Isolation and Characterization of the Testosterone–Estradiol-Binding Globulin from Human Plasma. Use of a Novel Affinity Column†

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ABSTRACT: This report concerns the purification and characterization of the testosterone–estradiol-binding globulin (TeBG) from human plasma. Cohn fraction IV was submitted sequentially to ammonium sulfate precipitation, affinity chromatography, gel filtration, and isoelectric focusing. The final product was homogeneous in polyacrylamide gel electrophoresis and sodium dodecyl sulfate gel electrophoresis. Its activity was demonstrated by the finding of slightly more than one binding site/mole for dihydrotestosterone. Association constants (M^{-1}) at 4 and 37°C were ascertained for three steroids: dihydrotestosterone, 2.4×10^9 and 0.99×10^9; testosterone, 1.1×10^9 and 0.35×10^9; estradiol, 0.60×10^9 and 0.22×10^9. TeBG is a glycoprotein having a molecular weight of 94000 and both the amino acid and carbohydrate content are presented along with other physical properties.

Human plasma contains a trace protein, testosterone–estradiol-binding globulin (TeBG),[1] which binds dihydrotestosterone, testosterone, and estradiol with high affinity (for reviews, see Westphal, 1971; Anderson, 1974). Efforts to isolate a homogeneous, fully active product in several laboratories (Guériguian and Pearlman, 1968; Van Baelen et al., 1968; Rosner et al., 1969; Burstein, 1969; Pizarro, 1969; Mercier-Bodard et al., 1970) have been unsuccessful because of the marked lability of the protein. We recently studied the kinetics of denaturation of TeBG (Rosner et al., 1974) which allowed us to evolve relatively simple ways to reduce drastically its rate of inactivation. The use of low temperature, calcium, glycerol, Tris buffers, and dihydrotestosterone during isolation has enabled us to prepare a homogeneous, completely active protein. A preliminary account of this work has appeared (Rosner and Smith, 1974) along with a report of the isolation of an active but 20% impure protein (Rosner and Smith, 1975). While this manuscript was in preparation, Mickelson and Pétra (1975) reported on the isolation of TeBG in small amounts but could not sustain activity for a long enough period to complete physical or physicochemical studies.

Materials and Methods

General. All procedures involving the manipulation of proteins were at 4°C unless otherwise specified. Protein concentration prior to complete purification was measured by the microbiuret method of Itzhaki and Gill (1964) using bovine serum albumin as standard. When this method was applied to solutions containing calcium, a precipitate formed, but the absorbance of the supernatant obeyed Beer's law up to a protein concentration of 0.45 mg/ml. All water used was deionized. Radioactivity was determined in a liquid scintillation spectrometer in either toluene or dioxane-based phosphors (Rosner et al., 1969). The efficiencies for tritium were 57% in toluene and 45% in 10 ml of dioxane with 0.5 ml of water or buffer. In those experiments where double label counting was done in dioxane–water, efficiencies were: ^3H efficiency, 30%; ^{14}C efficiency, 70%; ^{14}C crossover into ^3H channel, 11.5%; and ^3H crossover into ^{14}C channel, 0.073%. [1,2-^3H]-5α-Dihydrotestosterone, 44 Ci/mmol, [4-^{14}C]estradiol, 58 mCi/mmol, and [4-^{14}C]testosterone, 50.6 mCi/mmol (all from New England Nuclear Corp.), were >95% pure by thin-layer chromatography and used without further purification. Radioinert testosterone, 5α-dihydrotestosterone, estradiol, and epiandrosterone succinate (3β-hydroxy-5α-androstan-17-one 3-hemisuccinate) were obtained from Steraloids, Inc. (Pawling. N.Y.) and all migrated as single spots on thin-layer chromatography. Cohn fraction IV precipitate (Kistler and Nitschmann, 1962) was obtained from the New York Blood Center as a lyophilized powder.

Preparation of the Affinity Column. We examined a number of steroid derivatives for their potential usefulness as ligands in the affinity chromatography of TeBG and report here the synthesis of the one we found most useful. We also found that side arms containing amino groups linked to Sepharose by cyanogen bromide (Cuatrecasas, 1970) were too labile for use in this isolation and have therefore used epichlorohydrin (Porath and Fornstedt, 1970) for the coupling of amino groups to the functional groups of Sepharose 4B (Pharmacia). Finally, in order to be certain that TeBG which absorbed to the affinity column could be removed from it, we chose a novel side arm, 4,4′-azodianiline, Figure 1, containing an azo bond that could, if necessary, ultimately be cleaved with dithionite thus assuring removal of half the side arm along with the steroid succinate and the TeBG.

Synthesis of 5α-Androstane-3β,17β-diol 3-Hemisuccinate (Androstanediol Succinate). To 5 g of epiandrosterone succinate in 200 ml of absolute ethanol, slowly add 4 g of sodium borohydride maintaining the temperature at 0–4°C in an ice bath. After 2–3 hr, acidify to pH 3 with concentrated acetic acid and reduce the volume in vacuo until the product just starts to precipitate (volume is 20–30

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[1] Abbreviations used are: TeBG, testosterone–estradiol binding globulin; ADA, azodianiline; DHT, 5α-dihydrotestosterone; NMR, nuclear magnetic resonance.

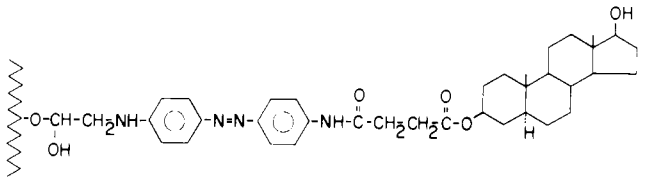

FIGURE 1: Structure of the affinity column.

ml). Add an equal volume of water acidified to pH 3 with acetic acid and wash the precipitate which forms with ice cold acidified water and then with ice cold water. Crystallize the final product from benzene–methanol.

Preparation of Azodianiline–Sepharose. On a funnel fitted with a coarse fritted disc, wash with suction 250 ml (packed volume) of Sepharose 4B (Pharmacia Fine Chemicals) with 2.5 l. of water. Transfer the moist cake of Sepharose to a round-bottomed flask, add 250 ml of 1 *N* NaOH, followed by 25 ml of epichlorohydrin (Aldrich Chemical Co.), and mix fairly vigorously with a magnetic stirrer for 2 hr at room temperature. Transfer the gel back to the funnel, wash with 8 l. of water, and again transfer the drained gel to a round-bottomed flask. Add 13 g of azodianiline (ADA, Eastman Kodak) in 500 ml of 50% (v/v) dimethylformamide and stir gently for 2 hr. Wash the coupled gel, which is a deep burnt orange in color, with 8 l. of 80% dimethylformamide and then with 8 l. of water. Ascertain the concentration of free amino groups with picrylsulfonic acid (Cuatrecasas, 1970) using azodianiline as standard.

Coupling of Androstanediol 3-Hemisuccinate to Azodianiline–Sepharose. To 250 ml of azodianiline–Sepharose suspended in 250 ml of 50% dimethylformamide, add 7.0 g of androstanediol hemisuccinate dissolved in 250 ml of 50% dimethylformamide followed by the addition, over 5 min, of 3.5 g of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (Aldrich Chemicals). Stir for 2 hr at room temperature and wash with 15 l. of 80% dimethylformamide and then 10 l. of water.

Assay for TeBG Activity. Throughout most of the procedure TeBG activity was assayed by a previously described method which involves precipitation of TeBG-bound [^{3}H]DHT with ammonium sulfate (Rosner, 1972). Results were expressed as micrograms of DHT bound at saturation. Dilute solutions of protein will not precipitate with ammonium sulfate and when this occurred, activity could be accurately determined by equilibrium dialysis. In addition, the value for bound DHT from any of the several Sephadex chromatographies (G150 and G50) used in the isolation procedure agreed well with the results obtained from the first two methods.

Assay of the Capacity of Androstanediol–Sepharose for TeBG. Cohn fraction IV was prepared for absorption to androstanediol–Sepharose by first dissolving 70 g in 30 l. of 50 m*M* Tris-HCl (pH 7.5) and 50 m*M* $CaCl_2$ and subjecting the solution to treatment with ammonium sulfate and Norit A as described below. Varying volumes of the crude preparation of TeBG were added to 0.5 ml of androstanediol–Sepharose or azodianiline–Sepharose (control) and mixed for 2 hr at 4°C. The supernatant was then assayed for TeBG and the amount absorbed to the gel calculated by subtraction. Leudens et al. (1972) have shown that substances which interfere with the assay may elute from the gels giving rise to falsely low concentrations of binding protein in the supernatant; we, therefore, assayed the supernatants both before and after passage through columns of Sephadex G-50 so as to remove any free steroid which might have been generated during the course of the exposure of the gel to the solution of TeBG.

Isolation of TeBG. A single buffer was used in all chromatographic steps unless otherwise specified: 50 m*M* Tris-HCl (pH 7.50) containing 50 m*M* $CaCl_2$. The pH of the buffer was adjusted and measured at the temperature at which it was to be used.

Ammonium Sulfate Precipitation. Make 700 g of Cohn fraction IV up to 30 l. in Tris buffer. Add 300 g/l. solid ammonium sulfate, stir for 30 min, and recover the precipitate by centrifugation at 16000*g* for 30 min. After washing the precipitate once with 50% ammonium sulfate, dissolve it in Tris buffer to a final volume of 20 l. Remove endogenous steroids by adding Norit A, 50 g/l., and shaking at room temperature for 30 min (Heyns et al., 1967). Remove the charcoal by centrifugation as above.

Affinity Chromatography. Add the protein from the above step to 250 ml of androstanediol–Sepharose and stir it in two 12-l. round-bottomed flasks at 4°C for 2 hr. Remove the supernatant by suction on a coarse sintered glass funnel and wash with 2.5 l. of Tris-Ca^{2+} buffer containing 1 *M* NaCl (the high salt concentration serves to minimize nonspecific adsorption of proteins to the affinity column). Transfer the gel to a glass column with a diameter of 8 cm and wash with Tris-Ca^{2+}–NaCl buffer until the absorbance (280 nm) of the effluent is negligible. Remove the NaCl by washing with 400 ml of NaCl-free buffer, and add one column volume (ca. 250 ml) of buffer containing 10% glycerol and 0.07 m*M* DHT (2000 cpm/μg). Bring the column to room temperature by removing it from the cold room and gently disperse the gel with a glass rod until the temperature at the center of the column is 22–24°C. Wait 1 hr and elute with an additional two column volumes of DHT buffer at 4°C.

Concentration and Readjustment of Specific Activity. Pool all the fractions containing absorbance and concentrate the solution of protein, ca. 800 ml, from the affinity column to 5 ml by sequential pressure ultrafiltration in cells TCF-10 and 8 MC (Amicon Corp.) using PM 10 membranes (Amicon Corp.). Remove the excess mass of DHT by gel filtration on a 2.5 × 30 cm column of Sephadex G-50, packed in Tris-Ca^{2+} buffer, and again concentrate the protein by pressure ultrafiltration to a volume of 5 ml. As the specific activity of the [^{3}H]DHT chosen to elute the affinity column was quite low, it is now necessary to increase that specific activity in order to detect tritium in small volumes in subsequent chromatographic procedures. Therefore, add about 10^6 cpm of [^{3}H]DHT to the concentrated protein allowing 18 hr at 4°C for equilibration to occur. The adjusted specific activity is 20000 cpm/μg. Prior to each subsequent chromatographic step, DHT of this specific activity was added to the protein in amounts sufficient to assure saturation of the TeBG, about a 10–20% excess of the estimated binding capacity.

Chromatography on Sephadex G150. Chromatograph the sample by upward flow on a 2.6 × 92 cm column of Sephadex G150 packed in and eluted with Tris-Ca^{2+} buffer. Pool the tubes containing bound [^{3}H]DHT and concentrate to a volume of 3 ml by pressure ultrafiltration (see above) in preparation for isoelectric focusing.

Isoelectric Focusing. Conduct isoelectric focusing experiments in a 110-ml capacity LKB Model 8101 column following the instructions supplied by the manufacturer. Focusing is achieved from pH 4 to 7 and the final ampholyte

Table I: Summary of Isolation of TeBG.

Procedure	Protein Applied (g)	Act.[a] Applied (μg)	Protein Recovered (g)	Act. Recovered (μg)	Sp[b] Act.	Purification from Previous Step	Cumulative Purification	Cumulative Recovery (%)
Cohn fraction IV	700	504			0.72			
Ammonium sulfate	700	504	400	505	1.30	1.8	1.8	100
Androstanediol–Sepharose	400	505	0.155	60.9	393	302	545	62[c]
Sephadex G150 I	0.155	60.9	0.0520	58.7	1130	2.9	1570	59
Isoelectric focusing I	0.052[d]	58.7	0.0210	47	2238	2.0	3108	48
Sephadex G150 II	0.021	47	0.0152	43.5	2862	1.3	3980	44
Isoelectric focusing II	0.0157	43.5	0.0119	34.8	2920	1.02	4050	35

[a] Activity is expressed as micrograms of DHT bound at saturation. [b] The specific activity is defined as: activity[a]/total protein (gram). [c] The gel bound 98 μg of which 60.9 μg was eluted while the remainder of the applied activity was recovered in the supernatant. Recoveries subsequent to this step are based on the 98 μg of activity which bound to the gel. [d] The 52 mg of protein obtained from Sephadex G150 I was divided into three portions and each was separately submitted to isoelectric focusing. The data presented are those resulting from pooling the results from the three focusing experiments.

concentration is 1%. Form the sucrose gradient (0–40%) with the LKB gradient marker after making both chambers 10% in glycerol. After half the gradient is formed, layer the sample (1 ml, ⅓ of the sample from the preceding step) mixed with an equal volume of heavy solution onto the column and complete the gradient. Cool the column at 4°C and set the initial voltage at 500 V. Do not exceed 1.5 W at any time. Increase the voltage to 800 ca. 18 hr later and end the run after an additional 5–7 hr. Drain the column with a peristaltic pump at 2 ml/min and determine pH, ^3H, and absorbancy.

Repetition of Sephadex Chromatography and Isoelectric Focusing. After pooling and concentrating the active fractions from the last step, they were rechromatographed on Sephadex G150 and then refocused in the same pH gradient as described above. After this second focusing, sucrose, glycerol, and ampholytes were removed on a 2.5 × 30 cm column of Sephadex G50 packed in and eluted with Tris–Ca^{2+} buffer. The TeBG was again concentrated, made 30% in glycerol, and stored at 4°C. The buffer can be changed and the glycerol removed on columns of Sephadex G-50 as necessary for determination of physical constants and binding parameters.

Results

Androstanediol Succinate. We obtained 4.5 g of crystals, mp 214–216°C, NMR[2] (ppm) 0.67 (s, 18-CH_3), 0.80 (s, 19-CH_3), 2.50 (s, $OCCH_2CH_2CO$), 3.43 (m, 17α-H), 4.91 (nm, 3β-H); ir (cm^{-1}) 1700 and 1730 (C=O of COOR), 2540, 2605, and 2700 (COOH), and 3338 (OH). Anal. Calcd for $C_{23}H_{36}O_5$: C, 70.36; H, 9.25. Found: C, 70.71; H, 9.21. The assignment of the structure was made from the ir and NMR spectra, the elemental analysis, and the known fact that the product of the reduction of the carbonyl with sodium borohydride yields predominantly the 17β-hydroxy compound (Clinton et al., 1957).

Capacity of Androstanediol–Sepharose for TeBG. Figure 1 shows the structure of the affinity column. Figure 2 illustrates the capacity of androstanediol–Sepharose for TeBG. The capacity was arrived at by subtracting the amount of TeBG present in the supernatant before and after exposure to the affinity gel.

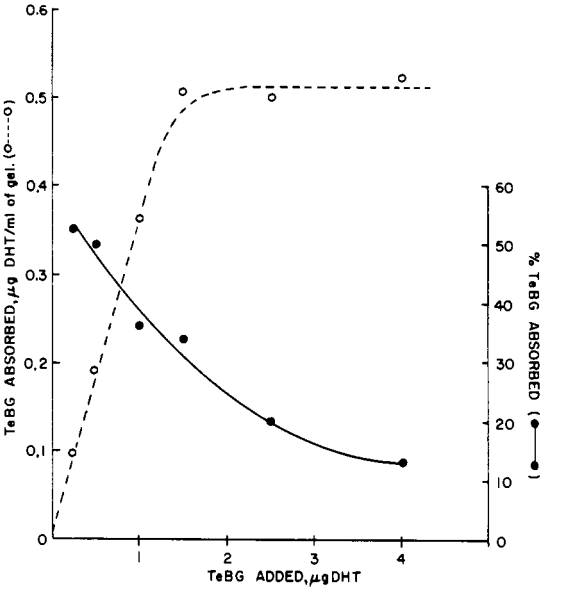

FIGURE 2: Capacity of androstanediol–Sepharose for TeBG. Cohn fraction IV was subjected to treatment with Norit A and ammonium sulfate and various volumes added to 0.5 ml of androstanediol–Sepharose. The supernatant was passed through Sephadex and assayed for TeBG activity. Results are expressed as the binding capacity for DHT (micrograms).

Isolation. The isolation procedure is outlined in Table I. Not shown in the table are the various concentration steps or G50 columns used to remove small molecules. There was no measurable loss of activity in either of these operations.

Elution of TeBG from the Affinity Column. Figure 3 illustrates the mode of elution of TeBG from androstanediol–Sepharose. The conditions for elution are in the figure and its legend. Note that the initial wash included 1 *M* NaCl in order to minimize nonspecific adsorption of protein and was conducted at 4°C in order to improve binding and stabilize the binding protein. The purpose of the second wash was to remove the NaCl. The third wash was the elution step and was accomplished by raising the temperature thus allowing

[2] The NMR spectra were obtained on a Varian EM-360 with Me_2SO-d_6 as solvent and Me_2SO as internal standard. The chemical shifts are given in δ parts per million: s, singlet; m, multiplet; nm, narrow multiplet. Ir spectra were obtained on a Beckman IR-9 spectrophotometer in KBr dispersion.

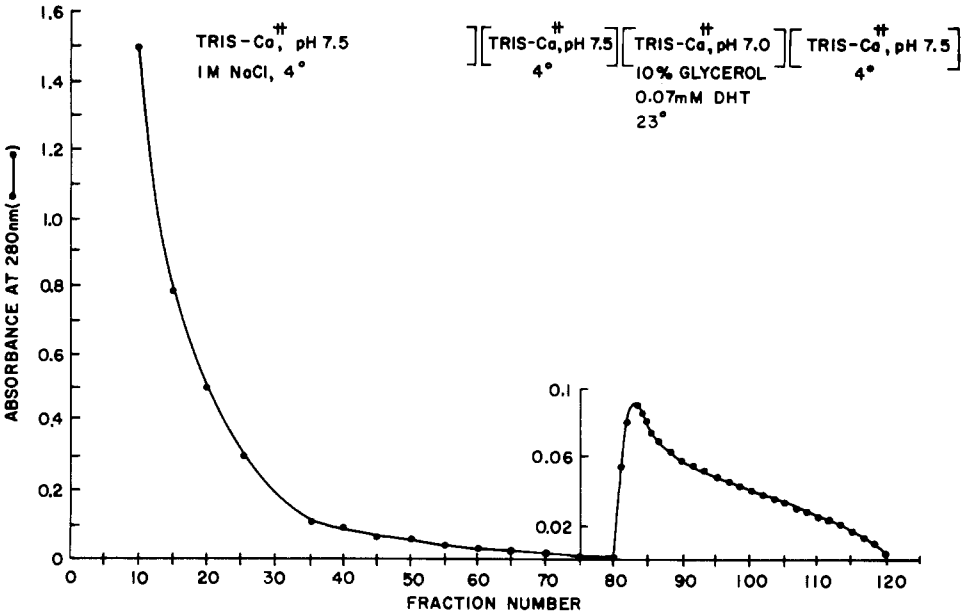

FIGURE 3: Elution of TeBG from androstanediol–Sepharose. Most of the protein was removed by decantation and washing on a funnel before the first optical density was measured. The conditions in brackets indicate the substances added to the column at the stage indicated. The fraction volume was 20 ml. An expanded absorbance scale is provided to make the elution pattern clear. Fractions 81–120 were pooled for further purification.

Table II: Some Physicochemical Properties of TeBG.

Mol wt[a]	94000
$s_{20,w}$	4.6
Mol radius[b]	2.95
Stokes radius[b]	5.55
f/f_0[b]	1.87
Partial specific vol[c], ml/g	0.692
$E_{1cm}^{1\%}$ at 280 nm[d]	5.5
Carbohydrate, %	32.1
Isoelectric point	5.51

[a] Determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The concentration of acrylamide was 4.59% and that of the cross-linker (methylenebisacrylamide) 1.96%. The proteins used for calibration were phosphorylase A, human serum albumin, pyruvate kinase, ovalbumin, and glyceraldehyde phosphate dehydrogenase. The plot of log mol wt against mobility was linear. [b] Calculated from data in this table with eq 6, 9, and 10 of Sherman (1975). [c] Calculated from amino acid and carbohydrate content. [d] Determined after extensive dialysis against distilled water, lyophilization, and drying at 110°C in vacuo.

column-bound TeBG to exchange with the DHT which had been added to the eluting buffer. The final wash served to elute the freed TeBG. An alternate method of eluting the TeBG by cleaving the azo bond in the side arm has been published previously (Rosner and Smith, 1975).

First Chromatography on Sephadex G150. Figure 4a illustrates the results of chromatographing fractions 80–120 (Figure 3) after concentrating and increasing the specific activity of the [^{3}H]DHT to 2×10^4 cpm/μg.

First Isoelectric Focusing. After pooling and concentrating fractions 37–45 from the first G150 column (Figure 4a), $\frac{1}{3}$ of the sample (ca. 15 mg of protein) was applied to an isoelectric focusing column and yielded the results illustrated in Figure 5a. This procedure was repeated two more times with the remaining $\frac{2}{3}$ of the sample with essentially identical results. As we did not explore the capacity of the focusing column, it is possible that the entire sample could have been handled in a single experiment. The data shown in Table I for this step were obtained by combining the results from the three separate focusing experiments.

Repetition of Sephadex G150 and Isoelectric Focusing. The results of repeating the preceding two steps are illustrated in Figures 4b and 5b. Fractions 37–46 from the second G150 column (Figure 4b) were applied to a single focusing column and fractions 19–25 from this column (Figure 5b) were used to characterize the purified TeBG.

Characterization. Polyacrylamide Gel Electrophoresis. Figure 6 illustrates the single band obtained when the final preparation of TeBG is submitted to polyacrylamide gel electrophoresis at two different gel concentrations. As Corvol et al. showed (1971) TeBG cannot be separated from transferrin at total gel concentrations less than 10% but can be separated at a gel concentration of 18%. Although the two gels shown in Figure 6 are not absolute proof of purity, they do furnish strong evidence that the most likely contaminating protein, transferrin, is not present in the purified TeBG. Further, in experiments in which the gels were sliced and counted, the [^{3}H]DHT migrated with the same R_f as the stained protein band.

Determinations of Sedimentation Coefficient and Molecular Weight. The sedimentation velocity of purified TeBG was determined in a Spinco Model E ultracentrifuge equipped with a monochromator and an ultraviolet photoelectric scanner. The protein was dissolved in 50 m*M* Tris–50 m*M* $CaCl_2$ (pH 7.4) at a final concentration of 1.3 mg/ml and centrifuged at 51000 rpm at 20°C for 60 min. Determinations were not made at multiple concentrations of the protein as the use of the ultraviolet (uv) scanner enabled us to do the experiment at a protein concentration of less than 2%. The $s_{20,w}$ obtained was 4.6 (Table II).

The molecular weight of the isolated TeBG was determined by a modification (see footnotes to Table II) of the sodium dodecyl sulfate–polyacrylamide gel method of Weber and Osborn (1969). TeBG was examined after the usual incubation in sodium dodecyl sulfate and 2-mercaptoethanol and, in addition, in the absence of 2-mercaptoethanol. The results obtained were independent of the presence of the reducing agent.

Amino Acid and Carbohydrate Composition. The amino

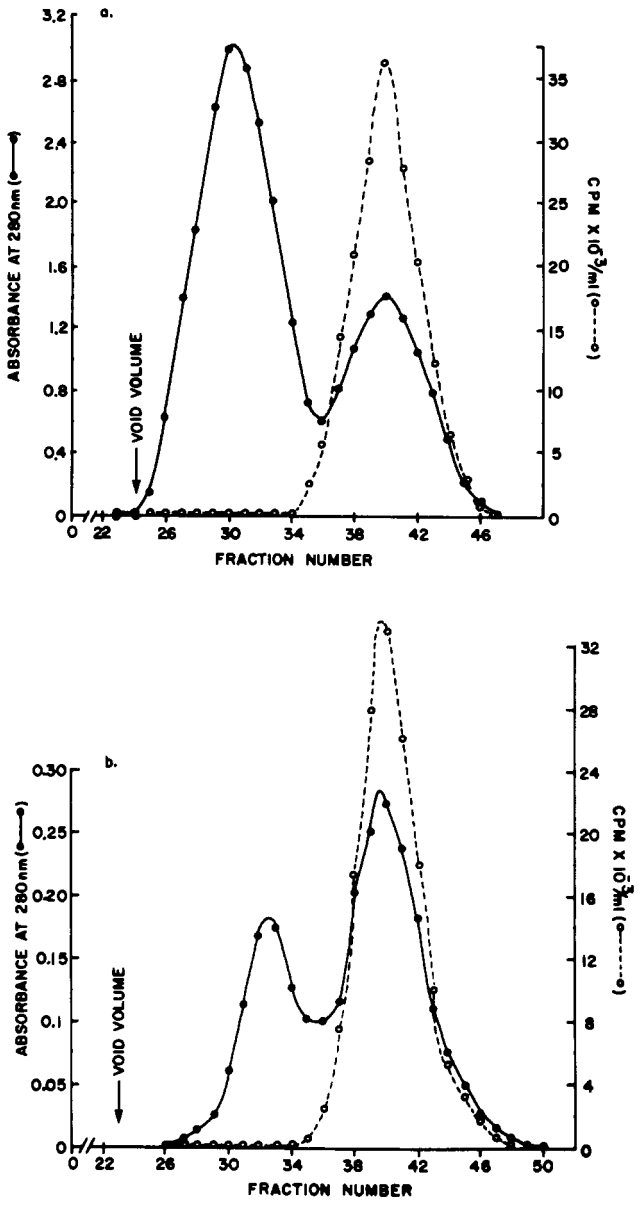

FIGURE 4: Chromatography on Sephadex G150. The flow rate was 12 ml/hr through a 2.5 × 92 cm column of Sephadex. Six-milliliter fractions were collected and radioactivity was determined in 25-μl aliquots: (a) fractions 81–120 from the affinity chromatography (Figure 3) were pooled, concentrated, and applied to the column; (b) fractions 18–26 from Figure 5a were pooled, combined with like fractions from two other isoelectric focusing columns, and applied to the column.

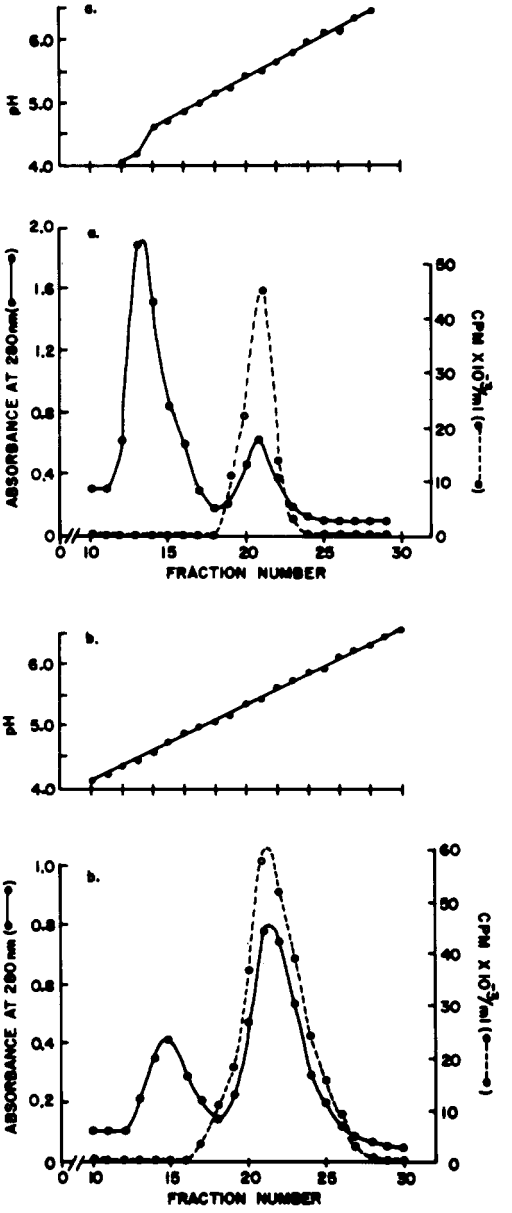

FIGURE 5: Isoelectric focusing. Fractions of ca. 3 ml were collected and radioactivity was determined in 10-μl aliquots: (a) fractions 37–45 from Figure 4a were pooled and concentrated and $\frac{1}{3}$ of the sample applied to the isoelectric focusing column as described under Materials and Methods; (b) the second isoelectric focusing column utilized fractions 37–46 from the second gel filtration (Figure 4b).

acid composition was determined on a Beckman Model 121 amino acid analyzer and the results are shown in Table III. In addition, free sulfhydryl groups were measured by the method of Ellman (1959) and gave a value of 6.3 mol/mol of TeBG. Disulfide bonds were estimated by the method of Iyer and Klee (1973) and gave a value of 8.0 mol of disulfide/mol of TeBG. We have no explanation for the 13 mol discrepancy in half-cystine between these results and the amino acid analysis except to point out the possibility of buried unreactive sulfhydryl groups. A mol wt of 94000 was used in both these calculations. The carbohydrate analysis shown in Table III was done by the gas chromatographic method of Mayo and Carlson (1974).

Binding Studies. All binding studies were conducted in duplicate on TeBG which was fully saturated with [^{3}H]DHT at the beginning of equilibrium dialysis. Equilibrium dialysis experiments utilized 2.4 μg of TeBG in 0.5 ml of 50 m*M* Tris buffer (pH 7.4) containing 50 m*M* $CaCl_2$ within the dialysis sac. Dialysis took place against 6 ml of the same buffer containing total amounts of DHT between 8 and 1300 ng. Scatchard plots of the data are presented in Figure 7 and the association constants derived from these plots are shown in Table IV. In order to determine the association constants for estradiol and testosterone, we applied the relationship (Edsall and Wyman, 1958):

$$\frac{r_a}{r_b} = \frac{k_a[a]}{k_b[b]}$$

where r_a = concentration of bound DHT; r_b = concentration of estradiol or testosterone; k_a and k_b = respective association constants; [a] and [b] = concentrations of the respective unbound steroid. As has been pointed out (Edsall

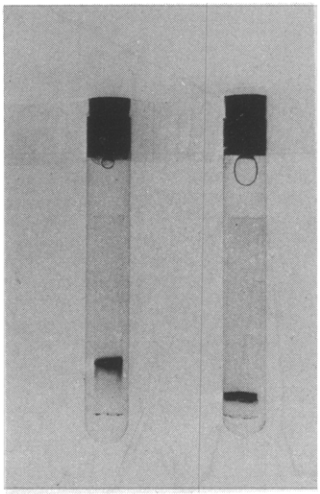

FIGURE 6: Polyacrylamide gel electrophoresis of purified TeBG at two different gel concentrations: (left) concentration of the separating gel was 7.2% with 2.6% cross-linkage; (right) concentration of the separating gel is 18% with 0.66% cross-linkage. Tris–glycine buffer (pH 8.3) was used in both anode and cathode chambers. Electrophoresis was at room temperature at 8 mA/gel for 75 min.

Table III: Chemical Composition of TeBG.

Substance	g per 100 g of Polypeptide	mol/mol of TeBG[b]
Amino Acid[a]		
Lysine	8.66	43
Histidine	3.07	14
Arginine	4.65	19
Aspartic	12.05	67
Threonine	4.17	26
Serine	4.67	34
Glutamic	11.27	56
Proline	3.93	26
Glycine	4.02	45
Alanine	5.36	48
Half-cystine	5.95[c]	37
Valine	5.62	36
Methionine	1.77[c]	9
Isoleucine	2.63	15
Leucine	9.49	54
Tyrosine	4.84	19
Phenylalanine	5.28	23
Tryptophan	2.57	9
Carbohydrate	g per 100 g of Carbohydrate	
Fucose	1.2	2
Mannose	14.9	25
Galactose	16.2	27
N-Acetylglucosamine	33.0	45
Sialic acid	35.8	35
% Carbohydrate	32.1	

[a] Hydrolysis of TeBG carried out in 3 *N p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole in evacuated sealed tubes at 110°C for 24 hr (Liu and Chang, 1971). No corrections are made for destruction with time. [b] Calculated using a mol wt of 94000. [c] Determined as cysteic acid and methionine sulfone on a separate sample after oxidation with performic acid (Moore, 1963).

and Wyman, 1958), if k_b is determined at several concentrations of b, then this is strong evidence that a and b are both reacting reversibly with the same set of sites on a binding protein. The association constants for each concentration of added competitor were in good agreement and they are shown in Table IV.

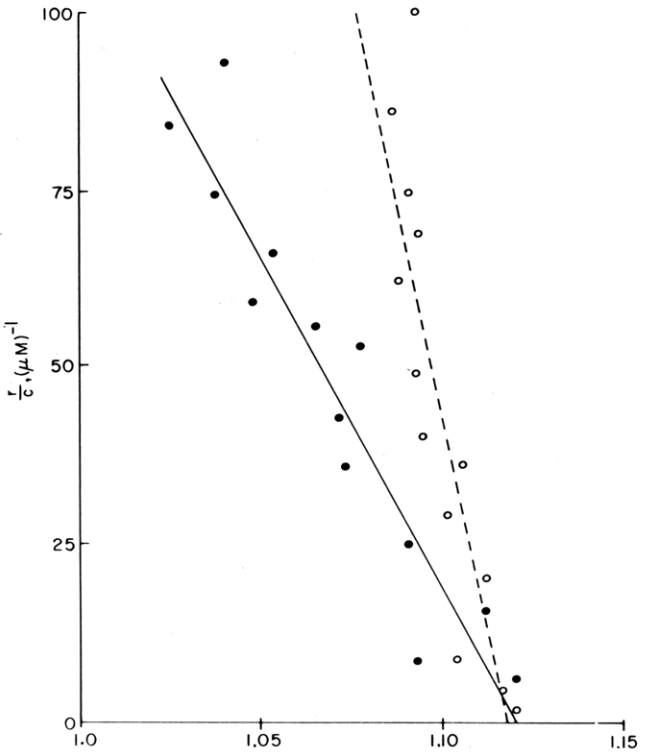

FIGURE 7: Scatchard analysis of the DHT–TeBG interaction. Equilibrium dialysis took place at 4°C (○) or 37°C (●) and each tube contained 2.4 μg of TeBG in 0.5 ml inside the dialysis bag; c = molar concentration of unbound TeBG; r = moles of DHT bound per mole of TeBG. The linear regression of r/c on r was calculated by the method of unweighted least squares. The limited range of values for r was unavoidable because the isolated TeBG was saturated with the DHT added to it during the purification procedure. The association constants derived from this plot are shown in Table IV.

Table IV: Association Constants for the Interaction of Steroids with TeBG.[a]

Steroid	K at 37°C (M^{-1})	K at 4°C (M^{-1})
Dihydrotestosterone	0.99×10^9	2.4×10^9
Testosterone	0.35×10^9[b] ± 0.0403	1.1×10^9[b] ± 0.0753
Estradiol	0.22×10^9[c] ± 0.0248	0.60×10^9[c] ± 0.113

[a] The association constants for DHT were derived from the slopes of the lines shown in Figure 7. The dialysis bags contained 2.4 μg of TeBG in 0.5 ml of buffer along with 28 ng of [^{3}H] DHT. The specific activity was adjusted so that the final $^3H/^{14}C$ ranged between 3 and 12 in the aliquots taken for counting. The results for testosterone and estradiol are shown ± standard deviation. [b] Four experiments were done in duplicate at each temperature with the addition of 28, 56, 110, and 230 ng of testosterone as the appropriate combination of the ^{14}C-labeled and radioinert steroid. [c] Five experiments were done in duplicate at each temperature with the addition of 55, 160, 265, 465, and 1200 ng of estradiol as the appropriate combination of the ^{14}C-labeled and radioinert steroid.

Discussion

The affinity column which we have devised for the isolation of TeBG is novel and might prove generally useful. The preparation of diazonium–Sepharose has been described (Cuatrecasas, 1970) but this Sepharose derivative requires a suitably reactive ligand to be of use. The azo bond in the present work is incorporated into the side arm of the affinity column and azodianiline can be substituted for any of the

other bifunctional diamino compounds commonly used in affinity chromatography. Although we did not use cyanogen bromide as the activating agent in this work, azodianiline can be coupled to Sepharose in good yield with this reagent. Incorporation of the azo bond in the side arm allows the protein to be isolated to be removed from the column, together with the ligand, under relatively mild conditions (Cuatrecasas, 1970; Rosner and Smith, 1975). We have previously furnished an example of this kind of elution from azodianiline–Sepharose in a previous attempt to purify TeBG (Rosner and Smith, 1975).

The present method for the isolation of TeBG has yielded what appears to be a pure and fully active protein. The major pieces of evidence for purity are single bands in acrylamide gel electrophoresis at two different gel concentrations, a single band in sodium dodecyl sulfate gel electrophoresis, and the observation of a single binding site per mole of the isolated protein. The binding studies can also be considered as indicating the full activity of the preparation. Were the final preparation partially inactive we would have seen a fraction of a binding site per mole of protein (Figure 7).

The association constants we found for testosterone, dihydrotestosterone, and estradiol are in substantial agreement with values derived from experiments on partially pure preparations and whole plasma (reviewed in Westphal, 1971, p 367) although no one else has furnished data on all these steroids at these temperatures. The most remarkable agreement is between the present data and that of Mercier-Bodard et al. 1970) who examined testosterone and estradiol at 4°C in an impure system, allowing a much greater spread of bound values than we could achieve in our pure, fully bound system. Their values were within 1.5 standard deviations of ours. Thus, it seems that the TeBG steroid interaction is not affected in any important way by virtue of the system being immersed in plasma.

TeBG is rich in half-cystine and this is consistent with its loss of activity after exposure to sulfhydryl or disulfide reagents (Pearlman et al., 1969; Rosner and Smith, 1974). The demonstrated sialic acid content supports the indirect evidence presented by Van Baelen et al. (1969) which indicated for the first time that TeBG contained sialic acid and was a glycoprotein.

There is in the literature an important disagreement about the molecular weight of TeBG. Guériguian and Pearlman (1968), Van Baelen et al. (1968), and Rosner et al. (1969), all using TeBG of varying degrees of impurity, determined molecular weights of about 100000 using Sephadex. Since glycoproteins frequently give falsely elevated values for molecular weight when studied by gel filtration this value has been open to some question. Corvol et al. (1971) determined a molecular weight, by polyacrylamide gel electrophoresis, of 98000 but did not comment upon the reliability of the molecular weights of glycoproteins in their system. The most confusing entry in this field has been the data of Mercier-Bodard et al. (1970). They obtained a seemingly pure but relatively inactive preparation of TeBG, submitted it to sedimentation equilibrium, and determined a mol wt of 52000. This figure is in obvious disagreement with those already quoted and with that found by sodium dodecyl sulfate electrophoresis (also subject to error with glycoproteins) in this paper. We therefore recalculated the data of Mercier-Bodard and found that the proper molecular weight, based on their own data, was 104000. Although they do not specify the buffer in which the equilibrium sedimentation was done and although there is some difficulty in calculating the slope of lines from published reproductions, there can be no doubt that the value we calculated is within 10–20% of the correct value. Hence, the controversy over the molecular weight of the TeBG seems to have arisen from a trivial error in calculation.

We have found that the purified TeBG is stable for at least 6 weeks stored in Tris–calcium buffer in 10% glycerol at 4°C. This stability should allow more extensive study of this protein in the future.

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Subcellular Redistribution of Seryl-Transfer RNA during Estrogen-Induced Phosvitin Synthesis and Specificity of the Estrogen Effect†

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ABSTRACT: The estrogen-induced hepatic synthesis of the serine-rich protein, phosvitin, in chickens is accompanied by an increase in the serine acceptance of total hepatic tRNA, which is limited to two serine isoacceptors. To investigate the role of the tRNA alterations in the synthesis of phosvitin, the relative amounts of various seryl-tRNA species in isolated nuclei and in free and membrane-bound ribosomes were determined. Estrogen treatment causes a shift in the subcellular distribution of hepatic seryl-tRNA. Of the four serine isoacceptors, the amount of $tRNA^{Ser}_{AGU,AGC}$ was specifically increased in nuclei and in membrane-bound ribosomes. Changes in total hepatic tRNA occurring during physiologic estrogenization were compared with those occurring by varying steroid hormones, times after estrogen administration, estrogen doses, animal ages, and tisssue types. The changes observed demonstrate that the seryl-tRNA alterations are closely correlated with the synthesis of phosvitin. The coincident change in seryl-tRNA levels and in phosvitin synthesis, together with the specific change in cellular localization, suggests that the amount and subcellular distribution of each tRNA species are separately controlled in a manner dependent upon its frequency of use in translation.

Alterations in the levels of specific transfer RNA species have been noted in association with changes in the rate of specific protein synthesis (for a recent review, see Garel, 1974). A well-documented example of this phenomenon is the increase in levels of liver seryl-tRNA associated with increased rates of hepatic synthesis of the serine-rich yolk protein, phosvitin, in laying hens and in chicks and roosters after estrogen administration (Carlsen et al., 1964; Mäenpää and Bernfield, 1969; Beck et al., 1970). Increased tRNA acceptance of additional amino acids has also been found (Busby and Hele, 1970; Klyde and Bernfield, 1973) and it has not been demonstrated whether the increase in seryl-tRNA level is related in a physiologically significant way to the augmented synthesis of phosvitin. Indeed, no change in the amount of liver seryl-tRNA was observed during the estrogen induction of a phosvitin-like protein in *Xenopus* liver (Wittliff et al., 1972), and despite the increase in total liver seryl-tRNA induced in chicks by estrogen, no change was observed in the *rate* of total liver $tRNA^{Ser}$ synthesis relative to the rate of total tRNA synthesis (Klyde and Bernfield, 1973).

In cell-free systems the relative abundance of a specific aminoacyl-tRNA controls the rate of messenger translation (Anderson, 1969), and a similar relationship has been observed in experiments using tissue explants (Sharma et al., 1973). The present experiments were performed to assess the mechanism by which estrogen evokes seryl-tRNA changes and the possible role of these changes in phosvitin synthesis. The distribution of seryl-tRNA was assessed in tRNA derived from isolated hepatic nuclei and from free and membrane-bound ribosomes. The pattern of total hepatic seryl-tRNA alteration was established in laying hens to determine the nature of the seryl-tRNA alterations during physiological estrogenization and phosvitin synthesis. This specific pattern of seryl-tRNA levels was then assessed in several other circumstances to determine whether the pattern of seryl-tRNA levels changes coincidentally with increased phosvitin synthesis.

Materials and Methods

Animals. White leghorn roosters and 1-week-old male chicks were obtained from local hatcheries and fed *ad libitum.* Hormones were dissolved (by heating if necessary) in sesame oil and injected into leg muscles. Heparinized blood samples were analyzed for protein phosphate as previously described (Mäenpää and Bernfield, 1969).

Chemicals. Estradiol benzoate [estra-1,3,5(10)-triene-3,17β-diol 3-benzoate] and ethynyl estradiol [estra-

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