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Purification and Characterization of Androgen Binding Protein from the Rat Epididymis†

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ABSTRACT: Androgen binding protein (ABP) was purified from rat epididymides by sequential ammonium sulfate precipitation, affinity chromatography, gel filtration, and DEAE ion exchange chromatography. The column matrix formed by coupling dihydrotestosterone-17α-(hexanoic acid) to agarose via diisopropylamine was stable during the extensive washing required following application of crude tissue extracts to the affinity matrix. In addition, when used under the optimal conditions, the column produced a 1600-fold purification in a single step. Apparent homogeneity of the final product was shown by polyacrylamide gel electrophoresis, sedimentation equilibrium, and constant specific activity across the peak of the final chromatograph. The molecular weight determined by sedimentation equilibrium at pH 7.4 was 85000. By contrast, the molecular weight determined by sedimentation equilibrium in guanidine·HCl and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was approximately one-half that of the native protein, suggesting that ABP is comprised of subunits.

The Sertoli cells of the seminiferous tubules from rat testes secrete an androgen binding protein (ABP),[1] which can be assayed by its ability to bind testosterone or dihydrotestosterone (DHT) with high affinity (for a review, see Hansson et al., 1975). After it is secreted into the lumen of the seminiferous tubule, ABP is transported along with the spermatozoa to the epididymis, where it is concentrated and degraded. Several investigators have demonstrated that ABP synthesis in and secretion by the Sertoli cells are stimulated by testosterone and follicle-stimulating hormone (FSH). ABP is thus one of the few specific markers for hormone action on this key cell of the seminiferous tubule. We, therefore, thought it pertinent to purify and characterize this protein, so that it would be available as a reagent for subsequent studies on the hormonal control of Sertoli cell function. In this report we present a method for isolating homogeneous ABP from rat epididymides in high yield and describe the physical–chemical properties of this protein.

Materials and Methods

General. Protein was measured by a modification (Bensadoun & Weinstein, 1976) of the method of Lowry et al. (1951) and by the dye binding method of Bradford (1976). Bovine serum albumin was used as a standard. The former procedure was used to monitor recovery and specific activity changes, while the latter was used to screen column fractions in order to locate binding activity.

1,2-5α-[^{3}H]Dihydrotestosterone ([^{3}H]DHT), 44 Ci/mmol, was obtained from New England Nuclear Corp. and purified on silica gel thin-layer plates developed in chloroform–methanol (25:2). Radioinert DHT was obtained from Sigma Chemical Co.

[^{3}H]DHT in aqueous solution was quantified by liquid scintillation spectrometry using a fluor (toluene–Triton X-100 (2:1) containing 4 g of PPO/L and 50 mg of POPOP/L) at 27% counting efficiency. [^{3}H]DHT in polyacrylamide gel slices was measured using toluene-based fluor (4 g of PPO/L and 50 mg of POPOP/L) at an efficiency of 37%. 3,3′-Diaminodipropylamine was purchased from Aldrich Chemical Co.; Sepharose and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl were obtained from Sigma Chemical Co. Frozen epididymides were obtained from Dr. A. Parlow (Harbor General Hospital, Torrance, CA).

Column fractions were concentrated at 4 °C with constant stirring in Amicon ultrafiltration cells (Models TCF10 and

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[1] Abbreviations used: ABP, androgen binding protein; DHT, 5α-dihydrotestosterone; $NaDodSO_4$, sodium dodecyl sulfate; TeBG, testosterone–estradiol binding globulin; BSA, bovine serum albumin; DHT-HA, 3-oxo-17β-hydroxy-5α-androstane-17α-(6-hexanoic acid); PAS, periodic acid Schiff; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; TEG buffer, 20 mM Tris, 2 mM EDTA, and 10% glycerol; TG buffer, 20 mM Tris and 10% glycerol; TDK buffer, 20 mM Tris, 10% dimethylformamide, and 1 M KCl.

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52) using UM-20 membranes.

Synthesis of 3-Oxo-17β-hydroxy-5α-androstane-17α-(6-hexanoic acid) (DHT-HA). The synthesis of DHT-HA has been described in detail previously (Musto et al., 1977). Briefly, 3α,17β-dihydroxy-5α-androstane-17α-(6-hexyn-1-ol) was produced by coupling a six-carbon unsaturated alcohol (6-hexyn-1-ol) at the 17α position of 3α-hydroxy-5α-androstan-17-one using conditions described by Miller & Christinsen (1967). The triple bond in the six-carbon alkyl side chain was then hydrogenated with 10% palladium on carbon as a catalyst. The product, 3α,17β-dihydroxy-5α-androstane-17α-(6-hexan-1-ol), was then oxidized to DHT-HA with Jones reagent (chromium trioxide in acetone). The crude product was recrystallized three times from ethyl acetate. The melting point was 162–163 °C, and the purity by thin-layer chromatography (benzene–acetic acid–water, 5:5:1) was greater than 98%. The infrared and NMR spectra of this material were consistent with the proposed structure.

Preparation of Affinity Column Matrix. 3,3′-Diaminodipropylaminoagarose was prepared as described by Mickelson & Petra (1975) utilizing cyanogen bromide activated Sepharose 4B prepared by the method of March et al. (1974). Albumin-substituted agarose was prepared as described by Cuatrecasas (1970). The coupling of DHT-HA to agarose derivatives was accomplished in 70% dioxane with the water-soluble carbodiimide procedure (Parikh et al., 1974) using 1 mg of ligand and 10 mg of carbodiimide per mL of packed Sepharose. In order to ensure complete removal of noncovalently bound steroid, the finished product was washed extensively with 70% dioxane and then 80% methanol as recommended by Sica et al. (1973).

Assays for ABP Activity. Androgen binding protein was quantified by the steady-state polyacrylamide gel electrophoresis method of Ritzen et al. (1974) with the following modifications: (1) endogenous steroids were not removed by charcoal treatment; (2) samples were not preincubated with [^{3}H]DHT; (3) the stacking gels contained 5×10^{-9} M [^{3}H]DHT. The presence of high concentrations of androgen in samples did not interfere with the binding assay, since unbound steroid does not migrate into the gel and the bound steroid rapidly exchanges with the [^{3}H]DHT in the gel matrix. In addition, this method allows for the differentiation between steroid binding by ABP, serum albumin, and androgen receptor (Ritzen et al., 1974).

Column fractions were monitored for ABP activity with an assay in which 0.1 mL of sample was incubated with 2×10^{-9} M [^{3}H]DHT at 4 °C for 1 h. Free steroid was then removed by absorption with dextran-coated charcoal, followed by centrifugation at 2000*g* for 10 min (Musto & Bardin, 1976), and total bound steroid was determined from the radioactivity in the charcoal-free supernatant. Nonspecific binding was determined in a second tube containing [^{3}H]DHT plus a 200-fold excess of unlabeled steroid. Specific binding was calculated by subtracting nonspecifically bound [^{3}H]DHT from total bound [^{3}H]DHT. This assay allowed location of binding activity in column fractions with low specific activities. In more highly purified fractions, this procedure underestimated ABP binding activity by as much as fivefold due to absorption of protein by the charcoal. This underestimation could be prevented by the addition of 0.1% gelatin to each sample.

Cytosol Preparation and Ammonium Sulfate Fractionation. Frozen rat epididymides were partially thawed, minced, and homogenized in 9 volumes of TEG buffer (20 mM Tris, 2 mM EDTA, and 10% glycerol (v/v), pH 7.4 at 22 °C) and centrifuged at 17500*g* for 2.5 h. This and all subsequent operations were performed at 4 °C. The supernatant (cytosol) was made 2.1 M with respect to ammonium sulfate by slow addition of 0.32 g of powdered ammonium sulfate per mL of cytosol (Wood, 1976). After the precipitate was stirred for 1 h, it was collected by centrifugation (17500*g*, 45 min) and redissolved in TG buffer (20 mM Tris and 10% glycerol (v/v), pH 7.4 at 22 °C) at 0.45 mL per g of original tissue and centrifuged to remove insoluble material.

Affinity Chromatography. The affinity resin was suspended in TG buffer and packed in a 5-cm diameter column with a final bed volume of 250 mL. The solubilized pellet from the ammonium sulfate precipitate, derived from 540 g of epididymides, was added to the column and allowed to percolate under gravity flow into the bed. The column was washed with 2700 mL of TDK buffer (20 mM Tris, 10% dimethylformamide (v/v), 1 M KCl, pH 7.4 at 22 °C). In order to elute the specifically bound ABP, 250 mL of TDK buffer containing DHT (20 μg/mL) was added to the column, and the matrix-bound ABP was allowed to exchange with the free DHT for 10–16 h at 4 °C. This ABP was then displaced from the column by another 250 mL of the same buffer, and a second incubation for 3 h was performed. The process was repeated until a total of 750 mL was collected. These fractions were then pooled and concentrated.

Gel Filtration Chromatography. Preparative gel filtration was performed on a 5 × 50 cm column of Sephadex G-200 packed and eluted with TG buffer. Fifteen-milliliter fractions were collected at a flow rate of 32 mL/h. Analytical gel filtration was performed on a 1 × 100 cm column of Sephadex G-200 in TG buffer containing 0.25 M KCl.

Ion Exchange Chromatography. A DEAE-agarose column (1 × 2.5 cm) was equilibrated with TG buffer. The sample (approximately 1 mg of protein) was applied, and the column was washed with 3 column volumes of TG buffer. ABP was eluted with a 35-mL linear 0–0.1 M KCl gradient in TG at a flow rate of 25 mL/h.

Molecular Weight Determination by Sedimentation Equilibrium Ultracentrifugation. Molecular weights were determined with a Spinco Model E analytical ultracentrifuge equipped with Rayleigh optics. Interference patterns were developed on Kodak spectrophotographic plates, type IIG, and measured with a computer-linked Nikon Model 6 microcomparator as described by Carlisle et al. (1974). High-speed sedimentation equilibrium experiments were performed with a 12-mm liquid column (Ansevin et al., 1970) using the technique described by Yphantis (1964). Corrections for window distortion were made by running appropriate water blanks following each equilibrium run. The effective reduced molecular weight (σ_w) at various positions within the cell was calculated with the computer program described by Roark & Yphantis (1969). Values of σ_w at various positions within the cell were converted to actual molecular weight averages by multiplying σ_w by the factor $RT/\omega^2(1 - \bar{v}\rho)$. The density of the medium was experimentally determined, and the partial specific volume was calculated from the amino acid composition (Cohn & Edsall, 1943).

Amino Acid Composition. The amino acid composition was determined on a sample of purified ABP which was hydrolyzed in vacuo with 6 N HCl at 110 °C for 24 h. The hydrolysate was analyzed on a Beckman amino acid analyzer using a single column. No corrections were made for losses of various amino acids which may have occurred during hydrolysis.

Determination of Stokes' Radius. Stokes' radii were determined by gel filtration on an analytical Sephadex G-200

Table I:[a] Effects of Various Conditions on Affinity Chromatography of Androgen Binding Protein (ABP)

expt	leash[b]	wash buffer[c]	elution buffer[d]	ABP recovery (%)	sp act. (pmol of ABP/mg of protein)
1	BSA	Tris-glycerol-KCl[e]	Tris-glycerol	5	700
2	BSA	Tris-glycerol-KCl	Tris-glycerol-KCl	28	570
3	BSA	Tris-glycerol-KCl	Tris-dimethylformamide-KCl	75	119
4	dipropyldiamine	Tris-glycerol-KCl	Tris-glycerol-KCl	34	707
5	dipropyldiamine	Tris-glycerol-KCl	Tris-dimethylformamide-KCl	82	280
6	dipropyldiamine	Tris-dimethylformamide-KCl	Tris-dimethylformamide-KCl	95	1680

[a] Small columns (cm × cm) were packed with the appropriate affinity matrix and washed with 5 mL of buffer. The cytosol (1 mL of a 1:9 homogenate of epididymis) was added to each column followed by 10 volumes of buffer. The bound ABP was eluted by buffer containing 15 mg/mL DHT and measured by steady-state polyacrylamide gel electrophoresis (Ritzen et al., 1974). The protein was measured by the method of Lowry et al. (1951). [b] Indicates the compound used to couple the dihydrotestosteronehexanoic acid to the column. [c] Each column was washed with 10 column volumes of wash buffer. [d] Elution buffers all contained 15 μg/mL DHT. [e] Tris, 20 mM, pH 7.4; glycerol, 10%; KCl, 1 M; dimethylformamide, when used, 10%.

column using the method of Siegel & Monty (1966). The column was calibrated with the following standard proteins: yeast alcohol dehydrogenase (46 Å), catalase (52 Å), urease (61 Å), and ferritin (78 Å) (all from Sigma).

Polyacrylamide Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed at 0 °C in gels of 5–12% total gel concentration (% T), 2% cross-linking of methylenebis(acrylamide) (% C_{bis}), with multiphasic buffer systems 2860.0.X (pH 9.63) and 1935 (pH 7.42) as previously described (Chrambach et al., 1976). Stacking gels of 3.125% T and 20% C_{bis} were used. Polyacrylamide gels containing tritiated steroid were sliced and counted as previously described (Ritzen et al., 1974). Electrophoresis on polyacrylamide gels containing $NaDodSO_4$ (system 1000) was performed as described by Laemmli (1970). After incubation in sample buffer for 5 min at 95 °C, samples were fractionated in $NaDodSO_4$ gels of 6–12% T and 2.6% C_{bis} with a stacking gel of 3.08% T and 0.6% C_{bis}. The dye marker on all gels was bromophenol blue. Proteins were stained with Coomassie blue R-250 and PAS (Chrambach et al., 1967; Fairbanks et al., 1971). Relative mobility (R_f) of ABP was calculated as the ratio of binding protein migration distance to that of the dye front. The retardation coefficient (K_R) and the Y intercept on the Ferguson plot (Y_0) were computed as previously described (Rodbard & Chrambach, 1971). The values of geometric mean radius ($\bar{R}$) and molecular weight derived from K_R and valence (net protons/molecule) derived from Y_0 were computed by using the computer program PAGEPACK (Rodbard & Chrambach, 1971).

Isoelectric Focusing on Polyacrylamide Gels. Isoelectric focusing was performed at 0 °C as described by Nguyen et al. (1978). Ampholines in the pI range 3–10 (LKB Produckter, Bromma, Sweden) at a final concentration of 2% and glycerol at a final concentration of 5% were incorporated into the gel. The gel concentration for isoelectric focusing on polyacrylamide gels was 5% T and 15% C using the cross-linker *N,N*-diallyltartardiamide (DATD) (Bauman & Chrambach, 1976). Evans blue (pI = 5.35) and methylene blue (pI = 3.6) were used as pI markers. The anolyte and catholyte were 0.05 M Tris base containing 0.01 M CaO and 0.05 M glutamic acid, respectively. The gels were focused at 100 V for the first 3 h and then at 200 V for 15–20 h. The pH gradient was measured by using a Bio-Rad pH profiler. Staining of protein bands was accomplished by the method of Otavsky & Drysdale (1975).

Results

Affinity Chromatography. In order to maximize the effectiveness of the affinity chromatography, a series of preliminary experiments was performed with microcolumns (1-mL bed volume). In this study, the effects of two leash compounds (bovine serum albumin and dipropylamine), two washing buffers, and two elution buffers were evaluated with regard to their effects on recovery and final specific activity of ABP. The results of these experiments are summarized in Table I. Dipropylamine appeared to be the best leash compound (experiments 2 vs. 4; 3 vs. 5; Table I). The addition of dimethylformamide (10%) and KCl (1 M) to both the washing and the elution buffers resulted in the highest specific activities and greatest recoveries (experiment 6; Table I). The conditions shown in experiment 6 (Table I) were used in all subsequent affinity chromatography. In another experiment the functional capacity of the matrix was determined by adding increasing amounts of ABP to a series of microcolumns of known volume until ABP appeared in the effluent. When we used this method, a 1-mL bed volume of affinity matrix was able to bind the ABP from 2 g of epididymis.

Purification of Androgen Binding Protein. (a) Column Chromatography. The ammonium sulfate precipitate from 540 g of rat epididymides was applied to an affinity column in TG buffer and washed with TDK buffer until the OD_{280} in the column effluent was constant (Figure 1). A single protein peak was then eluted with dihydrotestosterone (6.6 μM in TDK buffer). Fractions 146–155 from the affinity column were pooled, concentrated, and further fractionated by preparative gel filtration (Sephadex G-200). The androgen binding protein chromatographed coincident with one of the two protein peaks (Figure 2). Fractions showing androgen binding activity (fractions 30–40) were pooled and applied directly to a DEAE-agarose column. The profile from the DEAE column revealed that the major peak of ABP activity eluted between 0.02 and 0.08 M KCl (Figure 3) and a minor peak of activity at high salt concentrations. These observations suggest charge heterogeneity of this protein. Fractions 12–32 of the major peak of activity were pooled, concentrated, and reapplied to the preparative gel filtration column as a final purification step. One major peak of protein and androgen binding activity was eluted as shown in Figure 4.

The purification scheme for ABP is summarized in Table II. The mean specific activities for ABP in unfractionated cytosol and in the final preparation were 2.6 and 16 800 pmol/mg of protein, respectively. An overall purification of greater than 6400-fold was achieved with 50% of this increase evident following affinity chromatography. Although ABP was greater than 90% pure after the first gel filtration, two additional steps were required to eliminate trace protein contaminants which could be demonstrated by polyacrylamide gel electrophoresis.

Table II: Purification of Rat Epididymal ABP

procedure	ABP[a] total (pmol)	ABP[a] sp act. (pmol/mg of protein)	total[b] protein (mg)	cumulative recovery (%)	x-fold purification each step	x-fold purification cumulative
cytosol	13 502	2.7	5076	100		
$(NH_4)_2SO_4$	12 827	5.4	2375	95	2	2
affinity chromatography	9 450	8 800	1.07	70	1600	3200
G-200 I	9 046	16 600	0.545	67	2	6400
DEAE	6 616	16 800	0.394	49	1.01	6400+
G-200 II	5 974	16 800	0.355	44	1.00	6400+

[a] Determined by steady-state polyacrylamide gel electrophoresis (Ritzen et al., 1974). [b] Measured by modified Lowry procedure (described under Materials and Methods) using bovine serum albumin as the standard.

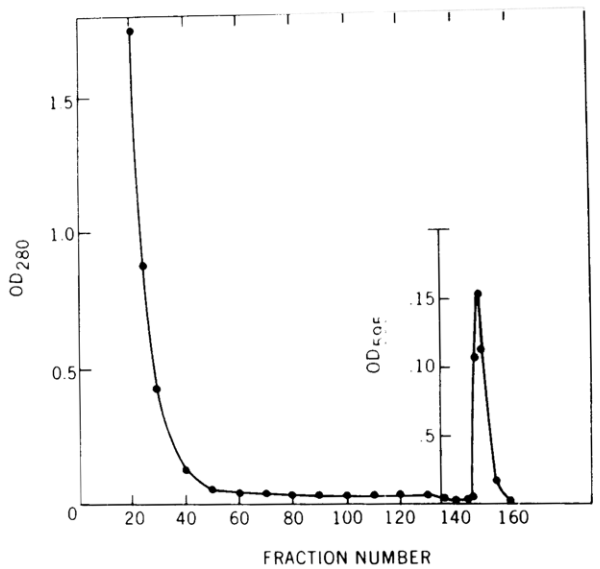

FIGURE 1: Affinity chromatography of rat epididymal androgen binding protein (ABP) on dihydrotestosterone-17α-hexanoic acid–Sepharose. The sample was applied to a 5 × 12 cm affinity column and washed with 2700 mL of TDK buffer using a flow rate of 200 mL/h. Twenty-milliliter fractions were collected. At fraction 135, DHT (20 μg/mL) was added to the buffer and the elution continued. The column-bound ABP was exchanged as described in the text. Protein was measured at 595 nm by the dye binding method of Bradford (1976). Fractions 140–160 were pooled and concentrated.

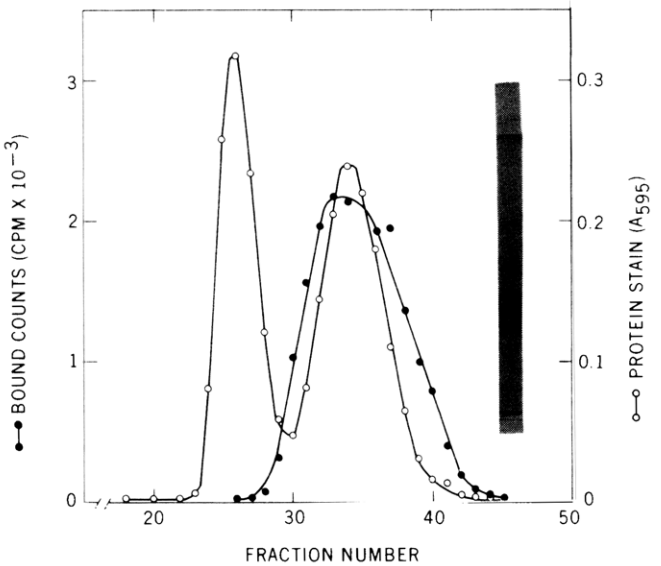

FIGURE 2: Gel filtration chromatography on Sephadex G-200. Fractions 140–160 from the affinity column were pooled, concentrated, and applied to a 5 × 50 cm column. Protein was eluted with TG buffer using a flow rate of 32 mL/h; 15-mL fractions were collected. Total protein and androgen binding protein were measured in an aliquot of each fraction by dye binding and dextran-coated charcoal assays, respectively, as described in the text. Fractions 30–40 were pooled and concentrated for further fractionation by ion exchange chromatography. The insert shows $NaDodSO_4$–polyacrylamide gel electrophoresis of this sample (load = 30 μg).

(*b*) *Assessment of Purity*. Fractions from the ascending side, top, and descending side of the protein peak eluted from the final gel filtration (Figure 4) were pooled. The specific activities of ABP in these three fractions could not be distinguished from one another. The pool of these three fractions was homogeneous in the ultracentrifuge (see below) and on native polyacrylamide gel electrophoresis at several acrylamide concentrations at both pH 9.63 and 7.42.

Although three independent techniques (gel filtration, ultracentrifugation, and polyacrylamide gel electrophoresis) suggested that the epididymal ABP was greater than 98% pure, examination by $NaDodSO_4$–polyacrylamide gel electrophoresis revealed two bands (45K and 41K). The mass ratio of these proteins as determined by amido black staining was 1:3 (Figure 5). This same ratio was also evident in the eluate from the affinity column and the fractions of each subsequent chromatography, as well as across the peak obtained from the last gel filtration.

Physical Properties of Androgen Binding Protein. (*a*) *Electrophoresis and Isoelectric Focusing on Polyacrylamide Gels.* The Ferguson plots of purified ABP were compared in native gel systems 1935 and 2860.0.X and in $NaDodSO_4$ gel system 1000. The physical properties calculated from these plots using the computer program PAGEPACK (Rodbard & Chrambach, 1971) are summarized in Table III. The mean molecular radii as determined in systems 1935 and 2860.0.X by comparison with appropriate protein standards were 3.56 and 3.20 nm, respectively. The molecular weights corresponding to these radii are 154 000 and 111 000, respectively. The accuracy of the molecular weight estimates for ABP depends on the goodness-of-fit of the Ferguson plots and on the validity of the slope and intercept of the standard curve of $\bar{R}$ vs. $(K_R)^{1/2}$ (where $\bar{R}$ is the mean molecular radius and K_R is the slope of the Ferguson plot). Since this curve has an appreciable 95% confidence interval due to the inherent error in the estimates of both $\bar{R}$ and $(K_R)^{1/2}$ for the standard proteins, the physical parameters of unknowns as estimated from the curve also have large confidence limits (Rodbard & Chrambach, 1971; Rodbard, 1976).

The individual weights of the two components (45K and 41K) observed in $NaDodSO_4$–polyacrylamide gel electrophoresis were constant at several gel concentrations (Figure 6). The molecular weights (41 000 and 45 000) determined at each gel concentration were similar and consistent with that determined from the Ferguson plots (Figure 6, Table III). This constant molecular weight at all gel concentrations together with the common Y intercept of the two ABP components and

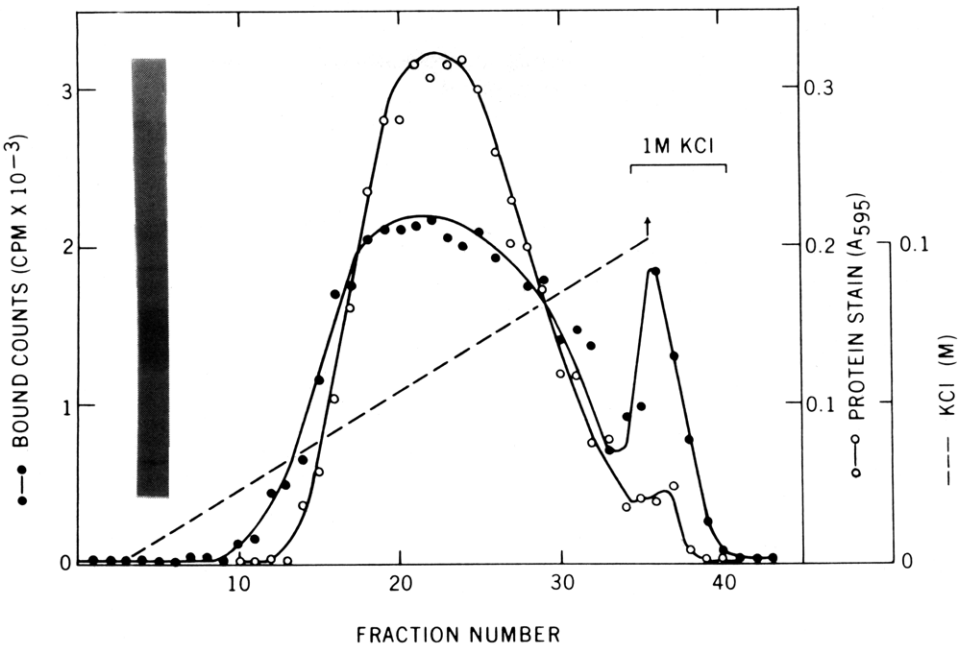

FIGURE 3: Chromatography on DEAE-agarose. The concentrated sample from the gel filtration was applied to a 1 × 2.5 cm column in TG buffer. The column was eluted with a linear 0–0.1 M KCl gradient at a flow rate of 25 mL/h. One-milliliter fractions were collected. The total protein and androgen binding protein were determined in each fraction as described in Figure 2. Fractions 12–32 were pooled and concentrated for further purification. The insert shows $NaDodSO_4$–polyacrylamide gel electrophoresis of this sample (load = 30 μg).

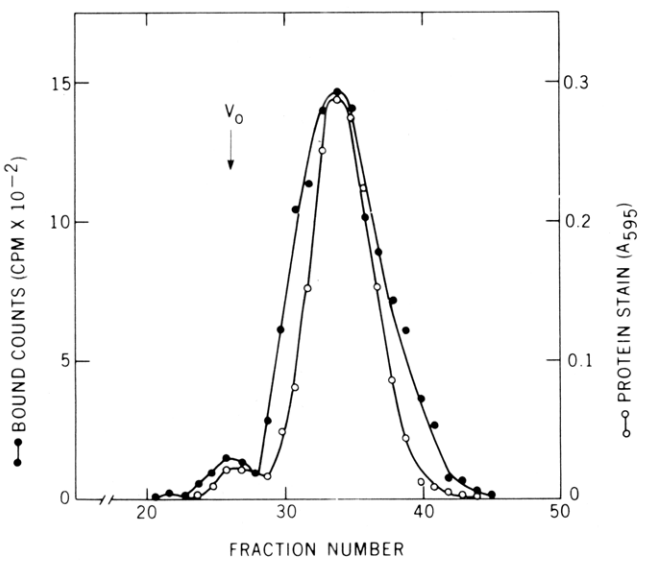

FIGURE 4: Second gel filtration on Sephadex G-200. The concentrated sample from the DEAE-agarose was applied to the same preparative column used in the first gel filtration step (Figure 2). Fractions 29–32, 33–36, and 37–41 were pooled separately for specific activity determination. In these three pooled samples, androgen binding protein was measured by steady-state polyacrylamide gel electrophoresis (Ritzén et al., 1974) and total protein by the method of Bensadoun & Weinstein (1976).

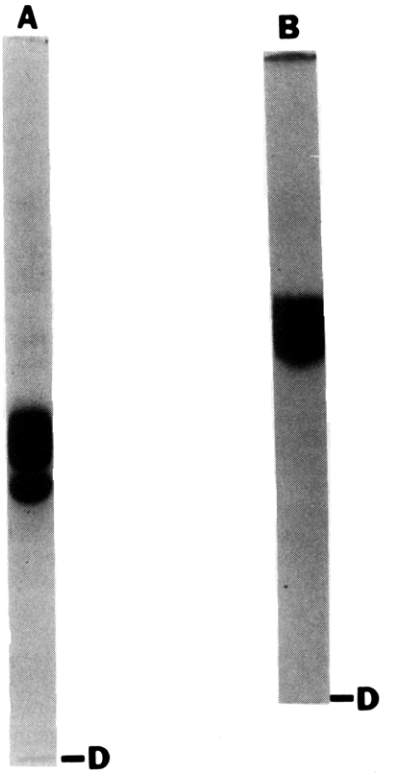

FIGURE 5: Polyacrylamide gel electrophoresis of purified rat epididymal ABP (30 μg) (A) with and (B) without sodium dodecyl sulfate. The total acrylamide concentration was 7.5% in gel A and 6.5% in gel B. Dye fronts are marked with a letter D.

the standards validated the use of $NaDodSO_4$–polyacrylamide gel electrophoresis for molecular weight estimates in these studies.

The purified protein demonstrated three isoelectric species of 4.7, 4.9, and 5.5 when focused in gels containing ampholines in the p*I* range of 3–10. The major band focused at pH 4.7 and two minor bands focused at pH 4.9 and 5.5.

(*b*) *Ultracentrifugation.* Examination of purified ABP by sedimentation equilibrium centrifugation under nondenaturing conditions revealed a homogeneous product with a molecular weight of approximately 85000 (Figure 7). By contrast, when ABP was examined by sedimentation equilibrium in 6 M guanidine hydrochloride, the product was much smaller (molecular weight approximately 40000) and was polydisperse (Figure 8).

(*c*) *Chemical Analysis.* Chemical analysis following hydrolysis of homogeneous ABP in 6 N HCl showed a large percentage of the charged residues to be acidic amino acids, which is consistent with the low p*I* for this protein. In addition, low amounts of tyrosine were detected, but amino sugars were

Table III:[a] Physical Properties of Androgen Binding Protein on Polyacrylamide Gels

system	K_R	Y_0	geometric mean radius (nm)	app mol wt	valence net protons/ molecule	free dispersion coeff (cm^2/s)
native gels						
1935	7.69×10^{-2}	2.54	3.56 (4.16-2.94)[b]	154 000 (2.4-0.89 × 10^5)	−10.89	4.3×10^{-6}
2860.0.X	8.87×10^{-2}	1.66	3.20 (3.72-2.68)	111 000 (1.75-0.65 × 10^5)	−16.11	
$NaDodSO_4$ gels						
1000	9.48×10^{-2}	2.90	2.38 (2.55-2.18)	45 000 (5.6-3.5 × 10^4)		
	9.15×10^{-2}	2.96	2.30 (2.49-2.09)	41 000 (5.2-3.1 × 10^4)		

[a] The retardation coefficient (K_R) and the Y intercept on the Ferguson plot (Y_0) were computed as described by Rodbard & Chrambach (1971). The values of geometric mean radius and molecular weight were computed from K_R and the valence was computed from Y_0. The molecular radius and molecular weight estimates are based on the following standards: bromophenol blue (670); ovine prolactin (22 550); ovalbumin (43 500); phosphorylase *b* (100 000); bovine serum albumin monomer (67 000); bovine serum dimer (134 000); ferritin (450 000). Standards for $NaDodSO_4$-polyacrylamide gel electrophoresis were as follows: collagenase (109 000); phosphorylase *a* (94 000); transferrin (80 000); bovine serum albumin (68 000); heavy chain IgG (55 000); ovalbumin (45 000); chymotrypsinogen (25 000). [b] 95% confidence intervals, calculated by using PAGEPACK, as described under Materials and Methods.

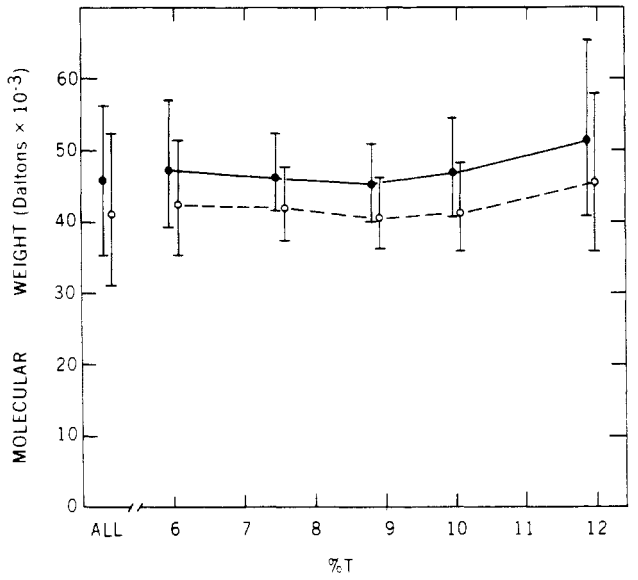

FIGURE 6: Molecular weight of androgen binding protein (ABP) subunits in $NaDodSO_4$-containing polyacrylamide gel as a function of total acrylamide (% T) concentrations. Homogeneous ABP and protein standards were electrophoresed in $NaDodSO_4$-containing gels at five different total acrylamide concentrations. The apparent molecular weights of the large and small subunits determined at each gel concentration are connected by the solid (●—●) and broken (O- - -O) lines, respectively. The apparent molecular weights determined from the Ferguson plot of all the data (ALL) are shown on the left.

Table IV: Physical Properties of Rat Epididymal ABP

molecular weight[a]	85 000
partial specific volume[b]	0.73 mL/g
molecular radius[c]	29.1
Stokes radius[d]	47.5
f/f_0	1.63
p*I*	4.7[e]
	4.9
	5.1
K_d[f]	5.8×10^{-9} M at 4 °C
N^f	13×10^{-9} mol/mg of protein

[a] Determined by sedimentation equilibrium centrifugation. [b] Calculated from the amino acid composition as described by Cohn & Edsall (1943). [c] Calculated from data in this table. [d] Determined by gel filtration (Siegel & Monty, 1966). [e] Major component. [f] Determined by Scatchard analysis (Scatchard, 1949) using dextran charcoal methods described in the text. Protein was assayed by the method of Lowry et al. (1951) using BSA as a standard.

not detected. The lack of detectable amino sugars or PAS staining suggests that this protein has very low carbohydrate content. The physical properties of homogeneous ABP are summarized in Table IV.

Discussion

Dihydrotestosteronehexanoic acid (DHT-HA) is a steroid derivative that can be conveniently coupled to Sepharose and used for affinity chromatography of proteins which bind androgens. This affinity matrix lacks labile ester bonds, which are subject to hydrolysis by nonspecific esterases found in many tissues (Huggins & Moulton, 1948). In previous experiments we attempted to use 17β-hemisuccinate derivatives of dihydrotestosterone to prepare the column matrix for affinity purification of androgen binding protein. Low yields from this column correlated with a time-dependent decrease in binding capacity of the matrix, a result of ligand cleavage from the matrix by esterases in extracts of rat epididymides. The use of DHT-HA circumvented this problem in the present study by providing a stable linkage so that ABP was quantitatively removed from crude epididymal cytosol. In contrast to columns made with 17β-hemisuccinate ester derivatives, those with DHT-HA can be reused a number of times without loss of capacity.

Affinity chromatography has shown great promise for the purification of trace proteins. With this procedure, several investigators (Rosner & Smith, 1975; Mickelson & Petra, 1975; Rosner & Bradlow, 1971) purified steroid binding proteins from plasma where their relative abundance is about 0.1%. However, one problem with affinity chromatography has been the inability to realize the full potential of the method (Mickelson & Petra, 1975, 1978; Rosner & Smith, 1975). Indeed, our first attempts to use this technique for purification of ABP resulted in only 50-fold purification with a 5–10% yield. Previous studies suggest this was due to nonspecific hydrophobic and ionic interactions between proteins and the column matrix (Aukrust et al., 1976). The experiments used to optimize the recovery of ABP from the affinity column (Table I) were consistent with this hypothesis. The addition of dimethylformamide to the washing and elution buffers reduced nonspecific hydrophobic interactions (Aukrust et al., 1976), and the presence of KCl prevented ionic absorption, thus markedly increasing the yield and purity of the eluted binding protein. The use of the affinity matrix under those

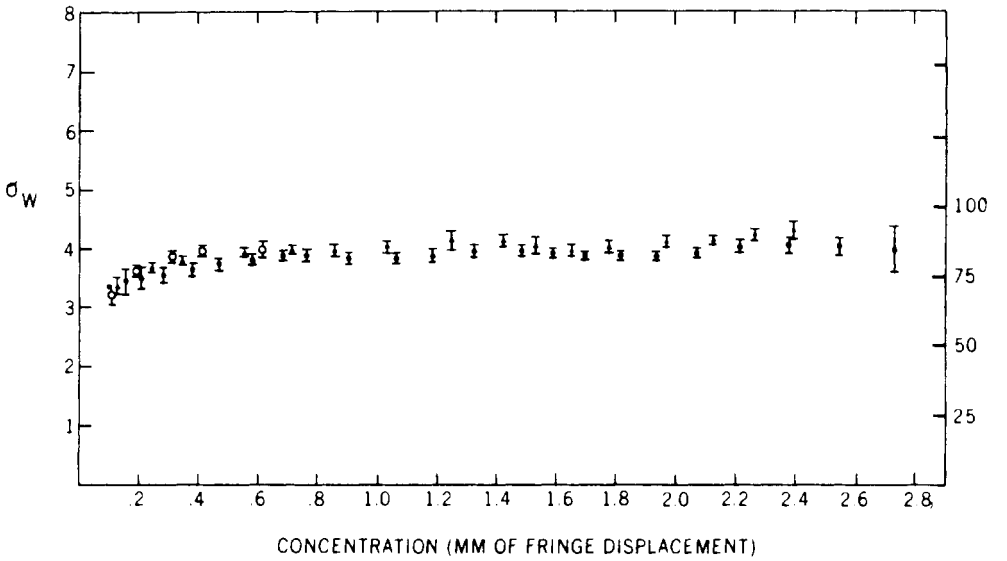

FIGURE 7: Molecular weight determination of purified ABP by sedimentation equilibrium in TEG buffer at 4 °C. Initial protein concentrations of 0.8, 0.4, and 0.2 mg/mL were centrifuged for 48 h at 21 000 rpm. Experimental values of σ_w were converted to molecular weight as described in the text. Molecular weight ($\times 10^{-3}$) is shown on the right ordinate.

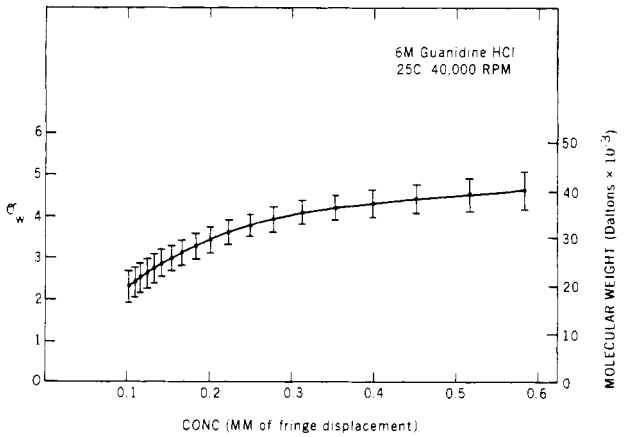

FIGURE 8: Molecular weight determination of purified rat epididymal ABP by sedimentation equilibrium in 6 M guanidine hydrochloride at 25 °C. An initial protein concentration of 0.7 mg/mL was centrifuged for 48 h at 40 000 rpm.

conditions which provided optimal yield and maximal increase in specific activity permitted purification of ABP to apparent homogeneity in only five steps. The fact that ABP was only 0.02% of the total protein in the starting epididymal extract demonstrates the power of this method.

The purified protein was shown to be homogeneous by polyacrylamide gel electrophoresis at multiple gel concentrations and resolving pHs. In addition, the specific activity of ABP was constant across the peak of the final G-200 chromatography. Finally, the analysis of ABP by sedimentation equilibrium ultracentrifugation indicated a high degree of homogeneity. In spite of these observations, heterogeneity was evident from three isoelectric species on focusing gels and by the presence of two bands on $NaDodSO_4$–polyacrylamide gels. These latter bands were approximately one-half the molecular weight of the native molecule, as determined by sedimentation equilibrium methods, and in addition appeared in a mass ratio of 1:3 (41K:45K). These observations are consistent with the postulate that the ABP isolated from the epididymis is a dimeric mixed hybrid system comprised of combinations of the 45K and 41K components. The distribution of hybrid combinations can be calculated to be 9 (45K + 45K) + 6 (45K + 41K) + 1 (41K + 41K) using the mass ratio above. Preliminary experiments suggest this may be the result of limited proteolysis of the 45K + 45K dimer. This is consistent with the observation that ABP activity disappeared during epididymal transit (French & Ritzén, 1973; Hansson et al., 1973). The possibility of steroid binding by individual subunits could not be tested, inasmuch as $NaDodSO_4$ and guanidine·HCl denatured the ABP with loss of binding.

Although the highly purified ABP was homogeneous by several criteria, there was some evidence for microheterogeneity. The diffuse band on polyacrylamide gel electrophoresis, multiple species on isoelectric focusing, and polydispersity in guanidine·HCl during ultracentrifugation are all indications of this heterogeneity. Such properties of a pure protein are often the result of variable carbohydrate content. The absence of detectable amino sugars in the amino acid analysis and the lack of PAS staining of the purified protein on polyacrylamide gel electrophoresis suggested that there was little or no carbohydrate in ABP. It is possible, however, that the inability to detect this constituent was due to the limited sensitivity of these methods. Although it is possible that variation in carbohydrate content could account for some of the observed microheterogeneity, limited proteolysis of one subunit cannot be excluded.

The difference between the apparent molecular weight as determined by polyacrylamide gel electrophoresis under nondenaturing conditions and that determined by sedimentation equilibrium ultracentrifugation and the difference in the molecular radius and Stokes radius are indicators of the nonglobular nature of this protein.

To date, several secretory androgen binding proteins have been purified and characterized. One of the most readily available is the serum β-globulin, TeBG. At present, the TeBGs from humans (Rosner & Smith, 1975; Mickelson & Petra, 1975), rabbits (Mickelson & Petra, 1978), and bovine (Suzuki et al., 1977) have been isolated in highly purified form. The proteins from cattle and rabbits are similar to ABP in the present study insofar as they are comprised of subunits. The observations on the structure of TeBG in man are conflicting. Rosner & Smith (1975) reported that TeBG isolated from lyophilized Cohn fraction IV had a molecular weight of 90 000 on $NaDodSO_4$–polyacrylamide gel electrophoresis. On the other hand, Mickelson & Petra (1975) purified a TeBG from

human pregnancy serum collected at delivery that showed a molecular weight of 54 000.

In contrast to TeBG, only one testicular ABP has been examined prior to the present report. Weddington et al. (1975) fractionated rabbit epididymides using conventional methods. This produced an ABP preparation of about 10% of theoretical purity on $NaDodSO_4$-polyacrylamide gel electrophoresis. From this analysis the authors reported the molecular weight for the major band to be about 65 000, with two minor bands of approximately 45 000. They assumed that the major band was ABP. However, if rabbit ABP has a subunit structure similar to that of rat, the monomer would have a molecular weight of about 45 000 (0.5 of 90 000), a molecular weight in the range of the minor components of their purified material. Thus, there is a possibility that rabbit ABP also exists as a dimer.

In conclusion, rat ABP has been isolated by affinity chromatography from the epididymis. This protein has some common and some unique features when compared to other androgen binding proteins. Antibodies prepared against homogeneous ABP will facilitate studies of the hormonal control of both the testis and epididymis (Gunsalus et al., 1978).

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