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Estrogen-Binding Proteins of Calf Uterus. Partial Purification and Preliminary Characterization of Two Cytoplasmic Proteins*

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ABSTRACT: The larger (~8 S) estrogen-binding protein (EBP) which is found in the soluble fraction of uterine cytoplasm has been purified about 500-fold from prepuberal calf. The smaller (~4 S) cytoplasmic EBP has also been purified about 1500-fold by a previous method. Using these partially purified preparations, the following molecular characteristics were assessed by sucrose gradient centrifugation combined with "gel filtration" on Sephadex G-200 and by electrofocusing. Larger EBP: $s_{20,w}$ = 8.6; Stokes radius, 67 Å; mol wt 238,000 (for $\bar{v}$ = 0.725); f/f_0 = 1.65; homogeneous at electrofocusing, with isoelectric point at 6.2. Smaller EBP: $s_{20,w}$ = 4.5; Stokes radius, 33 Å; mol wt 61,000 (for $\bar{v}$ = 0.725); f/f_0 = 1.25; heterogeneous at electrofocusing: two major components with isoelectric point at 6.6 and 6.8, respectively. As estimated by a method based on gel filtration, K_{ass} with estradiol of both EBPs is 1.5–3.5 × 10^9 l./mole at +4°. Relative affinity for ligands is as follows: diethylstilbestrol > estradiol > estrone > estriol; in comparison, no significant affinity for progesterone, deoxycorticosterone, testosterone, and cortisol is detected. Estrogen-binding activity of EBPs is irreversibly destroyed by acidic pH, +65° for 5 min at pH 7.5, cold ethanol or ether, 1.5–6 M urea, and 1.5–2.5 M guanidine hydrochloride at pH 7.5. In contrast, aspecific binding of estradiol by plasma albumin increases with increasing pH and temperature. It is suggested that the larger 8.6S EBP is a tetrad consisting of four 4.5S subunits and that the subunits possibly belong to at least two different types. Some data suggest that binding of estradiol is cooperative. The estrogen-binding subunit of cytoplasmic origin and the partially purified EBP which is extracted from the nuclear fraction of calf uterus homogenate were compared to find no significant difference in molecular and other properties as investigated by present methods.

The idea that the action of a hormone must involve specific interaction with a component (receptor) of the target cell is old (Ehrlich, 1902). Only recently, however, the binding of estrogens to target tissues was discovered (Jensen and Jacobson, 1962). It has been shown that the estrogenic molecule is bound by specific macromolecules in the cytoplasm (Talwar *et al.*, 1964; Toft and Gorsky, 1966; Jensen *et al.*, 1967; Erdos, 1968) and that the complex moves to the nucleus (Brecher *et al.*, 1967; Jensen *et al.*, 1968; Gorsky *et al.*, 1968) where an increased RNA synthesis takes place (Hamilton, 1968). These macromolecules are protein in nature (Toft and Gorsky, 1966) and there is ample evidence of their highest affinity for estrogenic ligands (Toft *et al.*, 1967; Puca and Bresciani, 1969a; Best-Belpomme *et al.*, 1970; Korenman, 1970).

Several estrogen-binding proteins (EBPs)[1] have been described in target tissue homogenates. In prepuberal calf uterus, which is a convenient source of these proteins, a larger and a smaller cytoplasmic EBP have been identified. They are also referred to as 8 S and 4 S, respectively, according to their approximate sedimentation coefficients on a sucrose gradient (De Sombre *et al.*, 1969). The larger EBP shows a marked

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[1] Abbreviations used are: EBP, estrogen-binding protein; BPA, bovine plasma albumin; RTF, receptor transforming factor.

tendency toward aggregation which hinders attempts at purification (Erdos, 1968; De Sombre *et al.*, 1969). Indeed, when purification procedures are applied which apparently circumvent aggregation by the use of high ionic strength in the presence of Ca^{2+} ions the invariable result is the smaller 4S EBP, while the larger 8S EBP is lost (De Sombre *et al.*, 1969; Puca *et al.*, 1970a). An EBP has also been extracted from the nuclear fraction of calf uterus slices previously incubated with estradiol-*6,7-t* (Puca *et al.*, 1969); this nuclear EBP sediments at about 5 S in the stage of crude nuclear extract and at 4.5 S after partial purification (Puca *et al.*, 1970b).

There seems to be little doubt that further elucidation of the phenomenon of estrogen binding and of its relevance to the mechanism of estrogen action is heavily dependent on progress in purification and characterization of estrogen-binding proteins and understanding of their interrelationships. As a contribution towards such a goal, this paper reports partial purification of the larger EBP by a procedure which circumvents both its tendency to aggregate and its tendency to change into smaller molecular forms during purification. Furthermore, both the larger and the smaller EBP of cytoplasm have been purified as estrogen-free molecules and, using these partially purified preparations, molecular parameters and other properties of both these proteins were investigated by suitable methods. Preliminary reports of this research have been given (Bresciani *et al.*, 1970; Puca *et al.*, 1971).

Material and Methods

Materials. All reagents were of analytical grade. Estradiol-*6,7-t* (New England Nuclear, specific activity 40 Ci/mmole) was >97 per cent pure at the time of experiment; its radiochemical homogeneity was checked by thin-layer chromatography as previously described (Puca and Bresciani, 1969b). Tris was obtained from Sigma Chemical; Sephadex G-25 and G-200 from Pharmacia; Sucrose from C. Erba. Standard macromolecules for Stokes radius and sedimentation rate determinations were obtained: γ-globulin (human plasma), albumin (bovine plasma), ovoalbumin, and myoglobin (horse muscle) from Serva; transferrin (human plasma) from Boehringer; Blue Dextran from Pharmacia.

Buffers. The following solutions were used: TE, pH 7.5 (Tris-HCl (10^{-2} M)–EDTA (1.5×10^{-3} M)); TK, pH 7.5 (Tris-HCl (0.1 M)–KCl (0.4 M)); TKE, pH 7.5 (Tris-HCl (10^{-2} M)–KCl (10^{-2} M)–EDTA (10^{-3} M)); TKC, pH 7.5 (Tris-HCl (0.1 M)–KCl (0.4 M)–$CaCl_2$ (10^{-3} M)).

Sucrose gradient centrifugation (Martin and Ames, 1961) was carried out in a I.E.C. B-60 ultracentrifuge on 5–20% gradients, and in some instances on 41–53% gradients. Gradients were stored at +4° for several hours before use. Preliminary tests of linearity of gradients were carried out by addition of phenol red to the sucrose solution of lower concentration. After centrifugation and routine collection of fractions, OD at 400 mμ was found to increase linearly with fraction number.

Gel Filtration. Sephadex G-200 was suspended in TKE buffer (pH 7.5) and allowed to swell for one week. During this time fine particles were decanted. Standard Pharmacia K 25/45 and K 50/100 glass columns were used; the columns were fitted with upward-flow adaptors and were packed with gel in a single step by the use of a removable funnel at the top of the column. The upward flow was generated by a peristaltic pump. Effluent was monitored for OD at 280 mμ by a LKB Uvicord II. Fractions were collected by means of an automatic fraction collector equipped with drop counter. Protein content and radioactivity of fractions were assessed as described below.

Electrofocusing. The 110-ml standard electrofocusing column (LKB) equipped with double-cooling jackets was used. A pH 5–8 gradient was achieved using 1 or 2% ampholine (LKB) in a 0–50% sucrose gradient. The starting solution was 52 ml of 50% sucrose containing 1.95 or 3.9 ml of 40% ampholine pH 5–8; the limit solution was 52 ml of H_2O containing 0.65 or 1.3 ml of the same ampholine solution. The temperature of the cooling water was kept constant at +3°. The pH 5–8 ampholine gradient was allowed to focus for 12 hr until milliamperage fell to a constant 0.9 mA (1% ampholine) or 1.8 mA (2% ampholine) at 800 V. At this stage power was turned off and the sample was introduced to replace an equal volume of the gradient in the upper third of the column, in the pH 7–8 range of the gradient. Power was turned on again and the increasing amperage was kept within about 3.5 mA by decreasing voltage; generally, after 6–8-hr amperage fell to a constant minimum 0.8 mA (1% ampholine) or 1.6 mA (2% ampholine) at 800 V. At this point the gradient was collected in the cold room at +4° in 2-ml fractions, at the flow rate of 1 ml/min. OD at 280 mμ, radioactivity, and pH were measured on aliquots of the collected fractions.

Protein Assay. Protein concentration was measured by the microbiuret method (Zamenhof and Chargaff, 1957). Routinely, chromatographic effluents were examined spectrophotometrically for protein.

Radioactivity Assay. Aqueous sample (0.1–0.2 ml) was added to 10 ml of toluene–phosphor solution containing 3.92 g % of 2,5-diphenyloxazole, 0.18 g % of *p*-bis(*o*-methylstyryl)benzene and 330 ml % of Triton X-100 in toluene; radioactivity was measured in a Mark I (Nuclear-Chicago) liquid scintillation spectrometer with a 35% efficiency (Radioactivity assay 1). When necessary because of low radioactivity, estradiol-*6,7-t* was extracted from aqueous solution as described elsewhere (Puca and Bresciani, 1969b) and dissolved in a toluene–phosphor solution lacking Triton X-100; radioactivity was measured in the spectrometer with a 45% efficiency (radioactivity assay 2).

Assay of Estrogen-Binding Activity. This assay is based on gel filtration experiments described in previous research (Puca and Bresciani, 1968, 1969a). It applies to macromolecular preparations, from high-speed supernatants of homogenate to preparations of higher purity, and is presently carried out as follows. The solutions (0.1–0.3 ml) to be tested, containing 0.01–1 mg of protein, are brought to 1 ml with TKE buffer (pH 7.5), containing 3×10^5 dpm of estradiol-*6,7-t* and colored with Blue Dextran. The mixture is incubated at +4° for 5 hr, a sufficient time to reach equilibrium. After incubation, separation of free from macromolecule-bound estradiol-*6,7-t* is accomplished by filtration on Sephadex G-25 at +4° and under standard conditions of column size (ϕ = 1.2 cm; total volume 20 ml) and flow rate (40 ml/hr); the *bound* hormone emerges associated with the first macromolecular peak, which is excluded by the gel (void volume) while *free* hormone is eluted as a peak emerging after the internal volume of the column. The excluded peak is easily identified by the color (Blue Dextran); there is no adsorption of estradiol to the dye. If the amount of bound hormone is an excess of one-tenth of that of free, the test is repeated after dilution of the sample in order to ensure saturation of binding sites. Under the above described conditions of gel filtration aspecific bindng of estradiol is negligible. By aspecific binding of estradiol we mean relatively low-affinity binding by macromolecules other than receptors; the highest known affinity for estradiol of a non-

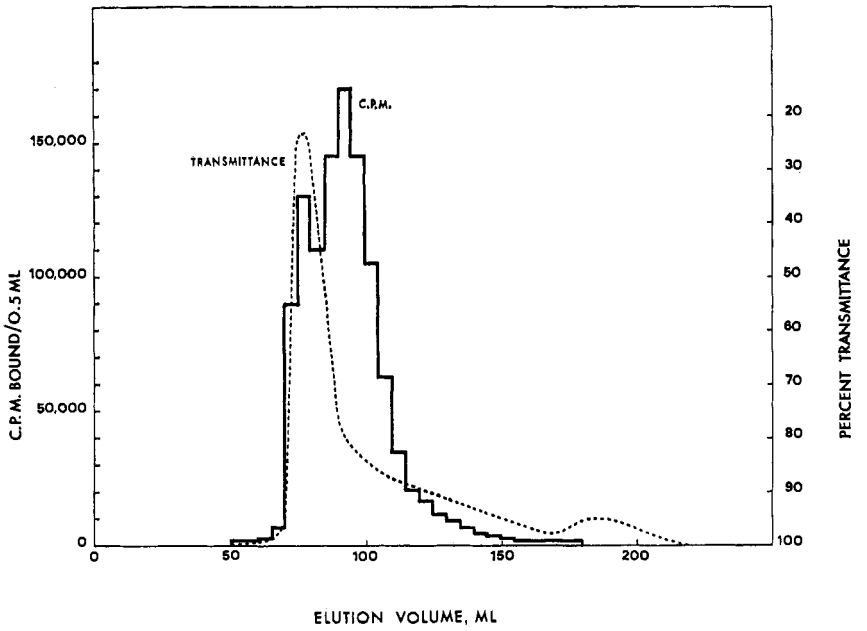

FIGURE 1: Preparation of larger cytoplasmic EBP (estradiol free). The ammonium sulfate precipitate of prepuberal calf uterus cytosol, prepared as described in Material and Methods, was dissolved in TK buffer (pH 7.5) and a 7-ml sample (6.5 mg of protein/ml) was applied to a 186.5-ml column of Sephadex G-200 equilibrated with TKE buffer (pH 7.5). Elution was performed at +4° using an upward constant flow (10–12 ml/hr). Transmittance at 280 mμ (- - -) was continuously monitored. Fractions (5 ml) were collected, of which 0.5 ml were withdrawn and assayed for estradiol-*6,7-t* binding activity (—). Fractions from 90 to 120 ml of column eluate were pooled together: sucrose gradient centrifugation showed that the EBP in these fractions sediments at an average of 8.6 S (see text). Binding activiy of this preparation of estrogen-free 8.6S EBP is 160,000 dpm/0.125 mg of protein, *i.e.*, about 500-fold purification over the homogenate.

receptor protein is that of plasma albumin, *i.e.*, about 10^5 l./mole (Sandberg and Slaunwhite, 1957) against more than 10^9 l./mole of receptor proteins (Puca and Bresciani, 1969a; Best-Belpomme *et al.*, 1970; Korenman, 1970). The method furnishes a linear response for binding activity up to 3×10^4 dpm of estradiol-*6,7-t* bound. Association constants between receptor proteins and estradiol-*6,7-t* were also estimated in this, as well as in a previous research (Puca and Bresciani, 1969a), using this gel filtration method. Affinity for other cold ligands were obtained indirectly from the study of competitive inhibition of hot estradiol binding by the cold ligand under investigation.

Preparation of Calf Uterus Homogenate. Uteri from immature calves were collected at the local slaughterhouse as soon as the animals were killed and kept in a plastic bag buried in crushed ice during transportation to the laboratory. Uteri of weight larger than 30 g were discarded. Within 3 hr after collection, uteri were minced in a meat grinder and homogenized in 4 volumes of TE buffer (pH 7.5) by means of an Ultraturrax homogenizer (Janke & Kunkel, Model TP 18/2) in six runs of 15 sec each, with 60-sec intervals. These operations were carried out at +4°.

Protein Purification Procedures. LARGER EBP. The following procedure circumvents fragmentation of the larger cytoplasmic protein into smaller components, a phenomenon which takes place when purification is carried out as described previously (De Sombre *et al.*, 1969; Puca *et al.*, 1970a). All operations were carried out at +4°. The uterine homogenate (~80 ml) was centrifuged for 1 hr at 60,000 rpm in an I.E.C. B-60 ultracentrifuge using the A-321 rotor (300,000*g*). $CaCl_2$ solution (0.2 M) was added to the decanted supernatant up to the final concentration of 4 mM; after 2 hr, 0.175 g/ml of finely powdered $(NH_4)_2SO_4$ (30% saturation) was added under continuous magnetic stirring. The precipitate was spun down for 20 min at 10,000 rpm in an I.E.C. B-20 centrifuge using the 870 rotor (19,000*g*), and then resuspended in a volume of TK buffer (pH 7.5) equivalent to one-tenth of the supernatant original volume. Undissolved material was sedimented by centrifugation for 20 min at 19,500 rpm in the B-20 using the 870 rotor (45,000*g*), and the resulting clear solution was applied to a Sephadex G-200 column (inverted flow 186.5 ml of bed volume) equilibrated with TKE buffer (pH 7.5); the column was equipped with continuous monitoring of effluent OD at 280 mμ (LKB Uvicord II) and with fraction collector with drop counter (LKB Ultrorac). The results of the Sephadex chromatography are reported in the appropriate section. It seems useful to point out that the significant difference between this and the next procedure, which yields smaller EBP, is the 2-hr interval between addition of $CaCl_2$ and of $(NH_4)_2SO_4$; why this makes a difference in the yields of larger or smaller EBP will be considered in Discussion. Other procedural differences result from the use of differing amounts of starting homogenate.

SMALLER EBP. This purification was carried out essentially as described previously (Puca *et al.*, 1970a). It will be briefly reported as it was actually applied in these experiments. All operations were carried out at +4°. The homogenate (~1000 ml) was centrifuged for 3 hr at 14,500 rpm in a I.E.C. B-20 centrifuge using the 872 rotor (28,800*g*). $CaCl_2$ solution (0.2 M) and KCl solution (3.5 M) were added to the decanted supernatant up to final concentration of 4 mM and 1 M, respectively. After 30 min, 0.113 g/ml of finely powdered $(NH_4)_2SO_4$ was slowly added to supernatant (20% saturation). After another hour, the precipitate was first spun down for 20 min at 10,000 rpm in the 872 rotor (15,000*g*) and then resuspended in a volume of TKC buffer (pH 7.5) equivalent to one-tenth of that of the original supernatant. Undissolved precipitate was removed by centrifugation for 60 min at 60,000 rpm in the B-60

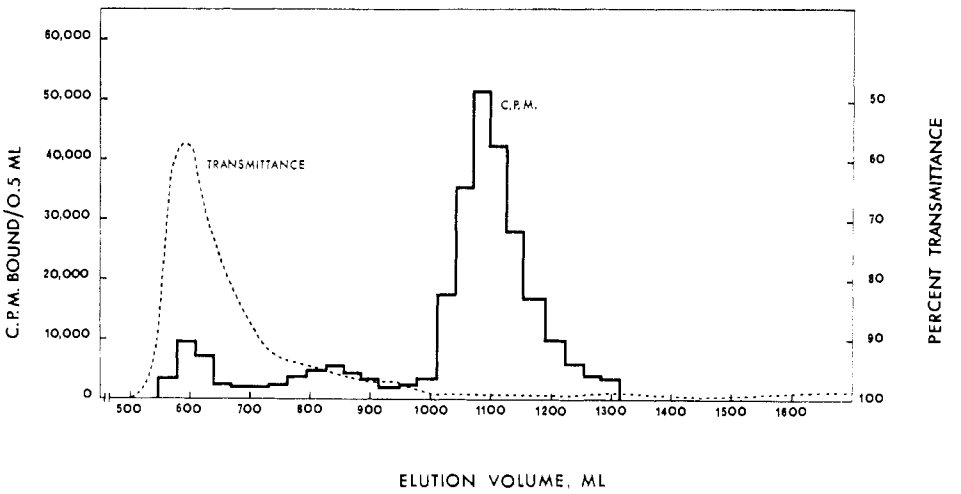

FIGURE 2: Preparation of smaller cytoplasmic EBP (estradiol free). The ammonium sulfate precipitate, prepared from prepuberal uterus cytosol as described in Material and Methods, was redissolved in TKC buffer (pH 7.5) and a 65-ml sample (2.4 mg of protein/ml) was applied to a 1800-ml column of Sephadex G-200 equilibrated with TKE buffer (pH 7.5). Elution was performed at +4° with TKE buffer, using an upward constant flow (30–35 ml/hr). Fractions (30 ml) were collected, of which 0.5 ml was withdrawn and assayed for estradiol-*6,7-t* binding activity (—). Transmittance of effluent at 280 mμ (- - -) was continuously monitored. Fractions from 1040 to 1160 ml of column eluate were pooled together; sucrose gradient centrifugation showed that the EBP in these fractions sediments at an average of 4.5 S (see text). Binding capacity of this preparation of estrogen free 4.5S EBP is 105,000 dpm/0.02 mg of protein, *i.e.*, about 1500-fold purification over the homogenate.

ultracentrifuge using the A-321 rotor (300,000*g*), and the resulting clear solution was applied on a 1800-ml column of Sephadex G-200 equipped as described for purification of the larger EBP. The results of the Sephadex chromatography are reported in the next section.

Results

Purification. LARGER EBP. Redissolved ammonium sulfate precipitate of calf uterus homogenate supernatant, prepared as described in Material and Methods for circumventing transformation of the larger EBP into smaller components, was applied to a Sephadex G-200 column. Transmittance at 280 mμ and estrogen-binding activity of the column effluent are shown in Figure 1. Most material absorbing at 280 mμ is excluded from the gel; *i.e.*, emerges with the void volume of the column (70 ml). However, only about 20% of total binding activity is associated with the excluded material; the remaining 80% is included and emerges with peak at 95-ml elution volume. Sucrose gradient centrifugation analysis of the excluded binding activity showed that this material consists of large, heterogeneous aggregates most of which are at the bottom of a 3.6-ml, 5–20% gradient after 4-hr centrifugation at 300,000*g*. In contrast, the included estrogen binding activity (fractions between 90- and 120-ml elution volume pooled together) sediments as a homogeneous peak at 8.6 S; further details are given later. The specific estradiol-*6,7-t* binding activity of the included peak of Figure 1 was 1,280,000 dpm/mg of protein, *i.e.*, about a 500-fold increase over the original uterus homogenate. Recovery was 32% of total binding activity of original supernatant. This preparation of larger EBP was used for all the characterization tests reported in this paper.

SMALLER EBP. Redissolved ammonium sulfate precipitate of calf uterus homogenate supernatant, prepared as described in Material and Methods for smaller cytoplasmic EBP, was applied on a column of Sephadex G-200. Transmittance at 280 mμ and estrogen-binding activity of column effluent is shown in Figure 2. Most material absorbing at 280 mμ is excluded from the gel, *i.e.*, emerges with the void volume of the column (~600 ml). Estrogen-binding activity, however, shows two smaller peaks and a larger one. The first peak is associated with the excluded material, while the other two are included. The excluded estrogen-binding activity, like in chromatography of Figure 1, sediments on a sucrose gradient as heterogeneous aggregates. Of the two included peaks, sucrose gradient analysis has shown that estrogen-binding activity of the small, first emerging one sediments at 8.6 S, *i.e.*, it corresponds to the larger EBP; while the largest and last emerging binding activity sediments as a homogeneous peak at 4.5 S; further details are given later. The specific estradiol-*6,7-t* binding activity of the larger and last emerging peak of chromatography in Figure 2 (fractions between 1040- and 1160-ml elution volume pooled together) was 5,250,000 dpm/mg of protein, which is more than a 1500-fold increase over the original uterus homogenate. Recovery was 27% of total binding activity of original supernatant. This preparation of smaller EBP was used for all characterization tests in this paper.

Sedimentation Rate. Centrifugation on 5–20% sucrose gradients gave the following results. For the included material of Figure 1: average sedimentation coefficient and standard error of mean, 8.61 ± 0.05 (8 independent runs). For the included material of Figure 2: 4.45 ± 0.01 (16 independent runs). Bovine plasma albumin (4.45 S) was the standard reference; human-IgG (7 S) was also run as a further check for the larger EBP. Typical sedimentation patterns are shown in Figure 3. The larger and smaller EBP are thus identified in the remainder of this paper by their approximate average sedimentation coefficients, *i.e.*, as 8.6S EBP and 4.5S EBP, respectively. The 8.6S and 4.5S EBPs also sediment into sucrose solution of at least 1.25-g/cm^3 density, *i.e.*, 53% sucrose at 0°, and therefore cannot have a hydrated specific volume larger than 0.80 (De Duve *et al.*, 1959); this is evidence that they do not contain significant lipid material. Sedimentation coefficient of crude cytosol-binding protein, under conditions un-

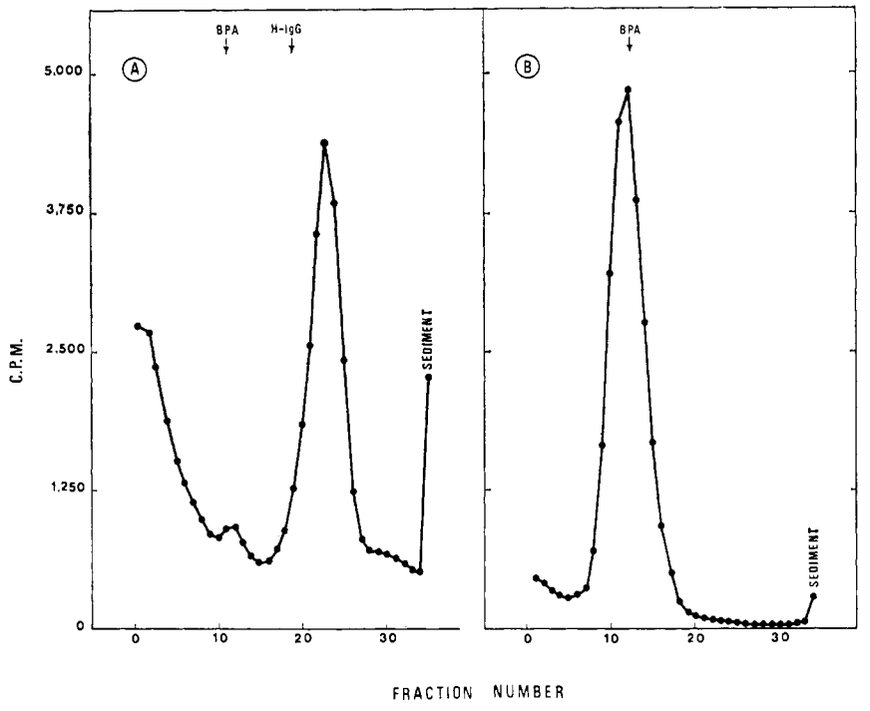

FIGURE 3: Sedimentation rate of larger and smaller cytoplasmic EBPs on sucrose gradients. Typical sedimentation patterns on 5–20% sucrose gradients of 0.2-ml samples from partially purified estrogen-free preparations of (a) larger EBP of Figure 1, after incubation at +4° with 0.004 μg/ml of estradiol-*6,7-t* (40 Ci/mmole); and (b) smaller EBP of Figure 2, after incubation at +4° with 0.002 μg/ml of the same tritiated hormone followed by reduction of volume to one-fourth by dialysis under reduced pressure. Sucrose was dissolved in TKE buffer (pH 7.5); centrifugation conditions were, 60,000 rpm at +2° for either 11.6 hr (a) or 12.5 hr (b) in the SB-405 rotor of the I.E.C. B-60 ultracentrifuge. Fractions of about 0.1 ml were collected by gravity after puncturing the bottom of the tubes and radioactivity in fractions measured by assay method 1. Fraction-labeled sediment consists of the bottom of the tube which was cut after collection of the gradient and dissolved in Soluene for counting. Sedimentation coefficients of EBPs were based on BPA (4.45 S) as standard reference; human IgG was also run as an additional reference. The positions of the sedimentation peaks of reference proteins were determined by the OD at 280 mμ and are shown by arrows. Average of 8 independent runs of (a) was 8.61 ± 0.05 S; and of 16 independent runs of (b) was 4.45 ± 0.01 S. See also text.

favorable to aggregation, has been previously estimated to be about 8.5 S (Puca *et al.*, 1969).

Stokes Radius. The 8.5S and 4.5S EBP preparations of Figures 1 and 2 were rechromatographed on a calibrated Sephadex G-200 column. The results are shown in Figure 4. To both preparations estradiol-*6,7-t* was added 1 hr before chromatography; no OD throughout this second chromatography was recorded by an expanded-scale LKB ultraviolet recorder. In Figure 5 the K_d's of standard proteins used for calibration of the column are plotted as a function of Stokes radius, according to Porath ($K_d^{1/3} = \alpha - \beta a$); as expected, a straight line is obtained, which is used to derive Stokes radius of EBPs from their respective K_d's. Stokes radius of the 8.6S EBP is 67 Å; and of the 4.5S EBP is 33 Å. There is no significant change in Stokes radius of both EBPs when estradiol free. It is worth emphasing (i) that lower K_d's and larger Stokes radius values are obtained for the 4.5S EBP when aggregates are present or when the preparation is stored for a long time; (ii) that the same Stokes radius value (33 Å) for the cytoplasmic 4.5S EBP was obtained when a highly purified preparation of this same protein (300 × 10^6 dpm of 40 Ci/mmole of estradiol-*6,7-t*/mg of protein) was analyzed on the same column; and (iii) that elution volume of the 4.5S EBP chromatographed on Sephadex G-200 columns equilibrated with buffer of high ionic strength (0.4 M KCl) is identical with that on standard columns; this is evidence that adsorption into Sephadex G-200 gel, which would lead to low values of Stokes radius, is quite unlikely.

Isoelectric Point. As shown in Figure 6, on electrofocusing on a pH 5–8 gradient the 8.6S EBP shows a single sharp peak at pH 6.2; free estradiol-*6,7-t* is focused at pH 8. In contrast, Figure 7 shows that the 4.5S cytoplasmic EBP has two major components with different electrical properties; a first peak is focused at pH 6.6, and a second peak at pH 6.8; a shoulder is also apparent at approximately pH 7.0.

A similar three-component pattern has been previously demonstrated also for the 4.5S EBP purified from calf uterus nuclear fraction (Puca *et al.*, 1970b). In these previous determinations the components of nuclear 4.5S EBP were found to focus at slightly lower pH with respect to the above data for cytoplasmic 4.5S EBP. However, present electrofocusing experiments carried out under exactly the same experimental conditions and with the specific aim of comparing nuclear 4.5S EBP to cytoplasmic 4.5S failed to show significant differences in their electrical patterns (Puca *et al.*, 1971).

Affinity for 17β-Estradiol. In previous research (Puca and Bresciani, 1969b; Puca *et al.*, 1970b) the association constant of nuclear EBP, both as native 5 S and as purified 4.5 S, was estimated at 1.5–2.5 × 10^9 l./mole +4°. By the same method, *i.e.*, utilizing gel filtration on Sephadex G-25, the affinity for estradiol of the 8.6S EBP and of the 4.5S cytoplasmic EBP has been now measured on an extended range of ligand concentration, including extremely low concentration not tested before.

Data are presented according to Scatchard in Figure 8. The curves for the 8.6S and 4.5S EBP are very similar. From extrapolation of the straight, intermediate segment of the curves to the coordinate axes, the association constant with estradiol can be estimated; for both 8.6S and 4.5S EBPs, K_{ass} is 3.5 ×

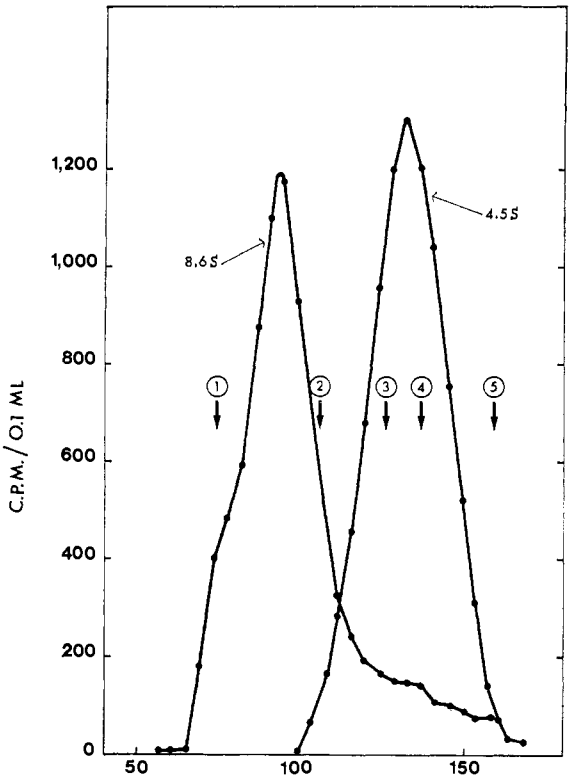

FIGURE 4: Determination of elution volume of 8.6S and 4.5S cytoplasmic EBPs on a calibrated Sephadex G-200 column. The arrows indicate the peak elution of (1): Blue Dextran, (2) human γ-globulin, (3) bovine plasma albumin, (4) ovoalbumin, and (5) myoglobin. Blue Dextran 2000 was chromatographed separately, while 5 mg of human γ-globulin together with 5 mg of ovoalbumin, and 10 mg of bovine plasma albumin together with 5 mg of myoglobin, were chromatographed in two separate runs. Buffer used for dissolving proteins was TKE (pH 7.5). The volume of samples containing the standard proteins, as well as that containing the two EBPs, was 2.5 ml. Total volume of the column was 186.5 ml. Column equilibration and elution were performed at +4° with the same buffer used for dissolving proteins. Upward constant flow was 7.5 ml/hr. Fractions of 2.12 ml were collected. The sample of 8.6 S consisted of 2.5 ml of the partially purified estrogen-free preparation of Figure 1; and the sample of 4.5 S of 10 ml of the partially purified estrogen-free preparation of Figure 2 reduced to 2.5 ml by dialysis under reduced pressure after incubation with estradiol-6,7-*t*. Both samples were incubated with estradiol-6,7-*t* prior to chromatography, as described in the legend to Figure 3. Uvicord II in expanded scale did not record any OD at 280 mμ. Radioactivity of fractions was measured by radioactivity assay 1.

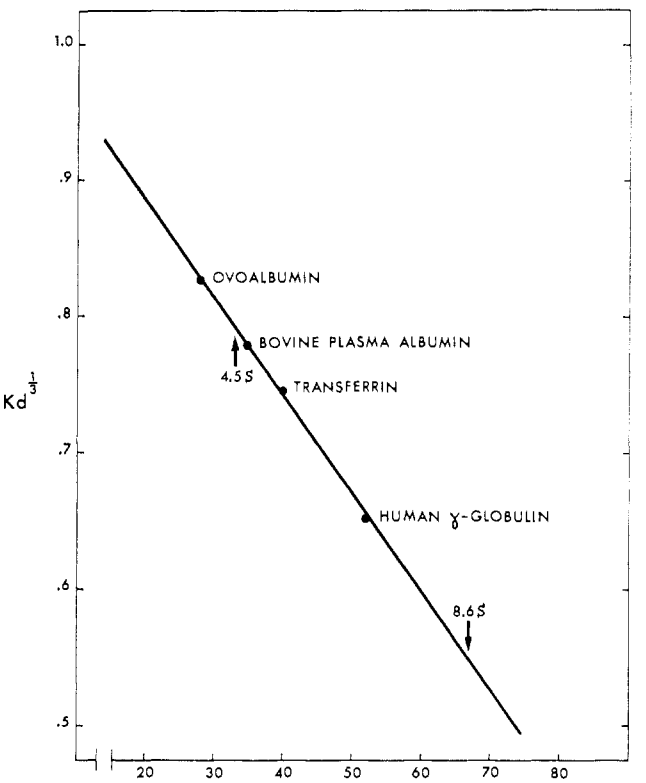

FIGURE 5: Correlation of K_d with Stokes radius. Standard protein gel filtration data, from chromatography as shown in Figure 3, and some additional data for transferrin, were plotted according to the correlation of Porath: $K_d^{1/3} = \alpha - \beta a$, where a = Stokes radius. The distribution coefficient, K_d, was calculated from the $K_d = (V_e - V_0)/(V_t - V_0)$, where V_e (elution volume) was the volume corresponding to peak elution of the protein, V_0 (void volume) was the volume corresponding to peak elution of the Blue Dextran 2000 (74 ml) and V_t (total volume) was 186.5 ml. In the calculation, V_g (volume of the gel) was neglected. Since the water regain of Sephadex G-200 is at least 20 g of H_2O/g of dry Sephadex, neglect of V_g introduces an error of less than 5%. Stokes radius values for the standard proteins were from the literature (Pagé and Godin, 1969). Stokes radius of the 8.6S and the 4.5S EBPs were derived by interpolation of respective K_d's.

10^9 l./mole at +4°. These data cannot be considered significantly different from the previous estimates reported above.

Affinity for molecules chemically or biologically related to estradiol was also investigated. With respect to estradiol, the 8.6S and 4.5S proteins have both an affinity 1.5 to 2.9 times higher for diethylstilbestrol, 10 times lower for estrone and 24 times lower for estriol. Binding of progesterone, deoxycorticosterone, testosterone, and cortisol is undetectable.

Effect of Denaturing Agents on Binding Activity. HEAT. Figure 9 shows the effect of heating, for 5 min at temperature ranging from +20 to +70° and in the presence of estradiol, on estrogen-binding activity of partially purified EBPs as well as on binding activity of BPA. Cytoplasmic 8.6S and 4.5S EBPs, as well as 5S EBP from nuclear fraction (Puca *et al.*, 1970b), were studied. No difference among the various EPBs was detected: when heated at pH 7.5, irreversible inactivation of their binding activity (tested at +4° *after* heating treatment) as a function of temperature was similar, with 50% inactivation at +52.5° and 100% inactivation at 65°; pH 7.5 was chosen because, as shown below, this hydrogen ion concentration does not affect stability of EBPs.

A significant difference in heat sensitivity is found when EBPs are heated free of their specific ligand. The protection afforded by the ligand is illustrated in Figure 9 for the cytoplasmic 8.6S EBP: when heated as estradiol free, the curve of inactivation is clearly shifted to lower temperatures; the largest differential effect is at +45°, where inactivation of EBP when ligand free is about 50% while it is virtually zero in the presence of estradiol. Figure 9 also shows that the heating increases BPA capacity for the hormone, an effect opposite to that on high-affinity binding.

pH. Both, cytoplasmic 8.6S and 4.5S EBPs were studied: no significant difference in pH sensitivity was found. Figure 10 shows binding activity of EBPs at +4° and pH 7.5, after ex-

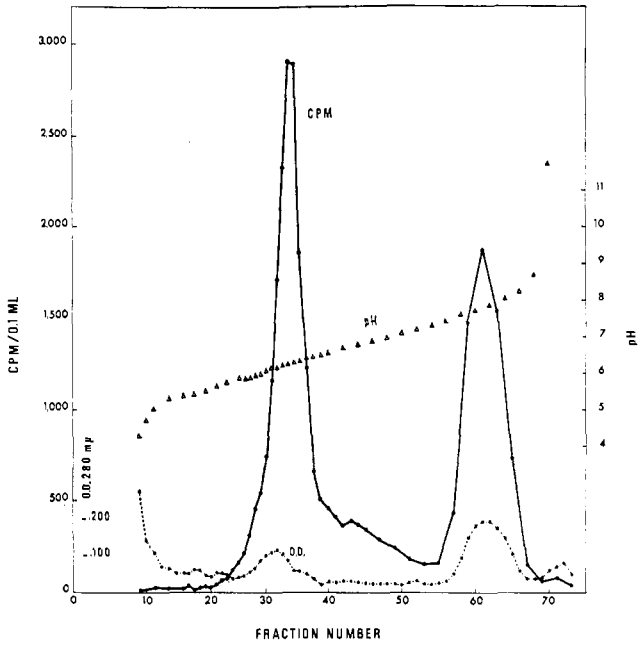

FIGURE 6: Isoelectric fractionation of the 8.6S EBP. A 0–50% sucrose gradient containing 2% (w/v) ampholine pH 5–8 was prepared in the LKB 110-ml electrofocusing column. The sample of 8.6S EBP was 2 ml of the partially purified estradiol-free preparation of Figure 1 after incubation with estradiol-6,7-*t* as described in legend to Figure 3. The gradient was allowed to focus until the milliamperage fell to a constant 1.5 mA at 800 V. Temperature of the cooling water was kept at +3° by a Colora thermostatic bath. Fractions were collected in a +4° room, at a flow rate of 1 ml/hr. The volumes of fractions were: fractions 1–15, 26 drops (32 drops ≃ 2 ml); fractions 16–36, 21 drops; fractions 37–75, 28 drops. From fractions 15 to 45 radioactivity was measured in each fraction; pH was measured in the cold room, soon after collection, by a Beckman expandomatic pH meter. Radioactivity of fractions was measured by radioactivity assay 1.

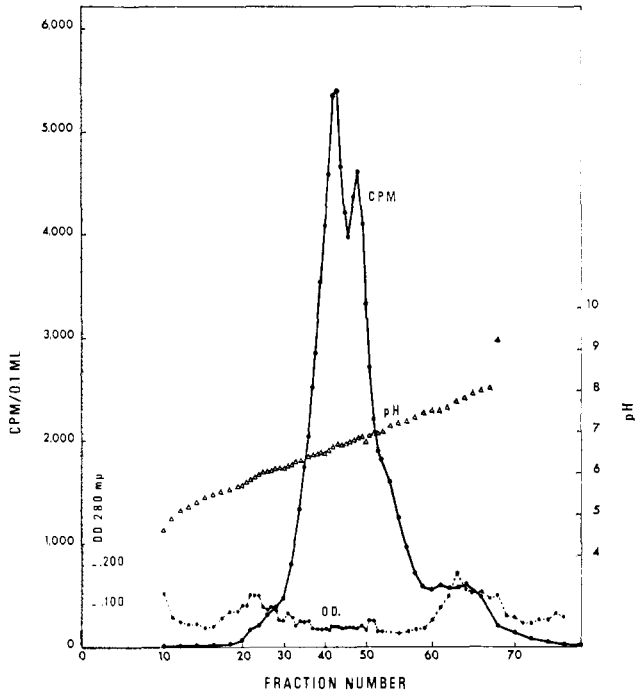

FIGURE 7: Isoelectric fractionation of the 4.5S cytoplasmic EBP. Experimental conditions were the same as those for the experiment described in Figure 5, except that 1% (w/v) ampholine was used and that at the end of the run mA = 0.8 at 800 V. The sample of 4.5 S was 15 ml of the partially purified estradiol-free preparation described in Figure 2, which was reduced to about 3 ml by dialysis under reduced pressure after incubation with estradiol-6,7-*t* as described in legend to Figure 3. Most of the unbound hormone was eliminated by the dialysis. Volumes of the fractions were: fractions 1–19, 32 drops; fractions 20–45, 16 drops; fractions 55–78, 32 drops. From fractions 35 to 56 radioactivity was measured in each fraction by radioactivity assay 1.

posure for 60 min to a range of pH values from 3 to 12 in the presence of estradiol. The curve of binding activity as a function of pH has a bell-shaped form, with a range of maximum stability between pH 7.5 and 8.5 and irreversible inactivation at lower or higher hydrogen ion concentration. EBPs appear to be particularly sensitive to acidic pH. Only a slight increase in sensitivity to extremes of pH is found when ligand-free EBPs are exposed. Also, pH sensitivity of nuclear 4.5S EBP is not significantly different from that of cytoplasmic EBPs. In contrast, binding of estradiol by BPA is increased by increasing pH values.

Urea and Guanidine·HCl. Figure 11 shows the effect of urea and guanidine·HCl, in the presence of estradiol, on binding activity of cytoplasmic 4.5S EBP. Exposure to guanidine·HCl for 1 hr at +4° inhibits binding activity as follows; the minimal active concentration is about 1.5 M and complete inhibition is obtained at 2 M. Urea is in general less effective than guanidine·HCl and shows no critical concentration; only at 6 M urea inhibition of binding activity reaches 90%. Quick dilution or dialysis of the amids to noneffective concentration (1 M or lower) does not restore binding ability except for a small fraction of lost activity (15–30%). Thus, at least under present experimental conditions, the effect of these amids is for the most part irreversible. Similar effects by guanidine·HCl and urea were obtained also with the 4.5S EBP from nuclear fraction, as well as with the 8.6S EBP from cytoplasm; furthermore, no significant difference was detected in trials with ligand-free EBPs. Aspecific binding of estradiol by BPA is affected by guanidine·HCl and urea in a fashion not too dissimilar from specific binding; in contrast to specific binding, however, specific binding is restored or even increased after diluting or dialyzing out the amids.

Some Other Characteristics. Stability at various temperatures of estradiol-free, partially purified 4.5S EBP from cytoplasm in dilute buffer solution at pH 7.5 has also been studied. At −20° there is rapid decrease of binding activity, to about 40% of original activity after 5-days storage; the rate of decrease is slower at +20° and only slight at +4°. Similar results were also obtained with the 8.6S EBP as well as the 4.5S EBP from nuclear fraction. When EBPs are stored in the presence of estradiol, there is some protection from inactivation by freezing at −20° and virtually no loss of activity for at least 1 month when stored at +4°. Attempts at fractional precipitation with organic solvents have shown that EBPs are destroyed by cold acetone, ethanol, or ether. EBP from cytoplasmic as well as nuclear fractions of calf uterus are precipitated, at pH 7.5 and +4°, by $(NH_4)_2SO_4$ concentrations between 15 and 30% saturation. It was confirmed that they are destroyed by incubation with protease, but not with DNase or RNase (Puca *et al.*, 1969).

Discussion

Purification of EBPs. The about 8S EBP present in the crude soluble cytoplasmic fraction of calf uterus homogenate (8.6 S,

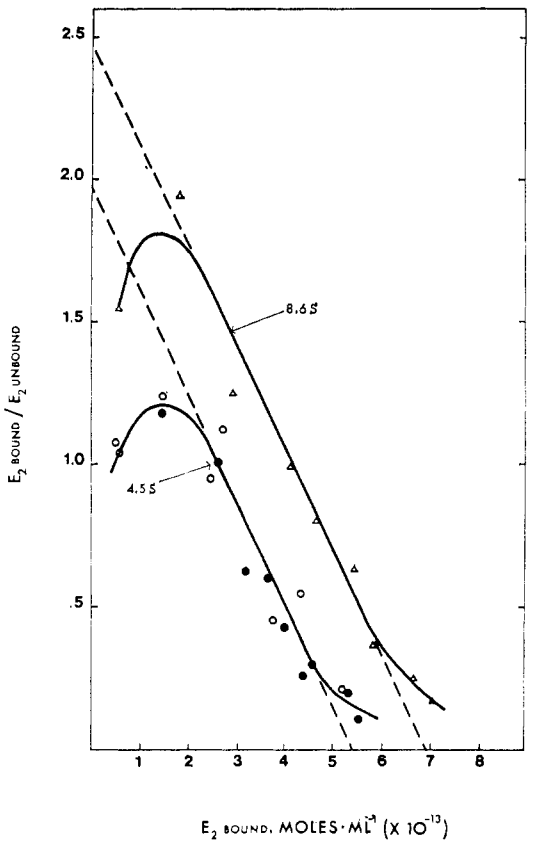

FIGURE 8: Determination of K_{ass} with 17β-estradiol of 8.6S and 4.5S cytoplasmic EBPs. Partially purified estradiol-free 8.6S preparation (0.2 ml) described in Figure 1, or 0.25 ml of the partially purified estradiol-free 4.5S preparation described in Figure 2, were brought to 0.9 ml with TKE (pH 7.5). To these 0.9-ml samples differing amounts of estradiol-*6,7-t* (40 Ci/mmole) were added in a final volume of 0.1 ml. Total radioactivity of samples was determined by counting 0.1 ml of each. After 5-hr incubation at +4°, separation of bound from free estradiol-*6,7-t* was obtained by filtration at +4° on standard Sephadex G-25 (fine) columns. Open circles represent an experiment carried out using Sephadex G-25 coarse instead of fine. Results were plotted according to Scatchard. K_{ass} were obtained by extrapolation of the straight segment of curves to *y* axis and *x* axis. Radioactivity was measured by radioactivity assay 2.

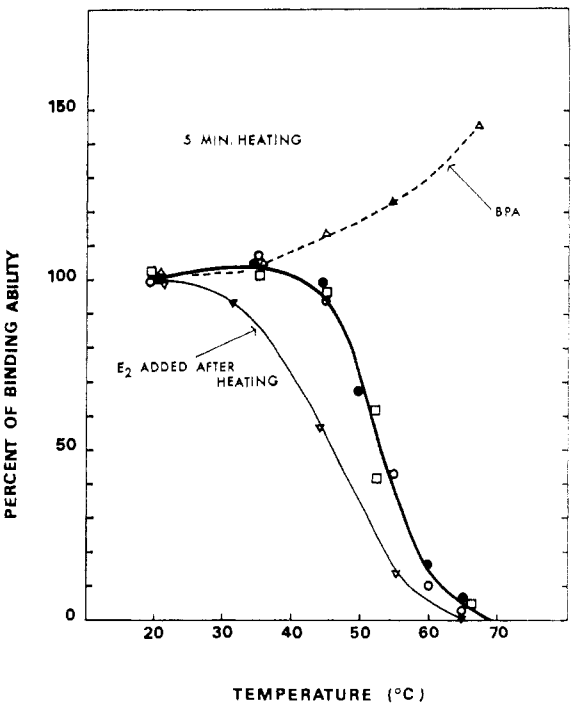

FIGURE 9: Effect of heating on 17β-estradiol-binding activity of EBPs from cytoplasm and nuclei and of bovine plasma albumin (BPA). 17β-Estradiol-*6,7-t* (8×10^{-9} M, 40 Ci/mmole) was added to 0.5-ml samples of (i) 8.6S EBP (○) prepared as described in Figure 1, (ii) 4.5S cytoplasmic EBP (●) prepared as described in Figure 2, (iii) 5S nuclear EBP (□) prepared as described elsewhere (Puca *et al.*, 1970b), and (iv) 1% solution of bovine plasma albumin (△). The samples solvent was TKE (pH 7.5). After heating for 5 min in water baths at the given temperature, the samples were quickly cooled in water at +4°, and bound hormone was separated from free hormone by standard gel filtration on Sephadex G-25 at +4°. In one experiment with 8.6S EBP, estradiol-*6,7-t* was added after heating (▽). Maximal estradiol-*6,7-t*-binding activity of preparations (=100%) was: 8.6 S, 130,000 dpm/0.125 mg of protein; 4.5 S, 105,000 dpm/0.02 mg of protein; 5 S, 45,000 dpm/1 mg of protein; BPA, 12,000 dpm/5 mg. Radioactivity was measured by radioactivity assay 2.

as shown in this paper) has resisted attempts at purification due to marked aggregation. Purification procedures employing Ca^{2+} ions and high ionic strength together apparently do circumvent aggregation but are incompatible with the 8.6S structure and the result is a smaller EBP sedimenting at about 4 S on a sucrose gradient (4.5 S as shown in this paper) (De Sombre *et al.*, 1969; Puca *et al.*, 1970a). Successful preparation of partially purified 8.6S EBP as reported in this paper was accomplished when Ca^{2+} ions were added first to the cytoplasmic soluble fraction and the increase in ionic strength followed after a 2-hr interval. The reason for this begins to be apparent. Preliminary results (Puca *et al.*, 1971) have shown that Ca^{2+} ions activate a macromolecular receptor transforming factor (RTF) which operates the transformation of the 8.6S EBP into the smaller and stable 4.5S derivative. Ca^{2+}-activated transforming activity is, however, labile and disappears rapidly. Furthermore, in the absence of sufficient ionic strength the Ca^{2+}-dependent 8.6S to 4.5S transformation does not take place effectively, while RTF rapidly undergoes the activation–inactivation cycle. Thus, the interval between addition of Ca^{2+} ions and increase of the ionic strength allows destruction of RTF before the condition required for an effective action of RTF on the 8.6S EBP is set.

Weight and Shape of EBPs. The elution position of a protein upon chromatography on a Sephadex G-200 column does not correlate with molecular weight but is instead a function of Stokes radius (Siegel and Monty, 1966). By measuring Stokes radius by gel filtration and sedimentation coefficient by sucrose gradient centrifugation, an unbiased estimate of molecular weight may be obtained also for a protein with uncommon axial ratio; furthermore, the frictional ratio of the protein may be derived (Siegel and Monty, 1966). Both, gel filtration and sucrose gradient centrifugation may be applied to protein in mixture and thus valuable information on molecular weight and shape of protein may be gained awaiting achievement of higher degree of purity.

The molecular weight (M) and frictional ratio are computed from the classical equations

$$M = 6\pi\eta Nas/(1 - \bar{v}\rho) \quad (1)$$

$$f/f_0 = a/(3\bar{v}M/4\pi N)^{1/3} \quad (2)$$

where η = viscosity of the medium, N = Avogadro's number,

TABLE I: Characteristics of Partially Purified Estrogen-Binding Proteins (EBPs) from Calf Uterus Cytoplasmic and Nuclear Fractions.

	Larger Cytoplasmic EBP	Smaller Cytoplasmic EBP and Nuclear EBP[b]
A. Physical Characteristics[a]		
1. $s_{20,w}$	8.6	4.5
2. Stokes radius (Å)	67	33
3. Molecular weight	238,000	61,000
4. f/f_0	1.65	1.25
5. Axial ratio		
Prolate	8.3	3.4
Oblate	0.09	0.31
6. IP	6.2	6.6, 6.8, (7.0)
B. Affinity for 17β-Estradiol and Other Ligands[c] (No Significant Difference among Different EBPs Is Detected)		
K_{ass} (17β-estradiol): 1.5–3.5 × 10^9 l./mole at +4°		
Relative affinity: dyethylstilbestrol > 17β-estradiol > estrone > estriol. No detectable binding of testosterone, progesterone, cortisol, and deoxycorticosterone.		
C. Chemical and Other Characteristics (No Significant Difference between Different EBPs Is Detected).		
1. Insoluble in 15–30% saturated $(NH_4)_2SO_4$ solution.		
2. Destroyed by proteases but not by DNase or RNase.		
3. Stable in dilute buffer solution pH 7.4 at +4° for at least 1 week. Rate of inactivation of solution stored at +22° is about 20% per week, and at −20° about 50% per week. In the presence of 17β-estradiol, stable at +4° for at least 1 month.		
4. Binding activity irreversibly destroyed by: (I) acidic pH, (II) +65° for 5 min, (III) iodination,[d] (IV) cold ethanol or ether, (V) 1.5–6 M urea, and (VI) 1.5–2.5 M guanidine hydrochloride.		
5. Protection from inactivation by iodine afforded by estradiol.		
6. Some protection from inactivation by heat, acidic pH, and freezing afforded by excess 17β-estradiol.		

[a] Sedimentation coefficients were obtained by sucrose gradient centrifugation according to Martin and Ames (1961) using bovine plasma albumin as reference. See text for standard error of sedimentation coefficients. Stokes radius was obtained by gel filtration according to Siegel and Monty (1966). Molecular weight, frictional ratio, and axial ratio were calculated as described in text, assuming a partial specific volume $\bar{v}$ = 0.725 cm³/g. Isoelectric points (IP) were measured by electrofocusing. [b] After partial purification the EBP from the nuclear fraction sediments at 4.5 S on a sucrose gradient. This and other characteristics on nuclear EBP are from previous research (Puca *et al.*, 1970b, 1971); molecular weight has been reassessed by the method described in this paper. Isoelectric points have also been measured anew under slightly different experimental conditions. Because no significant difference is found between partially purified EBP from nuclear fraction and the cytoplasmic EBP subunit, data are presented in a single column. [c] Affinity for 17β-estradiol was estimated by Sephadex G-25 dialysis. [d] Results of iodination experiments are from previous research (Puca and Bresciani, 1970a,b); they suggest presence of a tyrosyl and/or histidyl residue(s) at/or near the binding site.

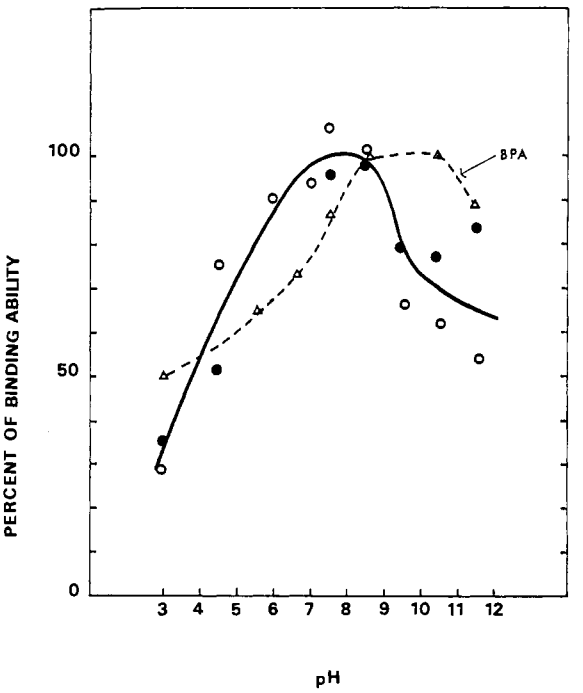

FIGURE 10: Effect of pH on estrogen-binding activity of the 8.6S and 4.5S cytoplasmic EBPs and of bovine plasma albumin (BPA). 17β-Estradiol-*6,7-t* (8 × 10^{-9} M, 40 Ci/mmole) was added to 0.5-ml samples of: (i) 8.6S EBP prepared as described in Figure 1 (○), (ii) 4.5S cytoplasmic EBP prepared as described in Figure 2 (●), and (iii) 1% solution of bovine plasma albumin (△). The sample's solvent was TKE (pH 7.5). The pH was slowly changed by addition of 1 N NaOH or 1 N HCl at +4° using a Mikro-Präzidensspritzen-Gerat (Braun). After 1-hr incubation at +4°, the pH was brought back to pH 7.5 in about 10 min and using 1 N NaOH or 1 N HCl. After an additional hour of incubation at pH 7.5 and +4°, bound hormone was separated from free by standard gel filtration at +4° on Sephadex G-25 (fine). Maximal estradiol-*6,7-t*-binding activity (=100%) of the different preparations was as reported in legend to Figure 9. Radioactivity was measured by radioactivity assay 2.

a = Stokes radius, s = sedimentation coefficient, $\bar{v}$ = partial specific volume, ρ = density of the medium. Axial ratio may then be derived from frictional ratios (Oncley, 1941).

Using the Stokes radii and sedimentation coefficients obtained as described in Results and assuming $\bar{v}$ = 0.725, the following values were calculated: for the 8.6S ESP, mol wt 238,000, f/f_0 = 1.65, axial ratio, 8 (prolate) and 0.09 (oblate); for the 4.5S EBP, mol wt 61,000, f/f_0 = 1.25, axial ratio, 3.4 (prolate) and 0.31 (oblate). In making these calculations the partial specific volume of 0.725 cm³/g was used because this value was selected by Martin and Ames (1961) as representative of most proteins in their sucrose density centrifugation studies; evidence that proteins under consideration do not contain significant lipid material are given in this paper. The above molecular parameters and other characteristics of EBPs of cytoplasmic and nuclear origin are summarized in Table I.

It is immediately apparent from data in Table I that the ratio of molecular weights of the larger 8.6S EBP and the smaller 4.5S EBP, *i.e.*, 238,000/61,000 is very close to 4. This finding suggests that the 8.6 S is a tetrad consisting of four 4.5S subunits. The tetrad hypothesis does not imply that the four subunits are identical. One may have here a situation similar to that of hemoglobin A, with four subunits all binding the ligand but belonging to two different types with

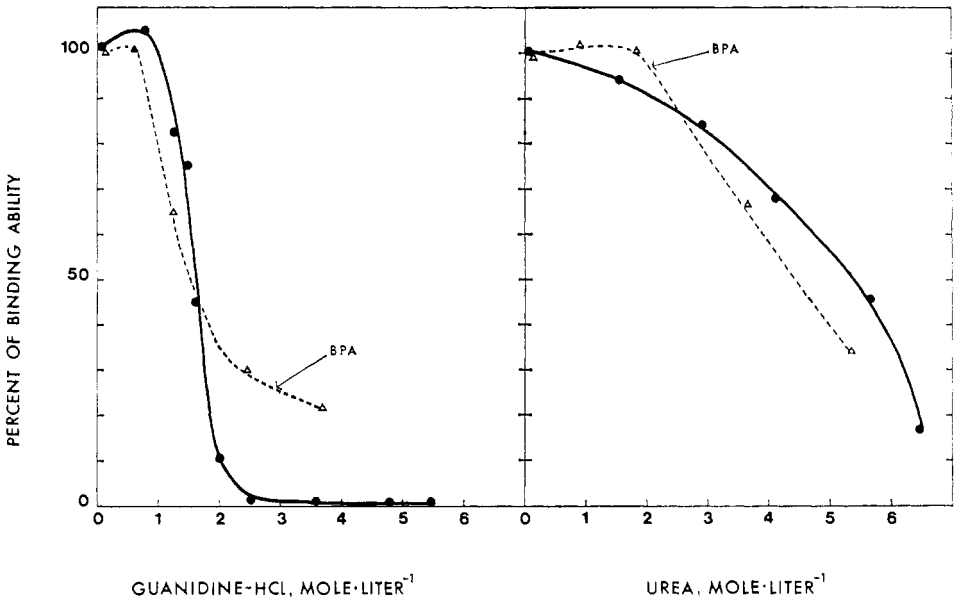

FIGURE 11: Effect of urea and guanidine·HCl on estrogen-binding activity of the 4.5S cytoplasmic EBP and of bovine plasma albumin (BPA). To 0.5-ml samples of 4.5S EBP and BPA prepared as described in legend to Figure 10, cold neutralized solutions of urea (8 M) or guanidine·HCl (7 M) in the same buffer of samples was added to reach a given molarity. The operation was carried out slowly and in an ice–water bath by means of Mikro-Präzidenssprizten-Gerat (Braun). After 1 hr at the given molar concentration, the samples were filtered at +4° through standard Sephadex (fine) G-25 columns in order to separate free from bound hormone. Binding activity of control samples in the TKE (pH 7.5) buffer (= 100%) is as reported in legend to Figure 9. Radioactivity was measured by radioactivity assay 2.

such slight differences in amino acid composition and molecular weight that they cannot be demonstrated by the methods applied here.

Another result which appears worth commenting is that while the frictional and axial ratios of the 4.5S ESP, *i.e.*, 1.25 and 3.4 (prolate), respectively, are within the range of values of most globular proteins, the same ratios for the 8.6S EBP, *i.e.*, 1.65 and 8.3 (prolate), show that this larger protein is rather asymmetric. The frictional and axial ratios of the two proteins suggest that the 8.6S EBP is a double-row, tetradic array of four 4.5S EBP subunits. One may easily compute axial ratios of single-row, linear arrays of 4.5S subunits to find that such configurations are at high discrepancy with the experimental data.

It is of interest that several similarities appear to exist between the estrogen-binding system and the progesterone-binding system of chick oviduct. The progesterone system is the only other steroid-binding system in an advanced stage of characterization (Sherman *et al.*, 1970) and also for this system the data indicate a monomer–tetramer conformation, a high f/f_0 prolate ellipsoid shape, similarities of cytoplasmic and nuclear binding proteins, etc. These analogies suggest the hypothesis that a fundamentally similar physicochemical receptor pattern is at the basic of the phenomenon of hormonal steroid binding by target tissues.

Heterogeneity of the 4.5S Subunit. Methods applied here cannot demonstrate slight differences in molecular size and shape of proteins. On electrofocusing, however, even small differences in electrical properties can be detected. While the 8.6S EBP shows a single homogeneous peak on electrofocusing (IP = 6.2), the 4.5S subunit consistently show two, and possibly three, components which focus at different pH (IP = 6.6, 6.8, and 7.0). One may think of explanations other than true protein heterogeneity for the multiple pattern of the 4.5S EBP at electrofocusing, including modification of specific protein molecules during isolation or nonequivalent binding of small molecules to the protein. However, contrary to these explanations one may cite (i) the consistent reproducibility of the pattern, (ii) no evidence of formation of other components when single peaks are refocused, and (iii) the lack of heterogeneity of the parent 8.6S protein under the same experimental conditions.

Affinity of EBPs for Ligands. Affinity of the 8.6S EBP for estradiol does not show any significant difference with previous (Puca and Bresciani, 1969a; Puca *et al.*, 1970b) at present estimates of 4.5S EBP affinity for the same ligand. This is in agreement with the 4.5 S being a subunit of 8.6 S. One should note, however, that the method applied here, *i.e.*, based on gel filtration, possibly furnishes a lower estimate of association constant. Thus, affinity for estradiol is probably in excess of present estimates which put K_{ass} at 1.5–3.5 $\times$ 10^9 l./mole at + 4°.

This high value of affinity, far above those characteristics of enzyme–substrate interaction, excludes a sufficient molecular turnover consistent with a chemical transformation mechanism. Rather an effect by stable occupation of the binding site by the ligand is more likely; such an occupation mechanism includes induction of physical conformation changes at molecular level (Bush, 1964). By present methods, no differences were detected in Stokes radius of EBPs when estrogen free or interacting with 17β-estradiol. However, this result does not exclude fine changes of physical conformation, and an allosteric effect is suggested by binding experiments at ligand concentration lower than tested before. Although at both the high and low ends of Scatchard plots the error increases drastically (Deranleau, 1969), the definite upward convexity of such plots of estrogen-binding data in the lower range of bound ligand concentration is indeed suggestive of a cooperative effect, i.e., the binding of a ligand molecule enhances binding of further ligand molecules by inducing a change of physical conformation of the macromolecule (Monod *et al.*, 1965). Of course, the conformational change

of the macromolecule revealed by the cooperative effect could well have also other consequence which could bear to a direct role of EBPs in the mechanism of estrogen action.

Denaturation and EBPs. Heat and pH allow discrimination between specific binding of estrogens by EBPs and aspecific binding of the same ligand by other proteins, as exemplified by bovine plasma albumin. Figure 9 shows that 5-min heating at temperatures ranging from +45 to +65° (pH 7.5) increasingly and irreversibly decreases specific binding, with complete inactivation at +65°; in contrast, binding by albumin increases up to about 150% at +65°. Also optimum pH is 7.5 while albumin binding increases with increasing pH. These effects may be reasonably interpreted as a consequence of loosening of the protein structure, with resulting loss of conformation of specific binding sites, on the one hand, and increase of protein surface and thus of aspecific binding, on the other hand.

In contrast to the differential effects of heat and pH, specific and aspecific binding are affected not too dissimilarly by two other denaturation agents, *i.e.*, urea and guanidine·HCl. The reason why these amids act equally well on both types of binding is not clear but it probably resides in an ability of these agents to also interfere *directly* in the interaction between protein and estradiol. This is suggested by the finding that while both types of binding are destroyed when these agents are present, after their dilution or removal by dialysis only aspecific binding is restored, or even increased. This direct action of the amides on estradiol–protein interactions may depend upon the ability of these agents to form hydrogen bonds with proteins, and thus interfere with binding of 17β-estradiol which is likely to involve hydrogen bonding (Engel *et al.*, 1964).

Comparison to Other Estrogen-Binding Entities. Mild tryptic digestion of the 8.6S EBP produces estrogen-binding fragments sedimenting at about 4 S on a sucrose gradient (Erdos, 1970). Preliminary experiments with these tryptic fragments have shown that although some molecular similarity exists, there is a drastic difference at electrofocusing between the estrogen-binding tryptic fragments and the smaller cytoplasmic EBP described in this paper: the tryptic fragments have a definitely more acidic isoelectric point. Also, the smaller cytoplasmic EBP here is definitely different from the approximate 5S EBP which can be reversibly obtained from the larger EBP by increase of the ionic strength of the medium only (Erdos, 1968, 1970). On the contrary, as shown in Table I, the smaller cytoplasmic EBP described here and the EBP extracted from calf uterus nuclear fraction, which was partially purified and characterized in previous research (Puca *et al.*, 1970b) do not show significant difference in any of the molecular parameters and other properties investigated by the methods applied in this paper. Their postulated identity is in agreement with the knowledge that they are both derived from the same cytoplasmic precursor. Based exclusively on gel filtration data, a slightly higher estimate of molecular weight for the nuclear receptor was previously given (Puca *et al.*, 1970b); the estimate by the present method is lower and coincides with that of the 4.5S cytoplasmic EBP.

The relevance of the transformation of the larger cytoplasmic EBP into subunits to the phenomenon of estradiol transfer from cytoplasm to the nucleus is unknown. However, the preliminary findings (Puca *et al.*, 1971) showing that in the uterus there exists a macromolecular, Ca^{2+}-activated cytoplasmic transforming factor which is responsible for the change of the 8.6S EBP into subunits, furnish a line of research which is presently being followed and it is hoped may contribute to elucidation to this problem.

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Characterization of Steroid-Binding Sites by Affinity Labeling. Further Studies of the Interaction between 4-Mercuri-17β-estradiol and Specific Estrogen-Binding Proteins in the Rat Uterus*

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ABSTRACT: The nature and extent of binding of 4-mercuri-17β-estradiol to a specific 8S rat uterine cytosol fraction and the subsequent transfer of this complex into the particulate fraction were investigated. When different amounts of the mercury steroid are incubated with cytosol samples, a constant level of steroid becomes bound in such a manner that it cannot be extracted with organic solvents. Preincubation with 17β-estradiol decreases the level of nonextractable steroid by an amount approximating that which sediments on sucrose density gradients as a complex with 8S protein. Incubation of cytosol with 4-mercuri-17β-estradiol eliminates subsequent binding of 17β-estradiol in the 8S region, and the sucrose gradient pattern resembles that observed when 17β-estradiol is incubated with preheated cytosol. Transfer of the mercury steroid from its 8S-complexed cytosol form into the particulate fraction does not occur under conditions where specific nuclear uptake of 17β-estradiol is demonstrable. The amount of 17β-estradiol which is specifically bound to 4–5S nuclear components is decreased proportionately when subsaturation levels of 4-mercuri-17β-estradiol are incubated with cytosol prior to the introduction of the 17β-estradiol. The results suggest that the previously observed estrogenic activity of the mercury derivative is not the result of intranuclear steroid–protein interaction, in contrast to the generally proposed mechanism of action of 17β-estradiol.

Uptake of estradiol by rat uterine tissue *in vitro* is accompanied by interaction of the steroid with a specific cytosol receptor protein having a sedimentation coefficient of 8 S (Toft and Gorski, 1966; Jensen *et al.*, 1968; Rochefort and Baulieu, 1969). Transfer of estradiol into the particulate fraction is occasioned by appearance of specific binding to a 5S component (Shyamala and Gorski, 1969; Puca and Bresciani, 1968; Jensen *et al.*, 1969) and to a 4S component (Jensen *et al.*, 1969; Musliner *et al.*, 1970). Although nuclear receptor complex formation is dependent on integrity of the cytosol receptor system (Jensen *et al.*, 1968; Brecher *et al.*, 1967), it has not been demonstrated clearly whether or not dissociation of the cytosol steroid–protein complex precedes nuclear uptake of estradiol.

The synthesis and affinity-labeling capability of 4-mercuri-17β-estradiol (4ME)[1] for estrogen-sensitive enzymes were described in the first paper of this series (Chin and Warren, 1968). Subsequent work established the inherent estrogenic activity of 4ME and its ability to interact with the uterine cytosol 8S estrogen receptor protein in the rat in a manner consistent with an affinity-labeling mechanism (Muldoon and Warren, 1969). The studies presented herein show that 4ME, in contrast to estradiol, interacts with the 8S receptor in an irreversible manner, and that the stable complex thus formed is not transferred to the particulate fraction. The fact that 4ME is estrogenic suggests that the primary intracellular events which precede manifestation of biological activity are different from those observed with the native hormone.

Materials and Methods

Female Holtzman rats, 21–23 days of age, were sacrificed by cervical dislocation. Uterine horns were excised, trimmed, rinsed in cold hypotonic Tris–EDTA buffer (0.01 M Tris and 0.0015 M Na_2EDTA, pH 7.4), and homogenized in the same buffer at a concentration of 3 uteri/ml. The homogenate was centrifuged for 90 min at 105,000*g* in a Spinco Model L2-65 ultracentrifuge. The resultant supernatant is referred to as the

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[1] The abbreviation 4ME is used to designate compounds in solution arising from added 4-acetatomercuri-17β-estradiol, with the understanding that the mercury steroid may exist in the free form or in combination with solution anions other than acetate.