Purification and Characterization of Androgen Receptor from Steer Seminal Vesicle†

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ABSTRACT: The androgen receptor has been purified from steer seminal vesicle cytosol by a combination of differential DNA–Sepharose 4B chromatography and testosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine–Sepharose 4B affinity chromatography. The procedure produced about 3 μg of receptor protein from 35 g of steer seminal vesicle, with a yield of 48%. The receptor protein, as a complex with unlabeled testosterone, was purified approximately 540 000-fold. A single band, migrating at 60 000 daltons, was observed following electrophoresis on a polyacrylamide gel containing sodium dodecyl sulfate ($NaDodSO_4$). This was confirmed by affinity labeling of the partially purified receptor with both 17-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one and 17β-hydroxy-[1,2,4,5,6,7,16,17-$^3H_8$]-5α-androstan-3-one 17-(2-bromoacetate), which showed a peak of radioactivity migrating at 60 000 daltons by $NaDodSO_4$ gel electrophoresis. The receptor had an estimated Stokes radius of 35 Å and a sedimentation coefficient of 3.8 S in the presence of 0.3 M NaCl. The calculated molecular weight and frictional ratio for the androgen binding activity were 57 000 and 1.42, respectively. Chromatofocusing of the purified receptor protein revealed an isoelectric point of 6.6. [^{3}H]Methyltrienolone, bound to the purified receptor, was displaced with methyltrienolone > testosterone > 5α-dihydrotestosterone ≫ 3β-hydroxy-Δ^5-androsten-17-one ≫ 5α-androstane-3α,17β-diol. The physicochemical properties of the purified receptor were similar to those of the receptor in crude cytosol. These results demonstrate that the androgen receptor from steer seminal vesicle was substantially purified without significant modification of its physicochemical or steroid binding properties.

The biological function of androgens in male accessory sex organs is mediated by androgen receptor molecules [review articles by Liao et al. (1975), Mainwaring (1978), and Chan & Tindall (1981)]. A detailed knowledge of the biological and physicochemical properties of this protein should provide a better understanding of the mechanism of androgen action. Attempts to purify the androgen receptor from rat prostate have met with limited success (Mainwaring & Irving, 1973; Hu et al., 1978; Mainwaring & Johnson, 1980). Recently, we have characterized the physicochemical and steroid binding properties of the androgen receptor in the cytosol fraction from steer seminal vesicle. We found this tissue to be an excellent source of androgen receptor for purification because it is readily available, is of large size, and contains low concentrations of endogenous androgens. Since two techniques—differential DNA chromatography (Coty et al., 1979; Wrange et al., 1979; Westphal & Beato, 1980) and steroid affinity chromatography (Kuhn et al., 1975; Govindan & Sekeris, 1978; Sica & Bresciani, 1979)—have been used successfully for purifying other steroid hormone receptors, we have combined these two techniques in a sequence for the purification of the androgen receptor from steer seminal vesicle. This report describes this purification procedure as well as some of the physicochemical and steroid binding properties of the purified receptor protein.

Materials and Methods

Materials. The following materials were purchased: [1,2,6,7-$^3H_4$]testosterone (83 Ci/mmol) from Amersham; 17-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one ([^{3}H]-methyltrienolone)[1] (87.0 Ci/mmol), nonradioactive methyltrienolone, and Enhance from New England Nuclear (all other steroids were from Steraloids); ammonium sulfate, sucrose, and Tris (base) from Schwarz/Mann; bovine γ-globulin, deoxyribonucleic acid (calf thymus), pyridoxal 5′-phosphate, and bovine serum albumin from Sigma; activated charcoal (Norit A) from J. T. Baker; gelatin from Knox-gel; Na_2EDTA and sodium chloride from Fisher; *N*,*N*′-methylenebis(acrylamide), acrylamide, and *N*,*N*,*N*′,*N*′-tetramethylenediamine from Eastman; X-oMat AR film from Eastman Kodak Co.; blue dextran, aldolase, ovalbumin, chymotrypsinogen A, dextran T-70, Sephacryl S-200, ribonuclease A, and CNBr-activated Sepharose 4B from Pharmacia. Leupeptin was a gift from the United States–Japan Cooperative Cancer Research Program.

Preparation of Cytosol. Seminal vesicles were obtained fresh from a local slaughter house and frozen quickly in liquid nitrogen. The tissue was pulverized and homogenized in TEDG buffer containing 10 μg/mL leupeptin by using a Polytron PT10/30 homogenizer (Brinkman, Inc.) at a setting of 5 with five 10-s homogenizations and by allowing 30 s between homogenizations for cooling. The homogenates were centrifuged at 42 000 rpm (105000*g*) for 60 min at 0–2 °C in a Beckman 45 Ti rotor. The lipid layer was removed, and the supernatant fluid (11–25 mg of protein/mL) was transferred to prechilled tubes.

Hydroxylapatite Assay. The hydroxylapatite assay described by Peck & Clark (1977) was used in some of these

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[1] Abbreviations: [^{3}H]methyltrienolone, 17-hydroxy-17α-[^{3}H]-methyl-4,9,11-estratrien-3-one; methyltrienolone, 17-hydroxy-17α-4,9,11-estratrien-3-one; [^{3}H]dihydrotestosterone bromoacetate, 17β-hydroxy-[1,2,4,5,6,7,16,17-$^3H_8$]-5α-androstan-3-one 17-(2-bromoacetate); [^{3}H]R5020, 17α-[^{3}H]methyl-21-methyl-19-norpregna-4,9-diene-3,20-dione; leupeptin, a mixture of *N*-acetyl- and *N*-propionyl-L-leucyl-L-leucyl-DL-arginine aldehyde hydrochlorides; $NaDodSO_4$, sodium dodecyl sulfate; TED buffer, 50 mM Tris-HCl buffer containing 1.5 mM EDTA and 1.5 mM dithiothreitol (pH 7.4 at 22 °C); TEDG buffer, TED buffer containing 20% glycerol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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experiments. Bio-Gel HT (Bio-Rad) was washed extensively with ice-cold TEDG buffer until a pH of 7.5 was reached. A slurry of 60% (w/v) hydroxylapatite in TEDG buffer was prepared. Samples (250 μL of cytosol plus 250 μL of TEDG buffer) were incubated at 4 °C for 3 h with 16 nM [^{3}H]-methyltrienolone with or without 100-fold excess methyltrienolone. Aliquots (0.5 mL) of washed hydroxylapatite were added, and the mixture was incubated at 0 °C for 15 min with vortexing every 5 min. The tubes were centrifuged at 5000 rpm for 5 min and washed 3 times with 1.5-mL aliquots of TEDG buffer. The steroid was then extracted with 1 mL of absolute ethanol in a 30 °C water bath for 20 min. The ethanol extract was decanted for radioactivity determination.

Charcoal Binding Assay. Charcoal solutions were prepared according to the procedure of Korenman (1969) with minor modifications. Dry, washed charcoal (1%) was suspended in a solution containing 0.5% dextran T-70, 0.1% gelatin, 1.5 mM EDTA, and 50 mM Tris-HCl (pH 7.4 at 22 °C). Receptor preparations were incubated with 16 nM [^{3}H]methyltrienolone, ±1600 nM unlabeled methyltrienolone, for 8 h at 2 °C. After 10 min of charcoal treatment, samples were centrifuged at 5000 rpm for 15 min. The supernatant fluid was decanted for radioactivity determination.

Steroid Exchange Assay. All receptor preparations, after steroid affinity chromatography, were assayed for binding radioactivity by incubation with an equal volume of charcoal solution (described in the previous section) for 10 min at 2 °C. After centrifugation at 5000 rpm for 15 min, the supernatant fluids were incubated with 16 nM [^{3}H]methyltrienolone, ±1600 nM unlabeled methyltrienolone, for 30 min at 30 °C. At the end of incubation, the incubate was subjected to charcoal treatment as described previously. The supernatant fluid was decanted for radioactivity determination. We obtained ~80% efficiency of exchange using this protocol.

Preparation of DNA–Sepharose. DNA–Sepharose was prepared according to a modification of the procedure described by Arndt-Jovin et al. (1975). One gram of highly polymerized DNA (calf thymus) was dissolved in 26 mL of 0.1 N NaOH and washed 3 times with 95% ethanol. The DNA was resuspended in 22 mL of 5 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA and added to 1 L of 10 mM phosphate buffer (pH 8.0). CNBr-activated Sepharose 4B (15 g) was added gradually to the DNA suspension. After being shaken at 100 rpm overnight, the DNA resin was washed with 1 L of 10 mM phosphate buffer (pH 8.2), 1 L of 1 M phosphate buffer (pH 8.2), 1 L of 1 M NaCl, and 2 L of glass-distilled water. The resin was suspended in 50 mL of TED buffer containing 0.04% NaN_3 and stored at 4 °C.

Preparation of Testosterone Affinity Resin. The synthesis of testosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine–Sepharose 4B was performed according to the method described by Mickelson & Petra (1978) with modifications. CNBr-activated Sepharose 4B (15 g) was resuspended in 1 L of 1 mM HCl (prechilled at 4 °C) and filtered through a Millipore prefilter with slight suction and gentle stirring at 4 °C over a period of 30 min. The resin was added to 38 mL of 3,3′-diaminodipropylamine (DADA)–$NaHCO_3$ (13:1 molar ratio). The mixture was shaken gently for 15 h at 4 °C. The DADA–Sepharose 4B was washed with 2 L of 1 M NaCl and then with 1 L of glass-distilled water at room temperature. The washed DADA–Sepharose 4B was resuspended in a flask containing 50 mL of 1.0 M ethanolamine (pH 9.0) and shaken gently for 2 h at room temperature. After incubation, the DADA–Sepharose 4B was washed with 2 L of 1 M NaCl and 1 L of glass-distilled water. The DADA–Sepharose 4B was washed with 150 mL of 70% dioxane. The resin was resuspended in 50 mL of 70% dioxane containing 250 mg of testosterone 17β-hemisuccinate. Three milliliters of a carbodiimide reagent [2.5 g of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide dissolved in 6 mL of glass-distilled water] was added to the mixture and shaken gently in a 250-mL siliconized flask with a mechanical shaker for 4 h at room temperature. Carbodiimide reagent (3 mL) was added at the end of the 4-h incubation, and the reaction was continued for 15 h at room temperature. The resin was washed with 2.5 L of 100% dioxane on a coarse disk sintered-glass funnel. The resin was packed in a 60-mL syringe and washed with 5 L of 80% aqueous methanol over a period of 3–4 days. The resin was removed from the syringe and washed with 2.5 L of glass-distilled water by using a coarse disk sintered-glass funnel with slight suction. After being washed with 1 L of TEDG buffer, the testosterone affinity resin was ready for use.

Chromatofocusing. A modification of the chromatofocusing technique described by Sluyterman & Elgersma (1978) was used to determine the isoelectric point of the androgen receptor. Polybuffer ion-exchange resin (PBE 94) (Pharmacia) was poured into a column (0.9 × 24 cm) and equilibrated with 250 mL of 25 mM imidazole hydrochloride buffer (pH 7.4). Polybuffer 74 (Pharmacia) was adjusted to pH 4.0, and 5 mL was applied to the column before sample application. [^{3}H]-Methyltrienolone-labeled samples, preequilibrated with 25 mM imidazole hydrochloride buffer, were applied to the column. Proteins were eluted with Polybuffer 74 (pH 4.0). Two-milliliter fractions were collected.

Gel Filtration. A Sepharcyl S-200 column (2.5 × 50 cm) was equilibrated with TED buffer containing 0.3 M NaCl. Samples of 2 mL were applied. Columns were eluted under a hydrostatic pressure of 15 cm, and fractions of 2 mL were collected. Aliquots of 200 μL from each fraction were assayed for specific binding activity by an exchange assay. Marker proteins were located by measuring their optical density at 280 nm. The column was calibrated with blue dextran and 10 mg/mL of each of the following proteins: ferritin (79 Å), bovine γ-globulin (52 Å), aldolase (47 Å), bovine serum albumin (35 Å), ovalbumin (28 Å), chymotrypsinogen A (22.5 Å), and ribonuclease A (18 Å). The Stokes radius was estimated by graphical analysis of $(V_e/V_0)^{1/3}$ vs. the Stokes radii of standard proteins, where V_e is the eluted volume of protein and V_0 is the void volume measured with blue dextran.

Sucrose Gradient Sedimentation. Linear 5–20% (w/v) sucrose gradients of 5 mL were prepared in TED buffer containing 10% glycerol and 0.3 M NaCl. Samples of 250 μL of supernate were layered onto the gradients. Ovalbumin (3.5 S) and bovine γ-globulin (6.9 S) at concentrations of 5 mg/mL were used as internal markers. Gradients were centrifuged for 1.5 h at 65 000 rpm in a Beckman VTi65 rotor at 0–2 °C. Fractions (200 μL) were collected from the bottom of the tubes and were assayed for specific binding activity by an exchange assay. Marker proteins were located by adding 1 mL of glass-distilled water to each fraction and measuring their optical density at 280 nm. Stokes radii and sedimentation coefficients of standard proteins were used to determine molecular weights and frictional ratios (f/f_0) of the binding proteins as described by Siegel & Monty (1966).

$NaDodSO_4$ Gel Electrophoresis. The procedure described previously by Laemmli (1970) for $NaDodSO_4$ gel electrophoresis was used. The total acrylamide concentration in the gel was 7.5%. The following protein standards were used for molecular weight calibration: phosphorylase A, 93 000; bovine serum albumin, 69 000; L-glutamic dehydrogenase, 53 000;

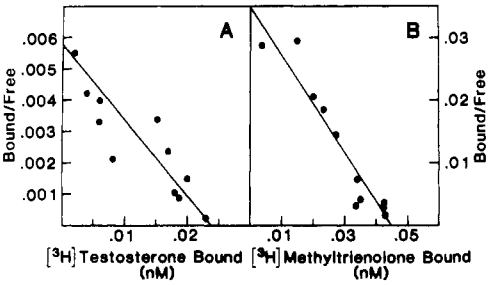

FIGURE 1: Scatchard analysis of androgen binding components in steer seminal vesicle cytosol. (A) Scatchard analysis of [^{3}H]testosterone binding. Cytosol (11 mg of protein/mL) was incubated with various concentrations of [^{3}H]testosterone ± 100-fold excess unlabeled testosterone for 4 h at 2 °C. The concentrations of [^{3}H]testosterone used were 0.1, 0.4, 0.7, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, 16.0, and 32.0 nM. Bound and free activities were separated by using hydroxylapatite as described under Materials and Methods. Binding analysis was performed according to that of Scatchard (1949). (B) Scatchard analysis of [^{3}H]methyltrienolone binding. Cytosol (20 mg of protein/mL) was incubated with [^{3}H]methyltrienolone ± 100-fold excess unlabeled methyltrienolone as described above, and Scatchard analysis was performed.

ovalbumin, 46 000; glyceraldehyde-3-phosphate dehydrogenase, 36 000.

Affinity Labeling. Bromoacetate derivatives of 5α-dihydrotestosterone were synthesized as described by LeGaillard & Dantrevaux (1977). Aliquots of partially purified receptor were resuspended in 10 mM phosphate buffer (pH 7.4) and incubated with either 20 nM 17-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one ([^{3}H]methyltrienolone; 87 Ci/mmol) or 20 nM 17β-hydroxy-[1,2,4,5,6,7,16,17-$^3H_8$]-5α-androstan-3-one 17-(2-bromoacetate) ([^{3}H]dihydrotestosterone bromoacetate; 147 Ci/mmol) in the presence or absence of excess unlabeled ligands, in an ice bath (0–2 °C) for 7 h. The incubation mixtures were further treated at 22.5 °C for 30 min, for the exchange of ligands and activation of covalent attachment. Samples incubated with [^{3}H]methyltrienolone were photolyzed for 30 min in an ice bath (0–2 °C) with an 85-W low-pressure mercury vapor lamp (General Electric H85A3). All samples were treated with equal volume of 20% trichloroacetate for 15 min and centrifuged at 4000 rpm for 20 min. The precipitant was redissolved in sample buffer and applied to an $NaDodSO_4$ gel electrophoresis (as described in the previous section). A 10% acrylamide gel, with diallyltartadiimide as a cross-linker, was used in $NaDodSO_4$ gel electrophoresis. Following electrophoresis, gels were washed extensively with 40% methanol–7% acetate to remove unbound steroid, treated with Enhance (New England Nuclear, Inc.), dried, and exposed to Kodak X-oMat AR film.

Protein Determinations. The quantity of protein in each sample was determined by using the procedure of Lowry et al. (1951). Bovine serum albumin was used as the protein standard.

Radioactivity Determinations. Scintillation fluid was prepared by mixing 160 mL of Liquiflour (New England Nuclear, Inc.) with 3.8 L of toluene. Samples were counted in a Beckman LS300 liquid scintillation counter having 40% counting efficiency as determined with [^{3}H]toluene of known specific activity.

Results

Characterization of Androgen Receptor from Steer Seminal Vesicle Cytosol. In order to determine if the steer seminal vesicle contained a binding component with high affinity for androgens, we incubated cytosol with increasing concentrations of either [^{3}H]testosterone or [^{3}H]methyltrienolone, in the presence or absence of excess unlabeled androgens. Scatchard

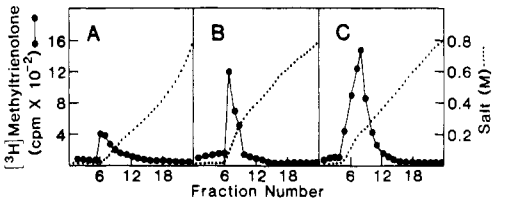

FIGURE 2: Activation and binding of receptor to DNA–cellulose. Cytosol (4 mL) from steer seminal vesicle was incubated with 16 nM [^{3}H]methyltrienolone for 4 h at 0 °C and then maintained at 0 °C (A), warmed at 21 °C for 30 min (B), or precipitated with ammonium sulfate (40% saturation) (C), before applying each sample to a DNA–cellulose column, as described under Materials and methods: radioactivity (closed circles); salt (NaCl) gradient (dotted line).

analyses (Scatchard, 1949) of both testosterone (Figure 1A) and methyltrienolone (Figure 1B) binding in steer seminal vesicle cytosol revealed single high-affinity binding components for both testosterone (K_d = 4.0 nM) and methyltrienolone (K_d = 1.3 nM). The number of binding sites for these androgens was between 20 and 60 fmol/mg of protein.

It is generally accepted that steroid receptor molecules have to undergo an "activation" phenomenon before they will bind to either nuclei or DNA. This has been demonstrated with many receptors in vitro by using DNA–cellulose resin and by activating with heat or ammonium sulfate. Therefore, we tested the ability of the seminal vesicle protein to bind to DNA–Sepharose before and after activation. When [^{3}H]-methyltrienolone-labeled cytosol was maintained at 4 °C, the majority of the binding activity was found in the flow-through fractions from a DNA–Sepharose column, and very little activity was eluted with salt (Figure 2A). When labeled cytosol was warmed to 21 °C for 30 min, the majority of the receptor bound to the DNA and was eluted at 0.15 M NaCl (Figure 2B). Precipitation of cytosol with ammonium sulfate at 40% saturation resulted in a 4-fold increase in DNA binding (as compared to that maintained at 4 °C), and a slightly higher salt concentration was required for elution (Figure 2C).

Since pyridoxal phosphate has been shown previously (Cake et al., 1978; Wrange et al., 1979; Cidlowski, (1980) to selectively elute glucocorticoid receptor proteins from DNA–resins, we determined whether androgen receptor could also be eluted with this reagent. Indeed, the receptor was eluted from a DNA–Sepharose column with a gradient of 0–50 mM pyridoxal 5′-phosphate in 25 mM sodium borate buffer (data not shown). Further elution with 0.5 M NaCl did not recover any receptor binding activity.

Purification of Androgen Receptor. Our laboratory has utilized androgen affinity chromatography previously for the purification of androgen binding protein (ABP) from rat epididymis (Tindall & Means, 1980). Therefore, a testosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine–Sepharose 4B resin was synthesized and tested for its ability to purify the steer seminal vesicle receptor.

First, optimal conditions for binding the receptor to the affinity resin were determined. Ammonium sulfate precipitated receptor (40% saturation) was incubated at 2 °C with the androgen affinity resin. At different time periods, the supernatant fluid was assayed for hormone binding activity remaining in solution. Approximately 60% of the receptor binding activity was bound to the affinity resin within 15 min, and this increased gradually to 85% after 75 min (Figure 3A).

Next, the time required to elute receptor from the affinity resin was determined. Ammonium sulfate purified receptor was incubated with the affinity resin for 2.5 h. After centrifugation at 2000 rpm for 10 min, the resin was washed extensively with 0.5 M NaCl in TED buffer. The resin was

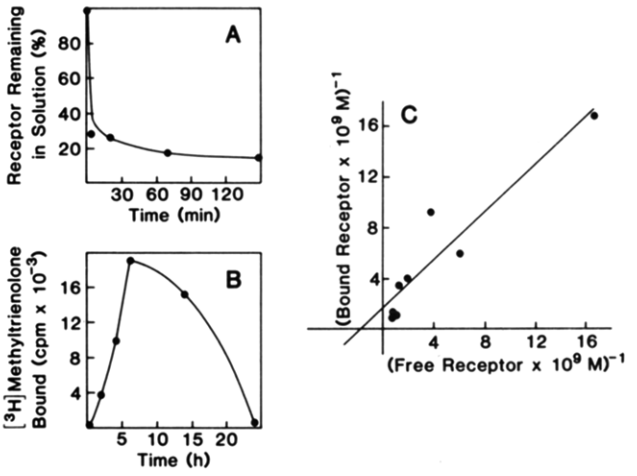

FIGURE 3: Kinetics of androgen receptor–testosterone affinity resin interaction. (A) Binding of androgen receptor to the testosterone affinity resin. Steer seminal vesicle cytosol was partially purified with 40% ammonium sulfate precipitation and resuspended in $^1/_6$ the original volume with TEDG buffer. The resulting solution (15 mL) was incubated with 10 mL of androgen affinity resin (testosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine–Sepharose 4B) with constant shaking. Aliquots (1 mL) were removed at different time periods and centrifuged. The supernatant fluid was labeled with 16 nM [^{3}H]methyltrienolone ± 100-fold excess unlabeled methyltrienolone for 3 h and assayed by charcoal adsorption. The specific hormone binding activity remaining in solution at 0 min of incubation was 9100 cpm and is represented as 100% in the figure. (B) Elution of the androgen receptor from the affinity resin. Affinity resin loaded with receptor was washed extensively with 0.5 M NaCl in TED buffer and eluted with TED buffer containing 100 μg/mL testosterone for varying intervals of time. For termination of elution, aliquots (1 mL) were taken and centrifuged at 2000 rpm for 15 min at different time intervals up to 18 h. The supernatant fluid was treated with equal volumes of charcoal solution and was assayed for binding activity by charcoal adsorption. (C) Binding affinity of receptor to testosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine–Separose 4B. Steer seminal vesicle cytosol was precipitated in $^1/_6$ the original volume. Increasing amounts of receptor solution in a constant volume of 3 mL were incubated with 0.5 mL of affinity resin (1:1 in TEDG buffer). After a 2-h shaking, the resin was removed by centrifugation, and 0.25-mL aliquots were labeled with 16 nM [^{3}H]methyltrienolone ● 250-fold excess methyltrienolone. The amount of specific binding activity remaining in solution was determined by charcoal adsorption assay. Lineweaver–Burk plot analysis was used to calculate the binding kinetics of the receptor protein–affinity resin interaction.

eluted with TEDG buffer containing 100 μg/mL testosterone. At different time intervals, aliquots of the resin were centrifuged, and the supernatant fluid was decanted for receptor assay by the exchange assay described under Materials and Methods. Approximately 50% of the maximal binding activity was eluted after a 4-h incubation. Maximal elution was achieved within 6 h. Thereafter, binding activity decreased, and by 24 h very little binding activity was detected, possibly due to receptor degradation (Figure 3B).

In order to determine the binding capacity of the resin, we incubated a constant amount of testosterone affinity resin (0.25 mL) with increasing amounts of ammonium sulfate precipitant and adjusted it to a constant volume with TEDG buffer. After a 2-h gentle shaking at 2 °C, the resin was centrifuged, and the receptor remaining in the supernatant fraction was measured by the charcoal binding assay. The amount of receptor that was removed from the solution by the affinity resin was determined by comparing the amount of receptor remaining in solution with that of a control solution that had been incubated with Sepharose 4B alone. Lineweaver–Burk analysis (Figure 3C) revealed that the testosterone 17β-hemisuccinyl-3,3′-diaminopropylamine–Sepharose 4B resin has a capacity of 7.2 pmol/mL of packed resin, with a dissociation

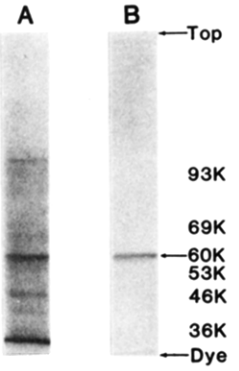

FIGURE 4: $NaDodSO_4$ gel electrophoresis of purified proteins from steer seminal vesicle cytosol. The electrophoresis procedure was described under Materials and Methods. (A) Testosterone affinity and differential DNA–Sepharose 4B chromatography (salt elution). Steer seminal vesicle cytosol was precipitated in $^1/_6$ the original volume. The ammonium sulfate precipitant was applied to the testosterone affinity resin and eluted with 100 μg/mL testosterone in TEDG buffer before being applied to the second DNA column. The receptor bound to the second DNA column was eluted with 0.5 M NaCl in TED buffer. Peak fractions of androgen binding activity were precipitated with 10% trichloroacetate, and 10 μg of protein was applied to an $NaDodSO_4$ gel. (B) Testosterone affinity and differential DNA–Sepharose chromatography (pyridoxal phosphate elution). The procedure is the same as described in Figure 4A except that 10 mM pyridoxal 5′-phosphate was used to elute the protein. Peak fractions of androgen binding activity were precipitated with 10% trichloroacetate, and 1 μg of protein was applied to an $NaDodSO_4$ gel.

constant of 0.5 nM. This latter value is comparable to the dissociation constant (4.0 nM) obtained for the binding of [^{3}H]testosterone to the cytoplasmic receptor (Figure 1), indicating that the receptor bound to the resin through the hormone binding site and not nonspecifically.

A pilot study was conducted to determine if both differential DNA chromatography and androgen affinity chromatography could be utilized to purify the receptor. Steer seminal vesicle cytosol was passed through a DNA–Sepharose 4B column, activated by precipitation with ammonium sulfate, and then applied to the testosterone 17β-hemisuccinyl-3,3′-diaminopropylamine–Sepharose 4B resin. After an extensive washing of the resin the receptor was eluted with 100 μg/mL testosterone in TEDG buffer. The eluate was applied to a second DNA–Sepharose 4B column and eluted with 0.5 M NaCl. This protocol resulted in an 8000-fold purification with a 50% yield. The $NaDodSO_4$ gel profile of the eluted proteins can be seen in Figure 4A. A major band at 60 000 daltons was observed with a number of minor bands.

Utilizing the previous protocol, we tried eluting the second DNA column with pyridoxal 5′-phosphate instead of sodium chloride. After the addition of 10 mM pyridoxal 5′-phosphate, one peak of androgen binding activity was eluted from the DNA II column. The peak tubes were pooled, precipitated with 10% trichloroacetate, and applied to an $NaDodSO_4$–polyacrylamide gel. The electrophoretic profile can be seen in Figure 4B. One band migrating at 60 000 daltons can be seen in this polyacrylamide gel.

Purification Protocol. On the basis of the pilot studies described above, we have established a rapid and efficient procedure for the routine purification of the androgen receptor, which takes approximately 2 working days to complete (see Table I). Cytosol was prepared from 35 g of steer seminal vesicle according to the procedure described under Materials and Methods. The cytosol (140 mL) was applied at a flow rate of 7.8 mL cm^{-2} h^{-1} to a column (5.5 × cm) containing 50 mL of packed DNA–Sepharose 4B (DNA I column), preequilibrated with TED buffer. The column was washed

Table I: Purification of Cytoplasmic Androgen Receptor from Steer Seminal Vesicle Cytosol

step	total protein (mg)	total receptor sites (total pmol)	sp act.[a] (pmol/mg)	yield (%)	x-fold purifcn
cytosol	3340[b]	77.6[c]	0.023	100	1.0
DNA I flow through	1960[b]	71.8[c]	0.037	93	1.6
40% pellet	925[b]	87.9[c]	0.095	113	4.1
affinity eluate	3.25[b]	43.2[d]	13.3	56	5.7×10^2
DNA II eluate (10 mM pyridoxal 5'-phosphate)	0.003[e]	37.4[d]	12500	48	5.4×10^5

[a] For a protein of M_r 60 000, the maximum theoretical specific activity is 16 600 pmol/mg. [b] Protein was assayed by the procedure of Lowry et al. (1951). [c] Receptor binding activity was assayed by the charcoal binding assay described under Materials and Methods. [d] Receptor binding activity was assayed by the steroid exchange assay described under Materials and Methods. [e] Protein was estimated from the Coomassie staining on the $NaDodSO_4$ gel as compared with bovine serum albumin at different concentrations.

with TED buffer until the optical density (280 nm) of the flow-through fractions approached zero. The flow-through fractions were pooled. This step resulted in a 1.6-fold purification and a 93% recovery of binding activity.

Next, the DNA I flow-through fractions were precipitated with ammonium sulfate at 40% saturation by the addition of ammonium sulfate crystals over a 30-min period. Precipitation was allowed to proceed for an additional 30 min, and the precipitant was centrifuged at 18 000 rpm for 15 min in a Beckman JA-20 rotor. This step resulted in an overall purification of 4.1-fold and a recovery of 113%. Whether this increased binding activity was a result of the removal of binding inhibitors is not known at the present time. As mentioned previously, ammonium sulfate precipitation serves to activate the receptor. The pellet was stored frozen at −90 °C overnight.

Next, the frozen pellets from the ammonium sulfate precipitation step were thawed quickly under running tap water (25 °C), resuspended in 22.4 mL of TEDG buffer, and stirred with 16 g of testosterone 17β-hemisuccinyl-3,3'-diaminodipropylamine–Sepharose 4B resin, which had been preequilibrated with TEDG buffer. After gentle stirring for 75 min at 2 °C, the resin was washed in a sintered-glass funnel with TED buffer containing 0.5 M NaCl until the optical density reading approached zero. The resin was resuspended in 22.4 mL of TEDG buffer containing 100 μg/mL testosterone and incubated for 6 h at 2 °C with gentle stirring. At the end of 6 h, the incubating mixture was filtered through a sintered-glass funnel and washed with 45 mL of TEDG buffer. A total of 65 mL of filtrate was collected. This step alone resulted in approximately 150-fold purification (Table I). The overall purification at this point was 570-fold, and a 56% yield of binding activity was obtained. However, it should be pointed out that binding activity may have been underestimated due to the presence of a large excess of testosterone.

The filtrate from the androgen affinity resin (65 mL) was applied to a column containing 5 mL of Sephadex G-75 as a plug and a 12-mL bed volume of DNA–Sepharose 4B (DNA II column) equilibrated with TED buffer. After the filtrate had entered the column, 50 mL of TED buffer was added to wash the column. This column was then equilibrated with 50 mL of sodium borate buffer (pH 8.1), and the receptor was eluted with 50 mL of 10 mM pyridoxal 5'-phosphate in sodium borate buffer. This final purification step resulted in an overall purification of 540 000-fold and a yield of 48% (Table I). Approximately 3 μg of purified protein was obtained by using this protocol. The purified receptor was stable for more than 2 weeks when stored at 2 °C.

The method described above allowed us to purify the receptor protein to near homogeneity. If a molecular weight of 60 000 for this protein band and one ligand site per molecule are assumed, the final specific activity is about 75% of the theoretical value. Since quantitative binding of ligand was not in all probability obtained, we expect that the purified material is essentially homogeneous and that the specific activity less than theoretical is due to denaturation of some of the hormone binding sites.

Table II: Specificity of Androgen Binding of Purified Receptors[a]

steroids	% bound[b] purified receptor	% bound[b] cytosol[c]
[^{3}H] methyltrienolone[d]	100	100
+4000 nM methyltrienolone	4	4
+4000 nM testosterone	9	8
+4000 nM 5α-dihydrotestosterone	20	11
+4000 nM 3β-hydroxy-Δ^5-androsten-17-one	72	48
+4000 nM 5α-androstane-3α,17β-diol	93	99

[a] Receptor was purified as described in the text and incubated with [^{3}H] methyltrienolone in the absence or presence of competitor under the exchange conditions described under Materials and Methods. [b] 100% bound radioactivity equals 1400 cpm and 1000 cpm for purified receptor and crude cytosol, respectively. [c] Cytosol was labeled as described in this table and assayed for binding activity by hydroxylapatite assay as described under Materials and Methods. [d] The purified receptor was labeled with 16 nM [^{3}H]-methyltrienolone. Cytosol was labeled with 0.25 nM [^{3}H]-methyltrienolone.

Characterization of Purified Receptor. The physicochemical properties of the purified androgen receptor were studied. First, the specificity of steroid binding was examined. The results of this study are shown in Table II. Methyltrienolone displaced 96% of the [^{3}H]methyltrienolone from the receptor. Testosterone displaced 91% and 5α-dihydrotestosterone displaced 80% of the [^{3}H]methyltrienolone. Neither 3β-hydroxy-Δ^5-androsten-17-one nor 5α-androstane-3α,17β-diol were very effective in competing with [^{3}H]methyltrienolone for receptor binding (28 and 7%, respectively). The relative affinities in which these steroids competed for receptor binding were similar to those found in crude cytosol (Table II).

The molecular charge of the androgen receptor was examined. Purified receptor was labeled with 16 nM [^{3}H]-methyltrienolone and applied to a chromatofocusing column. The column was eluted with a pH buffer gradient from 7.4 to 4. The binding activity eluted as a single peak at pH 6.6 (Figure 5), which was similar to the isoelectric point of the receptor in crude cytosol (solid arrow, Figure 5). Bovine serum albumin, used as an internal marker, was eluted at pH 5.1 from this column.

The Stokes radius of the purified receptor was determined. The purified receptor was applied to a gel filtration column (Sephacryl S-200) and eluted with TED buffer containing 0.3 M NaCl. Two peaks of binding activity were eluted from this column (Figure 6). A small peak of binding activity was eluted in the void volume while the majority of the binding

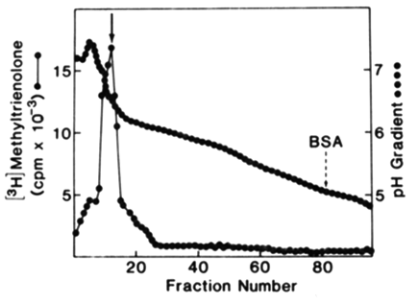

FIGURE 5: Chromatofocusing of purified receptor androgen binding activity. Purified androgen receptor was labeled with [^{3}H]-methyltrienolone by using the exchange assay described under Materials and Methods. The resulting supernatant fluid (approximately 0.1 μg of protein) was applied to a Sephadex G-25 column (0.8 × 20 cm) preequilibrated with 25 mM imidazole hydrochloride (pH 7.4). The column was eluted with 25 mM imidazole hydrochloride (pH 7.4). Peak fractions of bound radioactivity were pooled and applied to the chromatofocusing column as described under Materials and Methods: binding activities (closed circles); pH gradient (dotted line). Bovine serum albumin (BSA) was cochromatographed as an internal marker (broken arrow). The isoelectric point determined in cytosol preparation is denoted by the solid arrow.

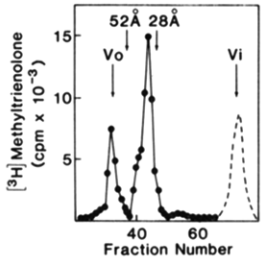

FIGURE 6: Gel filtration chromatography of purified receptor androgen binding activity. An aliquot (1 mL) of purified androgen receptor preparation was applied to a Sephacryl S-200 column (2.5 × 50 cm), equilibrated with TED buffer containing 0.3 M NaCl and eluted with the same buffer. The column was calibrated with standard proteins as described under Materials and Methods. Fractions of 2 mL were collected under a hydrostatic pressure of 15 cm. Aliquots (150 μL) of each fraction were assayed for specifically [^{3}H]methyltrienolone binding activity by using the exchange assay described under Materials and Methods. Pyridoxal 5′-phosphate was detected in the included volume (V_i).

activity was eluted with a calculated Stokes radius of 35 Å. Similar peaks of binding activity, eluting in the void volume and at 35 Å, were observed in seminal vesicle cytosol (unpublished data). The presence of binding activity in the void volume would suggest that the nondenatured receptor has an alternate form of greater than 79 Å or that aggregation of the purified protein has taken place (Sherman et al., 1970; Schrader & O'Malley, 1972; Sica et al., 1973; Schrader et al., 1977).

The sedimentation properties of the receptor were also studied. When purified receptor was centrifuged through a 5–20% sucrose gradient, multiple peaks of binding activity were observed (data not shown). Only one of these peaks (3.8 S) coincided with that observed in crude cytosol (unpublished data). The other peaks of binding activity, observed at higher sedimentation coefficients, suggested that aggregation had occurred under these conditions. Using the estimated Stokes radius of 35 Å and the sedimentation coefficient of 3.8 S, we obtained a molecular weight of 57 000 and a frictional ratio of 1.42.

The molecular properties of the androgen receptor were examined by affinity labeling of the active steroid binding site as shown in Figure 7. Since a bromoacetate derivative of progesterone had been demonstrated to bind covalently to the human progesterone receptor (Holmes et al., 1981), we synthesized [^{3}H]dihydrotestosterone 17β-bromoacetate and tested it for its ability to covalently bind to the androgen receptor. [^{3}H]Dihydrotestosterone 17β-bromoacetate was incubated with a preparation of partially purified receptor. Fluorography of the $NaDodSO_4$ gels showed two bands of radioactivity at 60 000 and 70 000 daltons (lane A). When excess unlabeled dihydrotestosterone 17β-bromoacetate together with [^{3}H]-dihydrotesterone 17β-bromoacetate was incubated with the same receptor preparation, very little radioactivity was observed in the 60 000 and 70 000-dalton bands (lane B). For confirmation of these results, a second affinity label with properties different from [^{3}H]dihydrotestosterone 17β-bromoacetate was needed. Dure et al. (1980) had demonstrated the covalent attachment of a tritiated synthetic progestin, 17α-[^{3}H]methyl-21-methyl-19-norpregna-4,9-diene-3,20-dione ([^{3}H]R5020), to the chicken progesterone receptor following photoactivation. Since methyltrienolone is similar to R5020 in its conjugated double bands, we chose to test [^{3}H]methyltrienolone for affinity labeling the androgen receptor. [^{3}H]Methyltrienolone was incubated with a partially purified receptor preparation and photoactivated with ultraviolet irradiation. Fluorography of the $NaDodSO_4$ gels showed two bands of radioactivity that were similar in size to the 60 000- and 70 000-dalton components labeled with [^{3}H]dihydrotestosterone 17β-bromoacetate (lane C). Labeling of the receptor in the presence of excess unlabeled methyltrienolone showed very little radioactivity in these two bands (lane D). No competition was observed with progesterone, 17β-estradiol, dehydroepiandrosterone, or 5α-androstane-3α,17β-diol.

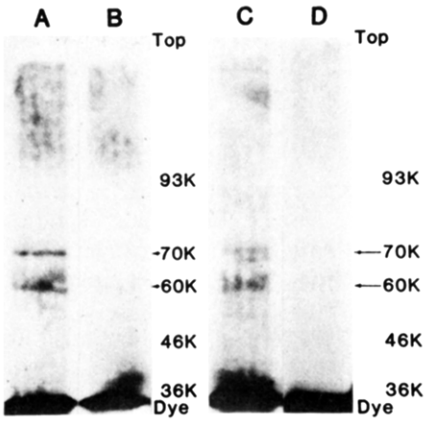

FIGURE 7: $NaDodSO_4$ gel electrophoretic profiles of the affinity-labeled androgen receptors. The receptor was sequentially purified by passing it through a DNA–Sepharose column, precipitating it with 40% ammonium sulfate saturation, and subjecting it to testosterone affinity chromatography. This partially purified receptor preparation was incubated with (A) 20 nM [^{3}H]dihydrotestosterone 17β-bromoacetate, (B) 20 nM [^{3}H]dihydrotestosterone bromoacetate plus 4000 nM unlabeled dihydrotestosterone bromoacetate, (C) 20 nM [^{3}H]methyltrienolone, or (D) 20 nM [^{3}H]methyltrienolone plus 4000 nM unlabeled methyltrienolone in an ice bath (0–2 °C) for 7 h. The incubation mixtures were further treated at 22.5 °C for 30 min. The [^{3}H]methyltrienolone mixtures were photolyzed for 30 min under an 85-W mercury lamp. Receptors were precipitated with an equal volume of a 20% trichloroacetate. The precipitant was redissolved in sample buffer, applied to an $NaDodSO_4$ gel, and electrophoresed. The autoradiographic treatment of the $NaDodSO_4$ gel was performed as described under Materials and Methods.

Discussion

This paper describes a rapid and efficient protocol for purifying the androgen receptor from steer seminal vesicle. The procedure was developed from a consideration of the biological and physicochemical properties of the receptor molecule. Because of the altered affinity of the receptor for DNA, before

and after activation, we have included DNA chromatography in the purification protocol. This technique has been used successfully to purify the progesterone (Coty et al., 1979) and glucocorticoid (Wrange et al., 1979; Westphal & Beato, 1980) receptors. We (Figure 4A) and others (Mainwaring & Irving, 1973; Kuhn et al., 1977; Yamamoto et al., 1974) have shown that elution of receptor with salt resulted in partial purification of the receptor. However, elution of the receptor from DNA–Sepharose with low concentrations of pyridoxal 5′-phosphate (10 mM) greatly improved the purification (as shown in Figure 4A,B). These findings agree with those of Wrange et al. (1979), who obtained optimal purification of glucocorticoid receptor from DNA–cellulose with pyridoxal 5′-phosphate.

Steroid affinity chromatography, utilizing the high affinity of receptor proteins for steroid molecules, is another powerful technique for receptor purification. Successful applications of this technique for receptor purification have been reported for progesterone (Kuhn et al., 1975), estrogen (Sica & Bresciani, 1979; Greene et al., 1980), and glucocorticoid (Govindan & Sekeris, 1978) receptors. We have, for the first time, combined both the differential DNA chromatography and the steroid affinity chromatography techniques in the purification of the androgen receptor from steer seminal vesicle cytosol. This procedure might be applicable for purifying other steroid hormone receptors.

The androgen receptor from steer seminal vesicle cytosol has a Stokes radius of 35 Å and a sedimentation coefficient of 3.8 S and was calculated to have a molecular weight of 57 000. This molecular weight for the androgen receptor in a crude cytosol preparation would suggest that the protein band observed in the $NaDodSO_4$ gel (Figure 4B) at 60 000 daltons is the receptor protein. Gel filtration and sucrose gradient analysis of the purified receptor also demonstrated peaks of binding activity at 35 Å and 3.8 S, respectively, which are in agreement with values for receptor in crude cytosol. However, larger molecular weight species were observed in both gel filtration and sucrose gradient studies. Even though these forms may represent larger forms of the nondenatured receptor, they most probably represent aggregates of the receptor in its purified form. This possibility is supported by the observations of others where aggregates of receptor proteins in both cytosol (Sherman et al., 1970; Schrader & O'Malley, 1972) and purified preparations (Sica et al., 1973; Schrader et al., 1977) were found on sucrose gradients and gel filtration columns.

The presence of a 60 000-dalton androgen binding component, as observed by Coomassie blue staining, was verified by two completely different androgen affinity labels. Affinity-labeling techniques have recently been used for characterizing the molecular weight of progesterone receptors from chick oviduct (Dure et al., 1980) and human uterus (Holmes et al., 1981). Recently, photoaffinity labeling of glucocorticoid receptors from cultured rat hepatoma (HTC) and mouse lymphoma (S49) cells has also been reported (Simons & Thompson, 1981; Nordeen et al., 1981). The chick oviduct progesterone receptor (Dure et al., 1980) was photoaffinity labeled with a tritiated synthetic progestin, 17α-[^{3}H]-methyl-21-methyl-19-norpregna-4,9-diene-3,20-dione. The $NaDodSO_4$ gel electrophoretic profiles of the photoaffinity-labeled receptor revealed multiple bands of radioactivity with prominent bands at 106 000 (B receptor), 78 000 (A receptor), and 39 000. In the present study, we also photoaffinity labeled the androgen receptor protein with [^{3}H]methyltrienolone and found two prominent bands at 70 000 and 60 000. When 21,16α-bis- and 11α-(bromoacetoxy)progesterones were used as the affinity-labeling ligands, the purified human progesterone receptor consistently migrated on $NaDodSO_4$ gels as a major binding component at 45 000 daltons. The present study, likewise, was able to demonstrate the affinity labeling of the androgen receptor with [^{3}H]dihydrotestosterone bromoacetate and revealed two prominent bands at 70 000 and 60 000, similar to those labeled with [^{3}H]methyltrienolone. Since the samples used for both affinity-labeling techniques had not been passed through the second DNA–Sepharose column, it seems probable that the 70 000 component was separated from the 60 000 component in this step and therefore was not observed in the pyridoxal 5′-phosphate eluate.

The purified receptor was found to have an isoelectric point of 6.6 (Figure 5), which was the same as that found for the receptor in crude cytosol. Thus, the charge properties of the receptor do not appear to be altered by the purification procedure. The isoelectric point of the seminal vesicle receptor is greater than that reported for the rat prostate (Mainwaring & Irving, 1973) and epididymal androgen receptors (5.8) in cytosol (Tindall et al., 1975). However, this discrepancy is probably due to differences in the techniques utilized in these studies. Indeed, bovine serum albumin, which has a reported isoelectric point of 4.7 by isoelectric focusing, was found in these studies to elute at pH 5.1 (Longsworth & Jacobsen, 1949). The fact that a single peak of binding activity was observed on the chromatofocusing column suggests that if aggregation of the purified receptor is indeed taking place, overall charge properties of the protein are not being affected.

This is the first report of an androgen receptor being purified to apparent homogeneity. However, the possibility cannot be excluded that we have isolated a breakdown product of the native receptor protein. This possibility exists because proteolysis of receptor proteins from a variety of tissues has been reported in the literature and because we observed in addition to the 60 000-dalton component a 70 000-dalton component that bound to both affinity labels. We have taken steps to ensure against proteolysis by including leupeptin in our homogenization buffer; however, there may have been proteolytic enzymes that were resistant to this inhibitor. Furthermore, a number of physicochemical properties of the purified receptor, including charge and molecular weight, are similar to those observed in cytosol. It should be pointed out that neither gel filtration nor sedimentation analysis would be able to differentiate between a 60 000- and 70 000-dalton species, and therefore both might be present in cytosol. Nevertheless, these studies demonstrate that we have isolated from the steer seminal vesicle a protein that contains both the androgen binding site and DNA binding region of the androgen receptor molecule. An understanding of the properties of the purified protein will contribute to a better understanding of the mechanism of androgen action in male accessory sex organs.

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