Estrogen-Binding Proteins of Calf Uterus. Interrelationship between Various Forms and Identification of a Receptor-Transforming Factor†

Giovanni Alfredo Puca,* Ernesto Nola, Vincenzo Sica, and Francesco Bresciani

ABSTRACT: At ionic strength, in the physiological range or higher, the 8.6S, 240,000 molecular weight, estrogen-binding protein (EB-protein) found in low-salt calf uterus cytosol changes into an EB-protein with the following characteristics: $s_{20,w}$, 5.3 S; Stokes radius, 54 Å; molecular weight, 118,000 (for $\bar{v} = 0.725$); f/f_0, 1.67. Molecular and frictional data suggest that this reversible transition consists of longitudinal dissociation of the 8.6S form into two halves. Smaller, low-salt-stable, 4.5S, 61,000 molecular weight, EB-protein derives from the 5.3S state by the action of a Ca^{2+}-activated, separate macromolecular factor of cytoplasm (receptor-transforming factor, RT-factor). The data suggest transversal splitting of the 5.3S EB-protein into about equimolecular 4.5S proteins. The RT-factor is at least 100,000 in weight, is destroyed by proteases, but not by nucleases, and is separated from EB-proteins by fractional precipitation by $(NH_4)_2SO_4$. Neither diisopropyl fluorophosphate nor phenylmethylsulfonyl fluoride inhibits RT-factor activity. Furthermore, the 4.5S EB-protein produced by the RT-factor is different from the estrogen-binding fragments of similar size produced by mild tryptic hydrolysis of 8.6S protein. The RT-factor, although enzymatic in nature, is thus not a protease of the trypsin group. Addition of Ca^{2+} to uterine cytosol starts an activation–inactivation cycle of RT-factor which, at maximal rate (4 mM Ca^{2+}), causes disappearance of receptor-transforming activity within 2 hr. We favor the hypothesis that formation of the 4.5S EB-protein by RT-factor is the step by which the confinement to cytoplasm of the larger 5.3S–8.6S native EB-protein system is overcome, thus allowing nuclear penetration of estrogen–protein complex.

After the discovery that estrogens are specifically taken up and retained by target tissues (Jensen and Jacobson, 1962), research aimed at identifying the physical basis of this property has brought to identification not one but several estrogen-binding proteins (EB-proteins)[1] endowed with the ability to complex estrogenic ligands with high specificity and affinity (Talwar *et al.*, 1964; Toft and Gorski, 1966; Toft *et al.*, 1967; Jensen *et al.*, 1967). Purification and characterization of EB-proteins of calf uterus and study of their interrelationship are present research aims of this laboratory; it is hoped that such studies will help elucidate the relevance of this protein system to the mechanism of estrogen action.

Previous papers (Puca *et al.*, 1971a,b) described partial purification and characterization of two cytoplasmic EB-proteins: a larger one, sedimenting at about 8.6 S and with a molecular weight of about 240,000, and a smaller one, sedimenting at 4.5 S and with a molecular weight of about 60,000. These proteins were previously identified as 8 S and 4 S, respectively (Jensen *et al.*, 1969). It was also shown (Puca and Bresciani, 1969; Puca *et al.*, 1970) that the estrogen–protein complex extracted from the nuclear fraction of calf uterus slices incubated with 17β-estradiol, which sediments at about 5–6 S in the stage of crude nuclear extract, after about 2000-fold purification, sediments instead at 4.5 S; this EB-protein from the nucleus is indistinguishable from cytoplasmic 4.5 S not only in sedimentation rate but also in Stokes radius, molecular weight, isoelectric pattern (electrofocusing), and all other properties which were investigated by methods suited to partially purified preparations (Puca *et al.*, 1971a).

The 8.6S and the 4.5S proteins are related. Indeed, when ionic strength and a Ca^{2+} concentration of 8.6 S containing uterine cytosol reach a sufficient level, 4.5 S is formed while 8.6 S disappears proportionally (De Sombre *et al.*, 1969). Preliminary evidence suggested that a cytoplasmic factor, the receptor-transforming factor (RT-factor), is involved in the formation of the 4.5S EB-protein (Puca *et al.*, 1971a). Continuing this line of research, we are now able to further describe the 8.6 S to 4.5 S conversion in uterine cytoplasm. This paper deals with the physical changes in the 8.6S protein brought about by simple increases in the ionic strength of the medium to physiological (or higher) levels; the different EB-protein form which results from the above increase is obtained in a partially purified state and characterized by its molecular parameters. We also wish to report that Ca^{2+} activates the RT-factor and to describe further the molecular and functional properties of this factor. The RT-factor is required for final formation of the 4.5S EB-protein, and it appears as a reasonable possibility that it is the 4.5S protein which subsequently penetrates the nucleus as a complex with the steroid ligand. A preliminary report of some of the findings in this paper has been made (Puca *et al.*, 1971a).

Materials and Methods

Materials. All reagents were of analytical grade. 17β-Estradiol-*6,7-t* (40 Ci/mmole specific activity) (New England Nuclear) was 97% pure at the time of the experiments. Tris was purchased from Sigma Chemical; Sephadex G-25 and G-200 from Pharmacia; sucrose (ACS) from C. Erba. Standard macromolecules for Stokes radius and sedimentation rate

† From Cattedra di Istituzioni di Patologia Generale, Università di Napoli, and Istituto Regina Elena per lo Studio e la Cura dei Tumori, Rome, Italy. *Received May 1, 1972.* Research supported by the Consiglio Nazionale delle Ricerche, Rome.

[1] Abbreviations used are: EB-protein(s), estrogen-binding protein(s); RT-factor, receptor-transforming factor; cytosol, cytoplasmic soluble fraction; TE, 10^{-2} M Tris-HCl–1.5 × 10^{-3} M EDTA; TKE-I, 10^{-2} M Tris-HCl–10^{-2} M KCl–10^{-3} M EDTA; TKE-II, 10^{-2} M Tris-HCl–0.4 M KCl–10^{-3} M EDTA; TKC, 10^{-2} M Tris-HCl–0.4 M KCl–10^{-3} M $CaCl_2$.

determination were obtained: IgG (human plasma), albumin (bovine plasma), albumin (*ex ovo*), myoglobin (horse muscle), from Serva; transferrin (human plasma) and crystalline trypsin from Boehringer; Blue Dextran 2000 from Pharmacia.

Buffers. The following solutions were used: TE, pH 7.5; TKE-I, pH 7.5; TKE-II, pH 7.5; TKC, pH 7.5.

Sucrose gradient centrifugation (Martin and Ames, 1961) was carried out in the SB-405 rotor of a I.E.C. B-60 ultracentrifuge on 5–20% sucrose gradients. Preliminary tests of linearity of gradients were carried out as previously described (Puca *et al.*, 1971b). Sucrose gradients were prepared in either low-salt TKE-I or high-salt TKE-II buffers. Bovine plasma albumin (4.41 S) was the standard reference when low-salt sucrose gradients were used. However, the sedimentation rate of albumin increases in high-salt gradients and, therefore, the reference was 4.5S EB-protein when high-salt gradients were used. The sedimentation rate of the 4.5 S is not modified by the high-salt TKE-II sucrose solution.

Collection of gradients and measurement of radioactivity in fractions and tube bottom were carried out as described earlier (Puca *et al.*, 1971b).

Gel Filtration. A standard Pharmacia K25/45 column packed with Sephadex G-200 and fitted with upward flow-adaptor was used for Stokes radius determination. Column equilibration and elution were performed at +4° using TKE-II, pH 7.5. Total volume of the column was 214 ml; the void volume was measured by Blue Dextran 2000. The volume of applied samples was always 2.5 ml. All other operations were carried out as described earlier (Puca *et al.*, 1971b).

Electrofocusing. The LKB 110-ml standard electrofocusing column equipped with double-cooling jackets was used. All operations were carried out as described in a previous paper (Puca *et al.*, 1971b).

Radioactivity Assay. Toluene–phosphor solution (10 ml) 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 was added to counting vials containing not more than 0.2 ml of aqueous sample. Radioactivity was measured in a Mark 1 liquid scintillation spectrometer with a 35% efficiency.

Protein Assay. Protein was measured by a microbiuret method (Zamenhof and Chargaff, 1957). Routinely, chromatographic effluents were examined spectrophotometrically for protein, either automatically by an Uvicord II (LKB), or manually by a Zeiss spectrophotometer.

Protein Purification Procedures. Partial purification of 8.6S and 4.5S EB-protein of calf uterus was carried out essentially as described previously (Puca *et al.*, 1971b). However, some modifications have since been introduced which definitely improve preparations; the modified procedures will be briefly described.

PREPARATION OF CYTOSOL. Unless specified otherwise all operations were carried out at +4°, either in a cold room or in refrigerated centrifuges. 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 weighing more than 30 g were discarded. The uteri were stripped of gross connective tissue, minced in a meat grinder, and homogenized in 4 volumes of TE buffer (pH 7.5) by means of an Ultraturrax homogenizer (Janke e Kunkel, Model TP 18/2) in six runs of 15 sec each, with 60-sec intervals. The homogenate was centrifuged for 1 hr at 60,000 rpm in an I.E.C. B-60 ultracentrifuge using an A-321 rotor (300,000*g*). To the decanted supernatant (cytosol) containing an average of 5–6 mg of protein per ml, dithioerythritol was added up to 1 mM. This thiol compound was found to be very effective in reducing the spontaneous formation of aspecific large aggregates, which include EB-proteins and occur with aging of the preparations; it does not interfere with binding of 17β-estradiol by EB-proteins, nor does it affect their molecular characteristics. When 17β-estradiol-*6,7-t* was also added to cytosol, to a final 10^{-8} M concentration, the preparation was referred to as "labeled uterine cytosol."

8.6S EB-PROTEIN. To the labeled uterine cytosol, KCl was added up to 0.4 M. After about 30 min, 0.25 ml of saturated and neutralized $(NH_4)_2SO_4$ solution per ml of cytosol was added at one time. After 10 min, the mixture was centrifuged for 10 min at 10,000 rpm in an I.E.C. B-20 centrifuge using rotor no. 870 (19,000*g*). The sediment was resuspended in TKE-II buffer (pH 7.5) containing 1 mM dithioerythritol added just before use; at this stage, the final volume was about 0.1 of the original cytosol. Undissolved material was sedimented by centrifugation for 60 min at 60,000 rpm in an I.E.C. B-60 ultracentrifuge using the A-321 rotor (300,000*g*). At this stage, the purification factor relative to original cytosol was 20- to 25-fold; this degree of purification was sufficient for the purposes of this research: further purification, up to about 500-fold or more, could be accomplished as described elsewhere (Puca *et al.*, 1971b).

4.5S EB-PROTEIN. To the labeled uterine cytosol, KCl up to 0.4 M and $CaCl_2$ up to 4 mM were added; after 60 min, 0.113 g/ml of finely powdered $(NH_4)_2SO_4$ was added slowly (20% saturation). After 60 min, the mixture was centrifuged for 10 min at 10,000 rpm in an I.E.C. B-20 centrifuge using rotor no. 870 (19,000*g*). The sediment was resuspended in TKC buffer (pH 7.5), and undissolved material was removed by centrifugation for 60 min at 60,000 rpm in an I.E.C. B-60 ultracentrifuge using the A-321 rotor (300,000*g*). The resulting clear supernatant was applied to a Sephadex G-200 column of an appropriate size (upward flow) equilibrated with TKE-I buffer (pH 7.5); the column was equipped for continuous monitoring of effluent optical density (OD) at 280 mμ (LKB Uvicord II) and with a fraction collector with drop counter (LKB Ultrorac); effluent was collected in 5-ml fractions, and radioactivity was measured by the radioactivity assay method. As shown elsewhere (Puca *et al.*, 1971b) and confirmed here, the large Sephadex G-200 included peak consists of 4.5S EB-protein. Recovery is about 30% of total binding activity of the original cytosol. The purification factor of this preparation is about 1500-fold. The 4.5S EB-protein preparation was concentrated to desired volume by dialysis under reduced pressure.

Trypsin-EB-Protein. Erdos *et al.* (1971) originally produced this artificial protein by incubation of uterine cytosol with trypsin. Trypsin-EB-protein has now been obtained in a partially purified state by using as substrate the 8.6S EB-protein purified as described above. Limited tryptic hydrolysis of 8.6 S was obtained by a 20-min incubation at +4°, with 5–10 μg/ml of trypsin. After incubation, $(NH_4)_2SO_4$ was added up to 20% saturation. The remaining procedure, including the Sephadex G-200 step, is as described above for the 4.5S protein.

Results

Effect of Increasing Salt Concentration on 8.6S EB-Protein. TRANSITION TO A REVERSIBLE 5.3S STATE. Since the initial investigations of Talwar *et al.* (1964) and Toft and Gorski (1966) on macromolecular binding of estrogens, uterine

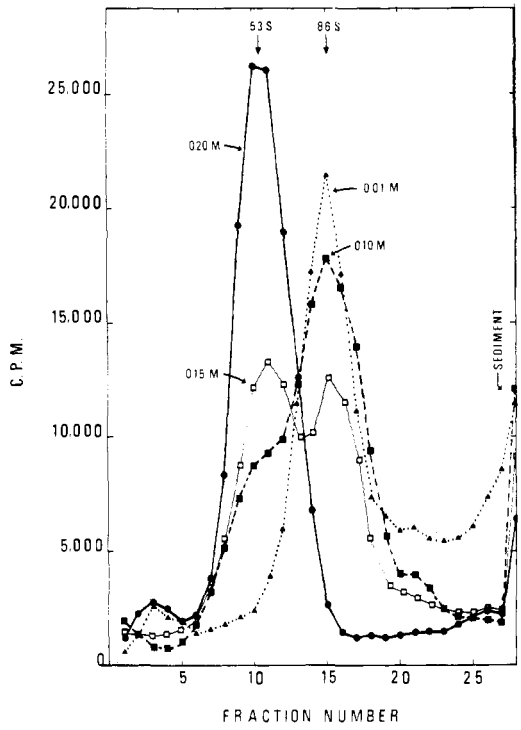

FIGURE 1: Effect of increasing KCl molarity of sucrose gradients on sedimentation rate of partially purified 8.6S (in low salt) EB-protein. EB-protein applied to the 5–20% sucrose gradients was partially purified as described in Methods. The buffer used to dilute sucrose was basal TE (pH 7.5) to which varying amounts of KCl were added. The volume of applied protein samples was 0.2 ml. Partially purified EB-protein from low-salt cytosol sediments at 8.6S at 0.01 M KCl (▲····▲). Increasing KCl molarity of sucrose results in a shift of sedimentation rate to 5.3 S, with complete disappearance of the 8.6S peak at 0.2 M KCl (●——●). Varying amounts of both estrogen-binding forms exist at 0.1 M KCl (■– – –■) and 0.15 M KCl (□——□). Sedimentation rate of partially purified 4.5S EB-protein (Puca *et al.*, 1971), contrary to that of bovine plasma albumin, does not change with changing KCl concentration and was thus used as reference. Centrifugation conditions were 60,000 rpm at +2° for 11 hr in the SB-405 rotor of the I.E.C. B-60 ultracentrifuge.

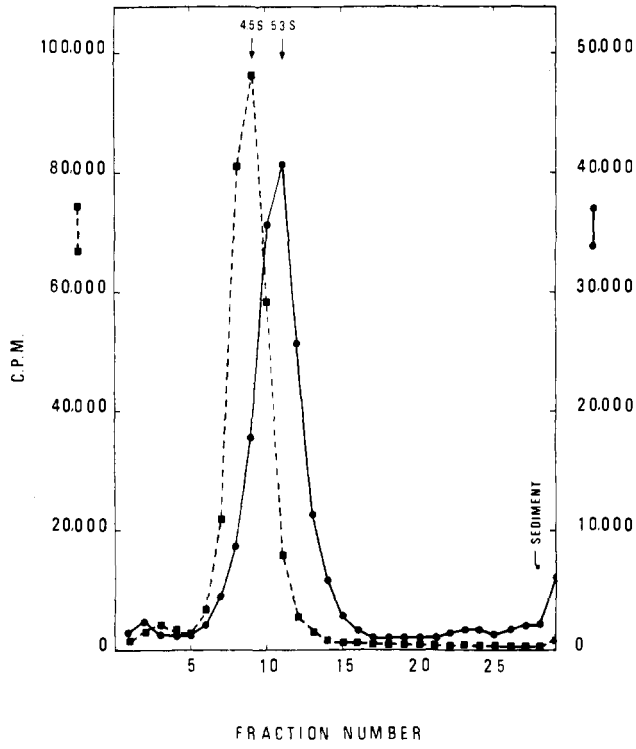

FIGURE 2: Sedimentation patterns on high-salt (0.4 M KCl) sucrose gradient of partially purified 4.5S and 5.3S (8.6S in low salt) EB-proteins. Typical runs of 4.5S and 5.3S EB-proteins. The 4.5S and 5.3S values are, however, the average of 16 and 6 separate runs, respectively. The standard errors were computed to be 0.01 for 4.5S and 0.1 for 5.3S. EB-proteins applied to the 5–20% sucrose gradient were partially purified as described in Methods. The volume of applied samples was 0.2 ml. Sucrose was dissolved with TKE-II buffer (0.4 M in KCl). Sedimentation rate of 4.5S EB-protein, contrary to that of bovine plasma albumin, does not change in high salt and is thus used as reference. Centrifugation conditions were the same as specified in the legend to Figure 1: (●——●) 5.3S EB-protein; (■– – –■) 4.5S EB-protein.

cytosol has been customarily prepared using low-salt buffers, *i.e.*, with ionic molarity between 10^{-3} and 10^{-2}. Thus, soluble cytoplasmic proteins in cytosol, including those binding estrogens, are in a hypoionic environment with respect to cytoplasm. Several researchers (Erdos, 1968; Erdos *et al.*, 1971; Korenman and Rao, 1968; Alberga *et al.*, 1971) have pointed out that when KCl concentration of low-salt uterine cytosol is increased (to 0.4–1 M KCl), the sedimentation rate of EB-protein on sucrose gradient is drastically decreased, from about 8 S in low-salt media to about 5 S at high-salt concentrations. This approximately 5S form is known only by its approximate sedimentation constant; furthermore, its relationship with the 4.5S EB-protein, previously purified and characterized (Puca *et al.*, 1971b), is unclear.

Taking advantage of available partially purified 8S EB-protein of calf uterus cytosol (actually 8.6 S as shown elsewhere, Puca *et al.*, 1971b), we tested the effect of increasing KCl or NaCl concentrations of the medium under less crude conditions. As shown by gradients in Figure 1, at KCl or NaCl molarity of 0.2 or higher, the 8.6S peak disappears and a peak sedimenting at 5.3 S appears. At 0.15 M salt the transformation is still incomplete. When KCl or NaCl molarity is decreased by dilution or dialysis back to 0.05 or less, 8.6 S reforms. In addition to its slightly higher sedimentation rate, this reversibility to original 8.6 S is a distinctive feature of the 5.3S state with respect to the previously purified 4.5S EB-protein, which is stable in low-salt media.

Molecular Parameters of 5.3S EB-Protein. In a series of six independent runs in high-salt (TKE-II) sucrose gradients, the sedimentation constant was assessed at 5.3 ± 0.1 S (average ± standard error of mean). The 4.5S EB-protein, purified as previously described (Puca *et al.*, 1971b), was used as standard reference. Albumin (bovine) is not an accurate reference when high-salt media are used, due to increased sedimentation rate with respect to low-salt solutions. Typical sedimentation patterns of 5.3S and 4.5S proteins are compared in Figure 2.

Stokes radius of 5.3S EB-protein was measured by chromatography on a calibrated Sephadex G-200 column. The resulting chromatographic pattern, together with the pattern of partially purified 4.5 S which was run later on the same column for comparison, is shown in Figure 3. In addition to the "included" peaks due to 5.3S and 4.5S protein one can see small "excluded" peaks; these latter peaks consist of exceedingly large protein aggregates, as shown by fast sedimentation to the bottom of a 5–20% sucrose gradient.

It seems worth emphasizing the large difference in elution volumes of the 5.3S and 4.5S proteins despite their comparatively small difference in sedimentation rate; this is, of course,

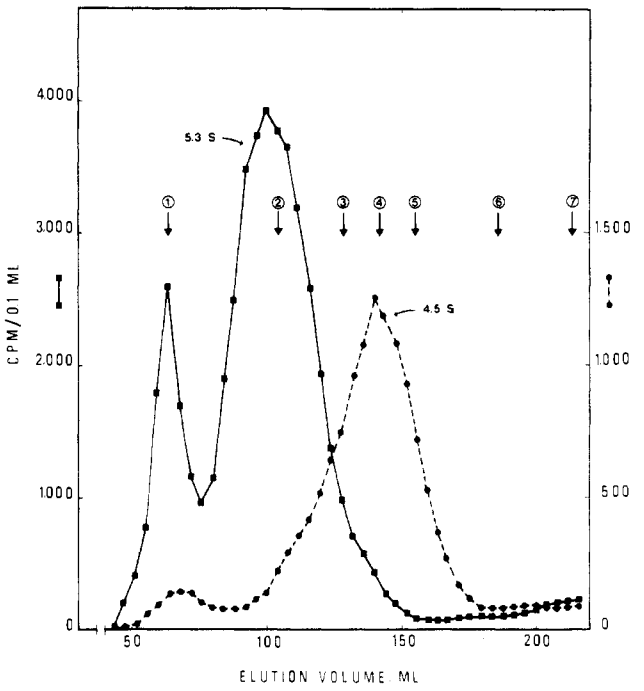

FIGURE 3: Elution from the same calibrated Sephadex G-200 column equilibrated with high-salt (0.4 M KCl) buffer of partially purified 4.5S and 5.3S (8.6S in low salt) EB-proteins. EB-proteins applied to the column were partially purified as described in Methods and derive from the same preparation used for sucrose gradient analysis in Figure 2. The buffer used was TKE-II (pH 7.5) (0.4 M in KCl). Proteins were applied in a standard 2.5 ml of TKE-II. Upward constant flow by a peristaltic pump was set at 12 ml/hr; 2-ml fractions were collected by a fraction collector equipped with drop counter, and radioactivity was measured in every other fraction: (■——■) 5.3S EB-protein; (●- - -●) 4.5S EB-protein. The arrows indicate peak elution of (1) Blue Dextran 2000, (2) human IgG, (3) transferrin, (4) bovine plasma albumin, (5) ovoalbumin, (6) myoglobin. Arrow 7 points out the total volume of the column (214 ml).

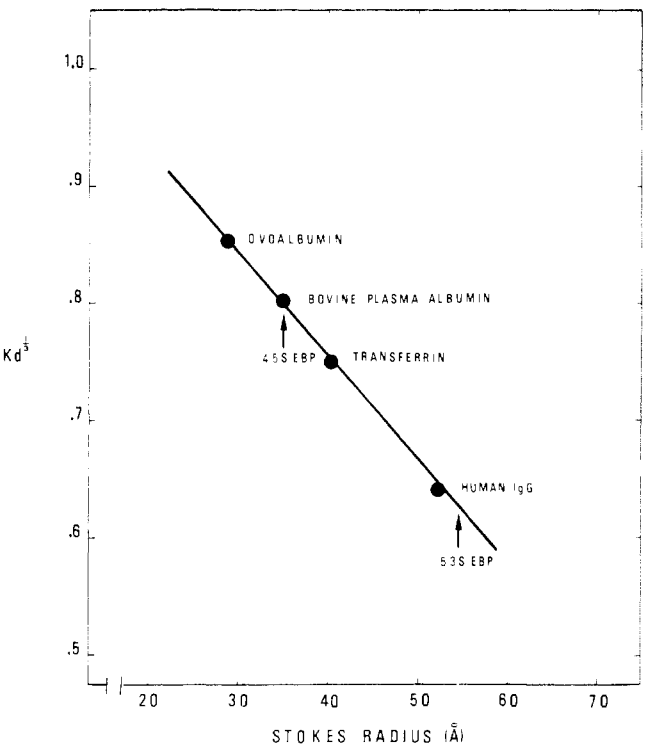

FIGURE 4: Correlation of K_d with Stokes radius. Standard protein gel filtration data from chromatography as shown in Figure 3 were plotted according to the correlation of Porath: $K_d^{1/3} = \alpha - \beta a$, where a = Stokes radius. The distribution coefficient, K_d, was calculated from $K_d = (V_e - V_0)/(V_t - V_0)$, 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 Blue Dextran 2000 (65 ml) and V_t (total volume) was 214 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 (Page and Godin, 1969). Stokes radius of 5.3S and 4.5S EB-protein were derived by interpolation of respective K_d's.

indication of a large discrepancy in their molecular symmetry and points out that direct derivation of the molecular weights of these proteins from a plot of log molecular weights against elution volumes (or K_d) would produce highly biased relative estimates. It is correct, however, to derive Stokes radii from Sephadex elution data (Siegel and Monty, 1966). In Figure 4, the Stokes radii of reference proteins used for calibration of the column were plotted *vs.* relative K_d according to Porath ($K_d^{1/3} = \alpha - \beta a$) and, as expected, a straight line was obtained. By interpolation of K_d's of proteins under test, the following Stokes radii were derived: 5.3S EB-protein, 54 Å; 4.5S EB-protein, 35 Å. The present estimate of the Stokes radius of 4.5 S in high-salt, *i.e.*, 35 Å, is in good agreement with a previous estimate in low-salt, 33 Å (Puca *et al.*, 1971b).

Using the above sedimentation coefficient and Stokes radius and assuming a partial specific volume of 0.725, the following estimates of weight and shape were derived by calculations described in detail in a previous publication (Puca *et al.*, 1971b): molecular weight, 118,000; f/f_0, 1.67; axial ratio, 10 (prolate) and 0.08 (oblate).

Formation Mechanism of 4.5S EB-Protein. REQUIREMENT OF A SEPARATE MACROMOLECULAR FACTOR, THE RECEPTOR-TRANSFORMING FACTOR (RT-FACTOR). We know that when, during the purification attempt, $CaCl_2$ is added to uterine cytosol followed by $(NH_4)_2SO_4$ (20% saturation), the 8.6S EB-protein is no longer present in the redissolved precipitate, being replaced by the low-salt-stable 4.5 S (De Sombre *et al.*, 1969). We have also found that the same phenomenon occurs in crude uterine cytosol, upon mere addition of KCl (0.4 M) and Ca^{2+} (4 mM).

Availability of partially purified 8.6 S now offers the opportunity to investigate the mechanism of 4.5S formation. As shown in Figure 5, when ionic strength and Ca^{2+} concentration of a solution of partially purified 8.6S EB-proteins were brought to the same levels effective with crude uterine cytosol, *i.e.*, 0.4 M KCl (or NaCl or $(NH_4)_2SO_4$) and 4–8 mM $CaCl_2$, 8.6 S was not changed, even after lengthy incubation (up to 60 min). The 8.6S peak was also not changed solely by addition of the residual cytosol fraction after precipitation of EB-protein by $(NH_4)_2SO_4$ at 25% saturation. If, however, $CaCl_2$ (4 mM), KCl (0.4 M), and the residual cytosol fraction were added together to the partially 8.6S EB-protein, complete transformation into 4.5 S took place within a few minutes. This effect is also produced by addition, together with the ions, of only the cytosol fraction which precipitates between 25 and 45% $(NH_4)_2SO_4$ saturation. The fraction of cytosol still soluble at 45% saturation is ineffective.

A series of trials was carried out to test the effectiveness in converting 8.6S EB-protein into the 4.5S form of various combinations of (i) 25–45% $(NH_4)_2SO_4$-saturated cytosol fraction; (ii) Ca^{2+} (4 mM); and (iii) KCl or NaCl (0.4 M).

TABLE I: Effect of Incubation with Different Combinations of Ionic Strength, Ca^{2+} Ions, and a Uterine Cytosol Fraction in Converting Partially Purified 8.6S EB-Protein into Slower Sedimenting Estrogen-Binding Forms.[a]

Cytosol Fraction[b]	$CaCl_2$ (4 mM)	KCl or NaCl (0.4 M)	Sedimentation Rate of EB-Protein (S) In High Salt[c]	In Low Salt[d]
−	−	−		
+	−	−		
+	+	−		
−	−	+	5.3	8.6
−	+	+		
+	−	+		
−	+	−		
+	+	+	4.5	4.5

[a] Experimental conditions were as follows. The cytosol fraction insoluble between 25 and 45% $(NH_4)_2SO_4$ saturation was dissolved in an amount of TKE-I buffer solution, containing 1 mM dithioerythritol, corresponding to 0.1× the original cytosol volume; this solution was dialyzed against the same buffer used for dilution and then centrifuged to remove $(NH_4)_2SO_4$ and undissolved precipitate. To 0.2 ml of partially purified 8.6S EB-protein, prepared as described in Methods, 0.2 ml of cytosol solution, 0.05 ml of 40 mM $CaCl_2$, and 0.05 ml of 4 M KCl or NaCl were added according to the combinations specified in the table. A standard final volume of 0.5 ml was always reached by addition of TKE-I buffer. After 1-hr incubation at +4°, the reaction was stopped by addition of 5 μmoles of EDTA and 0.2 ml of each sample was analyzed by gradient centrifugation in high- and low-salts ucrose. [b] Fraction insoluble between 25 and 45% $(NH_4)_2SO_4$ saturation. [c] 0.2 M or higher. [d] 0.05 M or lower.

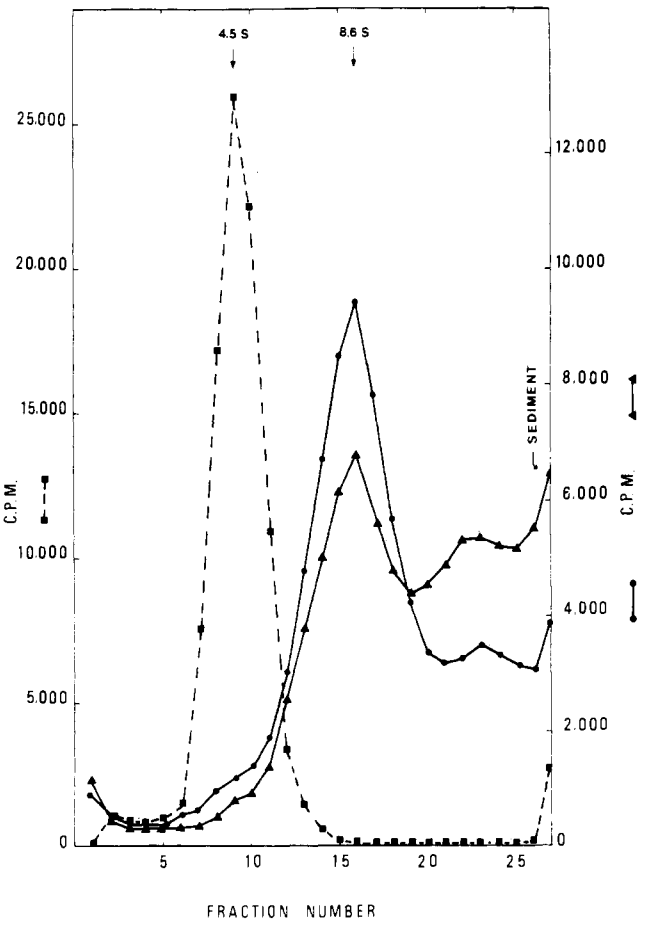

FIGURE 5: Mechanism of 4.5S formation. Centrifugation was carried out on 5–20% sucrose gradients prepared with TKE-I low-salt buffer (0.01 M in KCl). Centrifugation conditions were the same as specified in the legend to Figure 1: (●——●) partially purified 8.6S EB-protein (see Methods) after incubation with $CaCl_2$ (4 mM) and KCl (0.4 M); (▲——▲) partially purified 8.6S EB-protein after incubation with uterine cytosol fraction still soluble at 25% saturation (EB-protein free) with $(NH_4)_2SO_4$; (■- - -■) partially purified 8.6S EB-protein after incubation with Ca^{2+} (4 mM), KCl (0.4 M), and the fraction of uterine cytosol still soluble at 25% saturation with $(NH_4)_2SO_4$. Incubation was carried out at +4° for 1 hr; after incubation, 10 mM EDTA was added to reaction mixtures to stop the reaction before centrifugation. The ratio 8.6S EB-protein: cytosol fraction in reconstituted mixtures was about the same as in original cytosol.

The results are presented in Table I and show that for 4.5 S formation all 3 factors need to be present. It is thus clear that, in addition to sufficient ionic strength and Ca^{2+}, a separate, $(NH_4)_2SO_4$-precipitable factor is required for 4.5S EB-protein formation. This factor will be referred to as receptor-transforming factor (RT-factor). The finding that for 4.5 S formation a minimum ionic strength of medium is needed, which corresponds to the same minimum level required for 8.6 S to 5.3 S conversion, suggests that this latter form is the required 4.5 S precursor. Kinetics of formation of 4.5S EB-protein is shown in Figure 6.

Ionic Requirements for 4.5 S Formation. Using reaction mixtures similar to those described in footnote *a*, Table I, it was found that KCl can be substituted by the same molarity of NaCl, or by $(NH_4)_2SO_4$. Specificity for metals was also tested. In addition to Ca^{2+}, Mn^{2+} and Sr^{2+} were also effective, although to a much lesser extent; at an arbitrarily chosen time of incubation (15 min), Mn^{2+} and Sr^{2+} produce about 14 and 20% as much transformation, respectively, as Ca^{2+} at the same molarity. Other metals tested were Mg^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, Fe^{3+}; they were either ineffective or inhibitory (Cu^{2+}).

Preliminary Characterization of RT-Factor, a Protein. Tests of receptor-transforming activity are rather cumbersome. Indeed they require identification of 4.5 S formation by sucrose gradient centrifugation. Awaiting discovery of a method of assay of receptor-transforming activity which proves useful for routine work, the following limited experiments were carried out in order to give a preliminary characterization of the RT-factor.

The 25–45% $(NH_4)_2SO_4$ fraction of uterine cytosol was chromatographed through Sephadex gels of increasing pore size; RT-factor activity is excluded ($K_d = 0$) up to Sephadex G-75. According to exclusion limits of Sephadex gel, for carbohydrate-free globular proteins given by Andrews (1964), the RT-factor is thus a macromolecules of at least 110,000 molecular weight. Because the Sephadex G-75 excluded peak is active in transforming partially purified 8.6 S into the 4.5S form with addition of $CaCl_2$ and KCl only, one can also assume that other low molecular weight molecules (included by G-75) are not required for 4.5 S formation.

Also, RT-factor activity, before or after filtration through Sephadex G-75, is destroyed by proteases, but not by nucleases; thus RT-factor is, at least in part, protein. These and

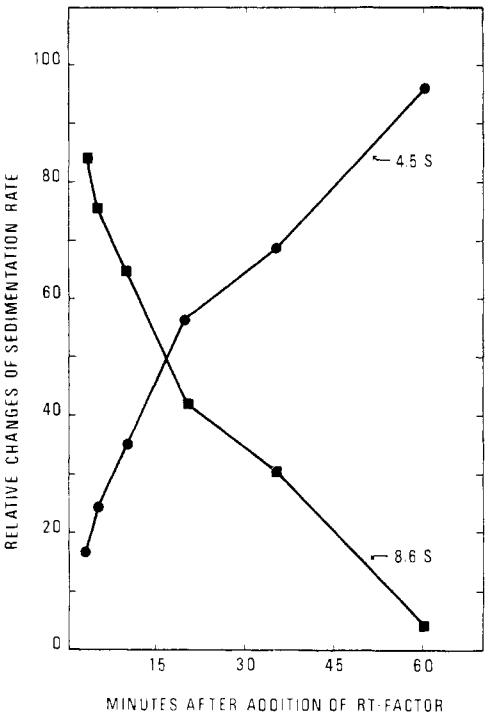

FIGURE 6: Kinetics of 4.5S EB-protein formation. To partially purified 8.6S EB-protein prepared as described in Methods, the following was added: RT-factor containing 25–45% $(NH_4)_2SO_4$-saturated uterine cytosol fraction (EB-protein free), $CaCl_2$ (4 mM), and KCl (0.4 M). After incubation at +4° for the given times, the reaction was stopped by addition of EDTA (10 mM) and amounts of residual 8.6S (■——■) and formed 4.5S (●——●) EB-proteins were estimated from the area of respective peaks on low-salt (TKE-I) sucrose gradient analysis. Sucrose gradient centrifugation and other conditions are as specified in the legend to Figure 5.

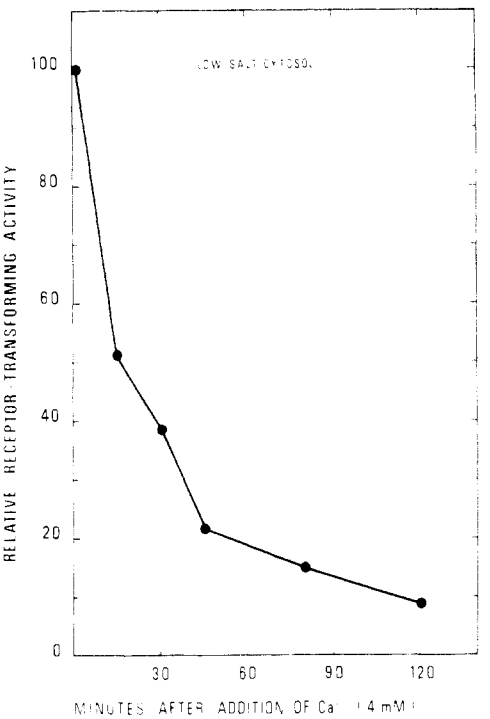

FIGURE 7: Kinetics of RT-factor inactivation after addition of Ca^{2+}. $CaCl_2$ (4 mM) was added to low-salt cytosol and after incubation for differing times receptor-transforming activity was assayed. The experimental sequence was carried out at 4° as follows: after the variable period of incubation with Ca^{2+}, KCl (0.4 M) was added and, after another standard 30 min at +4°, EDTA (10 mM) was further added in order to remove Ca^{2+} and stop the Ca^{2+}-dependent transforming reaction. The amount of 4.5S formed was then assessed by sucrose gradient centrifugation (from the area of the 4.5S peak). There is no decrease of RT-factor activity in parallel samples tested without prior incubation with Ca^{2+}.

other results of tests aiming at the characterization of RT-factor are as follows: (1) excluded by Sephadex G-75, minimum molecular weight 100,000; (2) insoluble in 25–45% saturated $(NH_4)_2SO_4$ solution; (3) destroyed by proteases (25 μg/ml of pronase or 100 μg/ml of trypsin for 30 min at 30°), but not by nucleases (25 μg/ml of deoxyribonuclease or ribonuclease for 30 min at 30°), and destroyed by heating at 60° for 20 min; (4) present almost exclusively in the 105,000*g* supernatant; (5) does not bind estrogen; (6) inactive and stable in the absence of Ca^{2+}; (7) Ca^{2+} starts an activation–inactivation cycle; (8) Mn^{2+} and Sr^{2+} may in part substitute for Ca^{2+}; Mg^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{3+} are ineffective; (9) activity is inhibited by: Cu^{2+} (1 mM), formaldehyde (0.08 M), *N*,*N*-dimethylformamide (10%), propanol (10%), or Tris (from 0.05 M); (10) activity is unaffected by phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (10^{-3} M) and cAMP and ATP.

Inactivation of the RT-Factor. An interesting peculiarity of the RT-factor is that while its transforming activity persists unhindered in EDTA-containing crude uterine cytosol, upon addition of Ca^{2+} it becomes unstable and rapidly disappears. The kinetics of this phenomenon in crude, low-salt cytosol is shown in Figure 7. Upon addition of $CaCl_2$ (4 mM), RT-factor activity of cytosol is found to decrease approximately exponentially with time, with about 10% residual activity after 2 hr. Similar inactivation of RT-factor was found to occur following addition of Ca^{2+} to partially purified RT-factor.

Thus cytosol preincubated for a sufficient time with Ca^{2+} is thereafter virtually incapable of transforming the 8.6S EB-protein. This explains a previous empirically found method of purifying the 8.6S EB-protein (Puca *et al.*, 1971b).

Trypsin-EB-Protein. Erdos *et al.* (1971) showed that after mild tryptic digestion of uterine cytosol, a low-salt-stable about 4S EB-protein replaced the 8S form. Using partially purified 8.6S EB-protein from calf uterus cytosol, we have confirmed this finding. This trypsin-EB-protein is indistinguishable from RT-factor-produced 4.5 S with respect to sedimentation rate on a sucrose gradient and elution volume on Sephadex G-200; however, it is distinctly different with regard to electrical properties (as shown by Figure 8)—on a pH 5–8 gradient it shows multiple spikes in the pH 5.8–6.5 range against the pH 6.6–6.9 focus (two spikes) for RT-factor-produced 4.5S EB-protein. In this context it seems important to report also the following findings.

Formation of trypsin-EB-protein is inhibited by protease inhibitors like diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride; these chemicals, however, do not interfere with formation of RT-factor-induced 4.5S EB-protein. On the other hand, neither high ionic strength nor addition of Ca^{2+} is required for trypsin-EB-protein formation in uterine cytosol.

Discussion

Several data from this and previous papers (Puca *et al.*, 1970, 1971a,b) are condensed in Figure 9, from which a co-

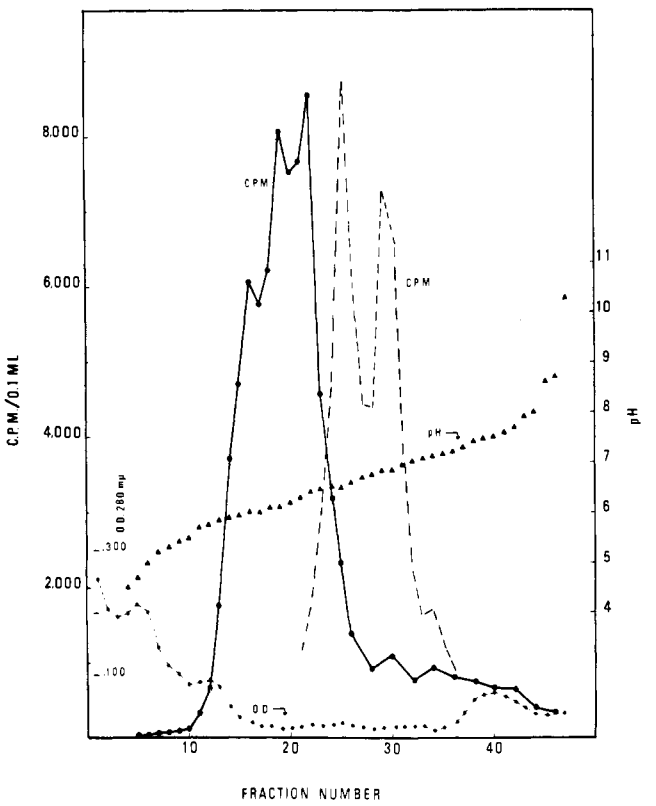

FIGURE 8: Isoelectric patterns of the product of mild tryptic hydrolysis of 8.6S EB-protein and of the RT-factor produced 4.5S EB-protein. Partially purified 8.6S EB-protein (see Methods) was incubated for 20 min at +4° with 5–10 μg of trypsin per mg of EB-protein preparation. Incubation was carried out in TKE-II (pH 7.5) buffer and was followed by filtration through Sephadex G-200 as described in Methods. The peak included by the gel sediments at about 4.5S, and it was this peak which was used for the isoelectric analysis (●——●). The figure also shows a parallel analysis of 4.5S EB-protein (– – –) produced by the RT-factor under the proper ionic conditions ($CaCl_2$, 4 mM; KCl, 0.4 M), with the same 8.6S EB-protein preparation used for tryptic digestion, as substrate.

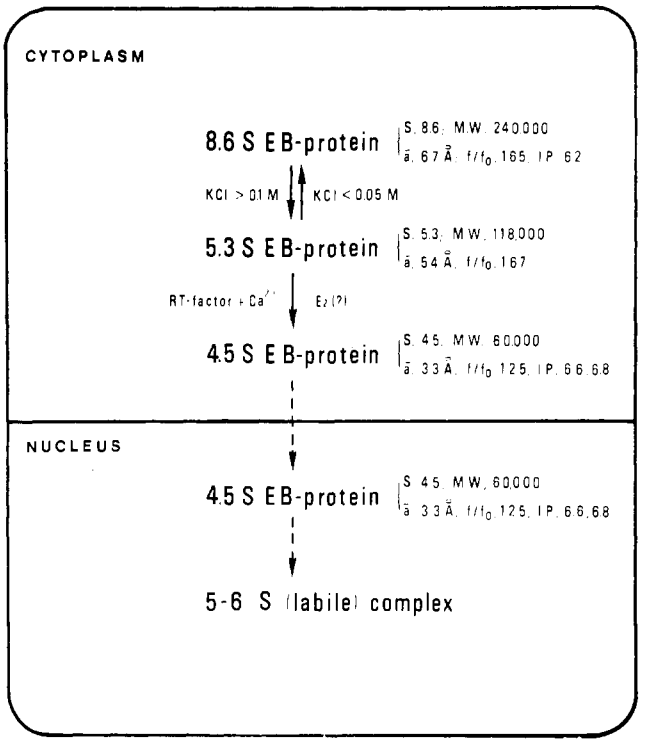

FIGURE 9: A summary of known facts and hypothesis regarding molecular transformation and intracellular transfer of EB-protein. See text for further specifications.

herent pattern of molecular interrelationship emerges for the EB-proteins of calf uterus. Several features in Figure 9 appear worth discussing, with regard to both molecular changes and mechanism of estrogen interaction with the uterine cells.

8.6S to 5.3S EB-Protein Conversion (Reversible). As shown by data in Figure 9, modification in size and shape which takes place in the 8.6 S to 5.3 S conversion consists of about halving the molecular weight, from 236,000 to 118,000, and slightly increasing the asymmetry, from an f/f_0 of 1.65 to one of 1.67. Calculations based on model prolate ellipsoids show that when an ellipsoid with an f/f_0 of 1.65 is split into two equal halves along the longitudinal (rotation) axis, the f/f_0 of resulting ellipsoids increases to about 1.7. If, on the contrary, the ellipsoid is split across the longitudinal axis, at the equator, the f/f_0 of the resulting ellipsoids drastically decreases to about 1.2. According to these calculations, the experimental data are consistent with 5.3 S resulting from longitudinal dissociation of the 8.6S form into two halves.

Ionic Strength and 8.6 S–5.3 S Equilibrium. From the earliest experiments toward identification of estrogen-binding cell components (Talwar *et al.*, 1964; Toft and Gorski, 1966), uterine cytosol has been customarily prepared from a homogenate diluted with low-salt buffer solutions (0.01 M) in a tissue:buffer ratio of 1:3–4. At such low-salt concentrations, only the large (8.6 S) form of EB-protein is present. That a drastic decrease in the sedimentation rate of the large EB-protein of low-salt cytosol takes place when KCl concentration is increased to 0.4 M or higher, as well as the reversible nature of this transition, has been shown for rat and calf uterus by others (Erdos, 1968; Korenman and Rao, 1968; Jensen *et al.*, 1969). In addition to the definition of the molecular parameters of the slower sedimenting form, we now know (i) that formation of 5.3 S does not require K^+ specifically but is the result of a specific increase of ionic strength, and (ii) that, at + 2°, formation of 5.3 S begins at 0.10 M and is complete at 0.2 M KCl or NaCl. Because total molarity of K^+ and Na^+ in uterus has been estimated at 0.16 (Cole, 1950) and sucrose gradients at this molarity show a preponderance of the 5.3S over the 8.6S form, and considering, furthermore, that electrical interactions at +37° are weaker than at +2°, the temperature at which gradients are run, it seems likely that the prevailing form *in utero* is 5.3 S, rather than 8.6 S. The 8.6 S state, as well as faster sedimenting aggregates, is possibly the result of experimental dilution of uterine cytosol with low-salt solutions (KCl or NaCl, 0.01 M).

5.3 S to 4.5 S Conversion (Irreversible). As shown by data in Figure 9, changes in size and shape which take place in the conversion of 5.3 S into low-salt-stable 4.5 S are the approximate halving of the molecular weight, from 118,000 to 60,000, and a drastic decrease of asymmetry, from a f/f_0 of 1.67 to one of 1.25. Calculations of frictional ratios of model prolate ellipsoids show that when an ellipsoid with an f/f_0 of 1.67 is split across the longitudinal axis, at the equator, into two equal halves, the resulting ellipsoids have an f/f_0 of about 1.3. Thus, 4.5 S formation would be consistent with splitting of the 5.3S protein into two, and only two, pieces of about half the parental length. Whether both these resulting proteins

bind hormone, and whether or not they are identical, cannot be answered on the basis of present data.

Some information pertinent to the latter question is, however, given by electrofocusing. While the 8.6S EB-protein is homogeneous at electrofocusing, with a single peak at pH 6.2 (for 5.3 S the required high-salt medium is incompatible with electrofocusing), the electrofocusing pattern of 4.5 S consistently shows two spikes at pH 6.6 and 6.8 (and possibly a shoulder). As already discussed (Puca *et al.*, 1971b), several explanations are possible for the finding, including that two different EB-proteins derive from 5.3 S cleavage, both having molecular weights of about 60,000 and sedimenting at 4.5 S but slightly different with respect to electrical properties. Also pertinent to the same question is the mechanism of 4.5 S formation. We know that sufficient ionic strength is only a *permissive* condition for 4.5 S formation. The *inducive* agent appears to be a Ca^{2+}-activated, separate protein factor, the RT-factor. The nature of the RT-factor will be discussed later in this paper and it will become apparent that this factor is likely to be an enzyme. If an enzymatic splitting of specific covalent bonds is involved in 4.5 S formation, it is indeed expected that 4.5 S formation be virtually irreversible, as found, and that different proteins derive from the 5.3S precursor.

RT-Factor. The results of preliminary trials of characterization have shown that this factor is a protein of molecular weight of at least 100,000; Ca^{2+} is specifically required for receptor-transforming activity, with Mn^{2+} and Sr^{2+} able to substitute in part for Ca^{2+}. An ionic strength of medium in the physiological range or higher is also needed for receptor-transforming activity. This last condition for 4.5 S formation seems connected to the requirement of 5.3 S as a precursor.

Presently known properties of the RT-factor are summarized in the Results section. All properties known to date are consistent with the RT-factor being endowed with enzymatic activity; but of course, much more work is needed for satisfactory molecular and functional characterization of the RT-factor. Such work requires a method simpler than sucrose gradient centrifugation for separation of 4.5S product from the precursor of higher molecular weight, and present research in this laboratory is directed toward such a goal. It is already possible, however, to exclude the possibility that the RT-factor is trypsin or an enzyme of the trypsin group, owing to the following results: (i) although similar in sedimentation rate and molecular weight, the product of mild tryptic hydrolysis of the 5.3S–8.6S EB-protein shows an electrofocusing pattern distinctly different from that of the 4.5S EB-protein produced by RT-factor; (ii) in contrast to RT-factor, trypsin is active in producing fragments from 5.3S–8.6S protein under low-salt conditions and in the absence of added Ca^{2+}; (iii) formation of tryptic fragments is inhibited by inhibitors specific to the trypsin group of proteases, while formation of 4.5 S by RT-factor is not.

RT-Factor Inactivation. In dilute cytosol and partially purified preparations with no Ca^{2+} content, the RT-factor is *inactive* and *stable*. Addition of Ca^{2+} up to 2–4 mM produces immediate receptor-transforming activity but also initiates a process which results in rapid and irreversible loss of such activity. Such loss of activity cannot be restored by further addition of Ca^{2+}. It thus appears that we are dealing with a case (and kinetic) of consecutive reactions of the type A → B → C, where A is inactive RT-factor, B is Ca^{2+}-activated RT-factor, and C is a product of B which has irreversibly lost receptor-transforming activity. The A to B and B to C reactions of our simple hypothesis are completely unknown in their nature except for the requirement of Ca^{2+}. One may envisage either a process of the type trypsinogen to trypsin active and inactive or intervention of another separate factor, which inactivates the RT-factor.

Demonstration of receptor-transforming activity furnishes an explanation for a previous empirically found method of purification of 8.6S–5.3S native protein by a preliminary 2-hr incubation of low-salt cytosol with 4 mM Ca^{2+} (Puca *et al.*, 1971b). Under these conditions the A → B → C reactions take place while 5.3 S, the required substrate for RT-factor, is not available due to insufficient ionic strength. When, after 2 hr, the inactivation of RT-factor is virtually completed, the cytosol can be manipulated at will, including increase of ionic strength by $(NH_4)_2SO_4$ or KCl as required for purification, without risk of 8.6S protein loss.

Mechanism of Estrogen Interaction with the Cell. Formation of a complex between estrogen and the calf uterus cytoplasmic 5.3S–8.6S native protein system occurs spontaneously on addition of the specific ligand to uterine cytosol. On the other hand, incubation of uterine nuclear fraction with 17β-estradiol does not give rise to any extractable EB-protein. Only extraction of nuclei prepared from calf uterine tissue exposed to radioactive 17β-estradiol, best extracted by 0.3–0.4 M KCl at pH 8.5, will yield an EB-protein–estradiol complex; when the crude nuclear extract is layered as such on sucrose gradients, this complex sediments at about 5–6 S (5 S) (Puca and Bresciani, 1969). After partial purification, however, the nuclear complex sediments at 4.5 S and is indistinguishable from the 4.5S cytoplasmic EB-protein in molecular parameters as well as in any other known properties (Puca *et al.*, 1970, 1971a). These data with calf uterus confirm previous research with the rat uterus (Jensen *et al.*, 1967; Puca and Bresciani, 1968). Formation of nuclear 5 S was further shown to be a temperature-dependent process which is accompanied by a decrease of 8 S (Brecher *et al.*, 1967; Gorski *et al.*, 1968; Jensen *et al.*, 1968, 1969).

The above results led to proposal of a two-step mechanism for the interaction of estradiol with rat uterus. According to the original postulation, estradiol first combines with the cytoplasmic 8S protein which then rapidly enters the nucleus by a temperature-dependent process to give rise to the nuclear 5S complex (Jensen *et al.*, 1968, 1969). The latest variation of this hypothesis (Brecher *et al.*, 1971) is that the 8.6S receptor protein is converted into a 4S subunit in the cytoplasm and that also the following 4 S to 5 S transformation occurs in the cytoplasm, before entering the nucleus.

On the basis of results in this and previous papers (Puca *et al.*, 1970; Puca *et al.*, 1971a,b) the following sequence of early events in the interaction of estrogen with the uterine cell may now be envisaged: (1) the estrogen enters the cytoplasm and forms a complex with the native 5.3S–8.6S EB-protein system which is confined in the cytoplasm; (2) the Ca^{2+}-activated RT-factor acts on the 5.3 S–estradiol complex producing 4.5 S–estradiol complex; (3) the derivative 4.5 S–estrogen complex penetrates the nucleus, possibly only in virtue of its smaller size; (4) the 4.5 S–estrogen complex forms labile interactions with nuclear molecules acquiring a faster sedimentation rate, 5–6 S (5 S), as in crude nuclear extracts. Recent results (Steggles *et al.*, 1971) showing that chromatin of estrogen target organs is endowed with sites having specific affinity for EB-proteins reasonably explains why the EB–estrogen complex concentrates in the nucleus once 4.5S EB-protein is formed and nuclear penetration is no longer impeded.

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