Purification of the Sex Steroid Binding Protein from Human Serum†

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ABSTRACT: The sex steroid binding protein from human pregnancy serum was purified to homogeneity by affinity chromatography and preparative polyacrylamide gel electrophoresis. The selective adsorbants were prepared by coupling [^{3}H]-5α-dihydrotestosterone 17β-hemisuccinate to 3,3′-diaminodipropylamine-agarose, poly(Lys-DLAla)-agarose, and albumin-agarose. The most effective adsorbant purifying for the binding protein was 5α-dihydrotestosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine-agarose. A preparative procedure with 5α-dihydrotestosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine-agarose yielded active material which was further purified by preparative polyacrylamide electrophoresis at pH 9.5. Homogeneity was shown by analytical disc gel electrophoresis at three different pH units. A single radioactive band corresponding to the stained band was shown by incubating with [1,2-^{3}H]-5α-dihydrotestosterone prior to electrophoresis. The radioactive peak corresponding to the pure sex steroid binding protein could not be detected when a 100-fold excess of 17β-estradiol was present in the incubation prior to electrophoresis demonstrating the specific sex steroid binding properties of this protein. The migration of this peak was identical with that obtained when diluted serum was electrophoresed under the same conditions in the presence of [1,2-^{3}H]-5α-dihydrotestosterone indicating that no significant changes in the molecular characteristics of the binding protein occurred during the purification procedure. The presence of carbohydrate in the pure protein was shown by the periodic acid–Schiff reagent procedure. Selective adsorbants containing 17β-estradiol linked at the 3 position were ineffective in retaining sex steroid binding protein activity.

Much research has established that the sex steroid hormones, 17β-estradiol and testosterone, are bound to proteins in the plasma of man and other species (for reviews, see Antoniades *et al.*, 1960; Crépy and Guériguian, 1970; Westphal, 1971; Anderson, 1974). Originally, albumin was thought to account for all the sex steroid binding capacity of serum (Eik-Nes *et al.*, 1954; Slaunwhite and Sandberg, 1959), but further studies using chromatographic and electrophoretic methods provided direct evidence for the existence of a specific sex steroid binding protein in the β-globulin fraction of human plasma (Mercier *et al.*, 1966; Rosenbaum *et al.*, 1966; Guériguian and Pearlman, 1968; Rosner *et al.*, 1969). Studies on the relative specificity of binding of various steroids in human plasma have revealed that the protein is highly specific for steroids having an hydroxyl group in the 17β position and a generally planar steroid structure (Vermeulen and Verdonck, 1968; Heyns *et al.*, 1969; Kato and Horton, 1968). 5α-Dihydrotestosterone was found to have the highest affinity for the protein when compared to estradiol and testosterone (Forest and Bertrand, 1972; Shanbhag *et al.*, 1973; Mickelson and Pétra, 1974b) and various assay procedures have been developed using this steroid as ligand to measure binding activity (Corvol *et al.*, 1971; Heyns and DeMoor, 1971; Rosner, 1972; Shanbhag *et al.*, 1973; Mickelson and Pétra, 1974a). The binding protein is present in the plasma of various mammals but exhibits different steroid specificity. For example, while the protein from human plasma binds both androgens and estrogens, the one from rabbit is much more specific for androgens (Rosner and Darmstadt, 1973), and that of rat serum has highest affinity for estrogens (Soloff *et al.*, 1971). The binding properties of the human protein have resulted in considerable confusion in the literature pertaining to nomenclature. The protein has been named: SHBG (sex hormone binding globulin), EBP (estradiol binding protein), TBG (testosterone binding globulin), TeBG (testosterone–estradiol binding globulin), SBβG (steroid binding β-globulin), and SBP (sex steroid binding plasma protein).[1] This paper retains the latter nomenclature which was assigned to the protein by Mercier-Bodard *et al.* (1970).

In view of the fact that the current concepts of the mode of action of sex steroids in various reproductive tissues requires specific binding to intracellular "receptors" (Jensen and De Sombre, 1973), we have purified SBP in the hope of using it as a model system to study the chemical nature of sex steroid binding sites. The specificity of SBP makes it a logical choice as a model system to study both androgen and estrogen protein interaction. Early attempts at the isolation of SBP by conventional methods have proved difficult and time consuming, and resulted in considerable losses during each stage of purification (Mercier-Bodard *et al.*, 1970; Guériguian and Pearlman, 1968; Rosner *et al.*, 1969). In one case, purification resulted in the formation of more binding proteins than were present in the original starting material (Rosner *et al.*, 1969). Our early attempts at purification revealed that conventional chromatographic procedures were not satisfactory to yield enough material to

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[1] Abbreviations used are: DHT, 5α-dihydrotestosterone; DHTS, 5α-dihydrotestosterone 17β-hemisuccinate; DADA, 3,3′-diaminodipropylamine. SBP, sex steroid binding plasma protein.

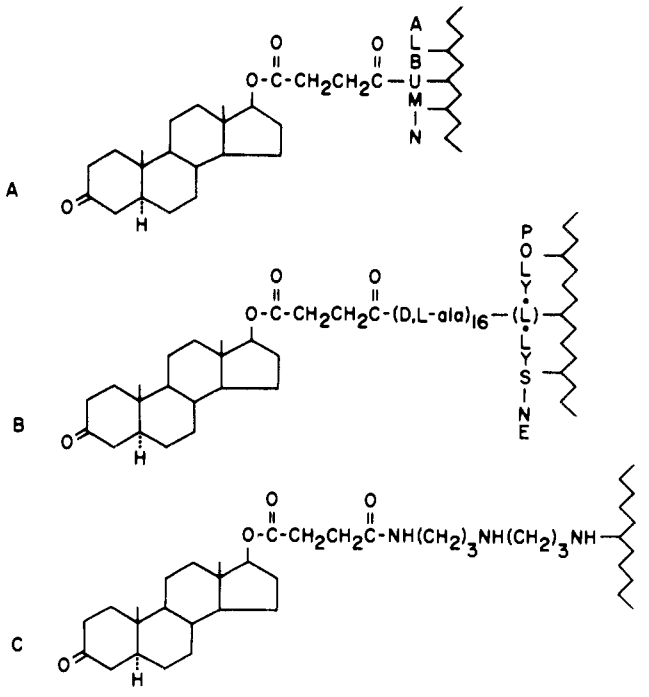

FIGURE 1: Structure of the 5α-dihydrotestosterone-agarose derivatives synthesized for SBP purification. (A) DHTS-albumin-agarose; (B) DHTS-poly(Lys-DLAla)-agarose; (C) DHTS-DADA-agarose.

carry out the proposed experiments. Consequently, we turned to affinity chromatography as a method of choice using the approach recently described for the purification of the estradiol "receptor" of calf uterus (Sica *et al.*, 1973). In this communication we report the first successful purification of human SBP by affinity chromatography. A preliminary account of this work has been recently presented (Mickelson and Pétra, 1974b).

Materials and Methods

Chemicals. [6,7-^{3}H]-17β-Estradiol (48 Ci/mmol), [1,2-^{3}H]-5α-dihydrotestosterone (44 Ci/mmol), and $^{59}FeCl_3$ (22 Ci/g) were all purchased from New England Nuclear. The radioactive steroids were at least 97% pure. All radioinert steroids were purchased from Sigma Co. except for 5α-dihydrotestosterone 17β-hemisuccinate which was from Steraloids Inc. The copolymer poly(Lys-DLAla), which consists of a polylysyl backbone containing an alanine to lysine ratio of 16:1, was obtained from Miles Laboratories Inc., and bovine serum albumin was purchased from Pentex. 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide was from Ott Chemical Co., and ethylenediamine, 3,3′-diaminodipropylamine, and cyanogen bromide were purchased from Eastman. Transferrin and putrescine were purchased from Sigma Chemicals. DEAE-cellulose filter paper discs (DE-81, 2.3 cm diameter) were purchased from Reeve Angel and Co. All other chemicals were reagent grade. Sepharose 4B and Sephadex G-25 were purchased from Pharmacia Fine Chemicals.

Synthesis of [1,2-^{3}H]-5α-Dihydrotestosterone 17β-Hemisuccinate (DHTS). [^{3}H]DHT (5μCi/mmol, 5 mmol) and 6 mmol of succinic anhydride were dissolved in 10 ml of freshly distilled toluene and refluxed for 4 hr. The material remaining after solvent evaporation was dissolved in 7 ml of acetone followed by 10 ml of distilled water. The solution was refluxed for 30 min. After cooling to 0° in an ice bath an oil formed. Ice-water was slowly added and the resulting precipitate was washed twice with 10 ml of water and dried. The residue was dissolved in 12 ml of methanol, filtered, and crystallized by the addition of 9 ml of ice-water. After two crystallizations the melting point was 137° which agrees with a DHTS standard (melting point 136–139°, Steraloids, Inc.). The yield was 70%.

Synthesis of Poly(Lys-DLAla)-Agarose. Sepharose 4B (100 ml) was equilibrated with 100 ml of 0.5 M potassium phosphate (pH 11) at 5–10° according to Porath *et al.* (1973); 30 g of finely divided cyanogen bromide were slowly added to the gently stirred gel solution. After 10 min at 5–10°, the product was washed under suction with 1 l. of 0.2 M $NaHCO_3$ (pH 9.0) at 4° on a Buchner funnel (coarse disc); 200 mg of poly(Lys-DLAla) were dissolved in 75 ml of 0.2 M $NaHCO_3$ at pH 9.0 and 4°. The solution was added to the freshly prepared activated agarose and gently stirred for 15 hr at 4°. The gel was washed at 25° with 2 l. of 1.0 M NaCl followed by 1 l. of distilled water. The amount of copolymer substituted was approximately 1.5 mg/ml of gel as determined by Lowry analysis of the unbound copolymer recovered from the washes of the derivatized gel.

Synthesis of Albumin-Agarose. Bovine serum albumin (400 mg) was dissolved in 75 ml of 0.2 M $NaHCO_3$ at pH 9 at 4° containing 10 M urea to promote multipoint attachment of the albumin to the matrix (Sica *et al.*, 1973). The solution was added to 100 ml of freshly prepared activated agarose and treated in the same manner as described for the poly(Lys-DLAla)-agarose derivative. The amount of albumin substitution was approximately 3.5 mg/ml of gel as determined by Lowry analysis of the unbound albumin recovered from the washes of the derivatized gel.

Synthesis of 3,3′-Diaminodipropylamine-Agarose. (DADA-Agarose). DADA (200 mmol) was dissolved in 75 ml of 0.2 M $NaHCO_3$ and the pH adjusted to 9. The solution was added to 100 ml of freshly prepared activated agarose and the mixture was treated as described above. The incorporation of amino groups was detected by trinitrobenzene sulfonate (Cuatrecasas, 1970).

Synthesis of DHTS-DADA-Agarose and DHTS-Poly(Lys-DLAla)-Agarose. The DADA-agarose and poly(Lys-DLAla)-agarose gels were each equilibrated with 70% (v/v) dioxane (Sica *et al.*, 1973); 1 g and 250 mg of [^{3}H]DHTS (5μCi/mmol) dissolved in 100 ml of 70% dioxane (pH adjusted to 5) were added respectively to 100 ml of DADA-agarose and poly(Lys-DLAla)-agarose; 5 and 1.25 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were added respectively to the DADA-agarose and poly(Lys-DLAla)-agarose mixtures in two equal portions 4 hr apart at 25°. The mixtures were gently stirred for 15 hr at 25°. The substituted gels were each washed on a Buchner funnel (without suction) with 5 l. of dioxane, poured into a column, and further washed with 10 l. of 80% (v/v) methanol. This step is necessary to remove unbound steroid (Cuatrecasas, 1970). Finally, 5 l. of distilled water was passed through the gels on a Buchner funnel with suction. The amount of substituted DHTS was 1 μmol/ml of packed DHTS-DADA-agarose and 2 μmol/ml of packed DHTS-poly(Lys-DLAla)-argarose determined as described in Radioactivity Measurements. The structure of these derivatives are shown in Figure 1.

Synthesis of DHTS-Albumin-Agarose. [^{3}H]DHTS (250 mg, 5 μCi/mmol) was dissolved in 100 ml of 70% (v/v) aqueous dioxane (pH adjusted to 5) at 25°, and 1.25 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was

added. The mixture was added to 100 ml of albumin-agarose and gently stirred for 15 hr at 25°. The rest of the procedure was identical with that described for the preparation of DHTS-DADA-agarose and DHTS-poly(Lys-DLAla)-agarose. The amount substituted was 2 μmol/ml of packed DHT-albumin-agarose gel. This derivative is shown in Figure 1A.

Synthesis of 17β-Estradiol-Agarose Derivatives. Four 17β-estradiol-agarose derivatives substituted at position 3 were prepared. [6,7-^{3}H]-17β-Estradiol 3-*O*-hemisuccinate was synthesized according to Sica *et al.* (1973) and coupled to ethylenediamino-agarose as previously described (Cuatrecasas, 1970). [6,7-^{3}H]-17β-Estradiol was coupled to three bromoacetyl derivatives of agarose:bromoacetamidoethyl-agarose, bromoacetamidobutyl-agarose, and bromoacetamido-3,3′-diaminodipropyl-agarose synthesized according to Cuatrecasas (1970); 100 ml of each of these derivatives were prepared, and the amounts of incorporated steroid were similar to published values. They are described in Figure 2.

Preparation of an Active SBP Fraction from Human Serum by $(NH_4)_2SO_4$ Precipitation. Human pregnancy blood lost during delivery was allowed to clot at 4°. Finely divided ammonium sulfate (295 g) was slowly added per liter of serum. The mixture was stirred overnight and the precipitate shown to contain SBP (Rosner, 1972) was recovered by centrifugation at 16,000*g* for 10 min. The precipitate was washed twice with a 295 g/l. of ammonium sulfate solution. The precipitate was dissolved in a minimum volume of cold distilled water and dialyzed against distilled water at 4° for 48 hr. The solution was centrifuged and the supernatant lyophilized and stored at −20°.

Preparative Affinity Chromatography Procedure. The active lyophilized powder (6.8 g, obtained by $(NH_4)_2SO_4$ precipitation of 1 l. of serum) was dissolved at 4° in 100 ml of 10 mM Tris-Cl-0.5 M NaCl (pH 7.4). The solution was gently stirred for 5 hr with 50 ml of DHTS-DADA-agarose diluted fivefold with unsubstituted Sepharose 4B. The mixture was poured into a column (5 cm diameter × 2.5 cm height) and elution with the same buffer was continued until the uv absorbance was negligible. The gel was then removed from the column and stirred 1 hr at 25° with 100 ml of the buffer containing 10^{-6} M [^{3}H]DHT (10^8 dpm, 0.44 Ci/mmol) and 10% glycerol. The mixture was poured back into the column and elution was continued at 25° until the uv absorbance was again negligible. The fractions were concentrated by ultrafiltration (Amicon Model TCF-10 thin-channel system with PM-30 membranes) and the resulting protein solution was frozen at −20°. SBP activity was measured by counting samples after removing unbound [^{3}H]DHT by Sephadex G-25 filtration (1-ml samples; 1.5 × 10 cm columns; 10 mM Tris-Cl and 10% glycerol (pH 7.4), 4°, 30 ml/hr flow rate).

Polyacrylamide Gel Electrophoresis Procedures. Analytical disc gel electrophoresis was performed at 4° using 5% acrylamide gels according to the method described by Shuster (1971) with the discontinuous buffer system of Davis (1964) for gels electrophoresed at pH 9.5. Analytical electrophoresis was also performed at pH 8.0 (Williams and Reisfeld, 1964), and pH 6.6 (Taber and Sherman, 1964). Samples (100 μl) containing 20-50 μg of protein saturated with DHT were applied to gels. The gels were stained with Naphthol Blue Black or with the periodic acid-Schiff reagent (Zacharius *et al.*, 1969) to determine the presence of carbohydrate.

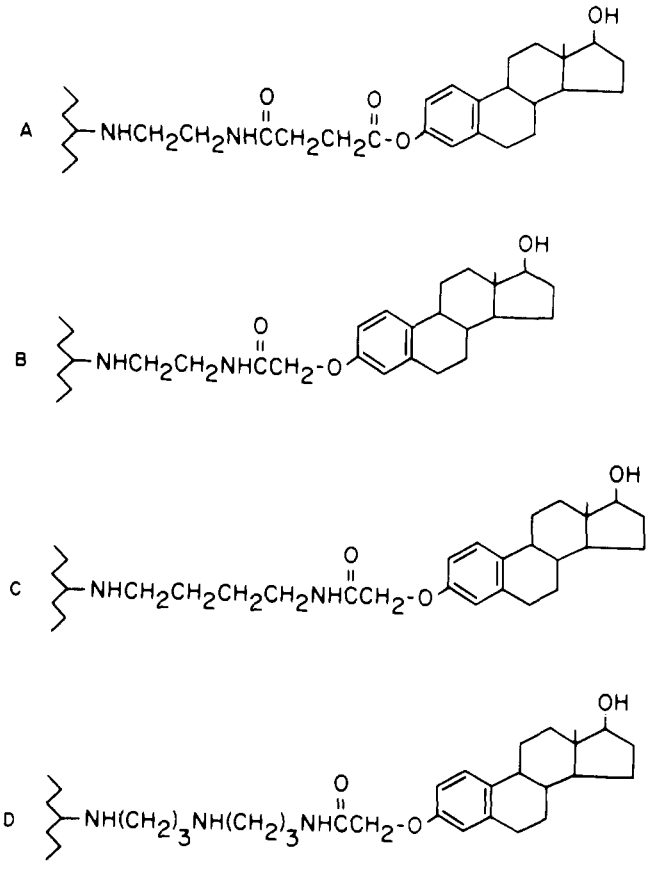

FIGURE 2: Structure of the 17β-estradiol-agarose derivatives which proved ineffective for SBP purification. (A) 17β-estradiol 3-*O*-succinate-ethylenediamino-agarose; (B) 3-*O*-acetamido-17β-estradiol-ethyl-agarose. (C) 3-*O*-acetamido-17β-estradiol-butyl-agarose; (D) 3-*O*-acetamido-17β-estradiol-3,3′-diaminodipropyl-agarose.

Location of the radioactive bands on gels was done by applying to the gels 100-μl samples containing 0.5 μg of SBP and 7 × 10^5 dpm [^{3}H]DHT (saturation conditions with respect to SBP). Gels were sliced transversely into 1.3-mm slices and digested with 0.1 ml of 30% H_2O_2 at 37° for 10 hr before addition of scintillant. Experiments with ^{59}Fe were done by applying to gels 100-μl samples containing 20 μg of SBP or 0.1 mg of transferrin and 0.2 μCi of $^{59}FeCl_3$.

Preparative polyacrylamide gel electrophoresis was performed with the Buchler apparatus as described in their instruction manual at 4°; 10 mg of protein saturated with DHT in 10 ml of buffer (20% glycerol) was applied on a 7.5% acrylamide separating gel and electrophoresed at pH 9.5, with the discontinuous buffer system described by Davis (1964). The eluted protein fractions were concentrated by ultrafiltration and stored frozen at −20° in the presence of 20% glycerol and DHT.

Assay of SBP Activity and Scatchard Analyses. SBP activity was measured with a new assay consisting of incubating protein solutions with [^{3}H]DHT in the presence or absence of 100-fold excess of radioinert steroid, followed by adsorption of the [^{3}H]DHT-SBP complex onto DEAE-cellulose filter paper discs (Mickelson and Pétra, 1974a). The batchwise Sephadex G-25 assay was also used according to published procedures (Pearlman and Crépy, 1967). The determination of binding constants was done at 4° by incubating 0.5-ml samples of diluted pregnancy serum (1:50 with 10 mM Tris-Cl (pH 7.4)) collected at term with varying

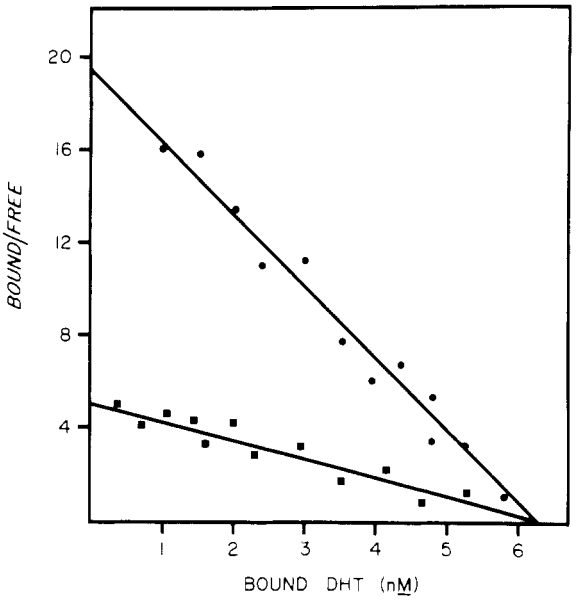

FIGURE 3: Determination of the equilibrium constants of association of DHT and DHTS by Scatchard analysis with the filter assay at 4°. (●) DHT, 0.5-ml samples of diluted pooled pregnancy serum (1:50 with 10 mM Tris-Cl (pH 7.4)) were incubated with 0.5–8 nM [^{3}H]DHT (44 Ci/mmol); 100-μl aliquots were removed for filter assay. The "efficiency" correction was 16%. (■) DHTS, same experiment carried out in the presence of 43.7 nM radioinert DHTS in every sample.

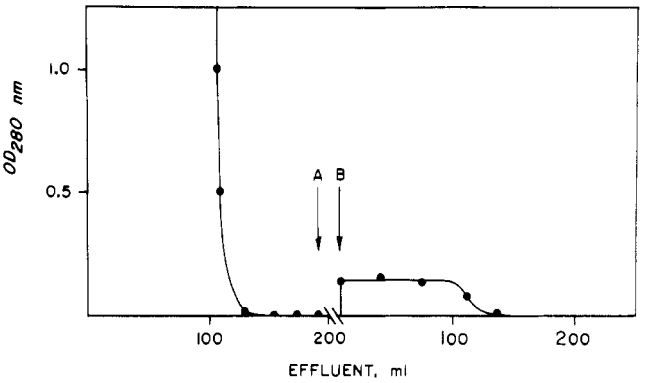

FIGURE 4: Elution pattern for the purification of SBP from 6.8 g of lyophilized $(NH_4)_2SO_4$ precipitate obtained from 1 l. of pregnancy serum on 50 ml of DHTS-DADA-agarose (column: 5 cm diameter × 2.5 cm height). Protein was dissolved in 200 ml of 10 mM Tris-Cl–0.5 M NaCl (pH 7.4), 4°, incubated for 5 hr, and eluted at 100 ml/hr. Gel was removed (arrow A), incubated with buffer containing 10^{-6} M [^{3}H]DHT (10^8 dpm, 0.44 Ci/mmol) for 1 hr at 25°, and repoured in the column (arrow B). Elution was continued at 25°. Active SBP was eluted in 140 ml. The column yield was 21%.

concentrations of [^{3}H]DHT (0.5–8 nM) in the presence or absence of 43.7 nM radioinert DHTS. Specific binding was determined with the DEAE-filter paper assay on 100-μl aliquots. The "efficiency" correction was 16% (Mickelson and Pétra, 1974b).

Radioactivity Measurements. Aqueous samples (0.5 ml) or filter discs were added to vials containing 10 ml of scintillant (5 g of 2,5-diphenyloxazole and 100 g of naphthalene/l. of dioxane) and counted in a Beckman LS-100 C scintillation counter at 34–37% efficiency. The incorporation of steroid into agarose derivatives was estimated by hydrolyzing 0.5-ml aliquots of gel with 0.5 ml of 1M NaOH for 10 hr at 37° followed by 0.5 ml of 1M HCl. Agarose derivatives containing phenyl ether bonds were heated with 0.5 ml of 30% H_2O_2 for 10 hr at 37° before adding scintillant.

Results

Determination of the Equilibrium Constant of Association of DHT and DHTS. The equilibrium constant of association, K_a, for DHT was determined by the filter assay using [^{3}H]DHT and diluted pregnancy serum at pH 7.4 and 4°. The upper line (circles) of Figure 3 represents a Scatchard analysis of the data from which $K_a = 3.1 \pm 0.5 \times 10^9$ M^{-1} is calculated from the slope. This value agrees well with other published values: $2.7 \pm 0.6 \times 10^9$ M^{-1} (Mickelson and Pétra, 1974a) and 2.8×10^9 M^{-1} (Shanbhag *et al.*, 1973). The equilibrium constant of association for DHTS, K_I, was determined by adding a constant amount of radioinert DHTS to each serum sample with increasing amounts of [^{3}H]DHT. A Scatchard analysis was performed in the same manner and from the data shown in the lower line (squares) of Figure 3, $K_I = 6.6 \times 10^7$ M^{-1} is calculated from $K_p = K_d(1 + K_I[I])$, where $K_p = 1.2 \times 10^{-9}$ M, apparent equilibrium constant of dissociation of DHT in the presence of competitive inhibitor, DHTS (reciprocal of the slope of the bottom line in Figure 3); $K_d = 3.2 \times 10^{-10}$ M, equilibrium constant of dissociation of DHT (reciprocal of the slope of the upper line in Figure 3); [I] = 4.4×10^{-8} M, concentration of the competitive inhibitor, radioinert DHTS; $K_I = 6.6 \times 10^7$ M^{-1}, apparent equilibrium constant of association of DHTS.

The results indicate that the incorporation of a succinate group at the 17β position of DHT reduces the affinity of this ligand to SBP by a factor of 50 when compared to DHT. However, the K_I of DHTS is still high enough to be considered as an effective ligand for the synthesis of specific adsorbants for the purification of SBP by affinity chromatography. The succinate side chain is needed to attach the steroid to the various agarose derivatives described in Figure 1.

DHTS-Agarose Derivatives. Figure 1 describes the three DHTS-agarose derivatives synthesized as possible specific adsorbants for SBP purification. They were tested at 4° by mixing each derivative with an excess of unsubstituted Sepharose 4B (1:5) and equilibrating with 10 mM Tris (pH 7.4) in columns (1.5 × 30 cm); 200 mg of lyophilized powder obtained from $(NH_4)_2SO_4$ precipitated serum were dissolved in 10 ml of buffer and pumped onto each column at 12 ml/hr flow rate. All three derivatives were able to retain the SBP activity applied onto the columns as determined by the Sephadex G-25 batchwise assay. The most effective adsorbant was the DHTS-DADA-agarose derivative (C in Figure 1). The DHTS-poly(Lys-DLAla)-agarose derivative (B in Figure 1) bound less SBP per ml of adsorbant than either of the other two derivatives, while elution with the buffer containing 10^{-6} M DHT resulted in a lower yield of SBP from the DHT-albumin-agarose derivative (A in Figure 1). Consequently, the DHTS-DADA-agarose derivative was chosen as the specific adsorbant for the purification of SBP on a preparative scale. No binding activity was retained by the derivatized agarose in the absence of covalently bound steroid.

Estradiol-Agarose Derivatives. Figure 2 describes the various agarose derivatives which were also tested using the same procedure described for the DHTS-agarose derivatives. None of these adsorbants proved effective in retaining the SBP activity applied onto the columns and therefore

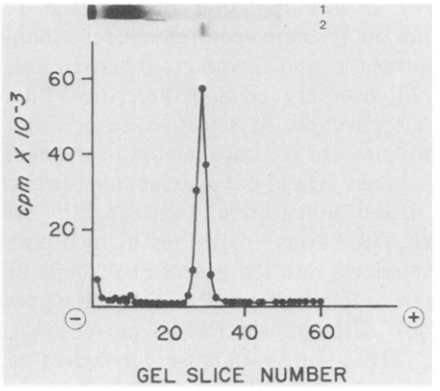

FIGURE 5: Analytical gel electrophoretic patterns of purified SBP at pH 9.5, 2 mA/gel tube. Gel 1, 120 μg of SBP recovered from the affinity column; gel 2, 20 μg of SBP purified by affinity chromatography followed by preparative gel electrophoresis. Both gels were stained with Naphthol Blue Black. Repeat of gel 2 with 100 μg of SBP yielded only one band. (●) 100-μl sample containing 0.5 μg of SBP and 7×10^5 dpm at [^{3}H]DHT (44 Ci/mmol). Gel slices (1.3 mm) were digested with H_2O_2 at 37° for 10 hr before addition of scintillant. Addition of 100-fold excess of radioinert estradiol on another gel caused disappearance of the radioactive peak.

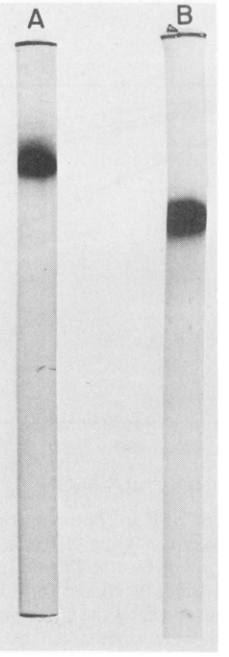

FIGURE 6: Analytical gel electrophoretic patterns of 50 μg of purified SBP at pH 6.6 (A) and 8.0 (B). The gels were stained with Napthol Blue Black. The bands appearing at the extremities of the gels do not represent protein, they are artifacts of photography.

further attempts at using these or any other estradiol derivatives for purification purposes were not undertaken.

Purification of SBP. Figure 4 illustrates the procedure used for the purification of SBP from 6.8 g of lyophilized powder obtained from 1 l. of pregnancy serum by $(NH_4)_2SO_4$ precipitation. After the removal of all uv adsorbing material the gel was removed from the column (arrow A, Figure 4), incubated with [^{3}H]DHT as described under Materials and Methods, and poured back into the column (arrow B, Figure 4). The active fraction separated in 140 ml of elution buffer; 21% yield of the SBP activity applied on the affinity column was obtained under these conditions. Analytical polyacrylamide gel electrophoresis of this material at pH 9.5 revealed the presence of slow migrating impurities as shown in gel 1 of Figure 5. These impurities were removed by preparative gel electrophoresis using identical pH conditions. Gel 2 in Figure 5 represents the final state of purity of SBP after the last step in the isolation procedure. One single band was obtained by analytical gel electrophoresis at pH 9.5 after staining with Naphthol Blue Black. Figure 6 represents the electrophoretic mobility of the purified protein in gels at pH 8.0 and 6.6 confirming homogeneity. Staining with the periodic acid–Schiff reagent of the material isolated from the affinity column reveals the presence of carbohydrate in SBP. Electrophoresis in the presence of [^{3}H]DHT revealed one single radioactive band corresponding with the stained band (Figure 5). The migration of the radioactive band is identical with that obtained when serum saturated with [^{3}H]DHT is electrophoresed under the same conditions indicating that the molecular characteristics of SBP are not changed during the purification procedure. The radioactive band corresponding to pure SBP disappeared when a 100-fold excess of radioinert 17β-estradiol was present in the incubation mixture prior to electrophoresis revealing the specific sex steroid binding properties of SBP. The yield of SBP at each step of the purification is shown in Table I.

The presence of transferrin which migrates similarly as SBP in gel electrophoresis (Corvol *et al.*, 1971) was ruled out by incubating fractions of pure SBP with $^{59}FeCl_3$ for 30 min at 25°. No iron binding was detected in the SBP band while a ^{59}Fe-transferrin control migrated normally under the same electrophoretic conditions.

Studies on the Stability of SBP. Preliminary experiments on the stability of SBP were undertaken in an attempt to explain the low yields obtained by the purification procedure. Figure 7 shows incubation studies carried out

TABLE I: SBP Purification.

Step	A (Total mg of Protein)	B[a] (Total ng of DHT Bound)	% Recovery (at each step)	Specific Activity B/A	Cumulative Purification	Cumulative yield (%)
Serum (1 l.)	41,000	51,250	100	1.25	1	100
$(NH_4)_2SO_4$ ppt	6,800	30,750	60	4.5	3.6	60
Affinity chromatography	20.5	6,458	21	315	252	12.6
Preparative electrophoresis	0.5	2,583	40	5166	4133	5

[a] The filter assay method (Mickelson and Pétra, 1974a) was used to measure total specific DHT binding activity under conditions where the binding protein is saturated with [^{3}H]DHT.

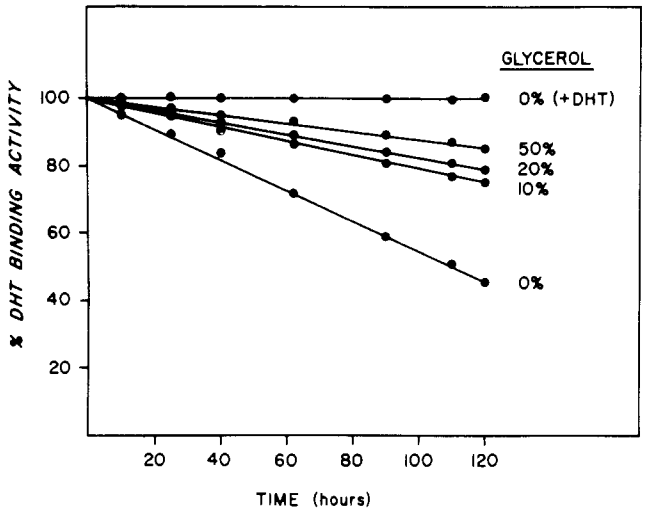

FIGURE 7: Stability of SBP in the presence of saturating concentrations of DHT and glycerol; 200 μg of lyophilized $(NH_4)_2SO_4$ precipitate was dissolved per ml of 10 mM Tris-Cl (pH 7.4), 4°. Aliquots were removed at indicated time, incubated for 15 min at 4° with 3×10^5 dpm [^{3}H]DHT, and assayed by the filter method. Stability studies in the presence of DHT were carried out by adding 3×10^5 dpm of [^{3}H]DHT at zero time and removing aliquots for assay at indicated time (specific activity 44 Ci/mmol).

with the 50% $(NH_4)_2SO_4$ precipitate material. Saturating concentrations of DHT completely stabilizes the SBP activity while increasing amounts of glycerol in the absence of DHT enhances the stability as measured by the filter assay.

Discussion

The purification of SBP has been attempted in various laboratories for the past 8 years. Most of the approaches undertaken were based on conventional chromatographic procedures. One of the earliest attempts utilizing affinity chromatography failed to yield significant quantities of SBP (Burstein, 1969). Only 0.4% of the serum SBP activity bound to the selective adsorbant was recovered. The author pointed out that either the elution conditions (1M guanidine · HCl (pH 2.1)) were not ideal for the removal of bound SBP, or the binding activity was irreversibly destroyed during the process. A few years ago, a brief report indicated that 17β-estradiol 3-*O*-hemisuccinate coupled to ethylenediamino-agarose (Figure 2A) could be used for the purification of SBP (Cuatrecasas, 1970). Unfortunately, no SBP activity could be retained by this particular agarose derivative or any of the other estradiol-agarose derivatives tested under our experimental conditions. The reason for the ineffectiveness of these derivatives is not apparent. Recently, Sica *et al.* (1973) reported the first successful purification of the estrogen "receptor" from calf uterus using estradiol coupled to the insoluble support at the 17 position. We therefore synthesized agarose derivatives with the steroid attached at the 17 position instead of the 3 position. This approach seemed rational since previous studies on the relative binding affinity of various steroids suggest that the SBP binding site has a specific requirement not only for the 17β-hydroxyl group of the steroid (17β-testosterone binds 25 times better than 17α-testosterone, and 17β-estradiol binds 15 times better than estrone), but also for the relative spatial position of ring A with respect to the rest of the steroid structure (5α-dihydrotestosterone binds 43 times better than 5β-dihydrotestosterone) (Vermeulen and Verdonck, 1968; Kato and Hornton, 1968; Heyns *et al.*, 1969; Mercier-Bodard *et al.*, 1970). Therefore, the 17β-hydroxyl group is not an absolute requirement for binding. Furthermore, Cuatrecasas and coworkers (Cuatrecasas, 1970; Sica *et al.*, 1973) have argued that the critical factors controlling the effectiveness of selective adsorbants for steroid binding proteins are not only related to the chemical structure of the bound ligand but also include the extent and stability of ligand substitution. Consequently, we also used macromolecular "arms" as means of incorporating multipoint attachments into the agarose backbone to provide an overall more stable linkage (P. Cuatrecasas, personal communication). Although the DHTS-poly(Lys-DLAla)-agarose and DHTS-albumin-agarose derivatives proved useful in the purification of SBP, they were found to be inferior to the DHTS-DADA-agarose derivative.

The purification procedure of SBP outlined in this paper resulted in a homogeneous protein by the criteria of gel electrophoresis. The molar concentration of SBP in our pools of pregnancy serum varied from 1.8×10^{-7} M (Table I) to 3.2×10^{-7} M (calculated from the abscissa on Figure 3). Although the cumulative per cent yield will be the same, the actual amount of SBP recovered for purification will vary from pool to pool. The low cumulative yield of 5% may be attributed to the instability of SBP in the purified state. In fact, removal of bound steroid from solutions of pure SBP results in rapid and irreversible loss of binding activity. This phenomenon was also found in the case of human corticosteroid-binding globulin (Muldoon and Westphal, 1967). The presence of DHT and glycerol and low temperature were found to retard the rate of inactivation of purified SBP. Other approaches to increase the stability of SBP are presently under investigation in our laboratory.

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Interaction of Steroids with Nucleic Acids†

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ABSTRACT: 17β-Estradiol and testosterone bind to both native and denatured DNA, and to RNA and poly(A) · poly(U). Binding affinity depends on the conformation of nucleic acid. Lowering the electrolyte concentration and raising the temperature increase the binding of 17β-estradiol to native DNA and decrease that to denatured DNA. In 0.01 *M* NaCl and at 37°, more 17β-estradiol is bound to native DNA than to denatured DNA. Higher binding of steroid to denatured DNA relative to native DNA at low temperature and high ionic strength is related to larger fraction of binding sites per unit nucleotide in denatured DNA. In addition to 17β-estradiol and testosterone, 17α-estradiol, 17β-estradiol-3-methyl ether and 19-nortestosterone also stabilize the structure of nucleic acids and poly(A) · poly(U) against thermal denaturation. The 17β-estradiol induced elevation of the T_m of DNA is diminished by methanol or high NaCl concentration. These results indicate the involvement of hydrogen bonding and hydrophobic interactions between steroids and nucleic acids. The results of binding isotherms and optical studies suggest a conformational dependence of the binding of steroids to nucleic acids.

Interaction of biologically active molecules with nucleic acids has been studied with a dual purpose: first, to serve as a probe for investigating the conformation which the nucleic acids are capable of adopting, and second, to contribute to our understanding of the mechanism of action of bioactive molecules. Several classes of molecules, such as dyes (Lerman, 1964), antibiotics (Muller and Crothers, 1968; Sobell, 1973), and carcinogenic hydrocarbons (Boyland and Green, 1962; Huggins and Yang, 1962) bind to nucleic acids; they often show preference for a particular conformation and base specificity. Biological effects of some of these molecules frequently can be correlated with their nucleic acid interactions.

Hormonal steroids play many regulatory roles in cellular proliferative and biosynthetic activities. Accumulating evidence suggests that the growth stimulatory effects of some of the steroids in the target tissue are mediated through their binding to a protein receptor(s) (see O'Malley and Means, 1974, for a recent review). Interaction of steroids with nucleic acids may also be involved in the diverse effects attributable to steroid hormones. Ts'o and Lu (1964) proposed that the binding of steroids in nucleic acids depends mainly on hydrophobic forces. Cohen and Kidson (1969), Cohen et al. (1969), and Kidson et al. (1970) concluded that steroids bind to denatured but not to native DNA, which involves both hydrogen bonding and hydrophobic in-

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