrated 1 day previously. The plot has been corrected for non-specific binding.

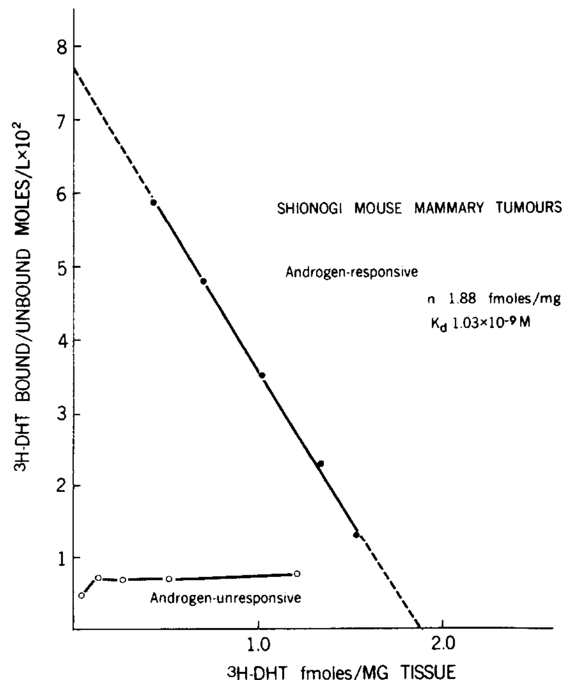

Fig. 5. Scatchard plots of $[^3H]$-DHT binding in androgen-responsive and unresponsive lines of the Shionogi 115 mouse mammary tumour. The plots are based on total binding.

Table 3. Binding of [^{3}H]-DHT by prostatic carcinoma specimens and response of the patients to therapy. ♂̸, orchidectomy; s.a.p., serum acid phosphatase; "local", local disease only, no evidence of metastatic spread. –, unresponsive to treatment; +, responsive to treatment; ±, disease under control, neither progressing nor regressing

Patient	Pre-op.	fmoles DHT/mg.	Post-op.	Response
J.C.	-	0	DES	± 6m Local
A.M.	-	1.2	DES, ♂̸	- Local
G.B.	-	1.4	DES, ♂̸	-
J.S.	-	1.7	TACE	± 6m Local
J.H.	-	3.2	♂̸	± 12m s.a.p.↑
A.C.	DES	1.1	DES	- Local
J.M.	♂̸	2.9	-	-
A.B.	DES	4.7	♂̸ Honvol, AG	Brief +
V.B.	TACE	5.6	TACE	± 10 yr. Local
A.D.	Estrogen	5.7	♂̸, DES	+
A.F.	DES	10.51	Honvol	- Local
A.N.	DES, ♂̸	12.28	DES	± 9 yr. Local

both species testosterone, and both 3α and 3β-androstane-diols displaced [^{3}H]-DHT from the binding molecule to approximately the same extent as non-radioactive DHT. U.28,048 (estr-5-ene-$3\beta,17\beta$-diol), which has been described by Murphy[29] to bind strongly to sex hormone binding globulin, displaced [^{3}H]-DHT to a large extent in the human tissue, but to a lesser extent in the rat prostate. Estradiol greatly reduced [^{3}H]-DHT binding in all specimens studied, while estrone and DES were moderately competitive in the rat prostate. DES was a poor competitor for [^{3}H]-DHT in the human prostatic specimens; results with estrone were variable. Progesterone was highly, and corticosterone moderately, competitive in the rat prostate, but neither reduced the [^{3}H]-DHT binding in the human prostate to a significant extent. The most striking difference between rat and human tissue was observed with C.A., which almost eliminated binding of [^{3}H]-DHT in the rat prostate, but was a poor to moderate competitor in the human specimens.

Table 4. Specificity of [^{3}H]-DHT binding in rat and human prostatic tissue. ♂̸; castrated. Numbers in parentheses indicate the number of specimens investigated with the appropriate steroid competitor. Where more than one specimen was investigated the values given are means; the variation between different specimens was small

	Rat v.p.	Human prostate		
	♂̸ 1d.	'Normal'	BPH	Treated ca.
Specific DHT binding (0.6×10^{-9} M)				
fmoles/mg	3.4 (2)	1.6	0.5	5.7 (3)
fmoles/μg DNA	0.7 (2)	0.4	0.08	1.1 (3)
+ Competitor 2.4×10^{-7} M	% reduction in binding			
Testosterone	101.8 (2)	98.7	84.8	98.4 (2)
Cyproterone acetate	99.5	14.2	-	29.7 (2)
5α - androstane - 3α, 17β - diol	90.8	90.2	-	97.8
5α - androstane - 3β, 17β - diol	102.6	98.0	-	99.4
U. 28,048 *	60.5	90.2	-	97.8
Estradiol	93.9 (2)	90.1	77.1	93.4 (3)
Estrone	36.6	0.2	-	75.0
Diethylstilbestrol	31.4	0.2	-	15.6
Corticosterone	55.6 (2)	0	0	6.2 (2)
Progesterone	91.6	-	11.4	8.3

* estr - 5 - ene - 3β, 17β - diol

The *Kd* value for both human prostate specimens investigated was of the same order as that found for the binding of DHT to SHBG by Forest and Bertrand[30].

DISCUSSION

The fact that human control tissues bound considerably more [^{3}H]-DHT than rat and mouse androgen-unresponsive control tissues; that the binding values for prostatic tissue from most of the untreated patients were approximately the same as those in control tissues, and that binding values in many estrogen treated patients were very high, indicated to us that we were measuring androgen binding to molecules other than an intracellular prostatic receptor. It has been shown that SHBG plasma levels are raised in elderly men [31] and that estrogen treatment (and possibly orchidectomy) raises these levels further [32]. As many of the highest binding values observed in these assays occurred in tissues from estrogen-treated patients we concluded that at least part of the androgen-binding measured was due to contamination by SHBG, which has an extremely high affinity for DHT, but which does not occur in the rat [33]. The results of the steroid specificity studies tended to confirm this. In particular, testosterone, the 5α-androstanediols, estradiol, and U.28,048 displaced [^{3}H]-DHT from the binding molecule effectively at the relative concentrations used, whereas DES, corticosterone and progresterone were poor competitors. This corresponds to the relative steroid affinities for SHBG, as reported by several authors [29, 34–38]. C.A. was a poor competitor in these assays: it has been reported to displace testosterone effectively from SHBG [39] but to the authors' knowledge, its ability to displace DHT has not been investigated.

In the rat prostate, the steroid specificity demonstrated here was similar to that reported by Baulieu *et al.* [40] as regards testosterone, estradiol and progesterone, but these authors found the androstanediols ineffective in displacing [^{3}H]-DHT from the cytosol receptor; however they were used at a considerably lower concentration relative to that of DHT than in our experiments. The moderate effectiveness of corticosterone in competing for [^{3}H]-DHT was unexpected, as it has been previously reported to be ineffective in this respect at similar relative concentrations [41].

We have concluded from these experiments that the [^{3}H]-DHT binding assay as described here is not sufficiently specific to distinguish between binding by SHBG and an intracellular prostatic androgen receptor similar to that in the rat prostate. This conclusion supports that reached by Walsh (personal communication) and by Steins *et al.*[23] who were unable to demonstrate any physiochemical differences by sucrose gradient centrifugation or by agar gel electrophoresis in the androgen-binding properties of human plasma and cytosol from benign hyperplastic glands. However, these authors observed higher androgen

binding by the prostatic tissue than could be accounted for by plasma contamination alone.

It may be possible to separate an androgen-binding receptor in human prostate from SHBG by iso-electric focussing, as suggested by Mainwaring *et al.*[42] and Hansson *et al.*[43]. An alternative approach may be to exploit the differential steroid affinities of the two types of binding molecules, particularly with regard to cyproterone acetate.

There are many factors to be considered in interpreting variations in androgen-binding by the human prostate gland. Thyroid status has been shown to affect SHBG levels [44]. Low endogenous plasma androgen levels brought about by orchiectomy or estrogen treatment would free more intracellular sites for androgen binding *in vitro* than would be available in the presence of normal plasma testosterone levels. Surgical stress has also been shown to lower plasma testosterone levels. There is some evidence that prolactin (PL) influences plasma testosterone levels and *in vitro* binding of androgen by the prostate gland in the rat [45, 46]. In man, it has been shown that plasma PL levels are raised by estrogen treatment [47], stress (including anaesthesia and surgical stress) [48], and several tranquillizing drugs, such as the phenothiazines and reserpine [49]. If high PL levels increase androgen binding by the human prostate *in vivo*, this may result in an increased number of androgen binding sites being occupied at the time of *in vitro* assay, resulting in an artificially low binding value.

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