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Research Article

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When the nuclear extract […]

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ABSTRACT The potent synthetic androgen methytrienolone (R 1881), which does not bind to serum proteins, was utilized to characterize binding to receptors in human androgen responsive tissues. Cytosol extracts prepared from hypertrophic prostates (BPH) were utilized as the source of receptor for the initial studies. High affinity binding was detected in the cytosol of 29 of 30 samples of BPH (average number of binding sites, 45.8±4.7 fmol/mg of protein; dissociation constant, 0.9±0.2 nM). This binding had the characteristics of a receptor: heat lability, precipitability by 0–33% ammonium sulfate and by protamine sulfate, and 8S sedimentation coefficient. High affinity binding was also detected in cytosol prepared from seminal vesicle, epididymis, and genital skin but not in non-genital skin or muscle. However, similar binding was demonstrated in the cytosol of human uterus. The steroid specificities of binding to the cytosol of male tissues of accessory reproduction and of uterus were similar in that progestational agents were more effective competitors than natural androgens. Binding specificities in cytosol prepared from genital skin were distinctly different and were similar to those of ventral prostate from the castrated rat in that dihydrotestosterone was much more potent than progestins in competition. Thus binding of R 1881 to the cytosol of prostate, epididymis, and seminal vesicle has some characteristics of binding to a progesterone receptor.

When the nuclear extract from BPH was analyzed, high affinity binding was demonstrated that conformed to the specificities of binding to an androgen receptor. Here dihydrotestosterone was a more potent competitor than progestational agents. Similar patterns of binding were detected in the crude nuclear extracts from seminal vesicle, epididymis, and genital skin but not in uterus, muscle, or non-genital skin. We conclude that the androgen receptor is not demonstrable in the cytosol of prostate, epididymis, or seminal vesicle of non-castrated men but can be measured in the cytosol of genital skin and the nuclear extracts of androgen responsive tissues. Because steroid hormones exert their major influence within the nucleus of target tissues, the measurement of nuclear receptor may provide valuable insight into the regulation of growth of target tissues.

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INTRODUCTION

The concept that steroidal hormones mediate their effects through binding to intracellular proteins, receptors, has gained wide acceptance. Based on the assumption that receptor proteins are required for hormonal action, it has been proposed that the endocrine sensitivity of a target tissue is related to the presence of cytoplasmic receptor proteins (1, 2). If this is true, then the measurement of the androgen receptor content of human tissues may give further insight into the mechanisms responsible for androgen mediated growth of various tissues. At the present time, it is unclear what role the androgen receptor plays in postnatal growth and maturation of the secondary sexual characteristics, the pathogenesis of benign prostatic hyperplasia, and the hormonal dependence and resistance of carcinoma of the prostate.

In the rat ventral prostate, it has been established that the binding of dihydrotestosterone to a specific receptor is an essential step in the sequence of androgen action (2). In man, the measurement of androgen receptors in prostatic tissue has been plagued with difficulties (3, 4). One major obstacle has been tissue contamination with a serum protein, testosterone-estradiol binding globulin (TeBG),[1] that is present in

[1] *Abbreviations and trivial names used in this paper*: androstanediol, 5α-androstane-3α,17β-diol and 5α-androstane-3β, 17β-diol; androstanedione, 5α-androstane-3,17-dione; androstenedione, 4-androstene-3,17-dione; BPH, benign prostatic

high concentration and that binds with high affinity to dihydrotestosterone. Recognizing this difficulty, a variety of methods has been devised to identify specific androgen receptors in human tissues. Several authors have attempted to purify the receptor utilizing ammonium sulphate precipitation (5, 6) or fractionation with Sephadex G-100–G-200 (6–9) or to provide steroid specific suppression of dihydrotestosterone binding through the use of cyproterone acetate (10). Although these techniques have provided some evidence for a specific receptor in human prostatic tissue, they fail to eliminate quantitatively all binding to TeBG. Other investigators have utilized sucrose density gradient centrifugation (7, 8, 11–15), ion-exchange chromatography (6, 14), or protamine precipitation assays (11) as means for specific identification of androgen receptors. Although these techniques are more specific for the measurement of androgen receptors, unfortunately they have failed to demonstrate reproducibly the presence of a cytosol receptor for androgen.

Recently, Bonne and Raynaud have reported on the use of methyltrienolone (R 1881), a synthetic androgen with a relative biological activity 24 times greater than dihydrotestosterone, that binds with high affinity to the receptor but not to TeBG (16, 17). A similar approach has been utilized for the identification of several other steroid receptors in human tissues, e.g. dexamethasone for the measurement of the cortisol receptor and R 5020 for the measurement of the progesterone receptor. This paper deals with our work utilizing R 1881 to characterize androgen receptors in human tissues.

METHODS

Materials. Ammonium sulfate (ultra pure) was purchased from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). Silica-Gel G-HY precoated, plastic-backed thin layer chromatography plates were obtained from Brinkmann Instruments, Inc. (Westbury, N. Y.). Protamine sulfate (histone-free, Grade I from Salmon) and bovine serum albumin (crystallized and lyophilized) were obtained through Sigma Chemical Co. (St. Louis, Mo.). The radioactive and nonradioactive compounds R 1881 (58.2 Ci/mmol) and R 5020, (51.4 Ci/mmol), were gifts from Dr. Jean-Pierre Raynaud, Centre de Recherches, Roussel-Uclaf (Romainville, France). Radioactive [1,2,4,5,6,7-^{3}H]5α-dihydrotestosterone (130 Ci/mmol) was purchased from Amersham Searle Corp. (Arlington Heights, Ill.). The nonradioactive steroids dihydrotestosterone, testosterone, androstenedione, androstanedione, and androstanediol were from Steraloids, Inc. (Pawling, N. Y.). Dimethylnortestosterone was a gift from Dr. John C. Babcock of The Upjohn Co. (Kalamazoo, Mich.).

Tissue storage. All human tissues, which were removed at open surgical procedure, were placed immediately in iced saline, examined by the surgical pathologist, and then transported directly to the laboratory. They were minced with a Weck blade, rinsed several times with saline to remove excess blood, dried with filter paper, and quickly weighed. Convenient amounts were placed in plastic vials and stored under liquid nitrogen.

For experiments utilizing rat ventral prostate, adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.), which had been castrated via the scrotal route under ether anesthesia 20 h earlier, were killed with chloroform. The ventral prostates were removed, pooled, minced with fine scissors and weighed. Tissue homogenization and preparation of subcellular fractions are described below.

Preparation of cytosol. Frozen tissue was pulverized under liquid nitrogen with a Thermovac tissue pulverizer, Thermovac Industries Corp. (Copiague, N. Y.). The powdered tissue was added to TED buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% (wt/vol) glycerol, pH 7.4 20°C) in 2:1 or 4:1 volume to weight ratio and homogenized with a Polytron PT 10-35 (Brinkmann Instruments, Inc.). The tissue was subjected to three bursts of 15–20 s each at increasing intensity settings (5–6, 6–7, 7–8) with a cooling off period between. All operations were carried out in a cold room at 4°C. The homogenate was spun at 100,000 *g* for 1 h at 0°C in a Beckman L5-65 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and the supernate (cytosol) was carefully removed with a Pasteur pipette to avoid the floating lipid layer.

Preparation of nuclear extract. In experiments utilizing nuclear extract, the homogenate was initially spun at 600 *g* in a Sorvall RC-5 superspeed refrigerated centrifuge (Du Pont Co., Instrument Products Div., Sorvall Biomedical Div., Wilmington, Del.). The supernate was decanted and the pellet was suspended in a 1:1 volume of TED with glycerol and respun at 600 *g* for a further 10 min. The supernate from this spin was discarded, and the pellet was resuspended in a 1:2 volume of TED containing 0.4 M KCl. This suspension was subjected to a short burst of 5 s with the Polytron PT 10-35 and then spun at 100,000 *g* in the Beckman L5-65 ultracentrifuge. The supernate from this spin constituted "crude nuclear extract."

In later experiments a purer nuclear preparation was generated in the following manner. Tissue stored in liquid nitrogen was allowed to thaw in ice-cold sucrose buffer (10 mM Tris, 1 mM dithrothoreitol, 1 mM $MgCl_2$, and 0.25 M sucrose-pH 7.4 at 20°C) until it was semifrozen, chopped into a coarse mince with razor blades, and passed through a Latapie tissue grinder (Arthur H. Thomas Co., Philadelphia, Pa.) The resulting pulp was mixed with additional buffer (final tissue: buffer ratio approximately 1-5 wt/vol) and was hand homogenized through a wire mesh screen using a teflon pestle. This crude cellular homogenate was collected in an ice-cooled beaker. White gelatinous stromal debris remained trapped in the interstices of the screen and were removed periodically by scraping and transferred to a beaker on ice. The cellular homogenate was next filtered through four layers of fine nylon gauze, and the resulting suspension was further homogenized by means of an all-glass Dounce homogenizer (Kontes Co., Vineland, N. J.) with 25 strokes of the loose-fitting pestle

hyperplasia; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; cyproterone acetate, 6-chloro-17-hydroxy-1α,2α-methylene-pregna-4,6-diene 3,20-dione acetate; dihydrotestosterone, 17β-hydroxy-5α-androstane-3-one; dimethylnortestosterone, 7α,17α-dimethyl-19-nortestosterone; estradiol, 1,3,5 (10)-estratriene-3,17β-diol; K_d, dissociation constant, progesterone, 4-pregnene-3,20-dione; R 1881 (methyltrienolone), 17β-hydroxy-17α-methyl-estra-4,9,11-triene-3-one; R 5020, 17,20-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; TeBG, testosterone–estradiol binding globulin; TED buffer, 10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, plus 10% (wt/vol) glycerol, pH 7.4; testosterone, 17β-hydroxy-4-androstene-3-one.

followed by 10 strokes of the tight fitting pestle. This homogenate was spun at 800 *g* for 10 min to obtain a nuclear pellet and a supernate; the latter was processed to cytosol by ultracentrifugation at 100,000 *g* for 1 h at 0°C. When viewed by phase contrast microscopy, the nuclei appeared free of cytoplasmic 'tags', but stromal fibers were present. Further purification of the nuclei by ultracentrifugation on discontinuous sucrose gradients (0.88 M, 1.6 M, 2.2 M sucrose) or through heavy (2.2 M) sucrose caused considerable loss of nuclei and did not eliminate fiber contamination. However, analysis of the stromal debris scraped off the wire mesh after washing it twice with sucrose buffer, homogenizing with a Polytron, spinning the homogenate of 800 *g* for 10 min at 0°C, and recentrifuging the supernate at 100,000 *g* for 1 h at 0°C, revealed no high affinity binding whatsoever. Therefore, purification of nuclei through discontinuous sucrose gradients was not utilized for subsequent assays.

The nuclei were washed twice in sucrose buffer, resuspended in high saltbuffer containing 10 mM Tris, 0.6 M NaCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol, and allowed to stand for 1 h in ice before centrifugation at 12,000 *g* for 10 min to obtain a partially purified nuclear extract.

Binding studies. Binding was measured by incubating 200 μl of cytosol or nuclear extract with 50 μl of steroid solutions. For Scatchard plot analysis, a set of steroid solutions was prepared containing either radioactive steroid (70 pM–5 nM) or radioactive plus excess nonradioactive steroid (0.5 μM) to correct for nonspecific binding. The cytosol-steroid mixtures in duplicate were allowed to incubate for 20 h at 0°C (except where noted). At the end of the incubation period, the unbound steroid was extracted by the addition of a volume of 0.25% dextran-coated charcoal (0.25% acid-washed activated charcoal, 0.0025% dextran in a buffer of 10 mM Tris, 1.5 mM EDTA, pH 7.4, 20°C) solution equal to twice the cytosol volume. The charcoal-cytosol mixtures were allowed to shake for 30 min at 0°C (18), and the charcoal was then pelleted by centrifugation at 7,000 *g* for 20 min. Finally, the entire supernate from each of the assay tubes was pipetted into a liquid scintillation vial, and 10 ml of Hydromix, Yorktown Research Inc. (S. Hackensack, N. J.) was added. Radioactivity was quantitated with a Delta 300 liquid scintillation counter, Searle Analytic Inc. (Des Plaines, Ill.) with a counting efficiency of 50% and corrected to disintegrations per minute by means of an external gamma standard. The data were analyzed by Scatchard plots, and the results were expressed as femtomole of binding per milligram of cytosol protein.

Sucrose density gradient analysis. Sucrose density gradients were prepared as described (11) by layering five different sucrose solutions (prepared with TED with glycerol) in cellulose nitrate tubes and allowing them to form a continuous gradient by diffusion; linearity was assured by measuring the refractive index of each of the fractions. Cytosol samples were incubated with [^{3}H]R 1881 (1 nM) or radioactive plus 100-fold excess nonradioactive R 1881 (0.1 μM) for 4 h at 0°C. Similar incubations were prepared using 5% human female serum with [^{3}H]R 1881 or [^{3}H]-dihydrotestosterone. To charcoal-extract the sample a 10% (vol/vol) of 5% dextran-coated charcoal (5 g acid-washed charcoal, 0.5 g dextran made up to 100 ml with Tris and EDTA buffer) was added to the sample. The mixture was allowed to stand for 15 min at 0°C. The charcoal was then pelletted by centrifugation at 4800 *g* for 20 min and the supernate carefully pipetted off free of charcoal. 300-μl samples of the supernatant were layered on top of the gradients, and they were centrifuged for 16 h in a SW 60 Ti rotor at 40,000 rpm (164,000 *g*) at 0° in a Beckman L5-65 ultracentrifuge. Bovine serum albumin was used as a marker. Fractions were collected after puncturing the bottom of the tubes by paraffin oil displacement in 200-μl aliquots. Radioactivity was monitored in a liquid scintillation counter, and bovine serum albumin was measured spectrophotometrically.

Ammonium sulfate precipitation. The 0–33% fraction was obtained by adding crystals of ammonium sulfate in the ratio of 0.196 g/ml of cytosol (19) over a period of 15 min at 4°C with constant slow stirring. The mixture was allowed to stand for 10 min and then centrifuged at 16,000 *g* for 20 min. The precipitate was then resuspended in a volume of TED buffer equal to the original volume of cytosol to constitute the 0–33% fraction. Ammonium sulfate crystals in the ratio of 0.107 g/ml were then added to the supernate with slow stirring. The mixture was allowed to stand for 10 min and then centrifuged at 16,000 *g* for 20 min. The supernate was discarded, and the precipitate was resuspended in the original volume of buffer to form the 33–50% fraction, which was then analyzed in the usual manner.

Protamine sulfate precipitation. The methods for the measurement of androgen receptor utilizing a protamine sulfate precipitation techniques were described earlier (11). Aliquots of cytosol (200 μl) were combined with 400 μl of protamine sulfate (1.5 mg/ml) and centrifuged at 1,700 *g* for 10 min, producing an even white coating of precipitate on the bottom of the assay tube. The supernate was aspirated, 200-μl aliquots of the steroid solution were added in concentration ranging from 70 pM to 5nM, [^{3}H]R 1881, with or without 0.5 μM R 1881, and the incubations were continued for 20 h at 0°C. At the end of the incubation period, the steroid solutions were aspirated, and the assay tubes and pellets were washed three times with TED buffer. The precipitate adhered to the glass throughout the incubation and buffer washes. The precipitate was then extracted with two washes of absolute ethanol. The ethanol was decanted into liquid scintillation vials and was evaporated to dryness. Hydromix was added to the vials, and radioactivity was measured in a liquid scintillation counter and corrected to disintegrations per minute by means of an external gamma standard.

Thin layer chromatography. The purity of the [^{3}H]R 1881 and [^{3}H]dihydrotestosterone was determined by applying 5 pmol of each of these compounds to 20 × 20-cm sheets precoated with silica gel without gypsum. In addition, 10 μg of each of the nonradioactive steroids androstanedione, androstenedione, dihydrotestosterone, testosterone 3α-androstanediol, and 3β-androstanediol was applied with each radioactive sample. The plates were developed with two ascents of the solvent system chloroform: methanol (97.5:2.5 vol/vol), air-dried and sprayed with anisaldehyde reagent, (100 ml glacial acetic acid, 2 ml concentrated H_2SO_4, 1 ml *p*-anisaldehyde), and heated at 100°C for 15 min. Within each lane, the areas corresponding to the reference steroids were marked, cut with scissors, and placed in liquid scintillation vials. The entire lane was assayed for ^{3}H using 10 ml Hydromix. It was determined that [^{3}H]R 1881 was 90% and [^{3}H]dihydrotestosterone was 93% pure.

To analyze cytosol samples for steroid metabolites, aliquots were extracted with 7 ml chloroform:methanol (2:1 vol/vol). The samples were frozen at −25°C, and the liquid chloroform-methanol phase was pipetted from the aqueous phase and evaporated to dryness. The residue was redissolved in 0.05 ml absolute ethanol. These samples were then applied to the silica-gel plates and developed as before.

Protein measurements. Protein was determined by measuring absorbance at 230 nm and 260 nm in a spectrophotometer, model 200, Gilford Instrument Laboratories, Inc. (Oberlin, Ohio) and correcting the values to mg/ml: $(A_{230} \times 184) - (A_{260} \times 76.1)$ = protein μg/ml.

RESULTS

Studies on cytosol. The initial studies to characterize the binding of [^{3}H]R 1881 were performed on cytosol prepared from specimens of benign prostatic hyperplasia. When cytosol was incubated at 0°C with 10 nM [^{3}H]R 1881, specific bound radioactivity increased steadily for 8 h and then approached a plateau that was maintained for 40 h (Figs. 1A and B). When incubations were performed at 15°C, the initial rate of binding was marginally more rapid, but the pattern of binding otherwise was quite similar to that at 0°C. The rate of dissociation of bound [^{3}H]R 1881 was studied by adding 100-fold nonradioactive steroid to cytosol preparations that had been pre-incubated at 0°C for 20 h with 10 nM [^{3}H]R 1881 (Fig. 1B). Displacement was seen as early as 1 h with 40% being displaced at 4 h and 80% at 24 h.

When these experiments were performed utilizing non-saturating (1 nM) concentrations of [^{3}H]R 1881, no appreciable difference was detected at 0°C. At 15°C however, although maximal binding was achieved at 4 h, 50% of the binding was lost at 20 h. This loss of binding at 15°C is probably secondary to the heat lability of the receptor in the absence of saturating concentrations of the ligand. At higher temperatures, binding was more unstable with a total loss of specific binding by 10 h at 25°C and by 30 min at 37°C (results not shown).

A typical saturation curve for the binding of [^{3}H]-R 1881 is shown in Figure 2A. Specific binding was saturable at low steroid concentrations (5 nM), indicating a limited number of binding sites. To evaluate the binding affinity and the apparent number of binding sites available for specific binding, 200-μl aliquots of prostatic cytosol were incubated at 0°C for 20 h with concentrations of [^{3}H]R 1881 ranging from 70 pM to 10 nM (Fig. 2A). Analysis of the data after the method of Scatchard yielded a dissociation constant of 1.0 nM, and the number of apparent binding sites was 37.8 fmol/mg of protein. Because saturation was achieved at 5 nM, in later experiments the highest concentration of steroid utilized was 5 nM. The mean dissociation constant (K_d) of 29 determinations on tissue from 18 patients was 1.3 nM (range 0.3–4.5 nM), and the mean concentration of available binding sites was 45.8 fmol/mg of protein (range 10.9–118.8). When 5% adult non-pregnant female serum was analyzed under identical conditions, no high affinity binding was detected (Fig. 2B). It was therefore concluded that the specific binding measured under these conditions was not present in serum and thus did not represent binding to testosterone-estradiol binding globulin.

To further characterize this binding as specific for a receptor the following studies were carried out: density

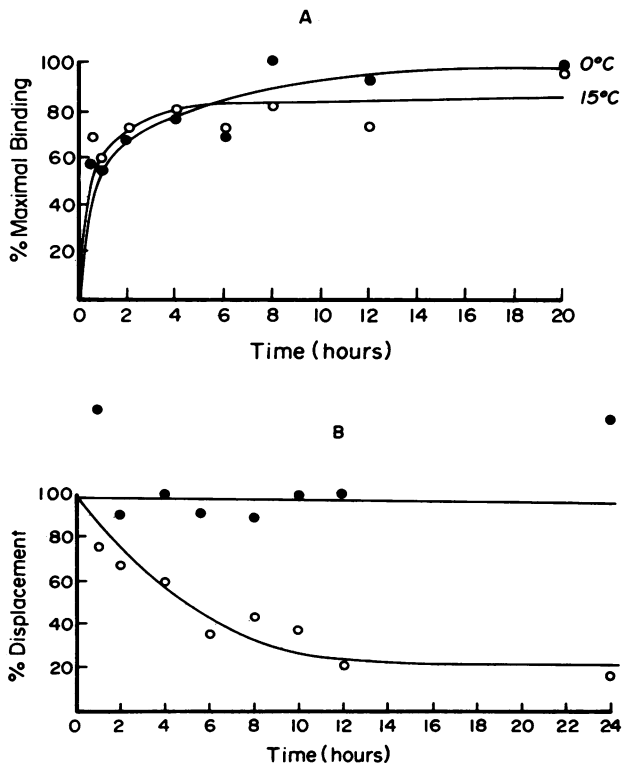

FIGURE 1 (A) Specific binding of [^{3}H]R 1881 by human prostatic cytosol as a function of time. Triplicate 200-μl aliquots of prostatic cytosol were incubated with 10.0 nM [^{3}H]R 1881 with and without 100-fold excess nonradioactive R 1881, at 0°C (● – ● – ●) or 15°C (○ – ○ – ○) for time intervals as indicated. Aliquots were extracted with dextran-coated charcoal, and bound radioactivity was measured as described. Nonspecific binding was subtracted in all instances. Results are expressed as percentage of maximal binding (binding at 20 h, 0°C). (B) Dissociation of specifically bound [^{3}H]-R 1881 as a function of time. Triplicate 200-μl aliquots of prostatic cytosol that had been pre-incubated with 10 nM [^{3}H]R 1881 for 20 h at 0°C were incubated at 0°C for longer intervals of time, as indicated, with either 1.0 μM nonradioactive R 1881 (○ – ○ – ○) or buffer (● – ● – ●). Nonspecific binding was subtracted in all instances. Results are expressed as percentage displacement.

gradient centrifugation, stability to heat, ammonium sulfate precipitation, and protamine sulfate precipitation. When samples of cytosol were incubated with 1.0 nM [^{3}H]R 1881 at 0°C for 4 h and subjected to sucrose density gradient centrifugation, a peak of radioactivity was demonstrated in the 8S region (Fig. 3). This peak was displaced by the addition of 100-fold excess non-radioactive R 1881. No peak was seen in the 4.6S region indicating that there was no binding to contaminating serum proteins. A similar pattern was seen in cytosol obtained from prostatic cancer and seminal vesicle. When 5% serum was incubated with 10 nM [^{3}H]R 1881 and analyzed under identical

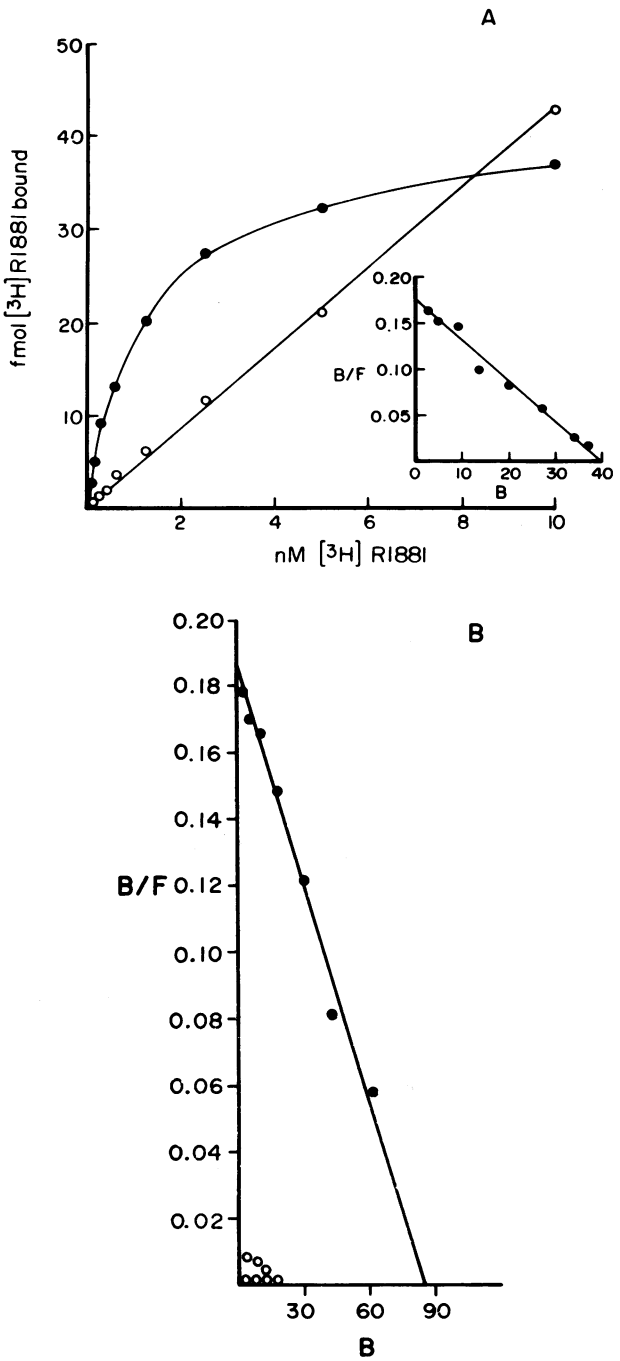

FIGURE 2 (A) Specific and nonspecific binding of [^{3}H]R 1881 in human prostatic cytosol. Cytosol (protein = 4.6 mg/ml) prepared under the standard conditions described in the text was incubated at 0°C for 20 h with concentrations of [^{3}H]-R 1881 ranging from 70 pM to 10 nM. Free steroid was removed with dextran-coated charcoal. Specific binding (● – ● – ●) is defined as the difference in counts bound in the absence and presence of 100-fold excess nonradioactive R 1881, and nonspecific binding (○ – ○ – ○) as the amount of radioactivity remaining in the presence of 100-fold excess nonradioactive R 1881. Data are also expressed in the form of a Scatchard plot. (B) Scatchard plot analysis of specifically bound [^{3}H]R 1881 by human prostatic cytosol. Cytosol (protein = 3.8 mg/ml) prepared under the standard conditions described in the text was incubated at 0°C for 20 h with concentrations of [^{3}H]R 1881 ranging from 70 pM to 5 nM. (● – ● – ●). Free steroid was removed with dextran-coated charcoal. Nonspecific binding was subtracted in all instances. The amount of specifically bound steroid in fmol/200 μl is plotted on the abscissa, and the ratio of bound-to-free steroid is on the ordinate. The estimated number of binding sites is 118 fmol/mg of protein, and the K_d is 1.8 nM. Binding of [^{3}H]R 1881 by serum, under identical conditions, is also shown (○ – ○ – ○).

conditions, no 8S peak of radioactivity was seen. After the incubation of serum with 10 nM [^{3}H]dihydrotestosterone, there was a single peak of radioactivity (4.6S) that represents binding to TeBG and albumin (Fig. 3B).

The stability of the receptor to heat was then studied. Aliquots of cytosol heated at 45°C for 1 h were incubated with increasing concentrations of radioactive steroid at 0°C for 20 h and then subjected to Scatchard plot analysis. All specific binding was destroyed, and no Scatchard plot could be generated. A control sample of cytosol analyzed under identical conditions without prior heating contained specific binding (K_d = 1.8 nM) of a magnitude of 118 fmol/mg of protein. (Fig. 2B). It is known that the androgen receptor is precipitated by the 0–33% fraction of ammonium sulfate, and by the polycation protamine sulfate. Utilizing a cytosol preparation containing 1710 fmol/g (of tissue) of high affinity (K_d = 1.8 nM) binding, 482 fmol/g of binding (K_d = 1.7 nM) was demonstrated in the 0–33% ammonium sulfate precipitate, and 53 fmol/g of binding was recovered from the 33–50% precipitate. Thus, 28% of specific binding was recovered in the 0–33% fraction, whereas only 3% was present in the 33–50% ammonium sulfate precipitate. Analysis of the supernate after precipitation with 33–50% ammonium sulfate revealed no specific binding. When aliquots of prostatic cytosol were precipitated with protamine sulfate and incubated with increasing amounts of [^{3}H]R 1881, specific binding (K_d 1.0 nM) of small quantities (12.2 fmol/mg protein) was detected. This represented 39% of the binding detected in a control assay that was performed in the standard fashion (Fig. 4). Ammonium sulfate and protamine sulfate precipitation caused substantial loss of recovery of receptor. However, the bulk of the receptor recovered was obtained from the 0–33% fraction with ammonium sulfate and the precipitate with protamine sulfate.

The linearity and reproducibility of the R 1881 assay were then tested. When cytosol prepared in the standard manner was diluted and assayed for high affinity binding, a series of Scatchard plots was obtained (Fig. 5). The assay was linear down to a protein concentration of 1.8 mg/ml. To test the reproducibility of the assay, tissue from a single patient was pulverized

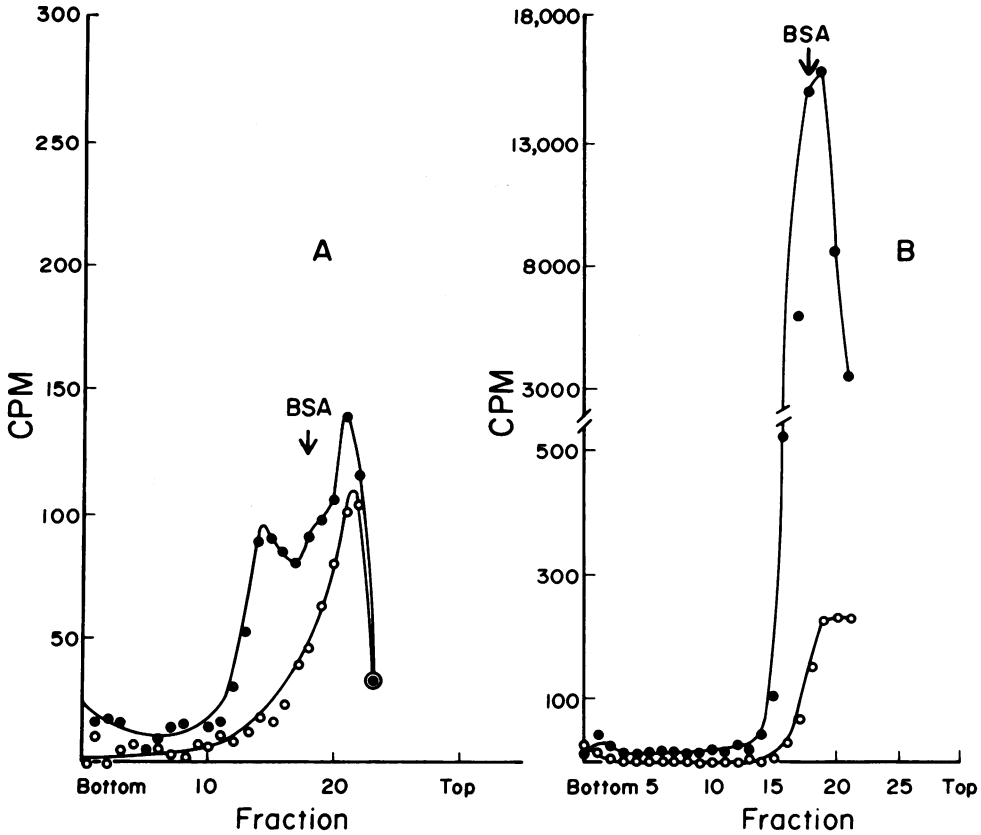

FIGURE 3 Density gradient centrifugation of prostatic cytosol (A) and serum (B) after incubation with radioactive steroids. (A) Aliquots of cytosol prepared under the standard conditions described in the text were incubated for 4 h at 0° with 1.0 nM [^{3}H]R 1881 with (○ – ○ – ○) and without (● – ● – ●) the addition of 100-fold excess cold competitor and extracted with dextran-coated charcoal. Aliquots of 300 μl were applied to 5–20% sucrose gradients containing 10% glycerol. The tubes were centrifuged at 164,000 *g* for 16 h, and 200-μl fractions were collected and assayed for radioactivity. The position of the bovine serum albumin (BSA) marker is indicated on the gradient. (B) Human serum (5%) was incubated with 10 nM [^{3}H]dihydrotestosterone (● – ● – ●) or 10 nM [^{3}H]R 1881 (○ – ○ – ○) for 4 h at 0°C and analyzed by density gradient centrifugation as described above.

and stored in liquid nitrogen. Samples of the mixed powder were analyzed for 5 consecutive days (Table I). The mean number of apparent binding sites was 34.9 fmol/mg of protein (±7.9 SD; range 24.1–43.1 fmol/mg of protein) and the mean dissociation constant, 0.99 nM (±0.16 SD, range 0.82–1.24 nM). To test the stability of the receptor, multiple tissue samples from a single patient were analyzed on eight separate occasions over a 3-mo period (results not shown). Although there was considerable variation in the number of apparent binding sites (64±30 fmol/mg protein; mean±SD; range 38–119 fmol/mg protein) there was no decline in binding with time. It is possible that this variability is secondary to regional differences in the tissue assayed. It is well known that marked variations in the histological appearance of benign prostatic hyperplasia exist, and these studies were not controlled with morphological examination of the tissue. These studies do suggest that the receptor is stable over long periods when whole tissue is stored in liquid nitrogen. However, when cytosol specimens were stored in liquid nitrogen, high affinity binding decreased rapidly.

To determine the tissue specificity of binding to [^{3}H]R 1881, the assay was utilized to analyze 30 samples of benign prostatic hyperplasia. These results were compared to measurements in 10 samples of seminal vesicle (removed at the time of radical cystectomy or radical prostatectomy), 4 samples of non-genital skin and muscle that were removed at the same time, 3 samples of epididymis and 4 samples of genital skin (scrotal skin = 3; infant foreskin = 1). Nearly all benign hyperplastic tissue showed receptor levels between 10 and 50 fmol/mg protein. Two specimens had binding > 50 fmol/mg protein, and one showed no

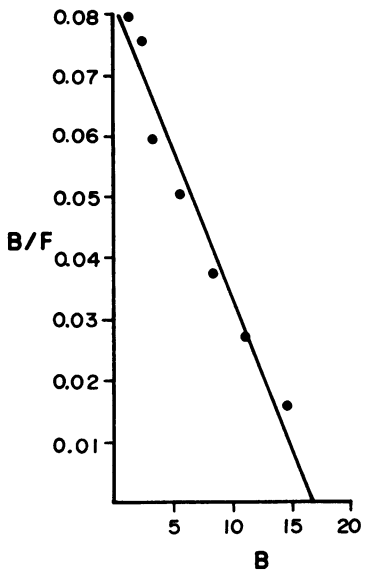

FIGURE 4 Scatchard plot analysis of protamine sulfate-precipitated cytosol. Cytosol (protein 6.9 mg/ml) prepared under the standard conditions described in the text was precipitated with protamine sulfate (1.5 mg/ml). The precipitate was then incubated for 20 h at 0°C with concentrations of [^{3}H]R 1881 ranging from 70 pM to 5 nM. The supernate was aspirated, the pellet was washed thrice with buffer, and bound radioactivity was extracted with ethyl alcohol. Nonspecific binding was subtracted in all instances. The amount of specifically bound steroid in fmol/200 μl is plotted on the abscissa, and the ratio of bound-to-free steroid is on the ordinate. The estimated number of binding sites is 12 fmol/mg of protein, and the dissociation constant (K_d) is 1 nM.

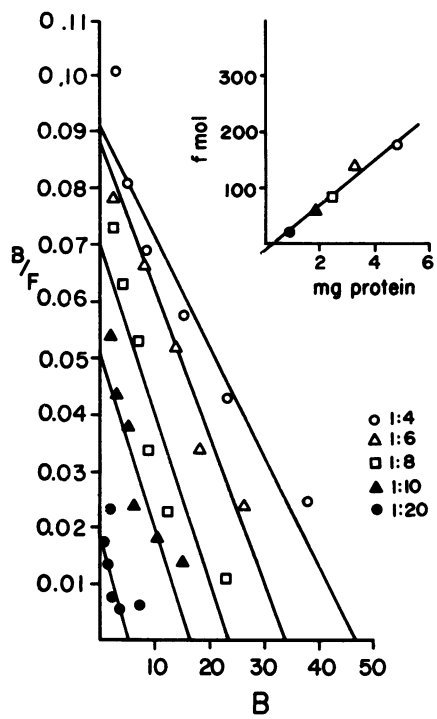

FIGURE 5 Effect of varying protein concentrations on Scatchard plot analysis of [^{3}H]R 1881 binding by prostatic cytosol. Cytosol (18.2 mg protein/ml), prepared under the standard conditions described in the text, was diluted to give effective protein concentrations of (*a*) 4.8 mg/ml (○ – ○ – ○) K_d = 1.4 nM, n = 36.9 fm/mg protein; (*b*) 3.2 mg/ml (△ – △ – △) K_d = 1.2 nM, n = 45.04 fm/mg protein; (*c*) 2.4 mg/ml (□ – □ – □) K_d = 0.8 nM, n = 34.4 fm/mg protein; (*d*) 1.8 mg/ml (▲ – ▲ – ▲) K_d = 0.9 nM, n = 36.2 fm/mg protein; (*e*) 0.9 mg/ml (● – ● – ●) K_d = 0.9 nM, n = 25.7 fm/mg protein. Incubations were performed for 20 h at 0°C, and bound radioactivity was measured after charcoal extraction. Nonspecific binding was subtracted in all instances. The insert depicts the available receptor sites calculated from the Scatchard plots as a function of protein concentration.

high affinity binding. The mean apparent number of binding sites in the seminal vesicle was 37 fmol/mg protein. No high affinity binding was present in either muscle or in non-genital skin. High affinity binding was present in three of four samples of epididymis (61±19 fmol/mg) and also in three of the four samples of genital skin (9–13 fmol/mg protein) as is shown in Table II.

Next, steroid specificity studies were performed. Cytosol prepared from human benign prostatic hyperplasia was incubated with 1 nM [^{3}H]R 1881 alone, or in the presence of 5-, 10-, 100-, or 1000-fold excess nonradioactive steroid. Nonspecific binding, defined as that binding not displaced by the addition of 1000-fold excess nonradioactive R 1881, was subtracted in all instances. The concentration of R 1881 required to displace 50% of specific binding was determined and assigned a relative binding affinity of 100. The concentration of other competing steroids causing 50% displacement was compared to this and constituted their relative binding affinity. The synthetic androgen 7α-17α-dimethyl nor-testosterone and R 1881 were equally potent in competing for specific binding of [^{3}H]R 1881 by the receptor. Steroids such as androstanediol (3α or 3β), Δ^4-androstenedione, and cortisol were ineffective in inhibiting binding (Figure 6A). Cyproterone acetate was more potent than testosterone and estradiol which were rather poor competitors. Surprisingly, dihydrotestosterone was only slightly better than testosterone or estradiol in competing for binding of [^{3}H]R 1881. To check the possibility that dihydrotestosterone was being converted to 3α-17β-androstanediol during the incubation, cytosol was incubated with 1 nM nonradioactive R 1881 and 5-, 10-, 100-fold excess [^{3}H]dihydrotestosterone. The radioactivity was then extracted with ethyl acetate and analyzed by thin-layer chromatography. It was seen that all of the radioactivity was co-chromatographic with dihydrotestosterone, indicating that metabolism was insignificant in the assay conditions used.

When 5% serum was incubated with [^{3}H]dihydro-

TABLE I

Results of Charcoal Binding Assays Performed on 5 Consecutive Days on a Mixed Powder Obtained from Prostatic Tissue Samples of a Single Patient

Experiment	High affinity [^{3}H]R 1881 binding	
	K_d (× 1 nM)	fmol/mg protein
1	1.24	43.1
2	1.00	37.8
3	0.97	24.1
4	0.82	29.3
5	0.91	40.1
Mean±SD	0.99±0.16	34.9±7.9

testosterone and similar competition studies were performed, an entirely different picture emerged (Fig. 6B). In this case, the binding was more characteristic of TeBG, showing good displacement with testosterone, estradiol, dihydrotestosterone, and 3β-androstanediol. R 1881 was a poor competitor, thus providing further confirmation that the binding of R 1881 to prostatic cytosol does not represent binding to contaminating serum proteins (Fig. 6B).

To further characterize the steroid specificity of binding of [^{3}H]R 1881 to prostatic cytosol, competition experiments were done utilizing progestational agents. When progesterone was used as the competing steroid, it was found that progesterone competed somewhat better than dihydrotestosterone for binding sites. The synthetic progestin R 5020 was an even more potent competitor (Table II). Because it has been suggested that R 1881 may bind to the progesterone receptor, the possibility that the protein being measured was a progesterone receptor was considered. Scatchard plot analyses were performed on prostatic cytosol comparing [^{3}H]R 1881 to [^{3}H]R 5020. Consistently, specific high affinity binding to [^{3}H]R 5020 was detected, although of lower affinity than the binding to [^{3}H]R 1881 (Fig. 7).

We next proceeded to compare the steroid specificity of [^{3}H]R 1881 binding to cytosol prepared from sex accessory tissues (prostate, seminal vesicle, and epididymis), to genital skin (scrotal skin and infant foreskin), to uterus, and to rat ventral prostate (Table II). In these studies the steroid specificity patterns of BPH, seminal vesicle, and epididymis were indistinguishable and exhibited striking similarity to that of uterine cytosol. In all these tissues, progestational agents were much more potent than dihydrotestosterone in competing for binding sites. In uterine cytosol, dihydrotestosterone was totally ineffective as a competitor. Binding to genital skin and rat ventral prostate was distinctly different. Here dihydrotestosterone was a highly potent competitor, whereas progestational agents were ineffective (Table II). These studies indicate that the steroid specificity of binding of [^{3}H]R 1881 to the cytosol of the BPH, seminal vesicle, and epididymis does not approximate the specificity of binding to the androgen receptor as classically described in ventral prostate from the castrated rat. Although the binding is heat labile, precipitable by 0–33% ammonium sulfate and protamine sulfate, exhibits high affinity and tissue specificity, and sediments in the 8S region on density gradient centrifugation, it has steroid specificities more

TABLE II

Binding Characteristics of [^{3}H]R 1881 to Human Cytosol Preparations and Rat Ventral Prostate

Affinity and receptor content	Benign prostatic hyperplasia	Seminal vesicle	Epididymis	Scrotal skin	Uterus	Rat ventral prostate
Number of positive assays	29	10	3	3	2	Not done
Number of assays performed	30	11	4	4	2	
Apparent K_d (× 1 nM)*	1.3±0.2	1.5±0.2	1.5±0.3	2.4±1.4	7.5±0.4	Not done
Available number of binding sites* (fmol/mg protein)	45.8±4.7	37.0±4.8	61.4±18.9	10.4±1.7	1494±74	Not done
Steroid binding specificity						
Number of assays	6	3	3	2	2	3
Relative binding affinity						
R 1881	100	100	100	100	100	100
DHT‡	2	0.3	0.2	82	0	88
R 5020	21	50	49	4	43	2
Progesterone	17	15	15	2	37	2
Cyproterone acetate	26	15	14	8	29	5

* Mean±SD.

‡ DHT, dihydrotestosterone.

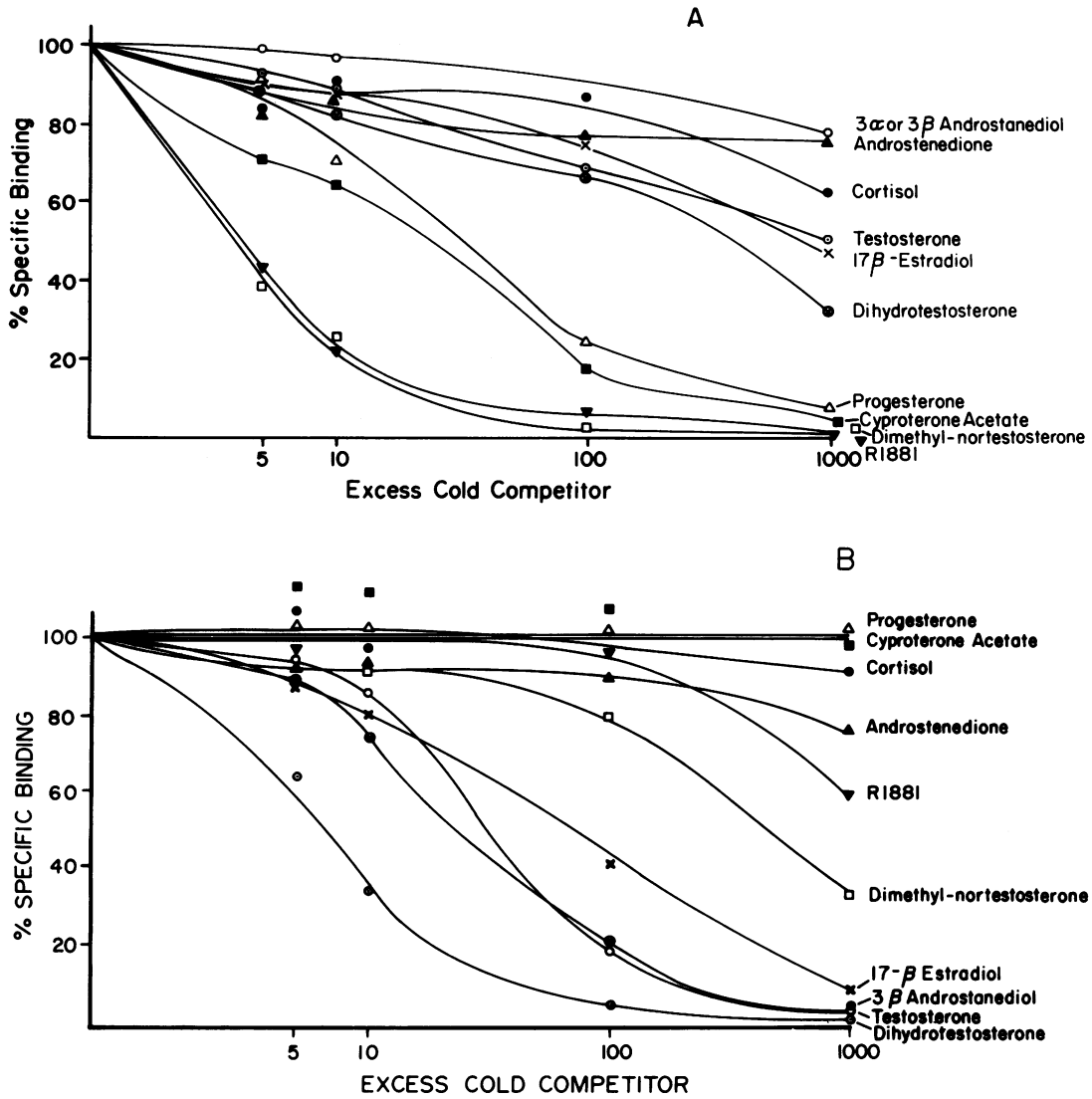

FIGURE 6 Steroid specificity of (A) [^{3}H]R 1881 binding by prostatic cytosol and of (B) [^{3}H]-dihydrotestosterone binding by 5% human serum. (A) Duplicate aliquots of prostatic cytosol were incubated with 1.0 nM [^{3}H]R 1881 with or without the addition of 5-, 10-, 100-, and 1000-fold excess nonradioactive competing steroid, as indicated in the figure, for 20 h at 0°C. Nonspecific binding, defined as that binding not displaced by the addition of 1000-fold excess R 1881, was subtracted in all instances. Bound radioactivity was calculated after dextran coated charcoal extraction. (B) Duplicate aliquots of 5% human serum were incubated with 1.0 nM [^{3}H]dihydrotestosterone with or without competing steroid as described above. Nonspecific binding was determined by the addition of 1000-fold nonradioactive dihydrotestosterone.

characteristic of a progesterone receptor than an androgen receptor.

Studies on nuclear extract. Having failed to demonstrate an androgen receptor in human prostatic cytosol, we directed our attention to the crude nuclear extract. Nuclear extract was prepared from specimens of benign prostatic hyperplasia and assayed for specific uptake of [^{3}H]R 1881. When nuclear extract was incubated at 0°C with 1 nM [^{3}H]R 1881, specific bound radioactivity increased steadily for 4 h and then approached a plateau that was maintained for 24 h. At 40 h there was minimal loss of specific binding. When incubations were performed at 25°C, the binding was more unstable with a total loss of binding by 4 h (results not shown). The rate of dissociation of [^{3}H]-R 1881 was studied by adding 1000-fold nonradioactive R 1881 to preparations of nuclear extract that had been preincubated at 0°C for 20 h with 1 nM [^{3}H]R 1881.

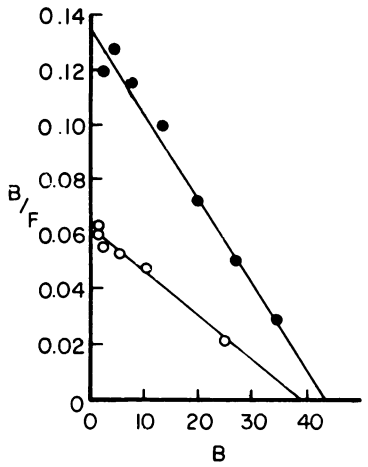

FIGURE 7 Scatchard plot analysis of specific binding of [^{3}H]R 1881 and [^{3}H]R 5020 by human prostatic cytosol. Cytosol (protein 3.8 mg/ml) prepared under the standard conditions described in the text was incubated at 0°C for 20 h with [^{3}H]R 1881 (● – ● – ●) or [^{3}H]R 5020 (○ – ○ – ○) in concentrations ranging from 70 pM to 5 nM. Free steroid was removed with dextran-coated charcoal. Nonspecific binding was subtracted in all instances. The amount of specifically bound steroid in fmol/200 μl is plotted on the abscissa and the ratio of bound-to-free steroid is on the ordinate. The estimated number of binding sites with [^{3}H]R 1881 is 59 fmol/mg of protein, and the dissociation constant (K_d) is 1.3 nM. The estimated number of binding sites with [^{3}H]R 5020 is 54 fmol/mg of protein and the dissociation constant is 2.7 nM.

Displacement was rapid and was seen as early as 1 h with total displacement being achieved at 20 h.

To evaluate the binding affinity and apparent number of sites available for specific binding, nuclear extracts prepared from BPH were incubated at 0°C for 20 h with [^{3}H]R 1881 in concentrations ranging from 70 pM to 5 nM and subjected to Scatchard plot analysis. The mean dissociation constant was 2.6 nM, and the mean concentration of available binding sites was 67.5 fmol/mg of protein. Upon conducting steroid specificity experiments, in sharp contrast to cytosol, dihydrotestosterone was much more potent than progestational agents in competition (Table III). The relative binding affinity of dihydrotestosterone was 83, whereas that of R 5020 was 6. Similar patterns of steroid specificity were seen with the nuclear extracts of seminal vesicle, epididymis, and scrotal skin. No high affinity binding was seen, however, in the nuclear extracts of muscle and non-genital skin. When nuclear extract from uterus was analyzed under similar conditions, although specific binding was detected, the steroid specificity was markedly different with dihydrotestosterone being an extremely poor competitor, whereas R 5020 was potent with a binding affinity of 75 (Table III). The steroid specificity of binding of [^{3}H]R 1881 to the nuclear extract of male sex accessory tissues is thus distinctly different from the binding to cytosol and bears the characteristics of binding to the androgen receptor.

It was apparent that binding studies with crude nuclear preparations may not in fact represent binding to the nuclear fraction, but may represent binding to a compartmentalized cytoplasmic fraction. Crude and purified nuclei, and their associated cytoplasmic fractions were prepared from the same patient and analyzed using Scatchard plots and steroid specificity reactions. Comparable results were obtained with both methods allowing for a loss of receptor on purification (Table IV). Analysis of the stromal debris did not demonstrate any high affinity binding to [^{3}H]R 1881.

DISCUSSION

The androgen receptor has been studied most extensively in the rat ventral prostate. On sucrose density gradient centrifugation, the receptor sediments as a polydispersed aggregate in low salt solutions not containing glycerol; in the presence of glycerol, it sediments as an 8S. It is completely destroyed by heating to 50°C for 1 h (20, 21). The receptor is precipitated

TABLE III
Binding Characteristics of [^{3}H]R 1881 to Human Nuclear Extract

Affinity and receptor content	Benign prostatic hyperplasia	Seminal vesicle	Epididymis	Scrotal skin	Uterus
Number of assays	3	2	2	1	1
Apparent K_d (× 1 nM)*	2.6±0.6	2.1±0.3	2.4	1.5	2.2
Available number of binding sites (fmol/mg of protein)*	67.5±34.5	73.5±5.5	56	40	417
Relative binding affinity					
R 1881	100	100	100	100	100
DHT‡	83	76	125	115	0
R 5020	6	0	0	0	75

* Mean±SD.
‡ DHT, dihydrotestosterone.

TABLE IV
Comparison of Binding [³H]R 1881 to Cytosol and Nuclear Extract Prepared by the Routine and Partially Purified Methods

	Routine preparation		Additional purification	
	Cytosol	Nuclear extract	Cytosol	Nuclear extract
K_d (× 1 nM)	1.0	2.0	0.4	1.5
n (fmol/mg) protein	70	88	24	53
Relative binding affinities				
R 1881	100	100	100	100
DHT*	8	91	6	48
Progesterone	53	0	52	0

* DHT, dihydrotestosterone.

by 30% ammonium sulfate or by the addition of polycations like protamine sulfate (11).

It is not known for certain whether the androgen receptor of human tissues has the same characteristics as that of rat prostate, and although a host of techniques have been applied to the characterization of the receptor in man, none has proved to be uniformly reproducible. Different investigators utilizing almost identical techniques have reported conflicting results. Some have been able to identify the receptor in all instances, others in most instances, and some, not at all (3, 4). It appears, therefore, that if strict criteria for identification of the receptor are used (sedimentation coefficient, steroid and tissue specificity, and precipitability by ammonium sulfate and by protamine sulfate), it is indeed exceedingly difficult to demonstrate the androgen receptor in human tissues with any degree of reproducibility. A variety of explanations has been proposed for this. It is recognized that receptor proteins are quite labile and that nodules of prostatic hyperplasia are difficult to homogenize and contain a large amount of proteolytic enzymes. It is also known that the human prostate contains large quantities of endogenous dihydrotestosterone and that therefore all receptor sites may be saturated (3, 4). In this study we have identified another factor, the presence of a cytoplasmic protein that binds progestational agents, that interferes with the demonstration of a soluble cytosol receptor for androgen.

Recently, Bonne and Raynaud demonstrated that [³H]R 1881, a synthetic androgen that does not bind to TeBG (16), binds with high affinity to human prostatic cytosol (17). We have confirmed this finding and have demonstrated that this binding has several characteristics of binding to a receptor: heat lability, precipitability by 33% ammonium sulfate or protamine sulfate, and a sedimentation coefficient of 8S on sucrose density gradient centrifugation. In addition, high affinity binding was also present in other androgen responsive tissues (seminal vesicle, epididymis, and genital skin) but not in non-genital skin and muscle. In recent studies, Asselin et al. found that the steroid specificity of binding of [³H]R 1881 to BPH was similar to that of uterine cytosol, with progestational agents being much more effective competitors than testosterone or dihydrotestosterone (22). They suggested that these data indicate the presence of either an atypical androgen receptor or a progesterone receptor in BPH. In this study we have demonstrated similar steroid specific binding in the cytosol prepared from human BPH, seminal vesicle, epididymis, and uterus. This binding is distinctly different from the binding present in cytosol prepared from human genital skin or rat ventral prostate where dihydrotestosterone is as potent as R 1881 and where progestational agents are poor competitors.

These data demonstrate the presence of a soluble receptor that binds progestins with great avidity in the cytosol of human BPH, seminal vesicle, and epididymis. It is unclear what function this receptor fulfills and why it is present. It is well known that circulating levels of progesterone are quite low (30 ng/100 ml) in men (23), and that progesterone is not considered a major tropic hormone for the male sex accessory tissues. However, it is accepted that progestational agents can modify androgen action by potentiation or inhibition. The mechanism by which these synandrogenic and antiandrogenic responses are mediated is not understood well, but this phenomenon may be related to the presence of receptor proteins for progestational agents in the cytosol of some androgen responsive tissues (24, 25). Finally, because the regulation of the progesterone receptor is under the control of estrogen (26), the presence of the cytosol receptor characterized here in some, but not all sex accessory tissues, may indicate that estrogen has a primary role in modulating the growth and function of these target organs. It is of interest that all tissues in which the cytoplasmic receptor was demonstrable were removed from aging males where one would predict that any estrogenic effect would be magnified, and it is conceivable that this receptor may not be present in tissues taken from young individuals. If this is true, this finding may provide important insight into the pathogenesis of BPH.

When the nuclear extract of benign prostatic hyperplasia was analyzed, high affinity binding was demonstrated likewise. This binding was both tissue- and steroid-specific. Dihydrotestosterone was a potent competitor, whereas progestational agents were not. Similar patterns of binding were also seen in the crude nuclear extracts prepared from seminal vesicle, epididymis, and genital skin. Although high affinity

binding was detected in extracts of uterine nuclei, this was competed for by progestational agents, but not by dihydrotestosterone. It appears, therefore, that the binding of [^{3}H]R 1881 to the nuclear extracts of prostate, seminal vesicle, epididymis, and genital skin indeed does represent binding to the androgen receptor.

Based on the known mechanism of steroid hormone action, in the presence of normal levels of endogenous steroid, most of the receptor molecules should be shifted to the nucleus. In the ventral prostate of the rat, it is difficult to demonstrate the presence of cytoplasmic receptor in the non-castrate animal (27). In man, it is well known that in the prostate (28) and possibly in other sex accessory tissues, the endogenous level of androgen is high. Consequently, it is not surprising that a large proportion of the demonstrable androgen receptor is present in the nucleus. Although these findings do not disprove the presence of a cytosol receptor for androgen in the prostate, they do suggest that it is exceedingly difficult to demonstrate. We therefore suggest that in man, studies of the androgen receptor should be directed at the nucleus. Because steroid hormones exert their major influence within the nucleus of target tissues, such studies may provide valuable insight into the role of androgen receptors in the regulation of the normal and abnormal growth and development of male sex accessory tissues.

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