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Isolation of Testosterone-binding Globulin from Bovine Serum by Affinity Chromatography and Its Molecular Characterization[1]

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The testosterone-binding globulin (TeBG) from bovine serum was purified by affinity chromatography and hydroxylapatite chromatography. The affinity column used was prepared by coupling 17α-carboxyethynyl-17-hydroxy-4-androsten-3-one to aminoethyl-Sepharose. The compound was replaceable by 17α-carboxyethynyl-17-hydroxy-5α-androstan-3-one, but not by testosterone 17-hemisuccinate, estradiol 17-hemisuccinate, or testosterone 3-(O-carboxymethyl)oxime. The TeBG isolated was homogeneous on analytical polyacrylamide gel electrophoresis and equilibrium centrifugation. The protein was a glycoprotein having a molecular weight of 89,500 and a carbohydrate content of 17%. The association constant (M^{-1}) at 4°C was 1.1×10^8 and the number of binding sites per molecule was 0.8. Treatment with guanidine-HCl dissociated the protein into subunits having a molecular weight of 28,400 (about one-third of that of the original molecule). SDS-gel electrophoresis showed that two of the three subunits were slightly larger than the other. The dissociation into subunits could also be accomplished by GEDTA treatment with concomitant loss of testosterone-binding activity. The activity and molecular size were reversibly restored by incubation with excess Ca^{2+}.

The presence in plasma of a protein which binds testosterone with high affinity and which is distinct from corticosteroid-binding globulin has been recently established. The protein (TeBG) is thought to be useful as a model system to study the interaction of " receptor protein " of prostates with

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Abbreviations used are: TeBG, testosterone-binding globulin; DHT, 5α-dihydrotestosterone; SDS, sodium dodecyl sulfate; GEDTA, glycoletherdiamine-N, N, N′, N′-tetraacetic acid.

androgen. Attempts to isolate it have been undertaken by a number of research groups starting from human (*1–5*), rat (*6*), guinea pig (*6, 7*), and rabbit (*8–10*) sera. Most of the approaches, however, were based on conventional chromatographic procedures which are usually difficult and time-consuming, and which involve considerable losses of the protein during each stage. The method of affinity chromatography which was recently developed by Cuatrecasas (*11*) appeared to be more suitable for isolating the protein, if proper conditions could be found. In this communication we report an isolation procedure for bovine TeBG in only two steps, one of which is an affinity chromatography with Sepharose-bound 17α-carboxyethynyl-17-hydroxy-4-androsten-3-one. The ligand is characterized by two free functional groups, 3-one and 17β-ol, which probably play a role in adsorbing TeBG from serum. The properties of isolated TeBG have been studied and compared with those of TeBG from other animals (*6–10*) and human TeBG, which was recently isolated on different affinity columns (*12, 13*). The study of the subunit showed for the first time that bovine TeBG dissociated reversibly into three subunit components, Ca^{2+} being indispensable for the recovery of testosterone-binding activity. A preliminary account of this work has recently been presented (*14*).

MATERIALS AND METHODS

Materials—[1, 2-^{3}H]Testosterone, specific activity 48 Ci/mmol, was provided by the Radiochemical Center, Amersham, Bucks, U.K. The compound was purified by thin-layer chromatography (chloroform: acetone=70 : 30, v/v) before use. All radioinert steroids were obtained from Sigma Chem. Co. All the reagents used were of analytical grade and were employed without further purification. Sepharose 4B was purchased from Pharmacia Fine Chemicals. Hydroxylapatite was prepared by the method of Tiselius *et al.* (*15*). Bovine blood obtained from a local slaughterhouse was allowed to clot at 4°C for 3 h. To the serum obtained by decantation, solid $CaCl_2$ was added to make a 20 mM solution. After standing overnight and being stirred while cold, the serum was centrifuged to remove insoluble substances and stored at −20°C until use. Redistilled, deionized water was used throughout this work.

Synthesis of Steroid Derivatives—The steroid derivatives used here to bind with Sepharose were as follows: 17α-carboxyethynyl-17-hydroxy-4-androsten-3-one (I), 17α-carboxyethynyl-17-hydroxy-5α-androstan-3-one (II), testosterone 17-hemisuccinate (III), testosterone 3-(O-carboxymethyl)oxime (IV), and estradiol 17-hemisuccinate (V) (Fig. 1). Of these compounds, the first two were newly synthesized as described below ((i) and (iii)), while compounds III, IV, and V were synthesized by the methods of Ruzicka and Wettstein (*16*), Erlanger *et al.* (*17*), and Ferin *et al.* (*18*), respectively.

(i) 17α-Carboxyethynyl-17-hydroxy-4-androsten-3-one (*I*) was synthesized from 17α-ethynyltestosterone (VI) (Fig. 1). Thus, compound VI (*19*) was treated with ethylene glycol in the presence of *p*-toluenesulfonic acid to give the ketal (VII). Treatment of the ketal (VII) with methyl-magnesium bromide followed by shaking in a carbon dioxide atmosphere (*20*) yielded the ethynyl carboxylic acid (VIII), which was hydrolyzed with hydrochloric acid to give (I).

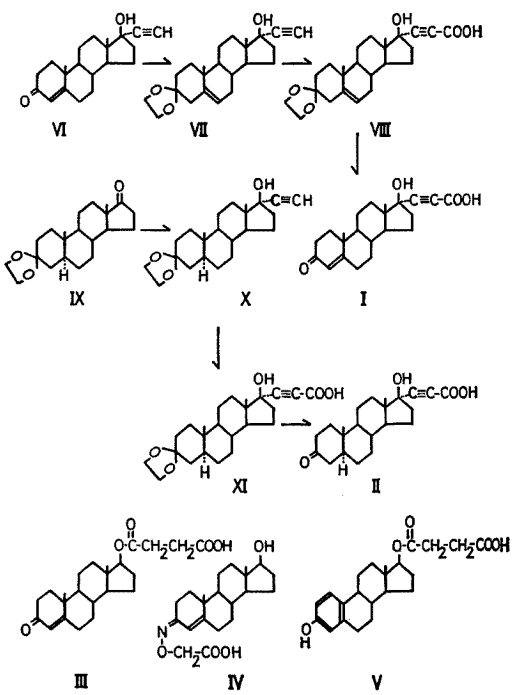

Fig. 1. The structure of steroids referred to in the organic synthesis procedures in the present paper.

The procedure for synthesizing the ketal (VII) is described below (ii) and the synthesis of compound I from the ketal was performed as follows. Four g of compound VII was dissolved in 80 ml of tetrahydrofuran and the solution was added dropwise to an ice-cold solution of Grignard reagent prepared from magnesium (4.0 g), methylbromide, and tetrahydrofuran (80 ml). The resulting mixture was refluxed overnight, and after cooling, was shaken in a carbon dioxide atmosphere for 5 h. After adding water and then 10% sulfuric acid, the organic solvent was removed by distillation *in vacuo*, and the resulting solid material was collected by filtration. A solution of the solid in acetone (200 ml) and 10% hydrochloric acid (20 ml) was refluxed for 1 h. The solvent was removed by distillation and water was added. The resulting oily material was collected and dissolved in tetrahydrofuran (30 ml). Trimethylamine (1.5 ml) was added and the mixture cooled with ice-water. The resulting solid salt (2.1 g) was collected by filtration. The salt was dissolved in dioxane (6 ml) and water (12 ml), and 10% hydrochloric acid was added. When a small amount of ether was added and the mixture was vigorously stirred, the free acid appeared as a colorless solid. Filtration and recrystallization from acetone-hexane gave 17α-carboxyethynyl-17-hydroxy-4-androsten-3-one (1.30 g), mp 257–261°C. $[\alpha]_D^{25}$ −9° (c=1.05, dioxane). NMR (in DMSO-d_6) δ: 0.79 (3H, s), 1.16 (3H, s), 8.62 (1H, broad s.). *Anal.* Calcd. for $C_{22}H_{28}O_6$: C, 74.12; H, 7.91. Found: C, 74.12, H, 7.88.

(ii) 3, 3-Ethylenedioxy-17α-ethynyl-5-androsten-17-ol (VII): A mixture of 17α-ethynyltestosterone (VI) (10.0 g) in benzene (800 ml) and ethylene glycol (160 ml) was distilled slowly to remove all traces of water. *p*-Toluenesulfonic acid (3.0 g) was added and the mixture was distilled for 5 h, during which time anhydrous benzene was added to maintain a constant volume. After cooling, pyridine (4 ml) and then water were added. The solid was collected by filtration, washed with water and dried to give the crude ketal (10.2 g). Recrystallization from acetone containing a trace of pyridine gave an analytical sample as colorless prisms, mp 253–255°C. $[\alpha]_D^{25}$ −66° (c=0.85, pyridine). *Anal.* Calcd. for $C_{23}H_{32}O_3$: C, 77.48; H, 9.04. Found: C, 77.37; H, 9.02.

(iii) 17α-Carboxyethynyl-17-hydroxy-5α-androstan-3-one (II) was synthesized as shown in Fig. 1. Thus, 3, 3-ethylenedioxy-5α-androstan-17-one (IX) (*21*) was transformed into the ethynyl compound (X) by reaction with potassium acetylide in *t*-butanol and tetrahydrofuran (*22*). Similar treatment of (X), as described in (i), gave the desired ethynylcarboxylic acid (II). The product obtained from 2 g of compound X was recrystallized to give 0.61 g of compound II as colorless leaflets. mp 286–289°C (decomp.) NMR (in DMSO-d_6) δ: 0.77 (3H, s), 0.98 (3H, s). *Anal.* Calcd. for $C_{22}H_{30}O_4$: C, 73.71; H, 8.44. Found: C, 73.87; H, 8.50.

(iv) 3, 3-Ethylenedioxy-17α-ethynyl-5α-androstan-17-ol (X): Acetylene gas was bubbled through an ice-cold solution of 3, 3-ethylenedioxy-5α-androstan-17-one (8.0 g) in tetrahydrofuran (250 ml) and *t*-butanol (140 ml) in which potassium metal (11.0 g) had been dissolved previously. After 4 h, the mixture was poured into water and the product extracted with methylene chloride. After washing with water and drying with anhydrous sodium sulfate, the solvent was removed by distillation to give the crude ethynyl compound, which was used in the next step without purification. Recrystallization from tetrahydrofuran-acetone containing a trace of pyridine gave an analytical sample in the form of colorless prisms, mp 266–271°C. $[\alpha]_D^{25}$ −23° (c=1.00, pyridine). *Anal.* Calcd. for $C_{23}H_{34}O_3$: C, 77.05, H, 9.55. Found: C, 77.18; H, 9.63.

Preparation of Steroid-Sepharose—Preparation of aminoethyl Sepharose and its coupling with the steroid derivatives, I, II, III, IV, or V, were performed essentially by the method of Cuatrecasas (*11*). Estimation of the amount of the steroid derivatives incorporated into the Sepharose was carried out in the case of preparation of I-Sepharose, employing [^{3}H]17α-(2-carboxyethynyl)-17-hydroxy-4-androsten-3-one (97 mCi/mmol): 0.15 μmol of the steroid derivative was usually incorporated per ml of the Sepharose matrix.

Preparation of TeBG—All procedures were carried out at 4°C unless otherwise stated. Four ml of I-Sepharose (designated as "$T_{17}S$") matrix was thoroughly mixed with 13.6 ml of Sepharose 4B matrix and the mixture was formed into a column (2.5 cm ×10 cm), which was then equilibrated using about 500 ml of buffer A (0.02 M Tris-HCl, pH 7.5, containing 0.3 M NaCl). About one liter of serum thawed from the stock was

applied to the column with a flow rate of 70 ml per h. The column was washed with buffer A until the absorbance at 280 nm of the effluent dropped below 0.1, then the Sepharose in the column was transferred to a beaker containing 5×10^{-6} M nonradioactive testosterone in 3 volumes of buffer A. The suspension was incubated for 15 min at 30°C with gentle stirring to release TeBG from the Sepharose and then poured back into the glass column used before. Elution from the column with the buffer was performed at room temperature within 10 min to obtain a solution (about 70 ml) containing TeBG (designated as "$T_{17}S$ eluate"). The volume of the solution was reduced to 2–3 ml by ultrafiltration and it was dialyzed overnight against 500 ml of buffer B (0.02 M sodium phosphate, pH 6.8). The protein preparation was applied to a hydroxylapatite column (1.2 cm×15 cm) previously equilibrated with buffer B. The flow-through fraction with the buffer (peak I) was used as the TeBG preparation or was concentrated by ultrafiltration for studies on physicochemical properties. The protein could be stored at 0°C for a week. Freezing at −20°C eliminated the testosterone-binding activity.

Binding Analysis—Testosterone-binding activity was measured by the charcoal-dextran method. The assay system contained 2.0×10^{-9} M testosterone labelled with ^3H (0.02 μCi) and an appropriate amount of protein (about 0.3–300 μg) in 0.2 ml of buffer A. After incubation for 5 min at 30°C and then for 30 min at 0°C, 1 ml of 0.25% charcoal–0.01% dextran suspension in buffer A was added. The suspension was centrifuged for 10 min at 3,000 rpm to remove charcoal and an aliquot (0.5 ml) of the supernatant was mixed with 0.2 ml of distilled water and 5 ml of scintillant (100 mg of 1, 4-bis-(5-phenyloxazol-2-yl)-benzene and 5.5 g of 2, 5-diphenyloxazole in 1 liter of Triton X-100/toluene (1/2, v/v) in a vial. The radioactivity was determined in an Aloka 651 liquid scintillation counter at 33–38% efficiency. Nonspecific binding activity was determined in the assay system containing 2.0×10^{-6} M nonradioactive testosterone and subtracted from the values referred to above.

Determination of the association constant was performed by an equilibrium dialysis method at 4°C. One ml of each of the sample solutions in buffer A (usually containing 3.3 μg protein) was placed inside a dialysis bag (Visking, 8/32 inch flat diameter) and the bags were then immersed in 6-ml solutions which contained 0.04 M sodium phosphate buffer (pH 7.5), 0.3 M NaCl, 0.5 mM mercpatoethanol, 1.09×10^{-10} M [^3H]-testosterone (48 Ci/mmol) and various amounts of nonradioactive testosterone. Equilibrium was achieved by gently stirring the contents with a magnetic stirrer for approximately 70 h at 4.0°C. An aliquot (0.5 ml) was taken from the inside and also from the outside for liquid scintillation counting. The association constant was determined from a Scatchard plot using the nonweighted least-squares method.

Chemical Analysis—For amino acid analysis, samples containing 2.2 mg protein were hydrolyzed at 110°C for 24 h under N_2 in sealed tubes containing 5 ml of 6 N HCl and 10 μl of phenol (*23*), and applied to a Hitachi KLA-3B amino acid analyzer. For the analysis of hexose and fucose, the method of Kim *et al.* (*24*) was used with a slight modification as follows. A mixture of the sample (3.0 mg protein), D-ribose (usually 60 μg, used as an internal standard) and about 20 mg of Dowex 50×2 (H-form) in 2 ml of 0.25 N H_2SO_4 was sealed in a tube and incubated at 100°C for 24 h. One drop of neutral red solution was added to the hydrolyzate, which was then passed through a short column of Dowex I (formate form, 5 mm length) to remove Dowex 50×2 and sulfuric acid. The effluent was dried in an evaporator. The sugars were reduced overnight with $NaBH_4$ at 4°C and acetylated with acetic anhydride in pyridine. Analysis of the acetylated sugars was performed in a Shimadzu GC-4 BPF gas chromatograph equipped with a flame ionization detector. Sialic acid and hexosamine was analyzed according to the method of Warren (*25*) and Gatt and Berman (*26*), respectively. Protein was analyzed by a spectrophotometric method (*27*). The dry weight of the protein was measured after drying *in vacuo* over P_2O_5.

Analytical Ultracentrifugation—The sedimentation velocity was determined using a Hitachi 282 ultracentrifuge equipped with a photoelectric absorption scanner and ultraviolet optical system. The protein was dissolved in buffer A, usually at a concentration of 0.42–1.67 mg per ml. The centrifuge was operated at 60,000 rpm at 20°C. Sedimentation equilibration was carried out according to the procedure of Yphantis (*28*). TeBG in buffer B was centrifuged at 20,000 rpm for 15 h at 20°C. To examine the subunits of TeBG, the protein

solution was dialyzed against 6 M guanidine-HCl (pH 7.5) for 3 days at room temperature, changing the outer solution 3 times, and then subjected to sedimentation equilibrium analysis.

Polyacrylamide Gel Electrophoresis Procedures—Analytical disc gel electrophoresis was performed at 4°C using 7.5% acrylamide gels according to the method described by Davis (*29*). Samples (100 μl) containing 50–100 μg of protein were applied to the gels. The gels were stained with Amido black or with the periodic acid-Schiff reagent (*30*). Location of the radioactive bands on gels was done by applying to the gels 100 μl samples containing 34 μg of TeBG which had previously been incubated with 6.5×10^{-8} M [^{3}H]testosterone at 30°C for 5 min and then at 0°C for 30 min, followed by removal of free steroid by charcoal-dextran treatment. Gels were sliced transversely with a gel slicer into 3 mm slices and digested with 0.5 ml of 30% H_2O_2 at 50°C for 24 h before the addition of scintillant.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Fairbanks *et al.* (*30*). Samples, which were pretreated with 1% SDS in 0.04 M Tris-acetate buffer, pH 7.4, for 2 h at room temperature, were applied to gel columns (5.5% polyacrylamide and 0.1% SDS) and migrated at 8 mA per column in 0.1% SDS–0.002 M EDTA–0.04 M Tris-acetate buffer, pH 7.4, Gels were stained with Coomassie blue or with periodic acid-Schiff reagent. Tracing of the stained protein was performed at 600 nm in a Gilford spectrophotometer equipped with a gel scanner.

RESULTS

Synthesis of 17α-Carboxyethynyl-17-hydroxy-4-androsten-3-one (I) and 17α-Carboxyethynyl-17-hydroxy-5α-androstan-3-one (II) and Their Coupling with Sepharose—Compounds I and II were newly synthesized as described above and their chemical structures elucidated by NMR and IR spectroscopy and elementary analyses. The carboxy groups of these compounds were coupled with aminoethyl Sepharose (Fig. 2) and the materials were washed completely to remove free steroids as described above.

Purification of TeBG from Bovine Serum—Compounds III, IV, and V were also coupled with Sepharose 4B, and these affinity columns, together with I- and II-Sepharose, were tested for efficiency

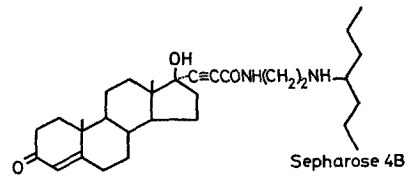

Fig. 2. The structure of the affinity column ($T_{17}S$) used in most experiments of this paper.

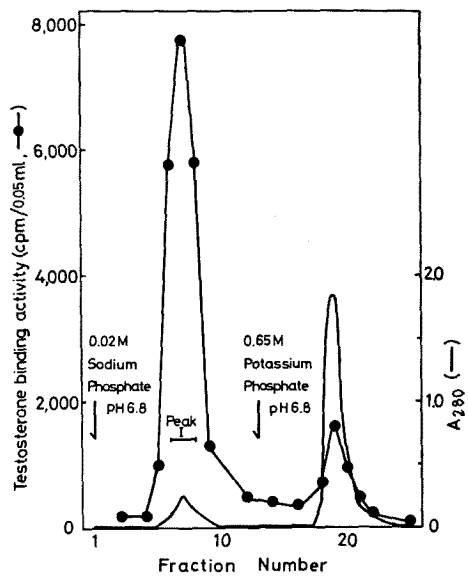

Fig. 3. Purification by hydroxylapatite chromatography. $T_{17}S$ eluate (3.2 ml) containing 10.9 mg of protein was applied to a hydroxylapatite column (1.2 cm × 15 cm) and eluted at 4°C with 40 ml of 0.02 M sodium phosphate buffer (pH 6.8), then with 50 ml of 0.65 M potassium phosphate buffer (pH 6.8), collecting fractions of 3 ml in a fraction collector. An aliquot (0.05 ml) was used for the assay of steroid binding by the charcoal-dextran method. The contents in the flow-through fraction (peak I) were combined to be used as TeBG preparation.

of specific binding of TeBG. Thus, when 50 ml each of serum was applied to a small column (1 cm × 3 cm) of I-, II-, III-, IV-, or V-Sepharose, the testosterone-binding activity of the flow-through fraction, as assayed by the charcoal-dextran method, was found to be 49, 25, 89, 98, or 87, respectively, as a percentage of that of the original serum (means of three to four experiments). This

TABLE I. Purification of TeBG from bovine serum.

Step	Volume (ml)	Total protein (mg)	Total activity (cpm $\times 10^{-7}$)	Specific activity (cpm/mg protein)	Recovery (%)	Purification (fold)
Serum	940	6.75×10^4	6.35	9.40×10^2	100	1
$T_{17}S$ eluate	68	32.7	0.44	1.35×10^5	6.9	142
Peak I	11	1.36	0.30	2.21×10^6	4.8	2,365

indicates that compound II is the best as a ligand for affinity chromatography, compound I being the second best, while III, IV, and V are rather inefficient. However, I-Sepharose ($T_{17}S$) was used throughout this work because testosterone is the predominant androgen in serum, and moreover, this column showed easier release of TeBG in the elution step.

In combination with affinity chromatography, the usefulness of various other methods including DEAE-cellulose chromatography, gel filtration, hydroxylapatite chromatography, *etc.*, was tested for the purification of TeBG. The procedure finally adopted is based on the binding of TeBG on $T_{17}S$ directly from serum followed by hydroxylapatite chromatography as described in " MATERIALS AND METHODS." A typical example of the purification is shown in Table I, giving about 2,400-fold purification over serum. A similar degree of purification was obtained reproducibly in a number of experiments. As shown in Figs. 3 and 4, unwanted proteins from the materials released from $T_{17}S$ were removed by the hydroxylapatite chromatography. Figure 5 shows that the final preparation gave a single band on gel electrophoresis, the protein of which is associated with [^{3}H]testosterone.

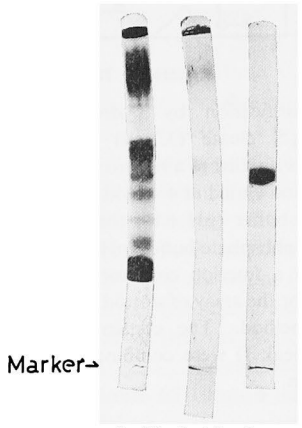

Fig. 4. Polyacrylamide gel electrophoresis of serum and TeBG. The concentration of the separating gel was 7.5%. Tris-glycine buffer (pH 8.3) was used in both anode and cathode chambers. Electrophoresis was performed at 4°C at 4 mA per gel for 3 h. Gel 1: diluted serum (0.1 mg of protein); Gel 2: $T_{17}S$ eluate (0.05 mg of protein); Gel 3: purified TeBG (0.05 mg of protein).

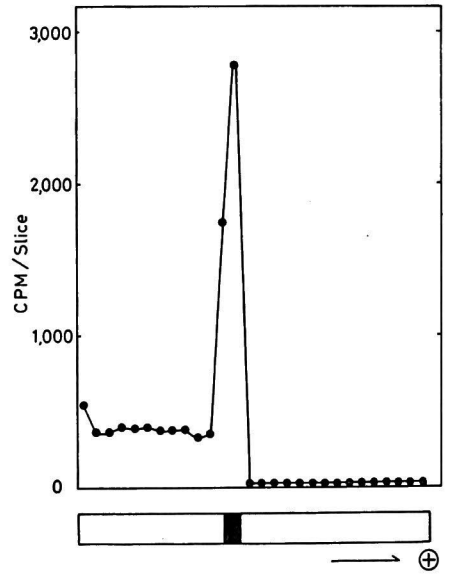

Fig. 5. Analytical gel electrophoretic patterns of purified TeBG. One-hundredth ml of [^{3}H]testosterone solution (48 Ci/mmol) was added to 0.17 mg of TeBG in 0.5 ml and the mixture was incubated for 5 min at 30°C and then for 30 min at 0°C. After free steroid have been removed by charcoal treatment, the [^{3}H]testosterone-binding TeBG (100 μl) was subjected to gel electrophoresis under the conditions described in the legend to Fig. 4. Gel slices (3 mm) were digested with H_2O_2 before addition of scintillant. Protein staining was performed with Amido black.

Physicochemical Properties of TeBG—From the sedimentation velocity of TeBG measured at four different concentrations, the value of $s_{20,w}$ was found to be 5.3 (Figs. 6 and 7). Figure 8 shows plots of equilibrium sedimentation analysis carried out by the method of Yphantis (*28*), indicating the presence of a homogeneous protein. From the slope, the effective reduced molecular weight parameter "σ" was calculated to be 4.55. By combination with an estimated value for partial specific volume, 0.730 ml/g, the apparent molecular weight was calculated to be 89,500. Other constants were also calculated using classical equations and are presented in Table II. The table also contains the value of pI, which was estimated by an electrofocusing method (*31*).

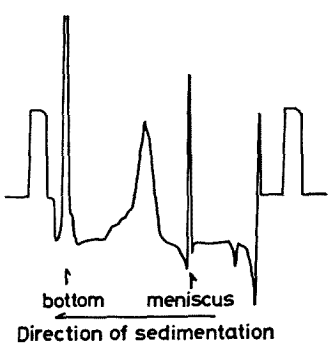

Fig. 6. Ultracentrifugation pattern of TeBG. Eight hundreds μl of a solution containing 0.5 mg of TeBG in buffer B was spun at 60,000 rpm at 20°C. The differential curve of absorption at 280 nm was traced after centrifugation for 24 min at this speed.

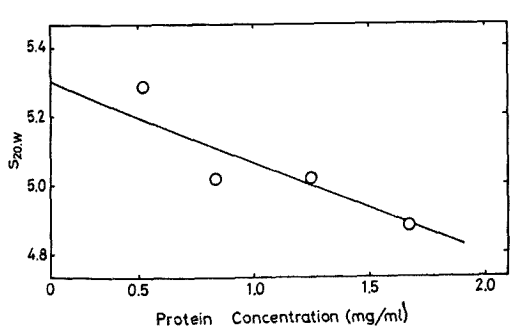

Fig. 7. Effect of protein concentration on the sedimentation coefficient of TeBG. The protein concentration varied from 0.42 to 1.67 mg per ml. Other conditions were as described in the legend to Fig. 6.

Amino Acid and Carbohydrate Compositions—The results of amino acid analysis are shown in Table III. Hydrolysis of the protein was carried out for 24 h and no correction was made for amino acid loss during the hydrolysis. Analysis of cysteine and tryptophan, which are destroyed during acid hydrolysis, was not performed. The amount of amino acid recovered was about 70% of the dry weight of the glycoprotein.

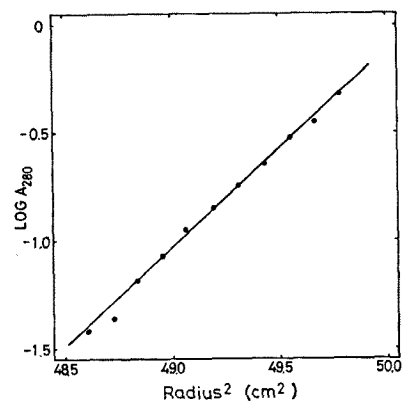

Fig. 8. Equilibrium centrifugation analysis of TeBG. TeBG dissolved in buffer A (0.53 mg/ml) was centrifuged at 20,000 rpm for 15 h at 20°C. Ordinate: logarithm of absorbance at 280 nm; Abscissa: square of the distance (cm) from the center of the rotor.

TABLE II. Some physicochemical properties of TeBG.

Molecular weight[a]	89,500
$s_{20,w}$	5.3
$D_{20,w}$	5.09×10^{-7} cm^2/s
f/f_0	1.42
Partial specific volume[a]	0.730 ml/g
$E^{1\%}_{cm}$ at 280 nm	10.1
A_{280}/A_{260}	1.38
Carbohydrate content	17%
Association constant for testosterone (4°C)	1.1×10^{8} M^{-1}
Binding sites per molecule	0.8
Molecular weight of subunit[b]	28,400
pI[c]	4.78

[a] Calculated from amino acid and carbohydrate contents. [b] Determined by equilibrium centrifugation analysis. [c] Determined by the electrofocusing method.

TABLE III. Chemical composition of TeBG.

	g per 100 g of glycoprotein	mol/mol of glycoprotein
Amino acid		
Lys	1.97	13.8
His	2.62	17.1
Arg	5.06	29.0
Asp	6.64	51.6
Thr	3.45	30.5
Ser	4.76	49.0
Glu	9.10	63.1
Pro	5.33	49.1
Gly	3.38	53.0
Ala	3.25	40.9
Val	3.36	30.3
Met	1.36	9.3
Ile	2.69	21.3
Leu	11.15	88.2
Tyr	0.61	3.3
Phe	3.59	21.8
Carbohydrate		
Fucose	0.18	1.1
Mannose	5.73	31.6
Galactose	5.98	33.0
N-Acetylglucosamine	2.94	12.9
Sialic acid	2.48	7.6

Carbohydrates were determined as described previously and the results are also listed in Table III, indicating that the glycoprotein is rich in mannose and galactose. The total amount of carbohydrate is 17% of the glycoprotein.

Testosterone-binding Activity—Equilibrium dialysis experiments were performed as described

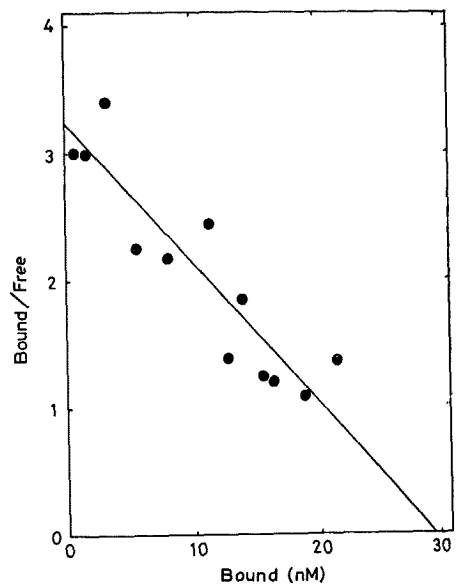

Fig. 9. Scatchard plots for the equilibrium dialysis of TeBG with [^{3}H]testosterone. Equilibrium dialysis was carried out in triplicate at 4°C. Each tube contained 3.3 μg of TeBG in 1 ml inside the dialysis bag.

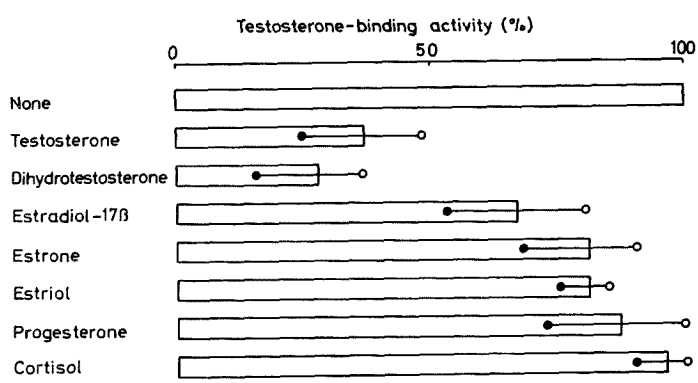

Fig. 10. Competitive binding experiments of various steroids with TeBG. TeBG dissolved in 1 ml of buffer A (open circles, 3.3 μg; closed circles, 4.0 μg) was dialyzed against 6 ml of buffer A containing 1.67×10^{-9} M [^{3}H]testosterone (0.02 μCi) and 2.5×10^{-8} M steroid as specified, in triplicate.

previously and Scatchard plots (*32*) of the data are presented in Fig. 9. The association constant and the number of binding sites were calculated taking a value of 89,500 for the molecular weight and are presented in Table II.

Release of [^{3}H]testosterone from TeBG on the addition of 2.5×10^{-8} M of nonradioactive steroids is shown in Fig. 10. The results indicate that DHT has the highest affinity for the protein and that estradiol 17β, estrone, estriol, progesterone, and cortisol exhibit partially competitive binding with respect to testosterone.

Subunits of TeBG—TeBG pretreated with 6 M guanidine-HCl was subjected to equilibrium centrifugation. As shown in Fig. 11, plots of the logarithms of absorbance at 280 nm *versus* the radius squared gave approximately a straight line. From the slope, taking values of 0.730 for the partial specific volume and 1.147 g/ml for the solvent density (*33*), the molecular weight was calculated to be 28,400, which is about 1/3 of that of the whole molecule. Polyacrylamide gel electrophoresis in the presence of SDS, however, showed two bands located very close together (Fig. 12). The amount of the slow component was estimated to be approximately twice that of the fast component (Fig. 12). Both components were stained purple with periodic acid-Schiff reagent. From these results, it appears that TeBG is composed of three subunits of almost equal size, two of which migrate slightly more slowly than the other on SDS-gel electrophoresis.

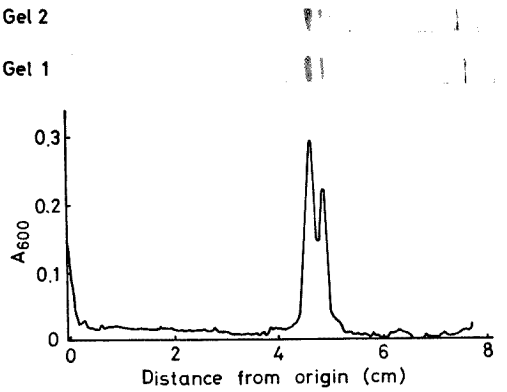

Fig. 12. SDS-polyacrylamide gel electrophoresis of TeBG. TeBG (50 μg) was incubated at 30°C for 2 h in 20 μl of 0.4 M Tris-acetate buffer (pH 7.4) containing 1% SDS and then subjected to SDS-gel electrophoresis (gel concentration, 5.5%). Electrophoresis was carried out at 8 mA/gel for 2 h. Gel 1 and gel 2 were stained with Coomassie blue and periodic acid-Schiff reagent, respectively.

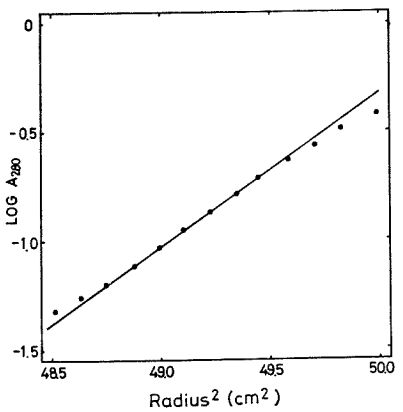

Fig. 11. Sedimentation equilibrium analysis of TeBG in 6 M guanidine-HCl. TeBG dissolved in buffer B (1.5 mg/ml) was dialyzed against 6 M guanidine-HCl (pH 7.5) at room temperature for 3 days and then centrifuged at 38,000 rpm at 25°C. When the equilibrium state was attained (about 15 h after the beginning of spinning), the distribution pattern of the protein was recorded.

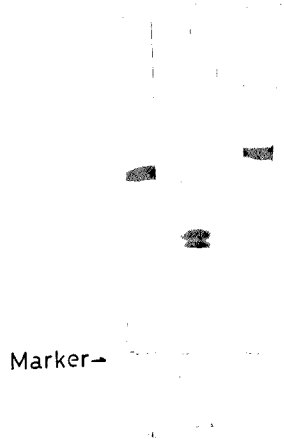

Fig. 13. Polyacrylamide gel electrophoresis of TeBG treated with GEDTA and $CaCl_2$. Gel 1: untreated TeBG; Gel 2: TeBG treated with 2 mM GEDTA; Gel 3: TeBG treated with 2 mM GEDTA and then with 5 mM $CaCl_2$. Conditions of electrophoresis were as described in the legend to Fig. 4.

TABLE IV. Effect of GEDTA and divalent cations on the testosterone-binding activity of TeBG. Testosterone-binding activity was measured as described in "MATERIALS AND METHODS" for (a) TeBG, (b) TeBG incubated with 2 mM GEDTA at 30°C for 5 min and then at 0°C for 30 min, (c) TeBG from (b) incubated further with 5 mM divalent cation as indicated at 30°C for 5 min and then at 0°C for 30 min. The binding activity of the original TeBG was taken as 100%. Each value is the mean of 4–7 experiments.

	Treatment	Relative binding activity (%)
(a)	No treatment	100
(b)	2 mM GEDTA	0
(c)	2 mM GEDTA and 5 mM $CaCl_2$	75
	2 mM GEDTA and 5 mM $SrCl_2$	71
	2 mM GEDTA and 5 mM $BaCl_2$	25
	2 mM GEDTA and 5 mM $MgCl_2$, $MnCl_2$, $HgCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_2$, or $NiCl_2$	0

Splitting into subunits also occurred on treatment with 2 mM GEDTA and the addition of Ca^{2+} regenerated the original protein (Fig. 13). Table IV shows that treatment with 2 mM GEDTA abolished the testosterone-binding activity of TeBG and that the addition of Ca^{2+} restored about 75% of the binding activity. Sr^{2+} was as effective as Ca^{2+}, but other divalent cations were ineffective in the restoration of the binding activity.

These findings suggest that TeBG is split into subunits by the removal of Ca^{2+}, with a concomitant loss of testosterone-binding activity.

DISCUSSION

The purification of bovine TeBG in the present experiments yielded a homogeneous protein by the criteria of gel electrophoresis and ultracentrifugation. The value of the molecular weight found by equilibrium centrifugation, 89,500, is similar to those of guinea pig TeBG, 82,800 (*7*), and human TeBG, 94,000–102,000 (*4, 5, 13*), although rabbit TeBG has a slightly smaller value, 74,000 (*8, 10*). The amino acid and carbohydrate compositions of the bovine TeBG, however, are somewhat different from those reported for the human (*13*) and guinea pig (*7*) proteins. Thus, the bovine TeBG is characterized by a higher hydrophobic amino acid content (46.5%) than human TeBG (39.5%) or guinea pig TeBG (42.5%). It is also noteworthy that the bovine TeBG contained more proline and less tyrosine and lysine residues than the others. The sialic acid content decreases in the order human, guinea pig, and bovine TeBG, while the amount of neutral sugars (mannose and galactose) decreases in the order guinea pig, bovine, and human TeBG. It is not clear how the differences in the amounts of these materials are related to their physiological roles, but they may reflect species differences in the plasma membranes of target organ cells.

The association constant at 4°C and the number of binding sites for testosterone of the bovine TeBG were found to be 1.1×10^8 M^{-1} and 0.8, respectively. The former value is in good agreement with that of guinea pig, 0.8×10^8 M^{-1} (*7*), but is somewhat lower than those of rabbit TeBG, 6.2×10^8 M^{-1} (*8*), and human TeBG, 4.5×10^8 M^{-1} (*2*), 1.1×10^9 M^{-1} (*12*), or 1.2×10^9 M^{-1} (*3*). The bovine TeBG had a stronger affinity for DHT than for testosterone, like other TeBG's (*13*). A low but distinct affinity for estradiol 17β was found in the protein, which is often called testosterone-estradiol binding globulin or sex steroid binding plasma protein (see ref. *12*).

The finding that the bovine TeBG consists of three subunit components of very similar size is interesting. The dissociation into subunits has been accomplished by treatment with guanidine-HCl, GEDTA or SDS, while no dissociation by SDS was observed in the case of human TeBG (*13*). The subunits were able to reassociate on dialysis in the presence of Ca^{2+}, recovering testosterone-binding activity. The effect of Ca^{2+} on the stability of TeBG during the isolation procedure has already been reported (*13*). It is likely that Ca^{2+} is essential for maintenance of a molecular configuration with high affinity for DHT and testosterone. This is the first report concerning subunits of TeBG of serum. In the case of the " receptor protein " of cytosol, the uterine protein was reported to dissociate into subunits (probably two) and to be stabilized by binding with estradiol 17β (*34*). The difficulty of obtaining the protein and its subunits in sufficient amounts has hampered investigations of the interaction of steroids with the proteins and their role in transportation into nuclei and gene transcription. Studies on the

isolation of TeBG subunits and their interaction with androgen may provide useful information in this respect.

The adsorbents used in the affinity chromatography of the present report were prepared by coupling 17α-carboxyethynyl-17-hydroxy-4-androsten-3-one to aminoethyl-Sepharose. Similar results were obtained using 17α-carboxyethynyl-17-hydroxy-5α-androstan-3-one, but not with testosterone 17-hemisuccinate, estradiol 17-hemisuccinate, or testosterone 3-(O-carboxymethyl)-oxime. This is in accord with the findings that TeBG binding requires a C-19, Δ^4-androstene or 5α-androstane steroid with an unhindered 17β-ol group and 3-ol or 3-one group (*35, 36*). The adsorbents used in the present experiments have two free functional groups and bind *via* a spacer to Sepharose at the 17α position, thus ensuring high affinity for TeBG. On the other hand, adsorbents recently used for the isolation of human TeBG which are bound to agarose at 17β-ol (*12*) or 3-one (*13*) were reported to give good results. To some degree, this may be ascribed to longer spacers used by these research groups and the spatial configuration of ring A (*12*).

Elution of the bound proteins from the adsorbents is often a difficult task and causes undesirable denaturation (*11*). In the present experiments, efficient release of TeBG from the affinity column was accomplished by incubation at room temperature in the presence of a large amount of testosterone. Thus, isolation of TeBG from bovine serum was easily and rapidly performed in only two steps. This method may be useful for isolation of " androgen receptor protein " in the cytosol of target organs, and, in fact, attempts to isolate a prostatic receptor protein are now under way.

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