A RAPID, SPECIFIC PROTOCOL FOR DETERMINATION OF AVAILABLE ANDROGEN RECEPTOR SITES IN UNFRACTIONATED RAT VENTRAL PROSTATE CYTOSOL PREPARATIONS

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Summary: A modification of the dextran-coated charcoal technique has been successfully employed for the measurement of androgen receptor binding of 5α-dihydrotestosterone in unfractionated rat ventral prostate cytoplasmic extracts. The addition of a small amount of ethanol to the dextran-coated charcoal solution during the adsorption of unbound ligand greatly facilitated charcoal adsorption of ligand associated with low affinity, high capacity binding components and reduced the contribution of the latter cytoplasmic binding components to less than 10 percent of the measured binding at near saturating concentrations, 10 nM, of 5α-dihydrotestosterone. The assay is facile, sensitive, and highly reproducible and a complete saturation curve can be obtained with as little as 100 mg of ventral prostate. This protocol therefore represents a unique procedure for the quantitation and characterization of the cytoplasmic androgen receptor of rat ventral prostate. The concentration of available cytoplasmic androgen receptor in ventral prostate from young mature (80-120 day old) albino rats, 24 hours post orchidectomy, was 10,300 ± 1780 sites per cell and the apparent binding constant for 5α-dihydrotestosterone was 6.49 ± 0.35 x 10^8 M^{-1}.

INTRODUCTION

The existence of a rat ventral prostate cytoplasmic androgen receptor is well established (1-4), and numerous reports of its physicochemical properties have appeared (5-7). The prostatic androgen receptor is unique among the steroid hormone receptors in that a rapid, sensitive protocol for the rigorous evaluation of its binding parameters and tissue concentration has not been reported. Previous reports concerning the quantitation and characterization of this receptor in crude tissue extracts have almost exclusively employed sucrose gradient centrifugation (1-3, 6-8) or gel chromatography (8-11) assay protocols. Neither of these procedures is well suited for the quantitation of available receptor sites by saturation analysis or for characterization

studies involving multiple determinations of receptor bound ligand due to time requirements and problems associated with peak integration, radioligand recovery, and receptor-ligand complex instability. Castañeda and Liao (12) have recently reported preliminary results of an assay procedure employing steroid antibodies to complex unbound and non-specifically bound ligand which in principle could be modified to provide a protocol for the quantitation of receptor-ligand complexes.

We recently reported a charcoal adsorption procedure for the measurement of receptor bound 5α-dihydrotestosterone in ammonium sulfate fractionated rat ventral prostate cytosols and the utilization of this protocol for the determination of ligand binding specificity of the prostatic androgen receptor (13). Although this assay also was useful for the determination of available receptor binding sites; the procedure possessed two inherent deficiencies: 1) fractionation of the cytosol, which was required to reduce the contribution of non-specific binding, resulted in a significant irretrievable loss of receptor binding sites and 2) assay insensitivity would not allow for adequate determinations of available receptor sites in tissue from a single animal. We now report a modified assay procedure which does not require cytosol fractionation and which is sufficiently sensitive to permit multiple determinations by saturation analysis of available receptor concentration in tissue from a single animal.

MATERIALS AND METHODS

All experiments employed ventral prostate tissue obtained from Sprague-Dawley rats (80-120 day old), 24 hours post orchidectomy. 5α-[1,2-$^3H_2$]dihydrotestosterone, specific activity 44 Ci/mmole, was obtained from New England Nuclear and was purified by paper chromatography before use (14). Human γ-globulin (fraction II) was from Miles Laboratories and Dextran T-70 was from Pharmacia Fine Chemicals, Inc. All other chemicals were the best reagent grade available and were used without further purification.

Cytosol was prepared in Buffer A (0.1 mM EDTA, 0.5 mM 2-mercaptoethanol, 50 mM Tris-Cl, pH 7.4) and radioactivity counted as previously described (13).

The assay for receptor bound 5α-[$^3H_2$]DHT,[1] was performed at 2° as follows: Freshly prepared cytosol (0.5 - 3.5 mg protein) was incubated for 2 hr in a total volume of 220 μl of Buffer A containing various concentrations of 5α-[$^3H_2$]DHT (0.25 - 10 nM). After this incubation, 100 μl of a dextran γ-globulin coated charcoal suspension, (5% charcoal, 0.5% dextran, 1% γ-globulin (w/v) in Buffer A) were added and each incubation tube was vortexed briefly. After 10 min, 25 μl of absolute ethanol were added per tube and the mixture incubated for an additional 20 min. The charcoal was then sedimented by centrifugation for 5 min at 1000 x g and 200 μl aliquots of the supernatants were counted for tritium. Non-specific binding of radioligand was determined in parallel incubations containing a hundredfold excess of 5α-DHT.[2] Sucrose gradient analysis (5-20% sucrose in Buffer A) was performed with 200 μl aliquots of similarly-treated samples and sedimentation coefficients determined according to the procedure of Martin and Ames (15) employing human γ-globulin as an internal sedimentation standard. Gradients were fractionated and radioactivity was determined as previously described (17).

The high speed nuclear pellets from cytosol preparations were precipitated with TCA and extracted as previously described (16). Pellet DNA content was determined by the procedure of Webb and Levy (18) using salmon sperm DNA as standard. Cytosol protein was determined by the method of Lowry *et al.* (19) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The data of Figure 1A represent typical sucrose gradient analyses of the binding of 5α-[$^3H_2$]DHT by prostatic cytosol after treatment with DGCC.[3] In agreement with the reports of others (3,5) the inhibition of 5α-[$^3H_2$]DHT binding by nonradioactive ligand demonstrated two distinct classes of binding activity. The binding of 5α-[$^3H_2$]DHT (10 nM) to the more rapidly sedimenting species (9.8S) was readily inhibited by the addition of a tenfold excess of nonradioactive ligand and therefore represented binding to high affinity, limited capacity androgen receptor sites. The absence of inhibition in the 3-4S region of the gradient indicated ligand association primarily with non-specific binding components. As we have previously reported (13), these data demonstrate the inadequacy of this DGCC protocol for the specific measurement of receptor bound 5α-[$^3H_2$]DHT in unfractionated cytosol.

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[1] 5α-[$^3H_2$]DHT: 5α-[1,2-^{3}H]dihydrotestosterone.

[2] 5α-DHT: nonradioactive 5α-dihydrotestosterone.

[3] DGCC: dextran-γ-globulin coated charcoal suspension.

The presence of a small amount of ethanol in the DGCC-cytosol incubations essentially eliminated the non-specific binding of 5α-[$^3H_2$]DHT in the 3-4S region of the gradients as shown in Figure 1B. Greater than 90% of the

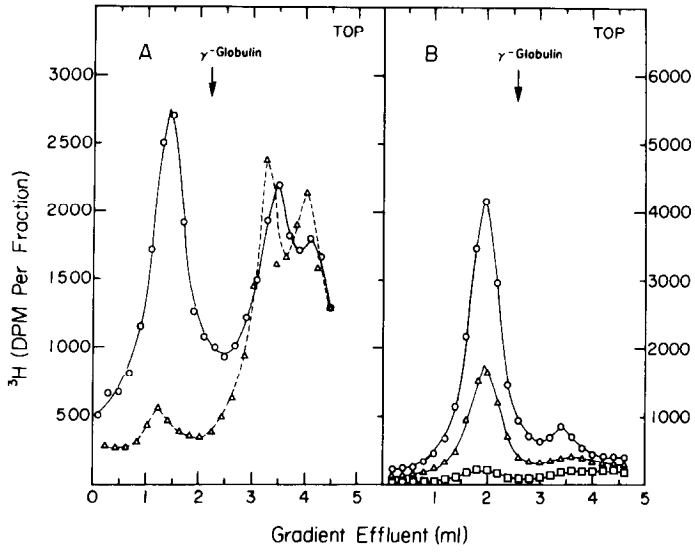

FIGURE 1. Sucrose gradient analyses of DGCC and DGCC/ethanol treated 5α-[$^3H_2$]DHT labeled cytosols. A. Cytosol (3.47 mg protein) was incubated with 10 nM 5α-[$^3H_2$]DHT in the presence (Δ-Δ) and absence (O-O) of 100 nM 5α-DHT and treated with DGCC as described in Methods (no ethanol). Samples (200 μl) of the DGCC supernatant were applied to sucrose gradients and developed at 100,000 x g for 20 hours at 2.5°. B. Cytosol (2.48 mg protein) was incubated with 10 nM 5α-[$^3H_2$]DHT in the presence of 15 nM (Δ-Δ), 200 nM (□ - □) or absence (O-O) of 5α-DHT and treated with DGCC and ethanol as described in Methods. Samples (200 μl) of the DGCC supernatant were applied to sucrose gradients and developed at 289,000 x g for 6.5 hours at 2.5°.

cytosol radioactivity remaining after DGCC/ethanol treatment sedimented as a single homogenous peak with a sedimentation coefficient, 9.1S, characteristic of the prostatic androgen receptor. Furthermore, near quantitative inhibition of 5α-[$^3H_2$]DHT binding was obtained in the presence of either a 1.5 or twentyfold excess of nonradioactive ligand. These data demonstrate that the DGCC/ethanol protocol was minimally 90% specific for the quantitation of receptor bound 5α-[$^3H_2$]DHT at a near saturating concentration of ligand (10 nM). It is not known at this time whether the small shoulder of

binding activity observed at 4.0S represents a distinct binding species or results from dissociation of ligand from receptor and reassociation with non-specific binding components which may occur during the gradient centrifugation.

Preliminary experiments similar to those previously described (13) indicated that a 2 hr incubation at 2° was sufficient to attain maximum receptor binding of radioligand and that the 20 min incubation of labeled cytosol with DGCC/ethanol did not result in a significant dissociation of receptor-ligand complexes (< 5%) and was adequate for the essentially complete charcoal adsorption of unbound and non-specifically bound ligand.

The results of a typical saturation analysis of ventral prostate cytosol as a function of the amount of cytosol protein assayed, employing the DGCC/ethanol assay protocol, is presented in Figure 2 according to the method of Scatchard (20). The linearity of the saturation data demonstrate that the protocol measures the binding of 5α-[$^3H_2$]DHT to a single class of binding sites. The validity of the protocol for determinations of total available receptor binding sites is further indicated by the direct proportionality

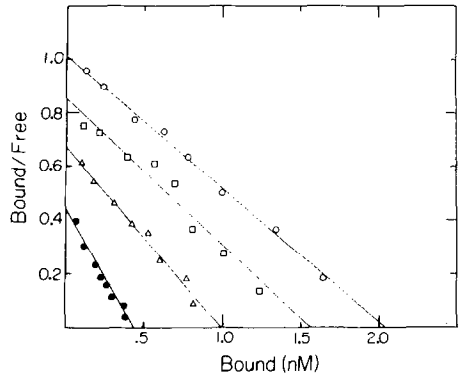

FIGURE 2. Saturation analysis of prostatic extracts at various concentrations of cytoplasmic protein. Aliquots of cytosol containing 0.84 (● - ●), 1.68 (Δ-Δ), 2.52 (□ - □) or 3.36 (O-O) mg protein were incubated with 5α-[$^3H_2$]DHT at the following concentrations: 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, and 10.0 nM and assayed with DGCC and ethanol as described in Methods.

between the apparent number of receptor sites and the amount of cytosol protein employed in the assay. As has been our routine observation, the apparent androgen binding constant progressively decreased as the amount of cytosol protein employed per assay tube increased. It is probable that the observation represents a reduction in the effective concentration of ligand available for receptor binding due to increased association with non-specific binding components at higher protein concentrations.

In Table 1 we compare the cytoplasmic ventral prostate androgen receptor

Table 1. Apparent Binding Constant and Concentration of Available Rat Ventral Prostate Androgen Receptor.

Assay Procedure	Number of Determinations	Receptor Sites per cell	Receptor Sites per mg protein ($\times 10^{-10}$)	K_B ($M^{-1} \times 10^{-8}$)
DGCC/ethanol	18	10,300±1780[b]	5.40±0.33	6.49±0.35
DGCC[a]	10	9,500± 780	5.15±0.31	6.87±0.24

a) Previously reported assay procedure (13).

b) Mean ± standard error.

content as determined by the presently reported protocol with the values obtained employing our previously reported assay procedure. Both protocols provide nearly identical estimates of the number of androgen receptor sites per cell or concentration per mg cytosol protein. Moreover, the latter estimates are very similar to the value of 7.23×10^{10} sites per mg cytoplasmic protein which can be calculated from the data of Wakeling and Visek (8) and support the validity of the present protocol. The data of Table 1 also demonstrate that the apparent androgen binding constant as estimated by either of our assay protocols is essentially identical.

We have also employed the DGCC/ethanol assay protocol to estimate the

number of available androgen receptor sites in ventral prostate cytoplasmic extracts prepared from tissue of intact animals. Two separate determinations indicated values of 960 and 850 sites per cell, demonstrating the marked sensitivity of the DGCC/ethanol protocol. This low level of available cytoplasmic androgen receptor in intact animals may represent 90 percent occupancy of receptor by androgen or cytoplasmic depletion subsequent to nuclear translocation.

The presently reported protocol has two very significant advantages relative to previously reported assay procedures (13). Firstly, the protocol does not require cytosol fractionation and the associated corrections for the loss of receptor binding sites. Secondly, assay sensitivity and specificity is increased due to a very marked reduction in the contribution of non-specific binding to the measured binding. Consequently a very broad range of ligand concentrations can be employed without requiring significant correction for non-specific binding. In routine assays we employ these corrections only when the non-specific binding contribution is greater than or equal to 10 percent of the observed binding at 10 nM 5α-[$^3H_2$]DHT. Employing this criteria, corrections are rarely required for analyses of cytosols from prostates of young mature animals obtained 24 hours post orchidectomy.

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