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Purification and Characterization of the Sex Steroid-binding Protein of Rabbit Serum

COMPARISON WITH THE HUMAN PROTEIN*

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The sex steroid-binding protein (rSBP) of immature rabbit serum was purified to homogeneity by the sequential use of DEAE-cellulose chromatography, affinity chromatography on 5α-dihydrotestosterone-17β-succinyl-diaminoethyl-(1,4-butanediol diglycidyl ether)-agarose, agarose (Bio-Gel A-0.5m) gel filtration, and preparative polyacrylamide gel electrophoresis. The cumulative yield is 13%. Homogeneity of rSBP was shown by the equilibrium sedimentation ultracentrifugation in 6 M guanidine HCl containing 0.1 M mercaptoethanol which yields an average molecular weight of 36,475 ± 865. Analytical gel electrophoresis in the presence of sodium dodecyl sulfate and gel filtration on agarose yield a molecular weight of 57,000 and 120,000, respectively. The variation is due to a 30% carbohydrate content. The amino acid composition is reported. Comparison of the rabbit and human SBP indicate that they are different in both their molecular and functional properties.

About 11 years ago a specific protein (SBP)[1] which binds the sex steroids testosterone and 17β-estradiol was discovered in the plasma of humans (1, 2). A large number of clinical studies relating the levels of hSBP to various physiological and pathological conditions involving sex steroid hormones in humans have been published (for review see Refs. 3 and 4). We have been interested in the physicochemical characterization of this protein isolated from human pregnancy serum (5, 6) in the attempt to develop a model system with which to study the chemical nature of the steroid binding process, with the ultimate goal of explaining the phenomenon of steroid binding specificity. In addition, the interest on the biological function of hSBP has prompted us and others to develop other animal model systems. SBP has been shown to exist as a molecular entity in the subhuman primates *Macaca nemistrina* (7), *Macaca speciosa* (8), *Macaca mulatta* (9), and *Papio cynocephalus* (baboon),[2] the dog (10, 11), the cat (12), and the rabbit (12–15). A preliminary study on the characterization of rabbit SBP has been published (16). In this paper, we report a purification method which leads to homogeneous rabbit SBP. We also report on the physicochemical properties of this protein, and we compare them to those of human SBP.

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[1] The abbreviations used are: SBP, sex steroid-binding plasma protein; rSBP, rabbit SBP; hSBP, human SBP; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DHT, 5α-dihydrotestosterone; DHTS, 5α-dihydrotestosterone-17β-succinate. The trivial names used are: 5α-dihydrotestosterone, 5α-androstan-17β-ol-3-one; 5α-dihydrotestosterone-17β-succinate, 5α-androstan-3-one-17β-succinate.

MATERIALS AND METHODS

Chemicals—Immature rabbit serum (type 1) was obtained from Pel-Freez, Rogers, Ark. Human serum was collected fresh from pregnant women. Transferrin (human), hemoglobin (human), ovalbumin, and catalase were purchased from Sigma Chemical Co. Bovine serum albumin was obtained from Miles Laboratories, Inc. Alcohol dehydrogenase (yeast) and glucose oxidase (*Aspergillus niger*) were purchased from Worthington Biochemical Corp. Carbonic anhydrase and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) were obtained from CalBiochem. Sepharose 4B was obtained from Pharmacia and microgrannular DEAE-cellulose (Whatman DE52) and DEAE-cellulose filter paper discs (Whatman DE81, 2.3 cm diameter) were from Reeve Angel. All reagents for polyacrylamide gel electrophoresis were obtained from Canalco, Inc. 5α-[1,2-^{3}H]Dihydrotestosterone (44 Ci/mmol) and Omnifluor were purchased from New England Nuclear. Radioactive steroids were at least 98% pure. Radioinert steroids were obtained from Sigma. Ethylenediamine was obtained Eastman Kodak. 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide was obtained from either Ott Chemical Co. or Aldrich. All other chemicals were reagent grade.

Assay of 5α-Dihydrotestosterone Binding Activity—The filter assay was used as previously described (17). The filter efficiency, which is a measure of the per cent of steroid·protein complexes remaining on the filter after the removal of nonspecific binding, was obtained by using rabbit serum (1:20 diluted) or partially purified rSBP. The efficiency was calculated to be 63% under conditions where 99% of the steroid was bound to SBP. This can be readily calculated by using K_D (4°C) of 0.86×10^{-9} M (Table I) for the DHT·SBP complex. Under the conditions of the assay, 37% of the DHT·SBP complexes are therefore not retained by the filter discs and a correction to 100% is applied to estimate the total concentration of SBP in a particular sample.

Synthesis of 5α-[1,2-^{3}H]Dihydrotestosterone-17β-succinate—This synthesis was carried out as previously described (6).

Synthesis of 5α-Androstan-17β-ol,3β-succinate—5α-Androstane-3β-ol-17-one (6.8 mmol) and succinic anhydride (20 mmol) were dissolved in 10 ml of warm, freshly distilled pyridine and refluxed for 6 h at which time the reaction had gone to completion as indicated by thin layer chromatography ($CHCl_3$:MeOH, 95:5). Concentrated HCl was then added until a precipitate formed. Ice-cold acidified water (100 ml, pH 3) was then added. The precipitate was washed with ice cold acidified water and then with ice cold 20% methanol. The yield was 98% and the product melted at 256°C. 5α-Androstane-17-one,3β-succinate obtained from Steraloids, Inc. had a melting point of 258–259°C. 5α-Androstane-3β-ol-17-one succinate (6 mmol) was dissolved in 150 ml of methanol and the mixture was chilled to 0°C.

[2] S. Neuhaus and P. H. Pétra, manuscript in preparation.

Sodium borohydride (1.9 g) was slowly added. After 1 h at 0°C, the solution was acidified to pH 3 with glacial acetic acid and the volume was reduced *in vacuo* until the product started to precipitate. Approximately 20 ml of water (pH 3) were added. The precipitate was washed with water. The product had a melting point of 212–213°C which agrees with that reported previously (18). The product was pure when analyzed by thin layer chromatography using two systems ($CHCl_3$:MeOH, 95:5 and C_6H_6:EtOH, 90:10).

Synthesis of Diaminoethyl-oxirane-agarose/3,3'-diaminodipropylamine-agarose, and Coupling of Steroids to Aminoalkyl Derivatives of Agarose—These procedures were carried out as previously described (5, 6).

DEAE-cellulose Chromatography of Rabbit Serum—Immature rabbit serum (500 ml) was dialyzed against 20 liters of 0.02 M sodium phosphate(pH 6.8, 4°C) for 4 days with one change of dialysis buffer. The dialyzed serum was pumped onto a column (5.0 × 55 cm) containing DEAE-cellulose previously equilibrated with the starting buffer. After elution of the protein not adsorbing to the column, the elution buffer was changed to 0.03 M sodium phosphate (pH 6.8, 4°C) and elution was continued until the absorbance at 280 nm became negligible. At that point, a linear gradient in sodium phosphate concentration was started. The limit buffer was 0.10 M sodium phosphate (pH 6.8, 4°C) and the total gradient consisted of 4 liters. Elution was performed at a flow rate of 330 ml/h using a peristaltic pump. The filter assay method was used to assay for DHT-binding activity. The active fractions were pooled, concentrated to 300 ml, dialyzed against water, lyophilized, and stored at −20°C.

Procedure for Affinity Chromatography—The lyophilized powder (8 g) partially purified by DEAE-cellulose chromatography as described above were dissolved in 200 ml of 10 mM Tris containing 0.5 M NaCl (pH 7.4, 4°C). The protein solution was gently stirred for 5 h with 50 ml of affinity adsorbent diluted 5-fold with unsubstituted Sepharose 4B. The mixture was poured into a column (5-cm diameter) and washed with 10 mM Tris continuing 1 M NaCl until the absorbance at 280 nm was negligible. The agarose was then removed from the column and stirred for 8 to 12 h at 4°C with 300 ml of 10 mM Tris containing 10^{-5} M [^{3}H]DHT (0.04 Ci/mmol), 0.5 M NaCl, and 10% glycerol. The mixture was then placed in a Buchner funnel (coarse) and washed with 100 ml of the above buffer at 4°C. The eluted protein was concentrated to 5 to 10 ml and dialyzed against buffer (10 mM Tris, 0.1 M NaCl, 10^{-5} M [^{3}H]DHT (0.04 Ci/mmol), 10% glycerol) using an Amicon model TCF-10 thin channel system with PM-30 membranes. In our most recent work we use PM-10 membranes which have better retention of SBP.

Agarose Chromatography—The protein fraction (16.8 mg) partially purified by affinity chromatography was concentrated to 2 ml and dialyzed against 5 ml of 10 mM Tris (pH 7.4, 4°C) containing 0.14 M NaCl, 10^{-5} M [^{3}H]DHT (0.04 Ci/mmol) using an Amicon model 12 cell with a PM30 membrane. The protein solution was pumped onto a column (2.5 × 75 cm) containing agarose (Bio-Gel A-0.5m) at a flow rate of 12 ml/h. The active fractions were pooled, concentrated to 2 ml, dialyzed against 5 ml of 10 mM Tris containing 0.14 M NaCl, 10% glycerol, and 10^{-5} M [^{3}H]DHT (0.04 Ci/mmol) using the Amicon model 12 cell. The yield from this procedure is 95% with an increase in specific activity of 10% (Table II). This procedure serves to remove substances which renders the protein solution opalescent and which interfere with protein stacking during the preparative gel electrophoresis procedure.

Preparative Polyacrylamide Gel Electrophoresis—Preparative gel electrophoresis was performed with a Canalco "Prep Disc" apparatus according to their instruction manual. Polyacrylamide gels (5%) were prepared by the method described by Davis (19), as modified by Shuster (20). Polymerization was performed at room temperature and the gels were allowed to remain at room temperature for 4 h before cooling to 4°C. A 1-ml solution containing up to 30 mg of protein was fractionated in a gel having a diameter of 2.5 cm and a length of 6 cm. Electrophoresis was performed at 20 mA (400 to 500 V) at 4°C. The proteins emerging at the bottom of the gel were removed continuously with buffer (0.38 M Tris-Cl, pH 8.9) at a rate of 72 ml/h. Six-milliliter fractions were collected. The active fractions were pooled, concentrated to 3 to 5 ml, and dialyzed against 10 mM Tris, 0.1 M NaCl, DHT (approximately 10^{-5} M), and 10% glycerol, and stored at 4°C. Calcium (1 mM) was added to SBP solutions.

Analytical Polyacrylamide Gel Electrophoresis—Analytical disc gel electrophoresis was performed by the method described by Davis (19) as modified by Shuster (20). Polymerization of the 5% acrylamide gels (100 × 5 mm inside diameter) was performed at room temperature. The gels were then subjected to electrophoresis at 4°C for 2 to 3 h at a constant current of 2 mA/gel. Samples were made 10% in glycerol, and bromphenol blue solution was added as the tracking dye. Electrophoresis was performed until the tracking dye had migrated approximately 7 cm (3 h at 4°C, 1.5 mA/gel). Gels were stained with napthol blue black (1%) overnight, then destained electrophoretically transversely. Gels to be assayed for binding activity were polymerized in the presence of 1 nM [^{3}H]DHT. Following electrophoresis, gels were severed at the dye front and sliced transversely into 1.3-mm discs. The discs were placed in counting vials with scintillant. Essentially all of the steroid was extracted after standing overnight. Analytical gel electrophoresis in the presence of SDS was performed according to the method described by Weber *et al.* (21). Protein samples were denatured in 1% SDS and 1% 2-mercaptoethanol at 100°C for 5 min. Samples (50 μl) containing usually 20 μg of protein were applied per gel.

Equilibrium Sedimentation Ultracentrifugation—Ultracentrifugation was performed in a Spinco model E ultracentrifuge using a 6-channel Yphantis centerpiece (22). Kodak II-G photographic plates were used to record the Rayleigh interference patterns. The data were analyzed on a Nikon 6C microcomparator controlled by a PDP-12 computer (23). Base-line data were obtained at the end of a run by vigorous shaking of the cells and re-centrifuging at low speed (24). Lyophilized protein was weighed out and dissolved in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol. The protein solution (1 ml, 1 mg/ml) was dialyzed for at least 48 h with 10 ml of 6 M guanidine HCl at 25°C. The appropriate sample concentrations (1.0, 0.67 and 0.33 mg/ml) were obtained by dilution with the dialysate. Molecular weights were calculated by computer by the method of Teller (25).

Other Methods—Apparent specific volume, determination of amino acid composition, carbohydrate analysis, and radioactivity measurements were all carried out as previously described (5, 6). Protein determinations were carried out by the method of Lowry *et al.* (26).

RESULTS

Equilibrium Constants of Dissociation (K_s) of DHTS and Other Steroids—K_s (DHTS) was determined by the "competitive" Scatchard method (5, 7). The following relationship holds:

$$K_P = K_D\ (1 + [S]/K_S)$$

where K_P = equilibrium constant of dissociation in the presence of competing steroid, K_D = equilibrium constant of dissociation of DHT, $[S]$ = concentration of radioinert competing steroid, and K_S = equilibrium constant of dissociation of the competing steroid. Fig. 1 represents the results for the

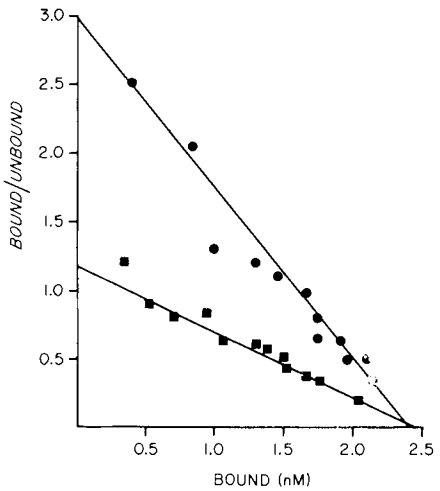

FIG. 1. Determination of the equilibrium dissociation constant, K_S, of DHTS by the "competitive" Scatchard method. The filter assay at 4°C was used. Aliquots (0.5 ml) of serum from immature rabbit diluted 200-fold with 10 mM Tris, pH 7.4, were incubated with 0.78 nM to 8.2 nM [^{3}H]DHT (40 Ci/mmol) with (■——■) or without (●——●) 3.46×10^{-7} M DHTS.

binding of DHTS to rSBP where $K_D = 8.0 \times 10^{-10}$ and K_S (DHTS) = 2.12×10^{-7} M as calculated from the equation above. The incorporation of a succinate group at the 17β position of DHT reduces the binding affinity to rSBP by a factor of 265. The affinity of DHTS, however, is still high enough to be used as a ligand for affinity chromatography. Also, since DHTS binds at the same site as DHT on the protein as shown by the convergence of both lines on the abscissa of Fig. 1, there is further assurance of a specific interaction on the agarose matrix. Table I shows the values of equilibrium constants of dissociation of DHTS as well as other related steroids as determined by the same method for both rSBP and hSBP.

DEAE-cellulose Chromatography of Rabbit Serum—The DHT-binding activity elutes at 0.04 M phosphate as shown in Fig. 2. The recovery of SBP is 80% with a 3-fold increase in specific activity (Table II). When a shallower salt gradient is used, the DHT-binding activity separates into two partially resolved peaks. Steady state electrophoresis, heat denaturation, steroid dissociation rates, and chromatography on agarose Bio-Gel A-0.5m carried out on each peak show no significant differences. Further work is in progress to explain the anomaly on DEAE-cellulose chromatography. Hansson *et al.* (16) have also observed the same phenomenon and have suggested that variability in the carbohydrate content may explain the heterogeneity.

Affinity Chromatography of rSBP—Two agarose derivatives were considered for the purification of rSBP (Fig. 3). One in which DHT is bound to the agarose matrix at the 17β position (derivative A), and the other at the 3β position (derivative B). On the basis of binding studies (Table I), it was expected that agarose derivative B would be the ligand of choice for the synthesis of an affinity adsorbent for rSBP. However, preliminary experiments showed that only 3% of rSBP could be adsorbed to this column. Consequently, the purification was carried out with agarose derivative A which had already been used successfully for the purification of hSBP (6). Partially purified rSBP obtained from the DEAE-cellulose step was incubated with agarose derivative A as described in the methods. Approximately 20% of the binding activity is retained by the affinity adsorbent, the rest can be dialyzed, lyophilized, and reapplied to a new batch of affinity adsorbent. The low 20% retention of rSBP by the column (compared to 80% in the case of hSBP, see Ref. 6) is probably due to the rapid rate of dissociation of the DHT·rSBP complex. The $t_{1/2}$ for the dissociation of this complex at 0°C is 5.2 min compared to 67 min for the DHT·hSBP complex (16). The retained rSBP was then specifically removed from the adsorbent by eluting with 300 ml of 10 mM Tris containing 10^{-5} M [^{3}H]DHT (0.04 Ci/mmol), 0.5 M NaCl, and 10% glycerol. The final yield of SBP in this step is 20% with 100-fold increase in specific activity (Table II).

Preparative and Analytical Polyacrylamide Gel Electrophoresis—After the agarose Bio-Gel step (see "Materials and Methods"), rSBP is purified to homogeneity by preparative PAGE. Fig. 4 shows the purification elution pattern of 14.4 mg of protein. The active fractions were pooled, concentrated to 4 ml, and stored at 4°C in the presence of 10^{-5} M [^{3}H]DHT (0.04 Ci/mmol) and 1 mM Ca^{2+}. Analytical electrophoresis of 50 μg of rSBP in 5% gels and in the presence of 1 nM [^{3}H]DHT (40 Ci/mmol) yields one single band of R_F 0.40 (Fig. 4, *Gel A*). When the gel is sliced into 1.3-mm sections and counted, one single radioactive peak appears at the same R_F as the stained band. The same R_F is obtained when electrophoresis is performed on unfractionated serum indicating that the purification procedure has not affected the molecular properties of rSBP.

TABLE I

Equilibrium dissociation constants of steroids to SBP

Experiments were performed with the "competitive" Scatchard method as shown in Fig. 1.

Steriod	Human (×10^9 M, 4°C)	Rabbit (×10^9 M, 4°C)
Dihydrotestosterone	0.42	0.86
Testosterone	1.89	3.7[a]
Estradiol-17β	4.79	84.6
Estrone	17.6	32.3
Estriol	115	68.3
Diethylstilbestrol	246	210
Progesterone	154	666
5α-Dihydrotestosterone-17β-succinate	250	212
5α-Androstan-17β-ol-3β-succinate	1430	78.2

[a] A value of 3.3×10^{-9} M was obtained when analysis was done directly with [^{3}H]testosterone instead of the "competitive" Scatchard method. The filter assay "efficiency" for [^{3}H]testosterone was 53%.

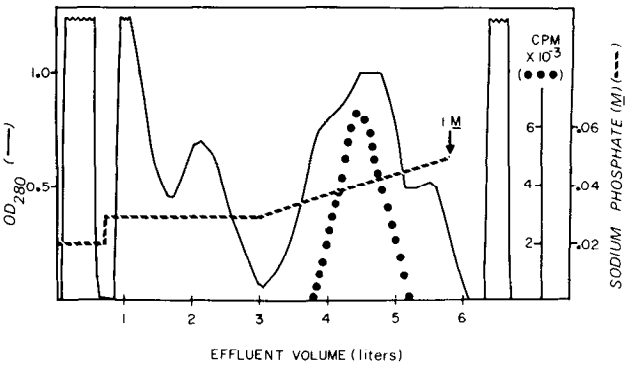

FIG. 2. DEAE-cellulose chromatography of rabbit serum. Five hundred milliliters were dialyzed against 20 liters of 0.02 M sodium phosphate (pH 6.8, 4°C) before applying to a column (5.0 × 55 cm) of DE52. The phosphate concentration in the column was changed to 0.03 M after the first peak was eluted. After the 0.03 M sodium phosphate, a linear gradient of sodium phosphate (4 liters, 0.03 to 0.10 M) was started. Elution was performed at 330 ml/h. The column was then washed with 1 M NaCl at the *indicated arrow*. Every 10th fraction was assayed for DHT-binding activity by the filter assay method after diluting 10-μl aliquots 1:50 with 10 mM Tris buffer (pH 7.4).

TABLE II

Rabbit SBP purification scheme

Step	A (total mg protein)	B[a] (total ng DHT bound)	% recovery (at each step)	Specific activity (B/A)[b]	Cumulative purification	Cumulative yield
						%
Serum (500 ml)	29,600	73,500	100	2.48	1	100
DEAE-cellulose	8,000	58,800	80	7.35	3	80
Affinity chromatography	16.8	12,348	21	735	300	17
Bio-Gel A-0.5m	14.4	11,731	95	815	329	16
Preparative PAGE	2.1	9,408	80	4,480	1,806	13

[a] The filter assay was used to measure specific DHT-binding activity.

[b] Specific activity is expressed as nanograms of DHT bound/mg of protein.

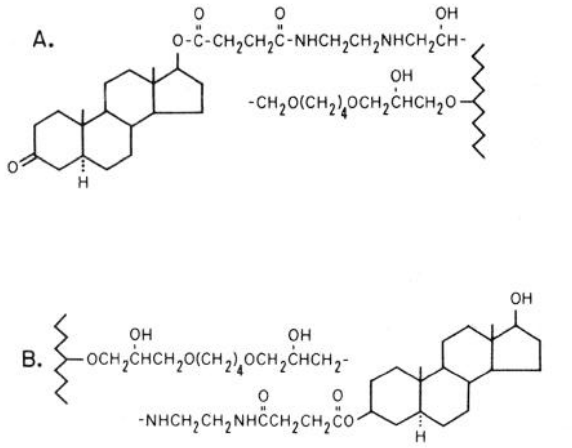

FIG. 3. 5α-Androstane derivatives of agarose used for the purification of rSBP. *A*, 5α-androstane-3-one, 17β-succinyl-diaminoethyl-(1,4-butanediol diglycidyl ether)-agarose. *B*, 5α-androstane-17β-ol,3β-succinyl-diaminoethyl-(1,4-butanediol diglycidyl ether)-agarose.

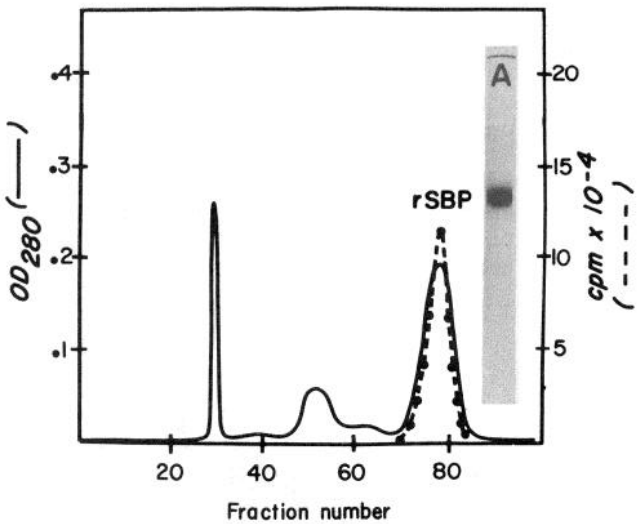

FIG. 4. Preparative polyacrylamide gel (5%) electrophoresis of 14.4 mg of rSBP obtained from agarose (Bio-Gel A-0.5m) gel filtration. The protein solution (2 ml), already dialyzed against 10 mM TrisCl, pH 7.4, containing 0.14 M NaCl, 10% glycerol, and 10^{-5} M [^{3}H]DHT (0.04 Ci/mmol), was layered onto a 5% acrylamide gel column (2.5 cm (diameter) × 6.0 cm (height)). Electrophoresis was performed at 4°C and 20 mA. Proteins were eluted at 72 ml/h with 0.38 M Tris-Cl, pH 8.9. Fractions (6 ml) were collected and 10-μl aliquots were removed and assayed by the filter assay after diluting 1:20 with 10 mM Tris-Cl, pH 7.4. The native gel represents 50 μg of rSBP obtained from the pooled area containing the radioactivity. $R_F = 0.40$, the gel was cut at dye front.

Molecular Weight and Apparent Specific Volume of Homogeneous rSBP—rSBP elutes with apparent molecular weight of 120,000 when applied to a calibrated column of agarose (Bio-Gel A-0.5m), the same value is obtained with unfractionated serum. Electrophoresis of 20 μg of rSBP in the presence of SDS (plus or minus mercaptoethanol) yields one polypeptide chain of 57,000 molecular weight (Fig. 5). Sedimentation equilibrium of rSBP in 6 M guanidine HCl containing 0.1 M mercaptoethanol indicates that the weight average molecular weight is 36,475 ± 865 (Fig. 6). The apparent specific volume is 6 M guanidine HCl is 0.698 ml/g as calculated from the amino acid analysis and carbohydrate content as follows:

The apparent specific volume of the polypeptide portion of rSBP is 0.736 ml/g as calculated by the method of Cohn and Edsall (28). Correction of this number for preferential interactions with guanidine hydrochloride yields 0.731 ml/g in 6 M guanidine hydrochloride as calculated by the method of Lee and Timasheff (29). From the carbohydrate content of rSBP of 30% together with an apparent specific volume of carbohydrate of 0.62 ml/g as computed from the data of Longsworth (30), a value of 0.698 ml/g is calculated for the apparent specific volume of rSBP in 6 M guanidine hydrochloride. This value was used for the determination of the molecular weight. An uncertainty of 5% in the apparent specific volume of carbohydrate propagates to a 5.2% uncertainty in the molecular weight reported here. A similar calculation was performed for the estimation of the apparent specific volume of hSBP in 6 M guanidine hydrochloride (6).

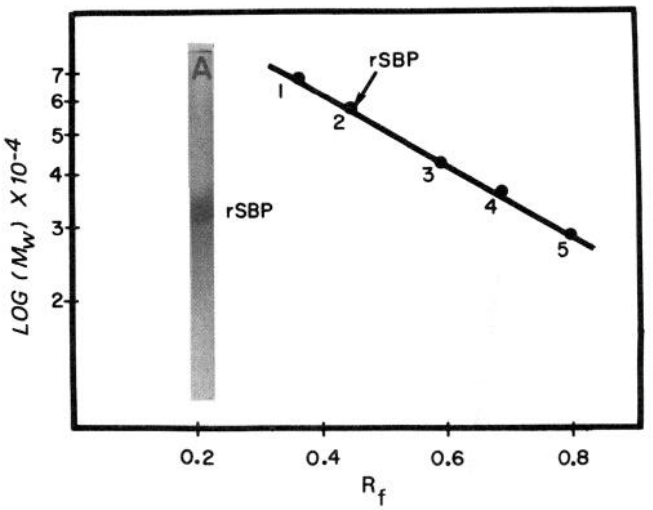

FIG. 5. Determination of molecular homogeneity and weight by SDS-gel (7.5%) electrophoresis. Calibration was done by applying 10 μg of each protein in 50 μl of SDS buffer. The standards were: *1*, human serum albumin (69,000); *2*, catalase (58,000); *3*, ovalbumin (43,000); *4*, glyceraldehyde-3-phosphate dehydrogenase (36,000); *5*, carbonic anhydrase (29,000). The SDS gel represents 20 μg of purified rSBP. $R_F = 0.44$. The gel was cut at the dye front.

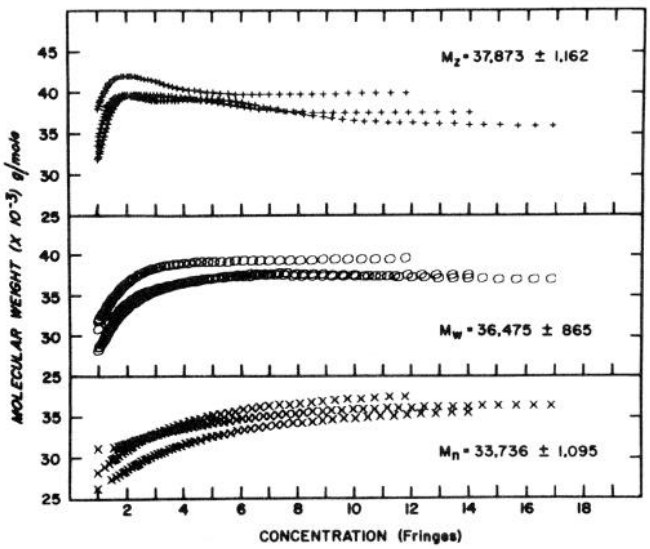

FIG. 6. Molecular weight distribution of rabbit SBP obtained by equilibrium sedimentation. Data presented are computer-generated plots of molecular weight *versus* concentration (fringes) from analysis of a Rayleigh plate. Initial loading concentrations of approximately 1.0, 0.67, and 0.33 mg/ml were centrifuged for 24 h at 28,000 rpm and 23°C in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol. M_n, M_w, and M_z represent the number average molecular weight, the weight average molecular weight, and the *z*-average molecular weight, respectively.

TABLE III

Amino acid and carbohydrate composition of SBP (mol/mol of SBP)

Values were calculated using a polypeptide molecular weight of 25,533 for rSBP and 29,795 for hSBP (6).

Amino acid	hSBP	rSBP
Half-cystine	6.0	3.2
Aspartic acid	25.1	21.7
Threonine	17.7	10.9
Serine	27.9	23.8
Glutamic acid	25.1	26.3
Proline	21.4	23.9
Glycine	33.1	21.4
Alanine	18.5	17.9
Valine	14.3	14.8
Methionine	3.1	2.9
Isoleucine	8.5	6.4
Leucine	35.3	34.1
Tyrosine	3.3	1.4
Phenylalanine	9.6	7.5
Histidine	8.0	3.5
Tryptophan	2.4	1.2
Lysine	11.3	9.6
Arginine	11.6	10.9
	%CHO	
Hexose	5.0	12.2
N-Acetylhexosamine	6.0	7.4
Sialic acid	6.5	9.3
Fucose	0.5	1.2
	18.0	30.1

Amino Acid Composition and Carbohydrate Content of rSBP—The carbohydrate content is 30% as shown in Table III. The amino acid composition was calculated on the basis of a polypeptide backbone molecular weight of 25,533 (70% of the average molecular weight of 36,475).

DISCUSSION

The procedure for the purification of rSBP described here is very similar to the one we recently reported for hSBP (6). In each case the crucial step for obtaining homogeneous protein is affinity chromatography. For the human protein the step is necessary to remove transferrin which cannot be separated from hSBP with conventional methods of chromatography or electrophoresis. Although the same probably applies here, we cannot be sure since pure rabbit transferrin was not available to us to use as standard. Nevertheless, affinity chromatography is necessary to remove another impurity detectable by SDS-gel electrophoresis which is present in the final preparation when we attempted to purify rSBP with conventional methods alone. The main disadvantage of affinity chromatography, however, is the low yield per preparation (Table II). The problem arises as a result of the high rate of dissociation of rSBP·steroid complexes, an inherent physicochemical characteristic of the rSBP steroid binding site. For instance, at 0°C the $t_{1/2}$ for the dissociation of DHT from rSBP in serum is 13 times greater than that of hSBP (16), even though both proteins have similar dissociation equilibrium constants for DHT (Table I). It is very likely that this inherent difference in the two binding sites will hold true for other steroids whether or not they are attached to agarose matrices. In fact, we had expected the agarose derivative B (Fig. 3) containing DHT bound at the 3β position to be a better affinity adsorbent for the purification of rSBP than agarose derivative A since the succinate DHT derivative of the former has a lower equilibrium constant of dissociation than the latter (Table I). However, the opposite was found, suggesting that the rate of dissociation of steroid derivatives having groups in the 3β position must be higher. Therefore, the important parameter to consider for steroid affinity adsorbents is the rate constant of dissociation rather than the equilibrium constant of dissociation. In view of these considerations, it is unlikely that better conditions will be found to improve the affinity chromatography step for rSBP. In the case of hSBP such a problem does not exist because the rate of dissociation of DHT at 0°C is slow ($t_{1/2}$ = 68 to 77 min, see Ref. 16 and 27). Although the 17β-succinate derivative of DHT would be expected to dissociate faster, the rate is still slow enough to permit the SBP complex to survive the chromatography. The result is a yield of 80% for that step (6). Although 20% yield for rSBP is low, the rest of the rSBP can be recovered and reapplied to another equilibrated column of affinity adsorbent; thus little rSBP is actually lost in the process.

Comparison of the physicochemical properties of rSBP and hSBP so far determined reveals a pattern consisting of more differences than similarities. Mobilities of homogeneous rSBP (Fig. 4) and hSBP (5) in analytical PAGE are significantly different, R_F (rSBP) = 0.40 and R_F (hSBP) = 0.32. These data agree with those reported for unfractionated sera (14, 16). The R_F (rSBP) is also higher than that of *Macaca nemestrina* SBP (0.32, see Ref. 7), dog SBP (0.32, see Ref. 11), cat SBP (0.34, see Ref. 12), and baboon (*Papio cynocephalus*) SBP (0.32).[2] These results indicate that rSBP behaves more like an α-globulin than a β-globulin in native gels and therefore is different from all the other SBPs so far examined.

Studies on the binding affinity of various sex steroids hormones to rSBP and hSBP indicate that the rabbit protein is mainly an androgen binding protein (Table I), consistent with published data. Even though estrogens bind weakly, their interaction is still a specific one since they compete with androgens for the same site as shown by the "competitive" Scatchard method. The situation is analogous to dog SBP which is also regarded as an androgen binding protein (10, 11). However, under physiological conditions it is unlikely that estrogens are significantly bound to these proteins in rabbit and dog plasma. The existence of an unhindered 17β-OH group is shown to be an important requirement for high affinity to both rSBP and hSBP since progesterone and DHTS bind weakly. Why hSBP binds progesterone 4 times more than rSBP is not apparent; however, in view of the high dissociation equilibrium constants, binding of this steroid to hSBP at physiological conditions is probably not significant. Another interesting finding involves the topology of the binding site where the 3β position of the steroid molecule interacts. rSBP can accommodate a bulky group in that position much better than hSBP as shown in Table I. On the other hand, when the succinate group is at the 17β position, the steroid binds equally well to both proteins. Since the difference between androgenicity and estrogenicity is dictated by the stereochemistry of ring A as shown in testosterone, 5α-dihydrotestosterone, and 17β-estradiol, chemical studies on the rSBP binding site will reveal structural elements which specifically determine androgenicity.

A comparison of the amino acid composition of rSBP and hSBP reveal significant similarities (Table III). The proline content is relatively high when compared to proteins of the same size and may perhaps play a specific role in the three-dimensional structure of the polypeptide chains. The low content of methionine will facilitate sequence studies provided they are located far from one another in the primary structure. Similarity in amino acid composition, however, does not necessarily mean that the two proteins will have similar tertiary structures. In fact, recent experiments indicate quite the opposite. A monospecific antibody directed against homogeneous hSBP does not cross-react with rSBP,[3] suggesting that

[3] S. Bordin, J. Lewis, and P. H. Pétra, submitted for publication.

significant portions of the native configuration of these two proteins will not be structurally related.

The molecular weight of rSBP varies according to the method used for its determination. The variation is due to the carbohydrate content which is generally known to produce anomalies during the experimental determination of molecular weights of glycoproteins by gel filtration, PAGE, and SDS-PAGE as discussed in our recent work on hSBP (6). The molecular weights of pure rSBP by agarose gel filtration and SDS-PAGE were found to be 120,000 and 57,000, respectively. The results might suggest that native rSBP be composed of two identical subunits which become dissociated in the presence of SDS. However, estimation of the molecular weight of the pure protein by sedimentation equilibrium in 6 M guanidine HCl revealed a weight average molecular weight of 36,475. Since a correction factor for the contribution of the carbohydrate side chains to the molecular weight as determined in 6 M guanidine HCl has been applied in this method, the value obtained is the most accurate to date. It should be realized, however, that 36,475 may not necessarily be the molecular weight of native rSBP unless the protein exists as a monomer in its native conformation. A dimer would yield a value of approximately 73,000 which correlates well with published results on unfractionated rabbit serum (13, 16). The estimation of the molecular weight of pure rSBP and hSBP in nondenaturation conditions is in progress. The data of Fig. 6 indicate that the preparation of rSBP shows polydispersity in the ultracentrifuge (M_n, M_w, and M_z are not equal). Since rSBP appears homogeneous by SDS-PAGE, we believe that the polydisperse nature of rSBP is caused by changes in the carbohydrate content. Further experiments are needed to substantiate this proposal.

In summary, the data reported here support the conclusion that rSBP and hSBP are significantly different in their molecular and functional characteristics. Consequently, studies on the biological role of SBP in the rabbit may not necessarily apply to the human model. In fact, recent findings indicate that rSBP decreases during the gestation period in the rabbit,[4] whereas it increases dramatically in the pregnant woman. Nevertheless, these and other data strongly support the contention that models such as the rabbit must be developed in order to better understand the chemistry of the steroid binding phenomenon and the role of SBP in nature. For example, studies in the rabbit may reveal biological roles for SBP different from those present in man. Furthermore, it is very likely that certain structural elements in the rSBP steroid binding site (as well as that of hSBP) will be found in androgen receptors, providing a model system for the study of these proteins.

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[4] K. E. Mickelson, Y. Kuwubara, and P. H. Pétra, manuscript in preparation.

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